Pierce's Disease Control Program

Symposium Proceedings

2007
Pierce's Disease
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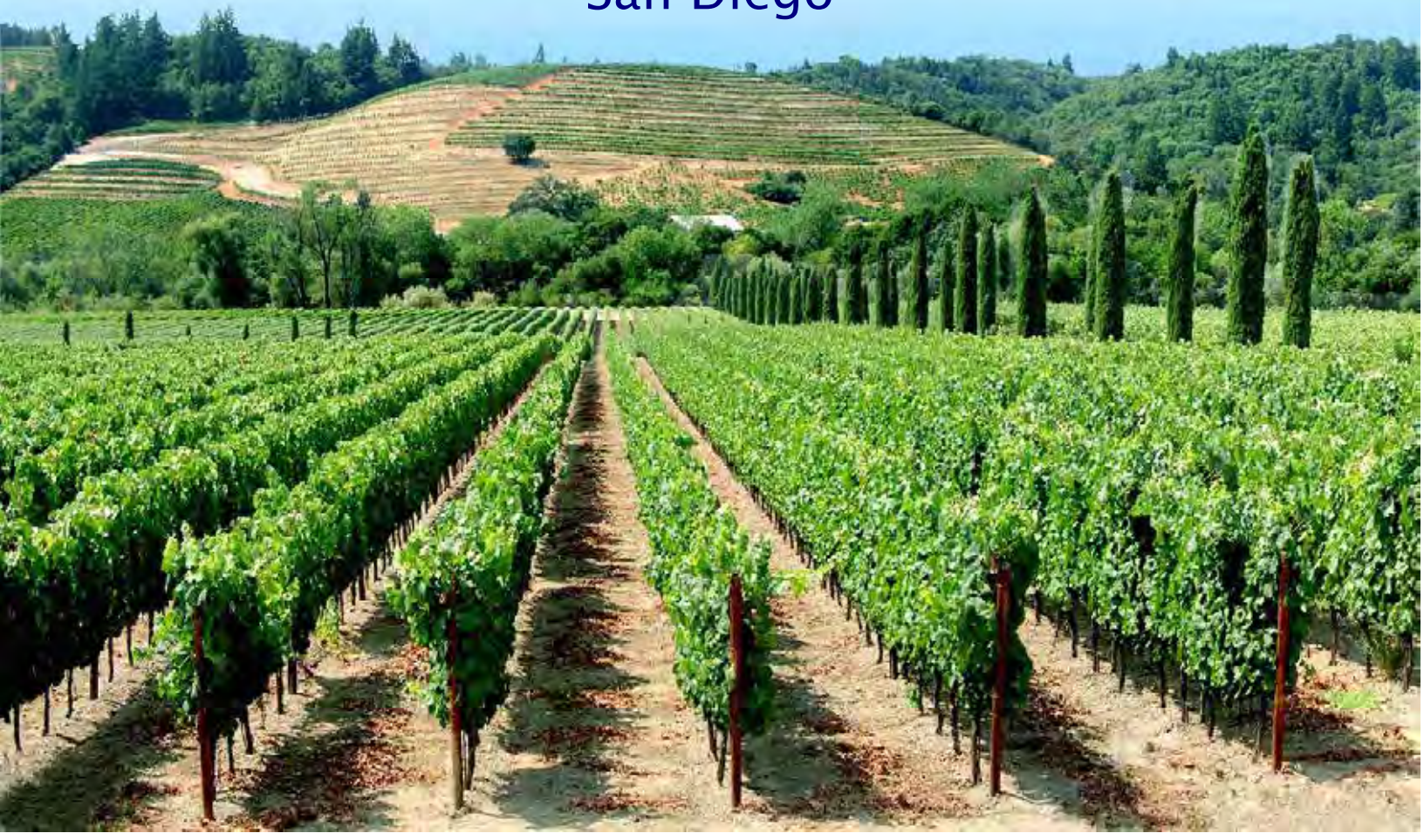
December 12-14, 2007
The Westin Horton Plaza
San Diego



California Department of Food & Agriculture

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Section 1:

Vector Biology and Ecology

HOST PLANT PREFERENCE AND NATURAL INFECTIVITY OF INSECT VECTORS OF *XYLELLA FASTIDIOSA* ON COMMON WEEDS AND CROP PLANTS

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Reporting Period: The results reported here are from work conducted October 2006 to July 2007.

ABSTRACT
Homalodisca vitripennis and *Spissistilus festinus* populations were surveyed bi-monthly in Kern County at sites with a variety of potential feeding and breeding hosts. Insects collected by sweeps and sticky traps were tested for *Xylella fastidiosa* (*Xf)* presence with vacuum-extraction and PCR. Comparisons of four techniques to detect *Xf* in insects are ongoing in greenhouse studies: live insect transmission to plants, vacuum-extraction PCR, culture of heads, and lyophilization with chloroform-phenol extraction. 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 and targeted monitoring to reduce insect populations and inoculum potential.

INTRODUCTION
Despite an area-wide insecticide spray program, endemic glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, populations are still present near Bakersfield, especially in abandoned vineyards, and along roadsides and windbreaks. Recently, three cornered alfalfa hopper (TCAH), *Spissistilus festinus*, populations increased in the San Joaquin Valley. Histological studies show that TCAH normally feed in phloem tissue with occasional probes to xylem. The highly polyphagous feeding habits of TCAH may be of concern to researchers worried about the spread of *Xf* between and within crops like alfalfa, grapes and almonds. Common vineyard weeds and windbreak species are hosts of *Xf* (Costa et al. 2004, Wistrom and Purcell 2005, Shapland et al. 2006). While GWSS and TCAH feed on a wide range of plans, quantitative host preference data for only GWSS has been collected, and the studies focused mainly on citrus, grapes and ornamentals (Daane and Johnson 2005, Mizell et al. 2005).

Here, we investigated host plant preference and natural infectivity of GWSS and TCAH. While there is little data about the natural infectivity of GWSS in agricultural settings, between 10% and 20% of GWSS transmitted *Xf* in greenhouse tests (Almeida and Purcell 2003) and 1.25% of GWSS tested positive for *Xf* when collected from urban landscapes in Bakersfield (K. Daane, unpublished data). GWSS can be tested for *Xf* presence by vacuum-extraction and PCR. *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 et al. 2005) of *Xf* in sharpshooter heads enabled more rapid, efficient, and convenient bacterial detection, compared to transmission tests (Purcell and Finlay 1980), or insect head culture (Hill and Purcell 1995). Lyophilization and maceration, followed by chloroform/phenol extraction, also reportedly sensitively detects *Xf* in sharpshooter heads. *Xf* transmission to grapevines is highly sensitive, so sharpshooter infectivity can be assessed when bacterial populations are below the detection thresholds of culture or PCR (Hill and Purcell 1995).

OBJECTIVES
1. Determine preference of insect vectors of *Xf* 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

Five collection sites in Kern County were selected based on the presence of endemic GWSS populations and diverse crop plants. Three sites were located in or near vineyards, and two had Pierce's disease present. Each site was divided into at least seven areas, which were sampled twice a month for one year, beginning in October 2006. Only one of seven sites received a specific insecticide treatment for GWSS, where Admire (imidacloprid) was applied to eucalyptus, jojoba, and citrus.

To date, 350 GWSS and 301 TCAH total were collected from the five field sites. Populations of TCAH are reported because they were unexpectedly high throughout the year and occurred on the same hosts at roughly the same populations as GWSS. TCAH were the only other Cicadomorph species regularly observed. The largest average populations of TCAH were on alfalfa and of GWSS were on willow and eucalyptus (Figure 2). Four percent of GWSS and two percent of TCAH collected were nymphs. The numbers of insects collected varied by site and host (Figure 1). There was greater variability in insect numbers collected by sticky traps than sweeps.

While the initial plan was to look at vector populations primarily on weeds, very few weeds inside and adjacent to the study sites. Over the year, there were no weeds at all for 22% of samples taken, less than 10% weedy ground cover for 72% of samples, and only 6% of samples had more than 10% cover. No GWSS were collected on sweeps of those weeds in October, November, and May, and only TCAH were collected on white sweetclover (*Melilotus alba*) in May. There was no relationship between percent cover of weeds and the number of GWSS (one-way ANOVA, $P = 0.95$), but there may be some relationship between TCAH populations and weeds (one-way ANOVA, $P = 0.07$). Instead, GWSS and TCAH were consistently collected on perennial crop plants.

Host plant condition influenced both TCAH and GWSS preference. An average of 0.23 GWSS/100 sweeps were found on hosts with mature fruit, compared to 0.14 GWSS/100 sweeps on plants without fruit, and 0.04 GWSS/100 sweeps for plants with green fruit (one-way ANOVA, $P = 0.003$). A similar relationship was found between fruit maturity and TCAH preference (mature fruit = 0.64, no fruit = 0.35, and green fruit = 0.08 per 100 sweeps; one-way ANOVA, $P< 0.0001$). Both GWSS and TCAH preferred unpruned plants (0.128 GWSS, 0.323 TCAH per 100 sweeps) to recently-pruned plants (0.015 GWSS, and 0.12 TCAH per 100 sweeps; two-sample t-test, $P = 0.05$ for both comparisons). On average, 0.14 GWSS and 0.37 TCAH were collected per 100 sweeps on hosts with suckers or new growth, and 0.02 GWSS and 0.08 TCAH were collected on plants without (two-sample t-test, $P = 0.005$ for GWSS and $P = 0.0003$ for TCAH).

The highest populations of GWSS were collected in fall of 2006, decreasing from mid-December through early February. Populations remained very low through July 2007, although collections in August and September 2007 indicate that GWSS populations are rising, in the same locations and on the same hosts as they were initially collected in fall 2006. TCAH populations collected by sweeps were similar to GWSS populations, though TCAH increased continuously in fall 2006, whereas GWSS fluctuated somewhat in that time.

Both insect populations decreased following unusually cold temperatures from 12 to 23 January 2005. Nighttime low temperatures averaged 26.5ºC, compared to an estimated historic average of 37.4ºC, 11ºC below normal (CIMIS temperature data; Arvin Station; near sites 1 and 2). New growth on the evergreen plants surveyed was delayed and/or damaged by the cold temperatures, and citrus trees were pruned extensively to remove damaged shoots. Populations of TCAH and GWSS declined from a peak population in mid-December to the lowest populations eight weeks later in February. The decline from cold temperatures was long-lasting but not immediate. At site 2, locations 2 (citrus), 3 (jojoba/ eucalyptus), 5 (jojoba), 8 (citrus), and 10 (eucalyptus) were treated with systemic imidacloprid during the first week of March 2007. Prior to that GWSS catches increased at a higher rate on sticky traps compared to collections with sweep nets. Following the spray, GWSS populations decreased to their lowest levels, but TCAH populations took another two weeks to peak and then declined.

To date, no *Xf* was detected in any of the 635 adult GWSS or TCAH tested by vacuum-extraction and PCR. Pierce's disease symptoms were observed at sites 3 and 5 in fall 2007. Ivy and oleander samples with leaf scorch, collected at site 4 in spring 2006, were negative for *Xf*.

Comparisons between detection techniques are ongoing. In experiments conducted in fall 2006, only two of 121 GWSS tested transmitted *Xf* to grapes, none of the 45 insects tested by culture and one of 29 tested by vacuum-extraction PCR positive for *Xf*. An additional 88 insects (blue-green sharpshooter; BGSS) were tested so far this summer, with none of the 42 tested by vacuum-extraction PCR and three of nine tested by culture positive for *Xf*. Lyophilization-PCR and culture of plants inoculated by insects are ongoing, as are additional replications of this experiment.

DISCUSSION

The results reported here are still preliminary. The major host plants used by both GWSS and TCAH are perennial crops and windbreaks. There was little ground cover at any of the sites, and populations of TCAH or GWSS were not appreciably

larger where it was present. Highest populations of TCAH and GWSS were on plants used as windbreaks: jojoba and eucalyptus. The weed control practiced by citrus growers for frost protection, combined with good sanitary practices and targeted irrigation resulted in few weeds at any of the study sites. Where there was ground cover, it was kept short through frequent mowing. When normal cultivation practices are followed, initial results suggest that it is unlikely that weeds or other groundcover play a role in hosting GWSS or TCAH, except weedy legumes, which may be breeding hosts of TCAH.

The results so far show that plant condition plays a role in TCAH or GWSS preference. Plants with suckers or lush growth were more attractive than plants that were recently pruned or had mature growth. This follows the idea that GWSS require very large volumes of xylem sap, and that host plant nutritional status is important for nymph development and survival (Andersen et al. 1989, Brodbeck et al. 2004). This would also show why plants with developing fruit were not as attractive to GWSS or TCAH; the green fruit may act as a nutrient and carbohydrate sink, decreasing levels of nutrients and sugar in the xylem and phloem tissues the insects feed upon. More data analysis and research into published literature is needed to speculate why plants with mature fruit were most attractive to GWSS and TCAH.

The cold snap in January also had unexpected results. GWSS and TCAH continued to be collected so the low temperatures did not kill the insects outright. Instead, GWSS populations began declining in December, and continued to decrease over the following two months. As the cold caused extensive damage to green, growing shoots preferred as feeding sources, perhaps there was reduced food available to the insects. In particular, nymphs would be affected by damage to feeding hosts since they are unable to move large distances to another host plant. This is suggested by the low GWSS populations lasting until late summer 2007. The usual springtime increase in GWSS populations was not observed in this study, suggesting that the cold had lasting impacts upon adult fecundity, nymph survival, and/or egg development.

The initial results from the field survey suggest that GWSS host plant use depends on the type and condition of host plants available as well as the time of year. Sweeps and visual inspection were different in the quantity of insects detected, although they roughly mirrored one another in monitoring trends in TCAH and GWSS populations. By detecting insects that are mobile, sticky traps may be helpful when GWSS are moving between hosts, for either nutritional or egglaying purposes, but underestimate GWSS populations when host plant conditions are attractive to GWSS, and thus remain on the plant for long periods of time.

Results from this project may help improve control decisions, by investigating the necessity of vegetation management or targeted insecticide sprays to reduce insect populations and inoculum potential, and providing some of the information required to develop a treatment threshold for GWSS populations in areas with endemic GWSS populations and Pierce's disease.

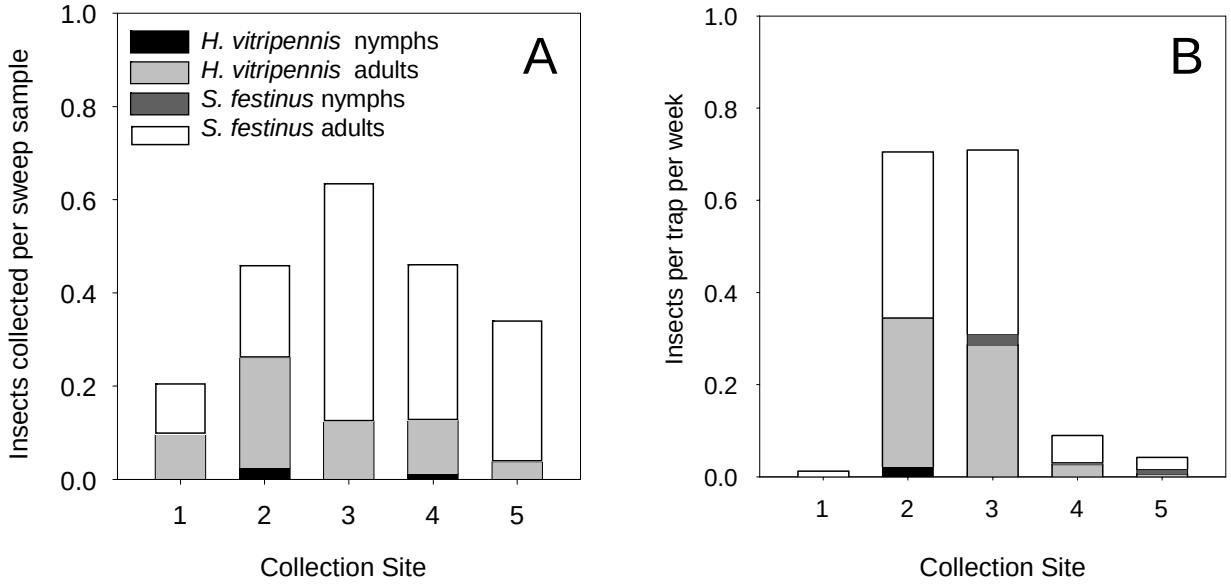

Figure 1. Average insect populations collected bi-monthly by A) 100 sweeps-beats or 30 second visual survey and B) sticky traps at field sites between July 2006 and July 2007.

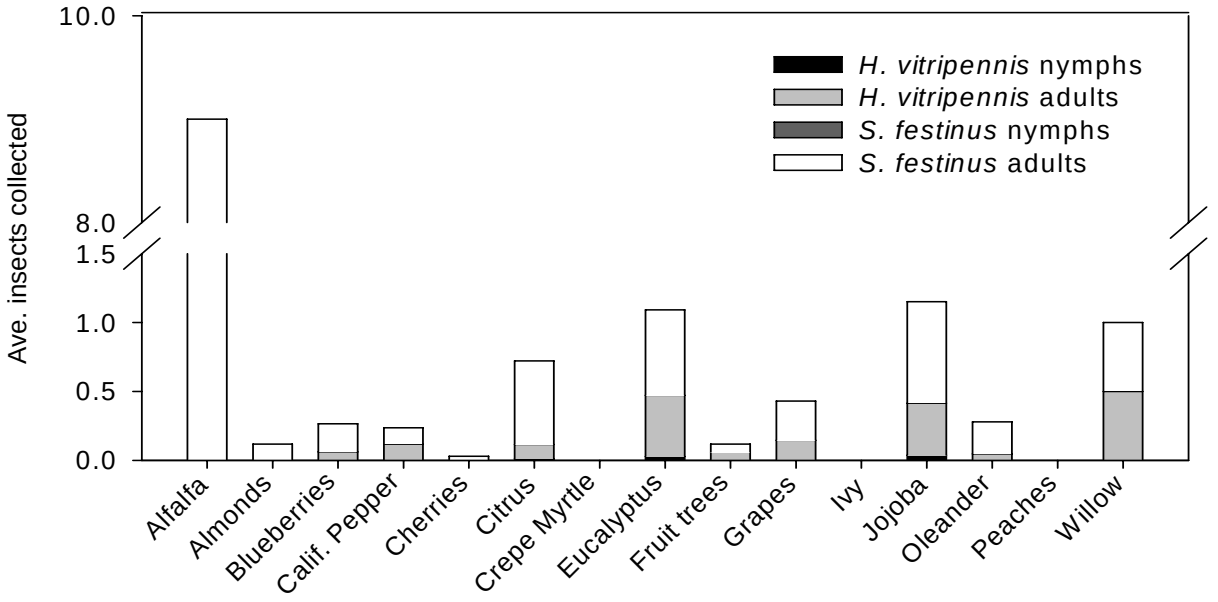

Figure 2. Average insect populations collected in 100 sweeps-beats or 30 second visual observation in bi-monthly surveys of plant hosts at all field sites between July 2006 and July 2007.

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FUNDING AGENCIES

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

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Reporting Period: The results reported here are from work conducted January 1, 2007 to September 30, 2007.

ABSTRACT
Surveys in the native range of *Homalodisca vitripennis* (*H. vitripennis*) are continuing to discover nymphal parasitoids and to determine the ecology and phenology of glassy-winged sharpshooter 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. Pipunculid adults have been recovered from hand collected *Oncometopia orbona* feeding on mustang grapes which have been identified as *Eudorylas* nr. *vierecki*. Populations of *H. vitripennis* began to increase in March and peaked in July. While populations peaked in July, a small proportion of these insects tested positive for the presence of *Xylella fastidiosa* (*Xf*) compared to the insects collected in later months (i.e. August, September, etc.). Only 7% of *H. vitripennis* tested positive in July, whereas 59% of *H. vitripennis* tested positive in September.

INTRODUCTION
The glassy-winged sharpshooter (GWSS), *H. 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, *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 have focused on egg parasitoids of GWSS (Mizell and Andersen 2003, Hoddle and Triapitsyn 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 *H. vitripennis*, including Pipunculidae, have not been evaluated as biological control agents. Skevington and Marshall (1997) review the natural history and rearing of Pipunculidae. 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 (unpublished). 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 & 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 pooled mean number of *H. vitripennis* and *Oncometopia* spp. adults from yellow sticky card traps for all locations are shown in Figure 1. Populations of sharpshooters were lower in 2007, even though all sites experienced above average rainfall. *Oncometopia orbona* populations peak in early spring followed by *H. vitripennis*. This phenology results in nymphal sharpshooter populations throughout the spring and summer which may be exploited by pipunculid parasitoids.

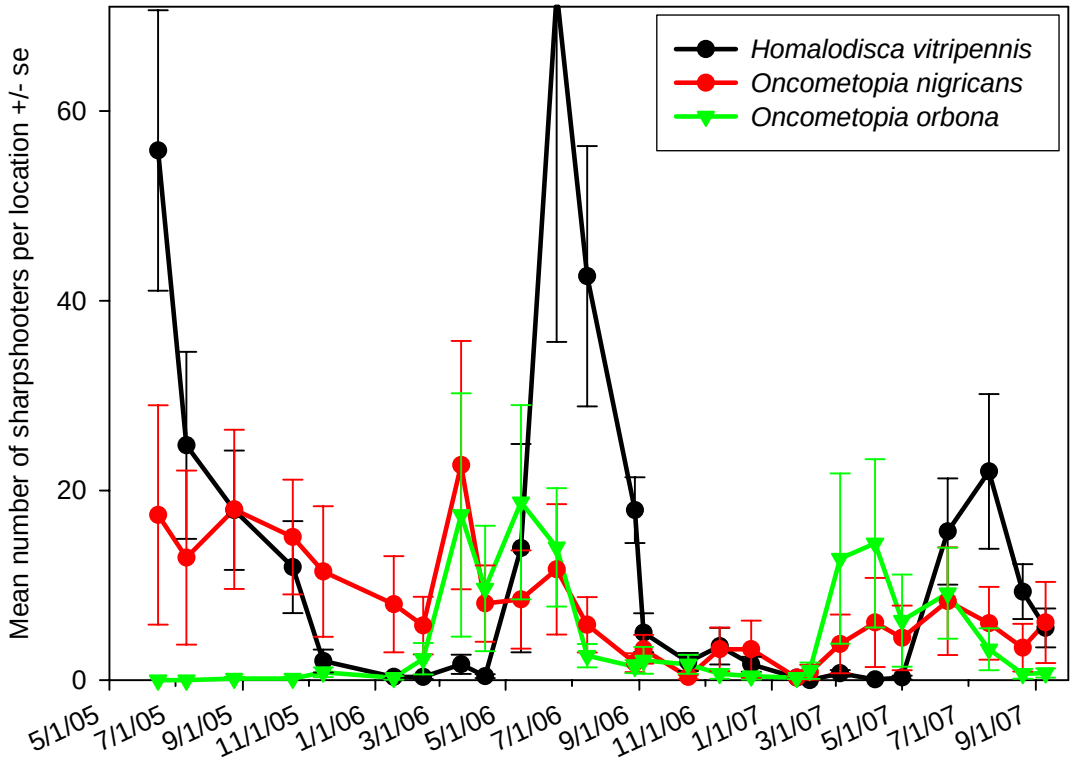

Figure 1. Yellow sticky trap catches of sharpshooter adults collected from mustang grape stands in southeastern, TX (Oct. 05- Sept 07).

Pipunculidae have been collected in yellow sticky traps and from hand collected sharpshooters from several survey locations in Southeast Texas. Many *Eudorylas* nr. *vierecki* have been recovered from *Oncometopia* spp., and possibly *H. vitripennis*, but the latter host association has not been confirmed with host remains.

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. Hand collecting of sharpshooter adults and nymphs has proven to be the most effective method for recovery of Pipunculidae. Despite our success at collecting *E.* nr. *vierecki* from hand collected *Oncometopia* spp., the apparent failure to rear pipunculids from *H. vitripennis* remains unclear. Pipunculids could be parasitizing small, early instars of *H. vitripennis* and then dropping them away from their feeding location, as per the published accounts by Jervis (1980) describing the life history a *Chalarus* species on a typhlocybine leafhopper in the UK. In this scenario, recovering parasitized *H. vitripennis* could be difficult. An alternative scenario could be that percent parasitism of *H. vitripennis* is very low and despite collecting thousands of nymphs from many different locations over time we have not collected the unique pipunculid parasitoid. Lastly, pipunculid parasitoids of *H. vitripennis* may be more common in other parts of its range, i.e. Florida where proconiine sharpshooter diversity is higher. To this end, Dr. Jesusa Legaspi is surveying for pipunculid parasitoids near Tallahassee, FL.

Sharpshooters collected from the traps were assayed for the presence of *Xf* using molecular techniques developed by Bextine et al. (2005). Analysis of these samples is not complete, so this data set will grow in the coming months. At this point, 95/345 (27%) *H. vitripennis* have tested positive from July to November. This figure is consistent with the other common sharpshooter species; *Oncometopia nigricans* (21%) and *Oncometopia orbona* (14%). Although there was little discrepancy in the percentage of insects testing positive, *H. vitripennis* was the most populous insect resulting on nearly four times the number of positive insects. Also, in July 2006, the percentages of *Xylella*-positive insects was low (*H. vitripennis* (7%), *O. nigricans* (0%) and *O. orbona* (10%)) in comparison to later months. For example, in September high numbers of *Xylella*-positive insects were found (*H. vitripennis* (59%), *O. nigricans* (57%) and *O. orbona* (71%)).

CONCLUSIONS

A pipunculid parasitoid, *Eudorylas* nr. *viereckii* has been recovered in fair numbers from the sharpshooter, *Oncometopia orbona* and *O. nigricans*. Despite intensive efforts spanning two years and multiple field sites a pipunculid parasitoid of *H. vitripennis* has not been positively recovered and identified. Further collecting will take place in the eastern range of *H. vitripennis* in northern Florida, where nymphal parasitoids of *H. vitripennis* may be more abundant. As potential vector species, all three sharpshooters have similar ratios of *Xylella*-positive insects and acquire greater amounts of *Xylella* as the season progresses. However, *H. vitripennis* occurs in higher numbers resulting in greater vectoral potential.

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

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

ABSTRACT
Glassy-winged sharpshooter (GWSS) developmental and reproductive biology has received relatively 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 research is to generate these fundamental biological data for GWSS to assist pest management programs, biological control efforts, and incursion risk management. These laboratory-derived data, once collected and analyzed, can be compared to similar data for the major egg parasitoids attacking GWSS. When taken together, the influence of temperature on pest development and reproductive output, and its natural enemies will enable pest managers to determine incursion risks and the expected level of biological control that natural enemies could provide as GWSS continues to expand its range within California.

INTRODUCTION
Day-degree accumulation for development and estimates of adult longevity, fecundity, survival rates, and seasonal generational turn over are the fundamental factors driving population growth. Scientifically-based insect pest management programs rely heavily on day-degree models to help with predictions on pest population growth so adequate control measures can be initiated well in advance of the development of economically damaging populations. While the role of day-degree research in pest management is well recognized and employed widely across a diverse group of insect pests, these data can also be invaluable for assessing invasion risk to areas currently uninfested by the pest, and the rate of spread and population growth intensity in newly invaded areas. Despite the high economic and pest profile of GWSS, relatively little is known about the degree-day requirements for this pest or the effect of varying temperature regimens on the survivorship rates of nymphs in various instars, the longevity of adults, adult fecundity and sex ratio of offspring. The research currently being conducted and reported here will address these important informational shortcomings for GWSS.

OBJECTIVES
1. Develop day-degree models for GWSS by quantifying the developmental biology at five different temperatures (20, 25, 27, 30, &33^{o}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 (Obj. 1), and demographic estimates (Obj. 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, *Gonatocerus ashmeadi* and *G. triguttatus*, thus enabling comparison to determine how well GWSS egg parasitoids will be able to track GWSS as this pest expands its range northwards in California.

RESULTS
Developmental times for GWSS nymphs, adult longevity, adult fecundity, and progeny sex ratio have been determined for eight-nine females at 25, 30 and 33^{o}C. At the time of writing this report, the final 260 nymphs were being reared to adulthood and for reproductive demographic studies. An additional 20 nymphs are being reared at 20^{o}C, 60 nymphs at 33^{o}C and 180 nymphs are being reared at 25^{o}C (90 nymphs) and 30^{o}C (90 nymphs).
Work is ongoing for 20^{o}C for all developmental and reproductive biology work. This low temperature is proving to be very slow and difficult to complete because nymphal development is extremely slow and adult sharpshooters are tardy when mating, maturing eggs, and ovipositing at 20^{o}C. In addition to the four experimental temperatures we are currently running (20, 25, 30, and 33^{o}C), we are planning to run a variable temperature experiment. For this variable temperature study, the mean daily maximum temperature will be set at 30^{o}C and the mean nightly temperature will be set at 20^{o}C. This variable temperature regimen will provide a mean temperature of 25^{o}C. Data from this experiment can be compared to those obtained

for GWSS reared at a constant 25°C. This additional temperature regimen is considered necessary for GWSS as previous peer reviews of similar studies completed for the egg parasitoids *Gonatocerus ashmeadi* and *G. triguttatus* have correctly pointed out that in nature animals are not subjected to constant temperatures in the environment. Some studies suggest that exposure to fluctuating temperatures may actually be beneficial for developmental times, survival times, and estimates of fitness (e.g., longevity and fecundity).

Data analyses for fecundity and subsequent demographic statistics have not yet been completed as replicates at four temperatures are still being run (20, 25, 30 and 33°C). Partial data analyses on developmental times are provided in Table 1. These data estimates will change as additional replicates are added to respective temperatures once completed. Data on the reproductive biology of GWSS at the four experimental temperatures has not been provided as these data are still being collected from reproductively active females and fecundity estimates and progeny development is currently insufficient for sex ratio analyses that are needed for jackknifing demographic statistics. We anticipate having this project completely finished, and analyzed by the winter of 2007, and a manuscript submitted for publication in early 2008.

Table 1. Partial developmental and reproductive statistics for GWSS at four different temperatures. All developmental times are presented in days. These data are preliminary and estimates will change as additional replicates are currently being run to bolster the number of GWSS used for analyses.

Temperature	Egg Devpt Time (Days)	Nymph to Adult Devpt Time (Days)	Adult Longevity (Days)	Female Fecundity & Progeny Sex Ratio
20°C	16.4	82.3	70.2	Ongoing
25°C	8.3	65.8	56.2	Ongoing
30°C	6.1	54.9	46.8	Ongoing
33°C	7.6	41.9	32.5	Ongoing
Variable Temp (20°C at night 30°C during day)	Yet to be started	Yet to be started	Yet to be started	Yet to be started

CONCLUSIONS

Upon the anticipated completion of this ongoing project at the end of winter 2008, we expect to have collected sufficient data to determine the day-degree requirements for GWSS, to have quantified survival and longevity for nymphs and adults, and to have robust estimates of life time fecundity and sex ratio for populations reared at various experimental temperatures. These data are necessary for the development of day-degree models for pest management, to predict incursion risk and population growth in new areas, and to determine what the overlap in range will be between GWSS and its mymarid egg parasitoids, for which similar studies have already been completed.

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EXPRESSED SEQUENCE TAGS FROM THE BLACK-WINGED SHARPSHOOTER: APPLICATION TO BIOLOGY AND VECTOR CONTROL

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ABSTRACT
The black-winged sharpshooter, *Oncometopia nigricans* Walker, is considered a highly competent vector of several strains of the xylem-inhabiting bacterium *Xylella fastidiosa (Xf)*, the causal agent of a number of economically important plant diseases including Pierce's disease, phony peach disease, plum leaf scald and periwinkle wilt. To better understand the biology of the this leafhopper on a molecular level, our lab undertook a large-scale 5' end expressed sequence tag (EST) sequencing project of cDNA clones derived from *O. nigricans* adults. Similar EST sequencing projects from other insect pests have definitively proven their worth in answering biological questions relating to organismal behavior, development and physiology. These data enables the identification of target genes with potential for development of novel genomics-based management strategies to reduce leafhopper impacts on agricultural crops. To that end, we report on our advances on leafhopper genomic research involving a vector of Pierce's disease, the sharpshooter, *O. nigricans.* We produced a dataset containing 4,411 high-quality ESTs, representing a set of 3,301 transcripts. Assembly identified 14 full length transcripts which have important biological functions. Research centering around the annotation, characterization, and application of the genomic information is demonstrated by work on a *delta 9 desaturase* and an *Imaginal Disc Growth Factor*, IDGF, isolated from *O. nigricans.* The sequences reported in this paper have been submitted to NCBI's dbEST under the following accession numbers: DR755012-DR759538

INTRODUCTION
Sharpshooter leafhoppers (Cicadellidae) are vectors of a number of destructive plant diseases. Although the glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* Germar, is the most notorious of these vectors due to its detrimental impacts to commercial crops in California, a second leafhopper commonly referred to as the black-winged sharpshooter, *Oncometopia nigricans* Walker, has also been associated with pathogen transmission. Like GWSS, *O. nigricans* is a highly polyphagous xylem-feeding leafhopper present throughout the southeastern United States (Tipping et al., 2004). Upon feeding, bacteria are taken up into the insect's mouthparts where they attach to the walls of the cibarium (Brlansky et al., 1983). During subsequent feedings, the bacteria are released into the plant. The transfer of xylem-inhabiting bacteria, including various *Xf* Wells pathotypes, is the cause of several economically important diseases including Pierce's disease (PD) of grape, phony peach disease, plum leaf scald, and periwinkle wilt. In addition, *O. nigricans* has the ability to vector citrus variegated chlorosis (CVC) *Xylella sp*. to citrus with a transmission rate of 20.3%, a much greater efficiency than most other species tested to date (Brlansky et al., 2002). These findings demonstrate the importance of *O. nigricans* as a vector species with significant implications for the sustainability of Florida's citrus industry. The importance of having genomic information generated from expressed sequence tag, EST, studies has been definitively demonstrated through such studies as *Drosophila*, Honey bee, and other organisms. Production of genomic information on *O. nigricans* which is now available (Hunter et al., NCBI) which was derived from single-pass sequencing of cDNA clones prepared from this sharpshooter and provides an invaluable resource for the identification of genes associated with the biology of the adult life stage. Annotation of the resultant dataset will also help in the identification of the gene/enzyme pathways involved in processes such as insect pathogen and insect-plant interactions. The availability of genomic data on *O. nigricans* provides the scientific community a foundation for future studies in functional genomics, and provides the genetic basis for tool development to advance creation of novel genomics-based management strategies for this and other leafhopper vectors of plant diseases. To that end, herein we report on full-length transcripts of 14 putative proteins, and the annotation of a dataset produced from *O. nigricans* and show their potential for quantification of global gene expression patterns, functional and comparative genomics studies.

OBJECTIVES
Use a genomics approach to advance our understanding of the genetic basis of sharpshooter leafhopper biology. The genetic products produced provide the needed information to conduct further functional genomic studies. The results from this study support development of emerging management strategies to reduce leafhoppers and the spread of Pierce's disease.

RESULTS
The assembled sequences were annotated using BLASTX, TBLASTX, and BLASTn analyses. Translated proteins were analyzed with BLASTP, and EXPASY. Sequences which had an E-value ≤-10 were considered as significant. Only one table is shown, Table 1, which list the significant matches to terms related to 'Biological Processes.' The putative full – length sequences which have been annotated and published in the NCBI database are shown in Table 2. The two genes highlighted in yellow, (Table 2) have been mined from the dataset, and used to characterize genes with important functions in leafhopper biology, IDGF and Delta-9 desaturase (Figure 1, and Table 3).

Imaginal Disc Growth Factor, IDGF, protein structure. Several families of peptide growth factors are implicated in regulating cell growth and proliferation of cells in culture. Genetic studies in *Drosophila* implicate some of these factors in growth control *in vivo* and report a new family of growth factors, related to chitinase enzymes, required by *Drosophila* imaginal disc cells in culture. The importance of IDGF in insect development, and in the correct formation of wings makes them an ideal target for disruption, wherein adult insects would be unable to fly, thus reducing their ability to disperse. As research on IDGFs and their interactions continues to advance, so will our understanding of insect development at the cellular level. New IDGFs are being identified but there are still only a small handful known (Huang et al., 2006; Kawamura et al., 1999). During larval stages, the cells of the imaginal disc primordia undergo extensive growth and proliferation, increasing in number by three orders of magnitude.(Bryant 1978). The rapid proliferation is accompanied by patterning events that control the organization of cells in the growing disc. Although the application of genomics approaches in recent years has produced significant advances in identifying the molecules and mechanisms involved in patterning, and the role of the imaginal discs, there is still much to understand about how cell proliferation is regulated, and the cell-to-cell signaling in the development of specific structures, such as wings.

Expression of Δ9 Desaturase in Sharpshooters- The importance of producing and comparing multiple species of sharpshooters is demonstrated in the annotation of two cDNA libraries from *Oncometopia nigricans* and the glassy-winged sharpshooters (GWSS), *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Comparisons lead to the isolation and description of a *delta 9 desaturase* transcript from each leafhopper. The desaturase gene expression data is shown for the GWSS leafhopper, covering the five developmental nymphal stages which feed on a variety of host plants. Sharpshooter leafhoppers are economically important agricultural pests due to their ability to transmit *Xf*, and other plant pathogens during feeding. Currently very little is known of leafhopper developmental physiology. Since desaturases play a key role in insect development and nutrition we chose to examine the expression of *Δ9 desaturase* in the GWSS.

CONCLUSIONS
Data mining of the genomic data produced from EST examinations provides rapid, cost effective insight into an organism's biology, pathology and development which would be difficult with any other methods. The genomic data set for *O. nigricans* has already produced valuable information on an important vector of plant diseases. This data also provides the first experimental access to these genes and builds the foundation for more in-depth molecular and functional genomic analysis by the research community. Moreover, it identifies genes that are critical in the physiology, reproduction, development, of leafhoppers. Genetic information is crucial to advancing our understanding of sharpshooter biology, and will play a major role in the development of future non-chemical, gene-based control strategies against leafhopper pests.

Table 1. Biological Process of ESTs from *O. nigricans* ESTs

Gene Ontology Term[a]	# ESTs	% of total ESTs represented[b]	# contigs	# singlets
[p] Behavior	8	0.41%	2	3
[p] Cellular Process				
[c] Cell communication				
[i] Cell adhesion	15	0.78%	2	9
[i] Cell-cell signaling	29	1.50%	2	2
[i] Signal transduction	60	3.10%	7	17
[c] Cell differentiation				
[i] Sporulation	1	0.05%	0	1
[c] Cellular physiological process				
[i] Cell death	3	0.16%	0	3
[i] Cell growth and/or maintenance				
[ii] Cell homeostasis	24	1.24%	2	1
[ii] Cell organization and biogenesis				
[iii] Cytoplasm organization and biogenesis	50	2.59%	6	13
[ii] Cell proliferation	29	1.50%	2	13
[ii] Transport	8	0.41%	2	3
[ii] General (no further information provided)	12	0.62%	1	2
[i] Cell motility	20	1.03%	2	6
[p] Development				
[c] Cell differentiation	124	6.41%	2	4
[c] Embryonic development	5	0.26%	1	3
[c] Growth	2	0.10%	0	2
[c] Larval or pupal development (sensu Insecta)	4	0.21%	1	2
[c] Mesoderm development	29	1.50%	6	4
[c] Organ development				
[i] Organogenesis				
[ii] Heart development	1	0.05%	0	1
[ii] Hemopoiesis	1	0.05%	0	1
[ii] Muscle development	12	0.62%	1	1
[ii] Neurogenesis	31	1.60%	5	4
[c] Pattern specification	1	0.05%	0	1
[c] Reproduction	2	0.10%	0	2
[c] General (no further information provided)	2	0.10%	1	0
[p] Physiological process				
[c] Coagulation	1	0.05%	0	1
[c] Death	43	2.22%	8	10
[c] Homeostasis	22	1.14%	4	3
[c] Localization	106	5.48%	11	21
[c] Metabolism				
[i] Alcohol metabolism	1	0.05%	0	1
[i] Amine metabolism	5	0.26%	1	0
[i] Amino acid and derivative metabolism	33	1.71%	5	15
[i] Biosynthesis				
[ii] Cuticle biosynthesis	2	0.10%	0	2
[ii] Nucleotide-sugar biosynthesis	1	0.05%	0	1
[i] Catabolism				
[ii] Macromolecule catabolism				
[iii] Protein catabolism	57	2.95%	9	30
[i] Cofactor metabolism	13	0.67%	3	4
[i] Electron transport	119	6.16%	6	5
[i] Heterocyte metabolism	1	0.05%	0	1
[i] Hormone metabolism	1	0.05%	0	1
[i] Lipid metabolism	72	3.72%	8	25
[i] Macromolecule metabolism				
[ii] Carbohydrate metabolism	172	8.90%	18	26
[ii] Protein metabolism				
[iii] Protein biosynthesis	232	12.00%	49	37
[iii] Protein complex assembly	1	0.05%	0	1

Table 1 (cont). Biological Process of ESTs from O. nigricans ESTs

Gene Ontology Terma	# ESTs	% of total ESTs representedb	# contigs	# singlets
[iii] Protein folding	13	0.67%	2	9
[iii] Protein modification	5	0.26%	0	5
[iii] General (no further information provided)	5	0.26%	0	5
[i] Neurotransmitter metabolism	1	0.05%	0	1
[i] Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	109	5.64%	11	38
[i] Organic acid metabolism	3	0.16%	0	3
[i] Phosphorous metabolism	282	14.59%	11	15
[i] Sulfur metabolism	1	0.05%	0	1
[i] General (no further information provided)	1	0.05%	0	1
[c] Muscle contraction	78	4.04%	6	5
[c] Organismal physiological process				
[i] Organismal movement	6	0.31%	2	2
[c] Response to stimulus				
[i] Response to biotic stimulus	67	3.47%	12	21
[i] Response to endogenous stimulus	1	0.05%	0	1
[i] Response to external stimulus	1	0.05%	0	1
[i] Response to stress	3	0.16%	0	3
[p] Regulation of biological process	2	0.10%	0	2
Totals	1933	100.00%	211	395

[a]Classification hierarchial: indented terms are children [c] of parent terms [p] listed above. All functional assignments of *Oncometopia nigricans* ESTs described here are the "inferred from electronic annotation" (IEA) using the top 5 BLASTX hits with an E-value of ≤-10

[b]% of total ESTs was calculated using only ESTs with a BLASTX hit at an *E*-value of ≤-10 and of known protein function.

Unknown Biological Process	481	10.90%	48	217
NSS	1307	29.63%	115	659
Virus	1	0.02%	0	1
Mt	1	0.02%	0	1
RRNA	686	15.55%	6	2
	4411	56.18%	381	1275

Table 2. Putative full-length protein sequences produced from *Oncometopia nigricans*, in silico characterization. Sequences published in the NCBI, public database. (Hunter et al., in prep).

Complete Putative Protein Sequences for WHON

Contig	NCBI Descriptor
[0010]	**IDGF-like protein**
[0015]	Actin, Muscle
[0017]	Actin, Cytoplasmic
[0018]	ADP/ATP translocase
[0021]	Rhodopsin
[0023]	**Delta-9 desaturase**
[0095]	Elongation factor 1-alpha (Posted)
[0124]	Ferritin GF2
[0158]	Fructose 1,6-bisphosphate aldolase
[0200]	Arginine kinase
[0409]	Glyceraldehyde 3-phosphate dehydrogenase
[0926]	Enolase
[1317]	CG7610
[1442]	RAB7

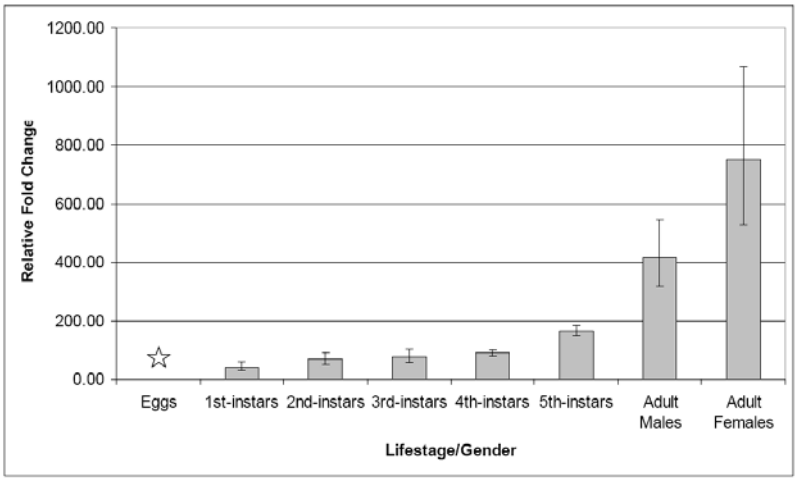

Figure 1. The isolated Δ9 desaturase cDNA from *O. nigricans* and GWSS. The cDNA encodes a 367 (aa) with 71% identity to *A. domesticus* desaturase, and significant (>50%) identity with other insect Δ9 desaturases. *In silico* analyses place GWSS Δ9 desaturase in the Family 1 of ProDomain fatty acid desaturases a Palmitoyl-CoA Δ9 desaturase-1. No detection of expression in eggs. All other instars expressed Δ9 desaturase at increasing levels for each instar. There was no significant difference in expression of Δ9 desaturase among nymphs, adults expressed significantly greater levels. Adult females showed a >7 fold increase over 1st-4th instars. There was no significant difference between adult sexes. Sequences published Acc. no. AAT01079.

Table 3. Sequences producing significant alignments:

			(Bits)	Value
gb|AAU95195.1|	delta-9 desaturase	*[Oncometopia nigrican*	744	0.0
gb|AAT01079.1|	delta-9 desaturase 1	*[Homalodisca vitripennis*	723	0.0
gb|AAK25796.1|AF338465_1	delta-9 desat.1	*[Acheta domesticus*	536	8e-151
gb|ABD72703.1|	fatty acid desaturase	*[Acyrthosiphon pisum*	518	2e-145

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GENOMIC SEQUENCING, DISCOVERY, AND CHARACTERIZATION OF VIRAL PATHOGENS IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
A new viral pathogen of the glassy-winged sharpshooter, GWSS, (*Homalodisca vitripennis*, aka *H. coagulata*) was discovered and characterized. Few pathogens of leafhoppers have been discovered which have potential for use as a biological control agent. To identify new pathogens of GWSS we used a genomic approach to isolate, sequence, and identify expressed sequence tags, ESTs, produced from field collected GWSS populations. Viral sequences were identified out of the initial 9,620 ESTs generated from single-pass 5' end sequencing of the GWSS expression library. Processing produced 8,795 ESTs which had lengths greater than 100 nucleotides post quality and vector trimming. The ESTs had an average read length of 689 bp, and an average inset size of 899 bp. Sequences shorter than 200 contiguous bases were removed from analyses. After assembly there were 3,008 sequences, 799 contigs with an average length of 1,113 bp, and 2,209 singlets. Using these sequences to get a start, it was possible to completely sequence the full virus genome, and the virus was labeled, HoCV-1. Further analyses and characterization of HoCV-1 demonstrated that it infected and crossed the midgut barrier of GWSS. The virus was classified as a member of the family *Dicistroviridae*, which are single-stranded RNA viruses which do not have a DNA stage. Two other viruses were also identified which are currently being characterized and which were taxonomically unrelated to HoCV-1. These leafhopper viral pathogens appear to induce increased mortality, 40% or more, during the nymphal stages of leafhopper development and may have further applications in the management of leafhopper pests to reduce the spread of Pierce's disease of grapes.

INTRODUCTION
Leafhoppers are the second most serious agricultural pest, after aphids, both of which transmit plant diseases. Few leafhopper pathogens are known and efforts to discover pathogens can be costly. Where it occurs the glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* Germar 1821 (Hemiptera: Cicadellidae) (Takiya et al., 2006) is the primary vector in the spread of Pierce's disease, PD, of grapes. Pierce's disease is caused by strains of the plant-infecting, *Xylella* bacteria, which cause severe economic losses to viticulture and other tree crops in the USA. The GWSS readily flies long distances, thus spreading PD throughout and across grapes growing regions. To maximize our efforts we chose an approach which would advance our understanding of the genetic basis of leafhopper biology, while optimizing efforts to discover leafhopper pathogens. Therefore, we chose to create a large-scale 5′ end sequencing project of cDNA clones produced from adult GWSS. The use of expressed sequence tags (EST) have proven to be a rapid method to generate important genomic information which permits researchers to address difficult questions concerning insect biology, pathology and disease transmission.

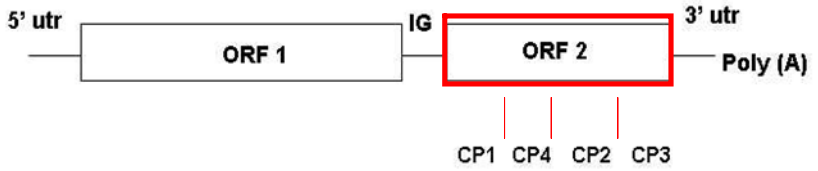
OBJECTIVES
Search for viral pathogens of sharpshooters using the molecular approach of cDNA libraries which provide a rapid, cost effective method that advances our understanding of an organism, plus identifies the invisible, unknown, internal/external organisms associated with the target species, the GWSS, or other leafhopper. Viral pathogens are tools for leafhopper management and open new avenues to reduce PD.

RESULTS & DISCUSSION
GWSS Genomics: Adult GWSS were collected from a citrus grove near Riverside, CA. Of the initial 9,620 ESTs generated from single-pass 5' end sequencing of the GWSS expression library, 8,795 ESTs had lengths greater than 100 nucleotides post quality and vector trimming. The ESTs had an average read length of 689 bp, and an average inset size of 899 bp. Approximately 500 of these sequences were identified as having significant homology to a virus. After assembly of the dataset there were 3,008 sequences, 799 contigs with an average length of 1,113 bp, and 2,209 singlets, average length of 681 bp. Of the total assembled 3,008 sequences, 1,574 or 52.3% corresponded with putative matches in GenBank at an E-value of <-10, while 1,434 sequences, or 47.7%, had 'no significant homology' to sequences currently listed in GenBank,nr database by *in silico* analyses (BLASTX, TBLASTX, BLASTN). The sequence data described in this paper have been submitted GenBank Accession Numbers CF194966 through CF195393. National Center for Biotechnology Information, NCBI. The capsid protein sequence of HoCV-1 was submitted into GenBank (accession number: DQ308403). The genomic architecture was determined (Figure 1) and phylogenetic analyses performed as in Hunnicutt et al., 2006, (Figure 2), (Table 1).

Genomic characterization of the virus: demonstrated that HoCV-1 was a close member of the Family: *Dicistroviridae*, which are single-stranded, RNA viruses, with no DNA stage. The complete nucleotide sequence of HoCV-1 infecting GWSS was determined and further *in silico* analysis revealed a genome containing 9,321-polyadenylated nucleotides encoding two large open reading frames (ORF1 and ORF2) separated by a 182-nt intergenic region (IGR) (Figure 1). The deduced amino acid sequence of the 5′-proximal ORF (ORF1, nt 420–5807) exhibited conserved core motifs characteristic of the helicases, cysteine proteases, and RNA-dependent RNA polymerases of other insect-infecting picorna-like viruses. These viruses are often reported to be in association with increased mortality of their infected host, as with fire ants, and honey bees. Virus analysis and detection from salivary gland (Sg) and midgut (Mg) tissues of *Homalodisca vitripennis* adults tested for presence of HoCV-1, using rtPCR. Both types of tissues from individual insects were dissected and analyzed in a pairwise fashion. Only midgut tissues were shown to test positively for virus presence. Tissues were then prepared for examination by transmission electron microscopy (Figure 3.). Virions were observed to be present in high numbers within midgut tissues of GWSS which tested positive for HoCV-1, and absent when GWSS tested negatively. The virions appeared to be taken up by the microvilli of the midgut and to propagate within the basal laminae (Fig 3).

Geographic Distribution: A host range for HoCV-1 was conducted. Adult leafhoppers were collected over a two year period throughout various geographic locations, including Florida, Georgia, South Carolina, North Carolina, California, and Hawaii. Other leafhopper species were also evaluated. The presence of HoCV-1 was detected in both sexes and all developmental stages of GWSS, including eggs (Hunnicutt et al., 2007). Although no mode(s) of transmission could be conclusively accepted/rejected, these results suggest that infection may be maintained through both transovarial and transstadial transmission. In Florida viruliferous samples were detected only in Gadsden and Suwannee counties, two of the three localities in which GWSS were most abundant. This account is similar with a distribution study conducted by Hoddle et al. (2003) which found that significantly more GWSS inhabit north Florida than central and south Florida. Infected GWSS were found in Georgia, South Carolina, and North Carolina with the incidence of infection ranging from 8–100% (Hunnicutt et al., 2007). Conversely, our assay failed to detect *HoCV-1* in any of the GWSS collected from the island of Oahu, HI. Virus infection was distributed among populations regardless of the host plant from which the insect was harvested. Adults of two additional sharpshooter vector species, *H. insolita* and *O. nigricans*, collected in north Florida were also demonstrated to be natural hosts for *HoCV-1* However, neither *D. minerva* nor *G. atropunctata* tested positive for the virus. These findings suggest that while *HoCV-1* is not limited to *H. vitripennis*, infection was not ubiquitous to all sharpshooter genera evaluated.

Figure 1. Genomic organization of HoCV-1, Capsid proteins are encoded at the 3′ end. Organization follows that of other Dicistroviruses. The complete nucleotide sequence of HoCV-1 infecting GWSS *in silico* analysis revealed a genome containing 9,321-polyadenylated nucleotides encoding two large open reading frames (ORF1 and ORF2) separated by a 182-nt intergenic region (IG).



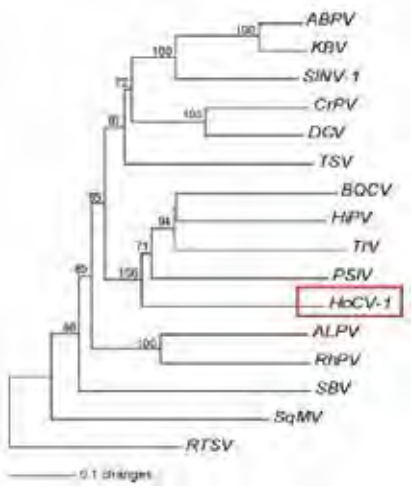


Figure 2. Phylogenetic analysis of HoCV-1 and other positive-sense ssRNA viruses based on amino acid sequence of the putative RNA-dependent RNA polymerase (RdRp). Neighbor-joining were produced using PAUP 4.0b software, 1000 bootstrap replicates. Outgroup was Sacbrood virus (SBV).

Table 1. Comparison of amino acid sequence of *Homalodisca vitripennis virus*, HoCV-1, capsid polyprotein to other viruses in *Dicistroviridae*. Percent identity and similarity from BLASTX.

Capsid Polyprotein		Identity %	Similarity %
HoCV-1	**905 aa**	**100**	**100***
PSIV	874 aa	25	44
TrV	915 aa	27	44
HiPV	889 aa	26	44
BQCV	885 aa	27	44
DCV	937 aa	24	40
CrPV	926 aa	23	39
ALPV	888 aa	26	40
RhPV	893 aa	26	41
ABPV	968 aa	22	38

Drosophila C virus (DCV) (Johnson and Christian 1998), *Cricket Paralysis virus* (CrPV) (Koonin and Gorbalenya 1992), *Aphid lethal paralysis virus* (ALPV) (Munster et al., 2002), *Rhopalosiphum pisum* virus (RhPV) (Moon et al., 1998), *Triatoma virus* (TrV) (Czibener et al., 2000), *Plautia stali intestine virus* (PSIV) (Sasaki et al., 1998), *Himetobi P virus* (HiPV) (Nakashima et al., 1999), *Black Queen Cell virus* (BQCV) (Leat et al., 2000), *Acute bee paralysis virus* (ABPV) (Govan et al., 2000). (From Hunter et al., 2006).

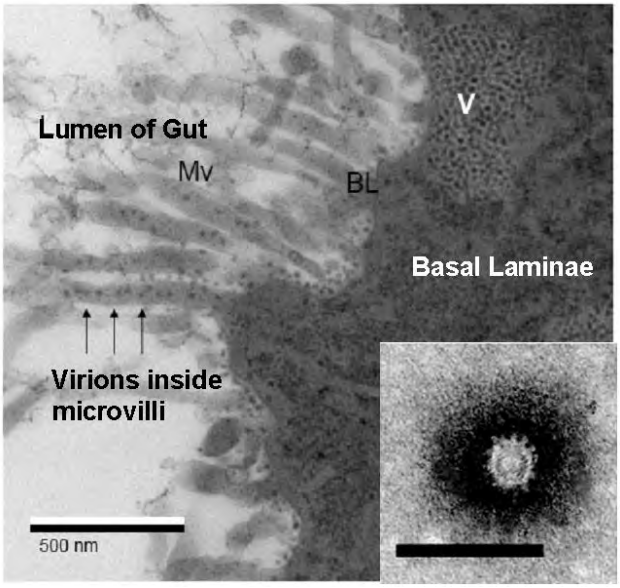


Figure 3. Electron micrograph of a single virion prepared from infected adult leafhoppers, *Homalodisca vitripennis*, glassy-winged sharpshooter. Scale bar = 100 nm.

CONCLUSIONS

The production of cDNA libraries provides rapid and cost effective methods that advance our understanding of an organism and the interactions of the invisible internal/external organisms associated with the target species, the GWSS. We have been using this method for the last four years to discover leafhopper pathogens, and to gather insights into the genetic basis of leafhopper biology, pathogen interactions. The first leafhopper virus characterized, HoCV-1, has demonstrated that it may have use to decrease GWSS population numbers. Insect viruses can cause indirect mortality by making the infected insects more susceptible to insecticide applications, and/or to parasitization and predation by reducing the activity of the leafhopper. Two other new leafhopper viruses which we have been discovered are currently being further characterized to determine if they too have application as biological control agents, and/or as gene delivery tools, to be used in the management of GWSS and other leafhopper pests, aimed as reducing the spread of Pierce's disease of grapes as well as other *Xylella* caused plant diseases.

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MICROARRAY ANALYSIS OF GENE EXPRESSION AND DIAPAUSE IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The condition of diapause in the glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis*, is poorly understood. Diapause is better known from other, non hemipteran insects. We used oligonucleotide microarrays to address the specificities of transcriptional responses of adult female GWSS, which were in 'diapause', to different lighting regimes. Two of these lighting regimes were known to induce oviposition in diapause females under greenhouse conditions during winter months. Thus we examined female GWSS gene expression during diapause and during the 'breaking' of diapause induced by light. Upon 'breaking' diapause, the females' ovaries became active, produced eggs and females oviposited similar to springtime conditions. The mRNA from 22 individual GWSS adult females was compared. Each individual was hybridized to a single chip. There were six individuals in the control group, and eight individuals in each treatment. Using strict criteria (a twofold change in expression), we determined that a definable number of genes was differentially expressed between the diapause females within the three lighting regimes. Of the 2,126 genes surveyed, five genes showed an increase in expression and two showed a decrease in expression (at least a 2.2-fold change) when comparing the control adult female GWSS to the GWSS exposed to the light treatments. Identification of the genetic basis of diapause will provide genetic targets which may be subjected to 'silencing' or 'down-regulation' by emerging technologies in plant improvement, or through virus delivery, or endophytic bacterial expression systems.

INTRODUCTION
Little is known about the genetic basis of diapause in the GWSS. As winter season approaches the GWSS becomes physiologically suppressed. The ovaries shrink and appear to become non active. These females, which survive the winter months, will emerge in the spring and become active again, thus ovipositing eggs in the early spring which become the next generation. Attempts to mass rear GWSS is difficult and often halts when winter arrived due to the lack of eggs. Some females were noticed to continue to oviposit when under a different light source. Having a condition which manifests a biological response provided a unique opportunity to examine the genetic basis of the effect of light wavelength on the induction of GWSS oviposition.

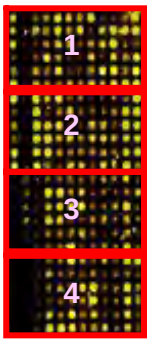
OBJECTIVES
Increase understanding of the genetic basis of diapause in sharpshooters, GWSS. Use of a genomics approach permits examination of leafhopper diapause at the genetic level. Discoveries from these results will advance our understanding of the triggers and biological pathways related to diapause.

RESULTS
The principles of Oligonucleotide and cDNA microarray assay of gene expression: Five genes were identified which responded to Far-Red light (Figures 2, 3). A microarray is a set of short Expressed Sequence Tags (ESTs) made from a cDNA library of a set of known (or partially known) gene loci. In this case the 'Unigene' set used in the production of this array consisted of 2,126 selected genes, produced by Hunter (database NCBI). The data set was mined from four different cDNA libraries produced from 1) adults, 2) 5^{th} instars, 3) midguts, 4) salivary glands. The ESTs produced from previous work are used as a template to prepare smaller oligonucleotides (35 bases long) which are mass produced and then spotted onto a cover-slip-sized glass plate. The GWSS microarray was produced by Combimatrix, Inc., (microarray slide format: 1 X 12,000 features) and was laid out in a repeat of four fields, each with the same 2,126 features to account for within microarray variation (Figure 1). Twelve chips were used for this experiment to evaluate 22 individual GWSS. Four slides in each of three treatments which were stripped and rehybridized with independent GWSS samples to account for between chip differences. A total of 22 individual GWSS were processed and hybridized to the arrays, six in the control, eight in treatment I., eight in treatment II. The control was: Normal grow lights-Phillips fluorescents, two banks of four blacklight 75Watt; Treatment I: was two Sylvania GroLux, 400 Watt, and Treatment II: was one AgroSun Gold Universal 1,000 Watt. Each light was raised or lowered to provide approximately the same light intensity, as determined by a hand held light meter. The mRNA transcriptomes are prepared by extraction from the whole body of a GWSS. Complementary DNA (cDNA) reverse transcripts are prepared and labeled with two different fluorescent dyes. The experimental and control libraries are hybridized to the microarray. Dual-channel laser excitation excites the corresponding dye, which fluoresces proportional to the degree of hybridization that has occurred. Relative gene expression is measured as the ratio of the two fluorescences:

"up-regulation" relative to the control will be visualized as a red "pseudo-colour," "down-regulation" shows as green, and constitutive expression (1:1 versus control) as a neutral black. Intensity of color is proportional to the expression differential.

CONCLUSIONS

The use of Oligonucleotide or cDNA microarrays can measure *life-stage and tissue specific patterns* of gene expression across tens, hundreds, or thousands of genes at a time, thus providing a powerful genomic tool. Genomic approaches to the investigation of diapause in insects have vastly expanded our knowledge about GWSS physiology. Leafhopper genetic analysis will undoubtedly expand and we will make new discoveries involved in the diapause of GWSS. Gene profiling of different leafhopper species, or even across insects, will assist in determining the complex pathways that comprise the response that we call diapause.



Four printed fields each repeats the same 2,126 genetic sequences.

An Oligonucleotide that is 35 bases long is designed to each gene sequence. The oligo is then mass synthesized and spotted onto the chip.

Total RNA is isolated from one insect, and labeled for each chip Hybridization.

Within chip variation, and between chip variation is controlled for, with significance being 2.5 fold difference of Increase or decrease of detection.

Figure 1. Microarray chip layout design, 1x12K

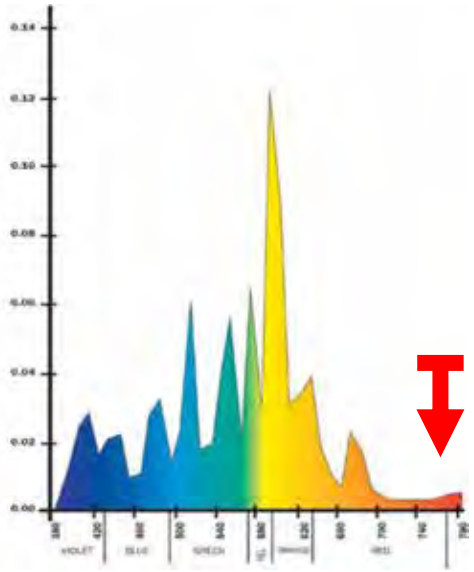


Figure 2. Wavelength spectrum chart from 1000 Watt bulb, Agrosun Gold Universal. Microarray analysis determined five genes were up regulated in response to stimulation to light in the Far Red spectrum (RED Arrow). GWSS which were in 'diapause' show a condition of reduced ovaries and no egg production. A light regime of two weeks, with an additional two weeks to start egg production produced females which were able to lay eggs and which had active, enlarged, ovaries.

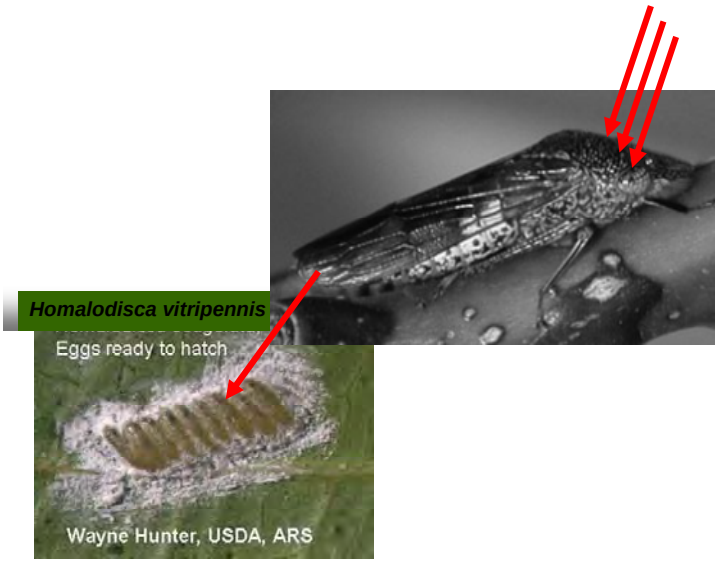


Figure 3. GWSS in diapause have reduced ovaries, and no egg production. In the spring the ovaries become revitalized and egg production starts again. Light and temperature affects the insect brain and physiology.

To identify the wavelength of light which may be involved in inducing the 'breaking' of diapause we evaluated gene expression in GWSS that were in diapause, after they were exposed to three different light sources which had different light spectra. We identified five genes which were significantly upregulated during GWSS exposure to far red light. These GWSS developed enlarged, active ovaries.

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PROTEIN IDENTITIES FROM BLUE-GREEN SHARPSHOOTER EXPRESSED SEQUENCE TAGS: EXPANDING LEAFHOPPER VECTOR BIOLOGY

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ABSTRACT
Although *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae) is the native 'blue-green sharpshooter,' BGSS, which has been a major vector of Pierce's disease in vineyards in California for nearly a century, only recently has any genomic information become available. Due to the importance of the BGSS as the principal native vector of Pierce's disease (Almeida et al., 2005), we chose to examine the biology of the BGSS using a genomics approach. A cDNA library was made from adult BGSS, and 8,160 expressed sequence tags, ESTs, were produced. After quality scoring 6,836 sequences underwent assembly which produced a set of 1,915 sequences that putatively represented distinct transcripts. Initial annotation of this dataset identified 44 putative protein sequences were characterized through *in silico* analyses, and published in the NCBI database (Accession numbers are listed in Table 1). BLASTX analysis identified 10 significant homology matches to heat shock proteins, HSP, which are the focus of this study due to their overall importance and functions in maintaining protein integrity and activity during stressful conditions, such as extreme heat, cold, drought or crowding. A putative full-length small heat shock protein was produced NCBI database accession DQ445538.1. Many other genes of interest which have various functions in leafhopper biology and physiology have also been identified but are not reported herein. The EST sequences reported in this study have been deposited in GenBank's dbEST under accession numbers EH655849-EH662328 and EH662332.

INTRODUCTION
Sharpshooter leafhoppers are vectors of a number of destructive plant diseases caused by the plant infecting bacterium, *Xylella fastidiosa*. The native leafhopper, *Graphocephala atropunctata* (Signoret) (Hemiptera) 'blue-green sharpshooter,' BGSS, is a major vector of Pierce's disease, PD, in vineyards in California and has been spreading PD for nearly a century. Unlike invasive glassy-winged sharpshooter (GWSS), the BGSS is smaller in size and prefers to feed in riparian habitats, thus keeping most infections in grapevine to the bordering plants. The crops grown in the San Joaquin Valley face extremely hot temperatures in the summers, often over 38°C, and freezing temperatures during the winter months which creates a highly stressful environment for sharpshooters and other insects. The importance of BGSS as the principal native vector of PD in grapes (Almeida et al., 2005), led us to examine the biology of BGSS using a genomics approach to determine how these sharpshooters are dealing with these harsh environmental conditions. The benefits gained from expressed sequence tag, EST, studies have been definitively demonstrated through many studies on insects (*Drosophila*, Honey bee, Aphids, Silk worm) and other organisms. Current production of genomic information on the BGSS is now available (Hunter et al., NCBI) which was derived from single-pass sequencing of cDNA clones. The identification of genes associated with leafhopper biology continues to expand as more ESTs are produced from different species. Annotation of these datasets advances current understanding of leafhopper biological pathways while providing clues to the genetic basis of such processes as insect-pathogen, and insect-plant interactions. The availability of genomic data on BGSS, which is one of three sets of genomic data on sharpshooters (Hunter, NCBI) provides a solid foundation for future studies in functional genomics to advance the creation of novel genomics-based management strategies for this and other leafhopper vectors of plant diseases. Herein we report on the production and annotation of 44 putative proteins from BGSS, and the annotation of heat shock proteins from BGSS.

OBJECTIVES
To produce, annotated, and identify genes critical to sharpshooter survival, such as heat shock proteins. Genomics advances our understanding of the genetic basis of leafhopper biology. The results build the foundation for functional genomic studies, aimed towards development of better leafhopper management strategies to reduce the spread and impact from Pierce's disease.

RESULTS & DISCUSSION
Adult *G. atropunctata* were obtained from a colony managed by Alexander Purcell at the University of California (Berkeley, CA). Founder BGSS were field-collected from mugwort (*Artemisia douglasiana* L) in Guerneville, CA (Sonoma Co.) and subsequently reared on sweet basil (*Ocimum basilicum* L.) at 25°C (+10°C/-5°C), 14 L: 10 D. First-generation progeny were macerated in RNA*later*® RNA Stabilization Reagent (Ambion, Austin, TX) and stored at -40ºC prior to shipment.

Sequence analysis - Base calling was performed using TraceTuner™ (Paracel, Pasadena, CA) and low-quality bases (quality score <20) were stripped from both ends of each EST. Quality trimming, vector trimming, and sequence fragment alignments were executed using Sequencher™ software (Gene Codes, Ann Arbor, MI). Sequencher contig assembly parameters were set using a minimum overlap of 50 bp and 90% identity. Contigs joined by vector sequence were flagged for possible miss-assembly and manually edited. Putative sequence identity was determined based on BLAST similarity searches using the NCBI BLAST server (www.ncbi.nlm.nih.gov) with comparisons made to both non-redundant nucleic acid and protein databases using BLASTN and BLASTX, respectively. Matches with an E-value ≤-10 were considered significant and were classified according to the Gene Ontology (GO) classification system. The 5′-single pass sequencing of a cDNA library derived from adult BGSS yielded 8,160 ESTs, of which 6,836 were designated as "high quality". Forty putative proteins identified from BGSS are listed in Table 1. Homologous matches to heat shock proteins, HSP 20, 40, 70, and 90, are shown in Table 2. A putative full-length protein was matched to a small heat shock protein Table 3. Protein sequence for the BGSS small heat shock protein, sHSP, to: Locust, Pink hibiscus mealybug, Honey bee, Parasitoid Nasonia, and the Mosquito showed most similar to Locus and mealybug.

Small heat shock protein - Small heat shock considered α-crystallin proteins, sHSP, are defined by a conserved sequence of approximately 90 amino acid residues, termed the α-crystallin domain (MacRae, 2000; Taylor & Benjamin, 2005). Functionally, most sHSP display *in vitro* chaperone-like activity, that is, the capacity to interact with other HSP to prevent aggregation and to keep proteins in a folded, competent state (Franck et al., 2004), they occur in all Kingdoms, but not in all organisms. Small HSP have been implicated in an astounding variety of processes, such as enhancing cellular stress resistance (Feder & Hofmann, 1999), regulating actin and intermediate filament dynamics (Wieske et al., 2001), inhibiting apoptosis, modulating membrane fluidity (Tsvetkova et al., 2002), and regulating vasorelaxation (Flynn et al., 2003). Amino acid sequence comparisons of the BGSS sHSP with other sHSP showed the common motif of the alpha-crystallin domain, NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast). The α-crystallin domain is a hallmark of the α-crystallin/small HSP superfamily. The putative α-crystallin domain was present at amino acid positions 64–146. The percentage identity among BGSS to other insect sHSP deduced amino acid sequences varied from 44% to 56%, with the highest similarity between *Locusta migratoria* HSP 20.7 and *Maconellicoccus hirsutus*, sHSP, (Table 3) and the lowest to *Rattus norvegicus* Alpha-crystallin A chain (not shown).

The occurrence of sharpshooters in high densities during summer months which may reach extremely hot temperatures as in CA and FL produce similar conditions of stress on sharpshooters. The insects must be able to prevent the crosslinking or deformation of proteins to maintain their function and life. Comparative genomics permits us to examine the full length cDNAs of HSP 20.5, 20.6, 20.7, 40, 70 and HSP 90 of the migratory locust which have been cloned and sequenced to make reasonable associations to similar proteins in the BGSS. The functions of HSP are well studied and further comparisons between the BGSS and other organisms provide key information for the examination and characterization of HSP in BGSS. We are using these findings in other insects, like locusts, to expand our understanding of the roles and pathways HSP play in BGSS survival.

CONCLUSIONS

The information gained from this study represents the first investigation regarding the transcriptome of *G. atropunctata*, BGSS. The resultant sequence data has produced valuable information on sharpshooter heat shock proteins, and identified many other physiologically important transcripts. The data has been made available to the public to facilitate the use of this information in further studies on sharpshooters. The important role of heat shock proteins to sustain protein integrity and other critical functions make them suitable for further examination as potential critical genetic targets which may be altered to reduce leafhopper populations. Collectively, these genetic sequences provide the strong foundation needed for further functional genomics studies which will enable the development of more biorational management strategies to reduce losses from the diseases spread by this and other leafhopper pests.

Table 1. Proteins from *Graphocephala atropunctata*, the blue-green sharpshooter, 44 Putative Protein Sequences, Published 03-03-06, NCBI. http://www.ncbi.nlm.nih.gov/sites/entrez

Definitions			Clone Accesion Number	
file	WHGA0016	(similar to CG2210).sqn:	WHGA0016	DQ445499
file	WHGA0091	(ribonuclease).sqn:	WHGA0091	DQ445500
file	WHGA0096	(cytochrome C oxidase polypeptide:	WHGA0096	DQ445501
file	WHGA0097	(s9e ribosomal protein).sqn:	WHGA0097	DQ445502
file	WHGA0105	(ubiquitin fusion protein).sqn:	WHGA0105	DQ445503
file	WHGA0114	(tropomyosin 1).sqn:	WHGA0114	DQ445504
file	WHGA0124	(thioredoxin-like protein).sqn:	WHGA0124	DQ445505
file	WHGA0140	(CSF signaling molecule).sqn:	WHGA0140	DQ445506
file	WHGA0151	(mitochondrial ATP synthase).sqn:	WHGA0151	DQ445507
file	WHGA0169	(cytochrome c reductase).sqn:	WHGA0169	DQ445508
file	WHGA0271	(LIM protein).sqn:	WHGA0271	DQ445509
file	WHGA0283	(tumor protein):	WHGA0283	DQ445510
file	WHGA0301	(oligomyocin sensitivity protein):	WHGA0301	DQ445511
file	WHGA0310	(cytochrome oxidase Va):	WHGA0310	DQ445512
file	WHGA0380	(ferritin):	WHGA0380	DQ445513
file	WHGA0381	(calmodulin):	WHGA0381	DQ445514
file	WHGA0392	(ADP-ATP translocase):	WHGA0392	DQ445515
file	WHGA0411	(NADH dehydrogenase 1 alpha):	WHGA0411	DQ445516
file	WHGA0412	(ribosomal protein L23):	WHGA0412	DQ445517
file	WHGA0430	(Histone3A):	WHGA0430	DQ445518
file	WHGA0449	(vacuolar ATPase subunit E):	WHGA0449	DQ445519
file	WHGA0585	(ribosomal protein 4e):	WHGA0585	DQ445520
file	WHGA0587	(ribosomal protein L37Ae):	WHGA0587	DQ445521
file	WHGA0762	(ribosomal protein S23e):	WHGA0762	DQ445522
file	WHGA0689	(elongation factor 1d):	WHGA0689	DQ445523
file	WHGA0199	(ribosomal protein 49).sqn:	WHGA0199	DQ445524
file	WHGA0225	(V-ATPase).sqn:	WHGA0225	DQ445525
file	WHGA0228	(NADH-ubiquinone reductase).sqn:	WHGA0228	DQ445526
file	WHGA0230	(cytochrome oxidase VIa).sqn:	WHGA0230	DQ445527
file	WHGA0257	(ribosomal protein L27Ae).sqn:	WHGA0257	DQ445528
file	WHGA0270	(mitochondrial ATP synthase).sqn:	WHGA0270	DQ445529
file	WHGA0783	(cytochrome oxidase Vb):	WHGA0783	DQ445530
file	WHGA0824	(cytochrome c):	WHGA0824	DQ445531
file	WHGA0900	(tropomyosin):	WHGA0900	DQ445532
file	WHGA0927	(PPIase):	WHGA0927	DQ445533
file	WHGA1072	(ribosomal protein L19e):	WHGA1072	DQ445534
file	WHGA1215	(GABA):	WHGA1215	DQ445535
file	WHGA1242	(mito. ATP synthase gamma):	WHGA1242	DQ445536
file	WHGA1340	(mito. porin):	WHGA1340	DQ445537
file	WHGA1462	(small heat shock protein):	WHGA1462	DQ445538
file	WHGA1611	(ribosomal protein L18A):	WHGA1611	DQ445539
file	WHGA2669	(mito. ATP synthase e):	WHGA2669	DQ445540
file	WHGA2689	(reductase complex QP-C):	WHGA2689	DQ445541
file	WHGA3412	(ribosomal protein S7e):	WHGA3412	DQ445542

Table 2. Heat shock protein homologs to transcripts in *Graphocephala atropunctata*, the blue-green sharpshooter, cDNA library. The full-length cDNA to a small heat shock protein, HSP 20.1, was sequenced and posted in NCBI database accession DQ445538.1. Partial sequences were identified homologous to HSP 40, HSP 70, and HSP 90.

Clone	Descriptor	E-value
Contig[1462]928 bp	gb|EAA04497.3| small HSP 20 *Anopheles gambiae*	8e-040
Contig[1012] 737 bp	gb|AAG42838.1| heat shock 70 kDa protein*Leptinotarsa decemlineata* (Colorado potato beetle)	6e-028
Contig[0130]1076 bp	ref|XP_623939.1| 90 kDa heat shock protein *Apis mellifera*	3e-084
Contig[1050] 741 bp	dbj|BAE44308.1| heat shock cognate protein 70 *Chilo suppressalis*	e-104
Contig[1901] 833 bp	gb|EAA08691.3| HSP cognate 70 *Anopheles gambiae*	6e-050
Contig[2381] 857 bp	gb|EAA03148.2| heat shock 40kD Anopheles gambiae	9e-022
WHGA057-76 560 bp	gb|AAL27404.1| 70 kDa heat shock protein *Artemia franciscana* brine shrimp	2e-045
WHGA051-87 578 bp	gb|AAO65964.1| heat shock protein 70 *Manduca sexta*	1e-054
WHGA079-33 749 bp	gb|AAO21473.1| hsp70 family member [*Locusta migratoria*]	2e-082
WHGA008-42 812 bp	dbj|BAD74196.1| heat shock protein hsp20.1 *Bombyx mori*	2e-037

Table 3. Alignment of conserved domain for Small Heat Shock Protein, Essential for life, from *Graphocephala atropunctata*, the blue-green sharpshooter. Conserved domain alignments were most similar to *Locusta migratoria* and *Maconellicoccus hirsutus*, sHSP (Expect = 5e-49), BLAST2, NCBI tools.

Similar alignment 182 aa	SCORE	P	ACCESSION	GI	PROTEIN DESCRIPTION
	Conserved Domain Database hits				
Locusta migratoria	569	27	ABC84492	85816366	HSP 20.5 **Expect = 5e-49**
Graphocephala atropunctata	500	18	AB D98776	90820038	small HSP
Maconellicoccus hirsutus	500	18	ABM55532	121543671	small HSP **Expect = 5e-49**
Apis mellifera	431	18	XP_001...	110750766	Protein lethal(2) essential for life (Protein Efl21)
Aedes aegypti	423	18	XP_001...	157135561	lethal(2)essential for life protein, l2efl
Aedes aegypti	423	18	XP_001...	157135559	lethal(2)essential for life protein, l2efl

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

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ABSTRACT

The purpose of this project is to define and quantify specific environmental constraints that influence the population dynamics and overwintering success of glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (formerly *coagulata*), and to field-test our laboratory findings. Using data collected in our laboratory tests and field studies, we developed a "cooling degree-day" (CDD) model to estimate the impacts of winter field temperatures on GWSS survival throughout California. Work on better understanding the feeding behaviors of GWSS at various temperatures is underway using an electrical penetration graph (EPG). Initial results from these studies indicate that GWSS adults may continue feeding during the two hours after the air temperature falls below 50ºF (= 10ºC) (i.e., our estimated feeding threshold), but eventually stop. Feeding activity data are currently being analyzed. Field studies on GWSS overwintering were initiated by releasing field-collected insects into the double-protected cages in secure areas in late November 2006 in the areas of Bakersfield (Kern County) and Riverside (Riverside County). Weekly monitoring was completed in February 2007. The caged GWSS adults at Bakersfield experienced 100% mortality in mid-January 2007 whereas 8.4% of GWSS at Riverside remained alive at that time. The number of surviving adults at Riverside in late February was equal to 0.4% of the initial cohort placed in the cages in early December 2006. Using the data from these field studies and those conducted by Don Luvisi, *Emeritus* Farm Advisor, during the winter season 2001-2002 in the Bakersfield area, we found that a non-linear model provided a good estimation of the impact of accumulated CDD on GWSS mortality. One hundred percent GWSS mortality was estimated when accumulated CDD reached 215 CDD.

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 earlier results from 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). We developed models 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 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 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

Sub-objectives on a) temperature dependent-survival of GWSS under different feeding conditions; b) survival under diurnal temperature cycles, and c) xylem excreta production bioassays have been completed with respect to data collection and analysis. A manuscript describing temperature dependent-survival of GWSS under different feeding conditions is being prepared for submission to *Environmental Entomology*. A second manuscript combing the results of the latter two sub-objectives will be produced and submitted for publication.

Variable temperature Electrical Penetration Graph recordings

Given difficulties in obtaining lab-reared GWSS individuals during the winter months, Electrical Penetration Graph (EPG) recordings were conducted using field-collected GWSS adults held within field cages. A GWSS adult was individually tethered to each Japanese euonymus plant used in the study (four replicates). To qualify and quantify real-time responses of GWSS feeding, environmental conditions (i.e., light intensity, temperature) were monitored using a data-logger (HOBO Pendant) to record climatic parameters. Recordings of EPG-generated waveforms were visually interpreted using the Windaq software program. Data analysis for the EPG experiment is in progress. A waveform (see Figure 1) may be categorized into pathway (p), ingestion (c), interruption (n), and baseline (z) (Joost et al. 2006). Preliminary results are provided below (Table 1, Figures 2B, C). Environmental conditions (i.e., light intensity and temperature) during the recording duration were presented in hourly means (Figure 2A). The waveform duration (mean ± SEM) of feeding activity (p and c waveform of four test insects) was also presented in hourly units (Figures 2B, C). During the EPG recording, temperatures ranged from 6.7 to 29.8ºC (mean SEM = 14.7 ± 0.4ºC) with an approximate photoperiod of 11:13 (L:D) hours. During the 24-hour period, GWSS adults spent 2.23 hours (133.8 min) feeding with substantial feeding occurring during the mid-night hours following initial set-up. Feeding intervals continued for two hours after the temperature fell below 10ºC. However, once stopped, feeding took about nine hours to resume as the temperature increased to greater than 10ºC. Following arsine data transformation [$X' = (X + 0.5)^{1/2}$], feeding activity (expressed as percentage of the observation time) was compared using a *t*-test for the period when temperature was above 10ºC versus below 10ºC. Overall, the GWSS adults spent less time feeding when temperatures were less than 10ºC, and three of the four test-insects did not feed during that time. GWSS spent approximately two-fold more time feeding when the temperature was above 10ºC, although the results were not significantly different. Waveform analysis should be completed by April 2008.

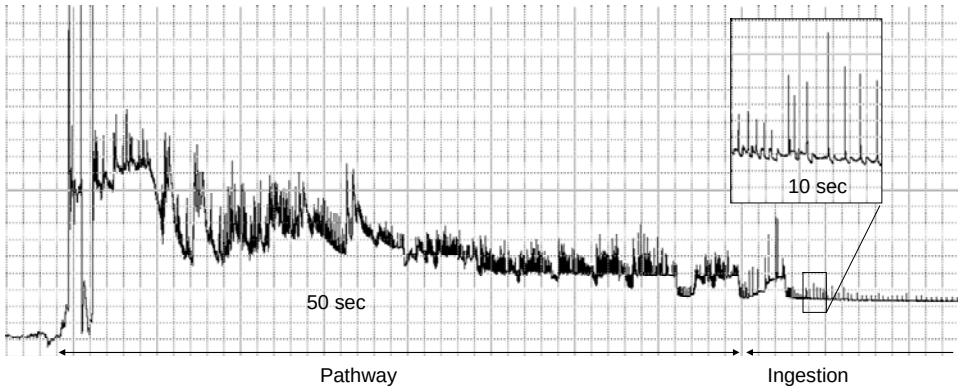

Figure 1. This chart represents a small interval of a 24 hr-recording of adult GWSS feeding at 30ºC. A typical EPG waveform is shown that represents two components of GWSS feeding activity: Pathway (p) and Ingestion (c). The larger output shows GWSS probing and continuous ingestion (p + c), and the enlarged portion of the output (enlarged box on right side of graph) shows the ingestion waveform (c) in finer scale.

Table 1. Duration (min and %, mean ± SEM) of feeding activity (Pathway = P; Ingestion = C) of GWSS in 24 hr-EPG monitoring under fluctuating temperature cycles.

Waveform	Waveform duration[a]	Temperature in daily cycle[b]		Total
		< 10ºC	> 10ºC	
P	Minutes	0.4 ± 0.4	12.1 ± 8.3	12.5 ± 8.6
	Percentage	0.1 ± 0.1	1.2 ± 0.8	0.9 ± 0.6
C	Minutes	20.2 ± 20.2	101.1 ± 77.1	121.3 ± 75.5
	Percentage	5.0 ± 5.0	9.8 ± 7.5	8.4 ± 5.2
C + P	Minutes	20.6 ± 20.6	113.2 ± 79.07	133.8 ± 79.4
	Percentage	5.1 ± 5.1	10.9 ± 7.6	9.3 ± 5.5

[a] Minutes indicate the waveform duration when temperature is above or below 10ºC and % indicates the percentage of waveform duration in a given time.
[b] As shown in Figure 2, temperatures below 10ºC occurred between 0040 and 0720 hours (405 min) and remained above 10ºC during the rest of time (1035 min).

Objective 2

Field study of GWSS overwintering survival

Survival of overwintering GWSS adults (using field-cages) and accompanying environmental conditions (using mini data-loggers) were monitored at two test sites in 2006-2007: Bakersfield (Kern County Extension Office at Mount Vernon Ave.) and Riverside (Agricultural Operations, UC Riverside). Test plants were maintained within fenced secured zones and were double-caged with yellow sticky traps and imidacloprid-treated citrus plants in the outer cages to reduce the chances of insects escaping. Field-collected GWSS adults were obtained from the CDFA Arvin Field Station. In late November 2006, 50 GWSS adults were released into each test cage, which enclosed potted grapevine and citrus plants. Weekly monitoring was conducted from December 2006 through February 2007. In each large cage, three smaller, test cages and one data-logger (HOBO Pendent Temperature/Light) were installed to record temperature data. The number of live adults per cage was monitored weekly. Insects were checked during the afternoon period (1300-1400), when the maximum daily temperatures (> 10ºC) permitted adults to feed on plants. Dead insects were usually found near the base of the plant or on the soil surface. There was a substantial reduction in the number of surviving adults at both sites during the first week after release. The high mortality appeared to result from stress induced during the preparation process (i.e., field-collection, aspiration, and anesthesia using CO_2, and transfer), and was similar to that commonly observed in field-collected colonies under laboratory conditions. Over the entire monitoring period, the number of surviving adults per cage was not significantly different between Riverside and Bakersfield (Repeated measures ANOVA, $P > 0.05$). However, all adults at the Bakersfield site died in mid-January 2007 whereas 8.4% of GWSS at Riverside still remained alive. In late February, the number of surviving adults in Riverside decreased to 0.4% of the original number. GWSS egg masses were observed in March 2007 in one Riverside cage.

Estimation of Cooling Degree-Days

Using the site-specific temperature data obtained from the data-loggers and monitoring data from two seasons (2001-2002 in Bakersfield conducted by Don Luvisi and 2006-2007 in Bakersfield and Riverside by this project), a cooling degree-day model was developed to describe the relationship between temperature condition and GWSS survival. The models provided good estimates of the percentage of GWSS overwintering mortality associated with accumulated CDD (Figure 3). The nonlinear model provided a better estimate than the linear model (r^2 of non-linear method > r^2 of linear method). The nonlinear model predicted that 90 and 100% of GWSS mortality would occur at 143 and 215 CDD, respectively. The linear model predicted that 90 and 100% of mortality would occur at 148 and 175 CDD, respectively. Given that the non-linear model does not account for ca. 24% of the variation, room for improvement exists. However, given that we only need to estimate those areas where 95 to 100% mortality occurs, the non-linear model may well serve our needs. Results from the non-linear model could potentially be mapped using GIS methods to provide a visual estimate of the impact of winter temperatures on GWSS survival.

CONCLUSIONS

Findings from our studies clearly indicate that survival and feeding activity of GWSS adults are significantly influenced by cool temperatures and exposure duration. In particular, low temperatures (< 10°C) caused rapid mortality. This project has generated significant new information regarding the thermo-biology of GWSS in California. Models generated from these data will permit spatial estimation of GWSS overwintering success to be expressed via GIS generated maps. These maps should be a valuable resource for individuals making decisions on where to apply pesticidal treatments to suppress spring populations of GWSS within California.

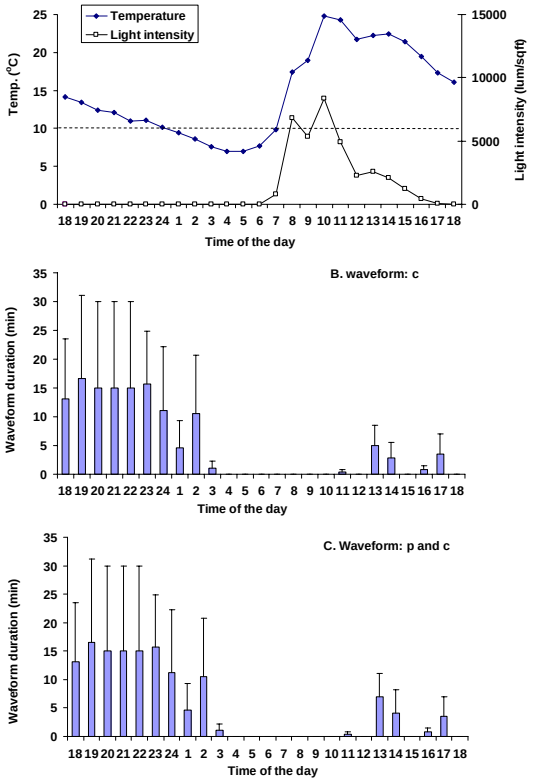

Figure 2. EPG waveform and environmental condition during 24-hr recording under outdoor condition: (A) hourly temperature (ºC) and light intensity (lum/ft^2), (B) duration in minutes of ingestion waveform (c) duration in hourly intervals, and (C) duration in minutes of feeding activity waveform (p and c) in hourly interval.

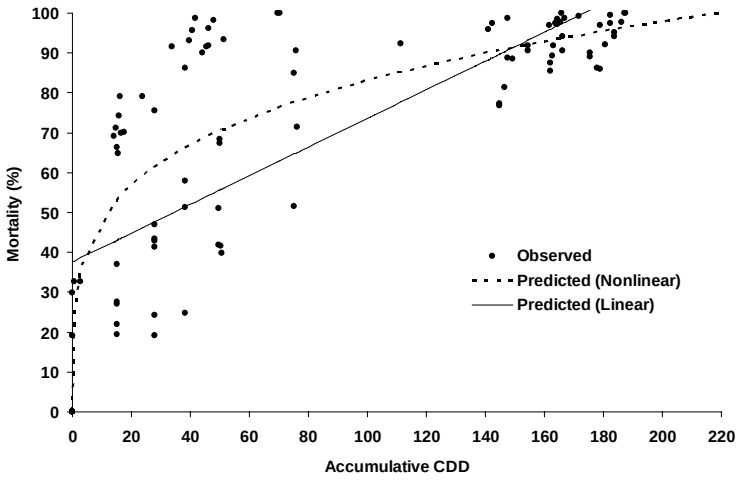

Figure 3. Linear (solid line) and non-linear (dotted line) regression models to predict the mortality (%) of GWSS adults based on cooling-degree days (CDD).

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ASSESSING THE POST-WINTER THREAT OF GLASSY-WINGED SHARPSHOOTER POPULATIONS

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ABSTRACT

After glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, arrived in California, it was believed that the insect would establish throughout much of the northern agricultural production areas. However, GWSS appears to be limited to discrete regions within the San Joaquin Valley where winter temperatures are mild and the temperature rarely drops below freezing. Prior research indicates that GWSS adults cannot feed at maximum daily temperatures below 50°F, thereby reducing its ability to survive cold winters. We plan to verify the impact of cool temperatures on GWSS adults by exposing them to a regime of seasonal temperatures (within temperature cabinets) that reflect some of the marginal areas where we expect GWSS to poorly survive California winters. The goal of the study would be to determine the ability of GWSS adults to survive winter conditions around the state by subjecting GWSS adults to low temperatures with daily temperatures below 10°C and nightly temperatures above 0°C. Using temperature records to calculate numbers of cooling degree days, we plan to construct maps to delineate areas where post-winter GWSS mortality should be substantial, thereby providing a tool to estimate the springtime GWSS threat to different regions. If successful, post-winter GWSS survival maps could be produced each spring (e.g., April) that would provide estimates of where GWSS populations should be absent or minimal because of winter conditions.

INTRODUCTION

The initial arrival of GWSS, *Homalodisca vitripennis*, into California's Orange and Ventura Counties was predicted to dramatically change Pierce's Disease (PD) epidemiology within infested areas (Varela et al. 2001). The insect soon spread into other southern California localities. PD devastated the wine grape industry in the Temecula Valley resulting in significant losses. First detected in Kern County in 1998, GWSS is now present in the San Joaquin Valley. However, the rapid population expansion first observed in southern California appears to be limited to discrete regions within the San Joaquin Valley coincident with citrus production areas where overwintering populations are highest and winter temperatures are relatively mild compared to locations elsewhere in the San Joaquin Valley. Additionally, persistent, localized GWSS populations are present within the urban areas of Fresno, Sacramento, and San Jose Counties where a range of perennial plant host types and slightly elevated daytime high and evening low temperatures might favor the survival and persistence of established populations.

Hoddle (2004) used the climate modeling program "CLIMEX" to estimate the potential worldwide distribution of GWSS. His reported estimates for California (when all localities received supplemental irrigation water) suggested that GWSS could establish reproducing populations along much of the California coast from San Diego north to the Eureka vicinity and within the Central Valley from Bakersfield north to the Redding vicinity. He did propose cold stress as a potential limitation to the establishment of GWSS in states north of California (i.e., Oregon, Washington). However, other observations and studies suggest that low winter temperatures may be the "bottleneck" that limits GWSS survival and distribution in the higher altitudes and northern regions of California. Pollard and Kaloostian (1961) observed that overwintering GWSS generally remained sessile at temperatures below 49°F (9.4°C) and that first flights occurred only after the ambient air temperature had reached or exceeded 52°F (~11.1°C). Russell Groves et al. (unpublished data from 2003) observed that seasonal patterns of GWSS capture on sticky traps within and among selected, perennial tree crops (e.g. navel, lemon, olive, avocado, sweet cherry, pomegranate, grape, peach, and plum) north of Porterville, CA, were surprisingly similar in the temperature requirements necessary for flights of adult insects. Specifically, regressions of logit-transformed, cumulative proportions of adults captured against estimated cumulative degree-day totals yielded peak correlation coefficients when a base flight temperature of 53°F (~11.1°C) was used in the development of the model. This value is very near to that estimated by

Pollard and Kaloostian (1961). CDFA-funded research showed that GWSS adults do not feed near or below 50°F (10.0°C), and that individuals will die if held below 50°F for long periods (e.g., 15 or more days) even in the presence of food and water (Johnson et al. 2006). Also of significant importance is the fact that the overwintering adult cohort is responsible for producing the offspring in the spring. Given this, if the daily maximum temperature infrequently surpasses the thermal activation threshold (50°F) necessary for GWSS ingestion, then GWSS survivorship may be curtailed by extended periods of cool temperatures in specific microclimatic regions of California. We have experimentally shown this phenomenon using programmable, temperature cabinets to simulate fluctuating diurnal temperature regimes based on January temperatures in the locations of Riverside (Riverside County.), Oakville (Napa County), and Buntingville (Lassen County), CA. In our study, the temperature for Riverside always exceeded 50°F (= 10°C), and about 20% of the test insects remained alive after 115 days; for Oakville it exceeded 50°F for 18 hours a day and only 10% of GWSS survived after 115 days; and for Buntingville the temperature never reached 50°F, and the entire test group died within 20 days (Youngsoo Son et al., unpublished data). We have applied the concept of cooling degree-days (CDD) to estimate the impact of cool temperatures on GWSS survival. The equation for CDD_{GWSS} may be expressed as:

$$\text{Daily } CDD_{GWSS} = \begin{bmatrix} |T_m - 50|, \text{if } T_m < 50\ ^{\circ}\text{F} \\ 0, otherwise \end{bmatrix}$$

where T_m = daily mean temperature in a given locality, $|T_m - 50|$ = absolute value of difference between T_m and the feeding threshold of 50°F when the mean daily temperature is lower than 50°F. Daily CDD_{GWSS} equals zero if the daily mean temperature (T_m) is higher than 50°F. By summing the CDD_{GWSS} for each day over an extended period, one can estimate the cumulative CDD_{GWSS} over the specified time period for that locality. Using unpublished field data collected by Don Luvisi, Farm Advisor *Emeritus*, in 2001-2002, we plotted the relationship between cumulative CDD_{GWSS} and GWSS survival at various sites in the vicinity of Bakersfield (Kern County). Based on a curvilinear regression, GWSS survival dropped to 10% or less when about 230 CDD_{GWSS} are accumulated. Further studies are needed to better refine the relationship between cumulative CDD_{GWSS} and GWSS survival.

Because most of our previous CDFA-funded work on the impact of cool temperatures on GWSS feeding and survival was conducted using constant temperatures, it was necessary to validate our findings under actual fluctuating temperatures in the field. Prior efforts to field-validate the impact of cool winter temperatures on caged GWSS adults in the crop production areas of the San Joaquin Valley (e.g., east and west of Bakersfield, central Fresno and Merced Counties) and farther north (Napa and Sonoma Counties) were prevented. This was due to concerns of growers and regulatory officials about potential escapes of GWSS individuals that might threaten nearby crops via *Xylella fastidiosa* (*Xf*) spread or the establishment of new GWSS populations. Fortunately, we were permitted to establish one field test comparing GWSS adults caged in the urban area of Bakersfield versus caged GWSS in Riverside (UCR Citrus Experiment Station). The GWSS individuals died in a shorter amount of time at the cooler Bakersfield site than the Riverside site. However, only one field test of our hypothesis using fluctuating temperatures is inadequate. Therefore, we proposed to test the impacts of fluctuating temperatures on GWSS survival using programmable temperature cabinets as we have done for the study mentioned above. These additional studies would provide insights into the benefits of using cumulative CDD_{GWSS} to estimate GWSS survival. We also plan to analyze historical temperature data for various locations within the agricultural production areas of California to determine if winter conditions (e.g. November to March) would permit significant GWSS survival based on CDD_{GWSS} accumulation. The eventual product that we aim to produce from these efforts will be the production of GIS maps that estimate CDD_{GWSS} accumulation over the winter months (i.e., November to March) to provide estimates of the ability of local GWSS populations to be a substantial threat to local agriculture in the following growing season (i.e., a risk assessment). As resources for GWSS management dwindle, government agencies will be forced to make decisions on which regions should receive area-wide treatment to suppress GWSS populations. Our studies suggest that the presence of the GWSS threat may vary with the severity of local winter temperatures (i.e., reduced GWSS densities correlated with cold winters). An annual estimation of overwintering GWSS survival across agricultural regions will provide insights into where resources for GWSS suppression should be most effectively allocated.

OBJECTIVES

1. Verify impacts of winter temperatures on GWSS survival from selected California sites;
2. Quantify and compare variation in "cooling degree day" accumulation within and among selected California sites using historical temperature data; and
3. Construct Geographical Information Systems (GIS) maps that estimate GWSS survival during the winter period

RESULTS

Objective 1

Verify impacts of winter temperatures on GWSS survival from selected California sites

Dr. Hannah Nadel was hired in mid-September 2007 to replace Dr. Youngsoo Son, who started a position with CDFA in early summer 2007. Given the departure of Dr. Son, the study research site was moved from the CDFA Arvin Field Station

in the San Joaquin Valley to the campus of the University of California at Riverside, where access to live GWSS would be easier and not a quarantine issue. Dr. Nadel has initiated activities to implement the experimental studies proposed under this objective. These studies will be conducted in temperature-controlled growth chambers on the UC Riverside campus. Studies will be conducted under laboratory conditions because using live GWSS in field-cage studies is prohibited outside of the GWSS-infested areas of California. Attempts to implement necessary field studies over the last two summers were impaired due to growers' concerns about potential escapes of field-caged GWSS individuals. Temperature cabinets are currently being procured for the studies. Cabinets will be programmed to run various fluctuating, diurnal temperature patterns that are representative of historical patterns from selected sites within California's agricultural regions. For ten CIMIS sites (e.g., Riverside, Santa Maria, Bakersfield, Porterville, Merced, Davis, Oakville, Chico, Gerber, and Glenburn), mean daily maximum and minimum temperatures will be calculated for the months of November, December, January, February, and March. Ten GWSS adults (five mated pairs) will be caged under a given temperature regime (e.g., Merced) for a five month period. In chronological order (November, December, January, February, and March), the temperature cabinets will be programmed to simulate the average maximum and minimum temperatures for the individual months (i.e., 30 days for November, 31 days for December, 31 days for January, etc.). GWSS adults are currently being field-collected for the laboratory studies. GWSS individuals will be provided with citrus and other host plants to feed upon. Numbers of live and dead individuals will be counted weekly until all insects die or the 5 month study period ends. Each temperature regime will be replicated 5 to 8 times. The cumulative CDD_{GWSS} will be calculated for each location regime (e.g., Merced) and percent survival compared among regimes using Repeated Measures ANOVA. The numbers of cumulative CDD_{GWSS} required to kill all GWSS individuals per cage will be compared across location regimes to determine if the value to kill all test insects remains fairly constant across different diurnal temperature patterns. Presently, we envision the actual laboratory experiments being started by 1 November 2007 as planned.

Objective 2

Quantify and compare variation in "cooling degree day" accumulation within and among selected California sites using historical temperature data

We are initiating activities on this objective. We have some of the necessary temperature data and will purchase additional data in the near future. Historical temperature data (last 10 years) will be used to quantify and compare variation in "cooling degree day" accumulation within and among selected California sites. For 20 CIMIS sites, the monthly accumulation of CDD_{GWSS} will be calculated for the individual months of November, December, January, February, and March for each winter season examined (e.g., winters of 1996-1997 through 2006-2007). We will statistically compare the sites and individual months to quantify the amount of variation in the accumulation of CDD_{GWSS} among sites and within sites. This exercise should provide insights into the amount of variation that occurs relative to probable survival of overwintering GWSS populations in various regions as a result of low temperatures. Based on our findings, it may be possible to reduce the number of regions in California that must be monitored for GWSS establishment. Certain northern or high altitude areas may consistently have temperatures so low that even annual temperature variation will not produce conditions under which significant numbers of GWSS individuals would survive the winter cold. Additionally, we should be able to compare our estimates of cumulative CDD_{GWSS} with historical CDFA records on GWSS sticky trap counts within specific areas. Dr. Mark Sisterson reports that CDFA will provide us access to their records on GWSS sticky trap counts within specific areas. We expect to find low numbers of GWSS trapped in those areas with high cumulative CDD_{GWSS} values.

Objective 3

Construct Geographical Information Systems (GIS) maps that estimate GWSS survival during the winter period

No progress to report at this time because data collection will not begin until November 2007. Using temperature data collected between the months of November 2007 to March 2008 by CIMIS and the Western Regional Climate Center (WRCC), we will estimate the accumulation of CDD_{GWWS} for about 340 temperature monitoring sites. We will then construct GIS maps of California that show: 1) the variation in cumulative CDD_{GWSS}; and 2) the estimated the risk of GWSS populations surviving the winter period. Spatial statistics techniques using ESRI ArcGIS® Geostatistical Analyst will be used to create interpolated surface maps using one of two analysis strategies: Inverse Distance Weighted or Krig surface generation. Risk will be expressed as a simple rating system such as: 0 = less than 0.1% possibility of the GWSS population surviving; 1 = possibility of between 0.1 and 1.0% of the GWSS population surviving; 2 = possibility of between 1.0 and 5.0% of the GWSS population surviving; and 4 = possibility of greater than 5% of the GWSS population surviving. For the time being, regions with ratings greater than 0 would probably require allocation of resources for GWSS suppression. However, standard GWSS monitoring should continue in all areas where GWSS populations are routinely found or might be expected to appear (e.g., areas along a transportation corridor, e.g., Hwy 65 in Tulare County). With an improved understanding of the climatological limits of GWSS overwintering survivorship, these risk estimates can 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.

CONCLUSIONS

This project has a high probability of success both in terms of generating significant new information regarding the impact of California winter temperatures on GWSS survival and also by providing a practical tool to use in the decision making process for GWSS management. Objectives outlined in this project address gaps in our present understanding that must be filled if we are to develop a comprehensive management plan to best manage GWSS over regional areas.

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FUNDING AGENCIES

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

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ABSTRACT

Host-plant water status is thought to influence dispersal of the glassy-winged sharpshooter (GWSS). Preference of adult GWSS for citrus plants maintained under different water deficit regimes was studied under laboratory and field conditions. In laboratory studies, settling and oviposition preference were studied on potted 'Washington navel' orange in cage choice tests, and feeding activity was estimated via insect excreta production. A field study was conducted in a citrus orchard ('Valencia') to determine the influence of plant water stress on population dynamics of GWSS. Experimental treatments in this study included irrigation at 100% of the crop evapotranspiration (ET_c) and continuous deficit-irrigation (CDI) regimes at 80% and 60% ET_c. Plant conditions were monitored by measurements of leaf surface temperature, water potential, and fruit quality and yield. GWSS population density and activity were monitored weekly by visual inspections, beat net sampling, and trapping. In laboratory tests, insects settled, oviposited, and fed significantly more on surplus-irrigated plants than on plants under moderate CDI. Plants under gradual deficit irrigation became less preferred after 7 d. Citrus water consumption at this point declined to 40% of the control and xylem-fluid tension was estimated at 9.3 Bar. GWSS populations in the field study were negatively affected by severe plant water stress; however, population density was not linearly related to decreasing water availability in plants. Citrus trees irrigated at 60% ET_c had significantly warmer leaves, higher xylem matrix potential, and consequently hosted smaller numbers of GWSS eggs, nymphs, and adults than trees irrigated at 80% ET_c. Citrus trees irrigated at 100% ET_c hosted the same number of insects as trees irrigated at 60% and 80% ET_c. Although the adult GWSS population was reduced, on average, by 35% in trees under severe water stress, the total number of fruit and number of fruit across several fruit grade categories were significantly lower in the 60% ET_c than in the 80% and 100% ET_c irrigation treatments.

INTRODUCTION

The GWSS is a highly polyphagous leafhopper with over 100 known hosts (Turner and Pollard 1959). Citrus is the most common overwintering and first generation reproductive host found in southern California (Blua et al. 1999) and plays an important epidemiological role in Pierce's disease incidence in adjacent vineyards (Perring et al. 2001) because it influences the spatial distribution of GWSS populations (Park et al. 2006). Therefore, with over 109,384 ha of citrus distributed throughout the state and nearly 13.1% of these hectares (14,356 ha) treated with imidacloprid in 2006 alone (CDFA 2006), integrated management tactics that are considered more ecologically sustainable and have less overall reliance on area-wide insecticide applications are warranted.

During the last 40 years, a considerable volume of information has been generated to characterize the impact of plant water stress on insect outbreaks and regulation of insect population dynamics. In general, resulting responses often appear to be insect feeding-guild dependent (Larsson 1989). In a recent analysis, which included results from 116 published studies, Huberty and Denno (2004) found strong negative effects of water stress on phloem-, xylem-, and mesophyll-feeders. Among the selected studies, only one study investigated the effect of plant water stress on the performance of a xylem feeder. Andersen et al. (1992) found that rates of GWSS adult feeding on water-stressed crape myrtle plants, *Lagerstroemia indica* L., were observed to decrease exponentially with increases in water stress and GWSS feeding ceased above a xylem tension of about 2.1 MPa. In further laboratory studies, the feeding rate and survival of the xylem-feeding sharpshooter *Oncometopia facialis* (Signoret), one of the vectors of *Xylella fastidiosa* (*Xf*) in citrus in Brazil (Krugner et al. 2000), were significantly reduced in sweet orange seedlings (cv. 'Caipira'), that were maintained under continuous water deficit irrigation

(Pereira et al. 2005). While the effect of plant water stress appears to be deleterious to xylem feeding sharpshooters, deficit irrigation regimes applied during less vulnerable phenological stages of citrus fruit development have caused little to no impact, and in some instances, increased gross yields, fruit loads, and fruit quality (Goldhamer and Salinas 2000).

Although significant new information is becoming available regarding the host selection behavior of xylem feeding cicadellids, little is understood regarding the effect of plant water stress on GWSS host selection behavior and population dynamics, which is critical to improving our understanding of vector ecology. The goal of this research was to generate novel information useful in the development of sustainable management strategies for control of GWSS populations, which might limit the spread of *Xf* into susceptible crops.

OBJECTIVES

1. To evaluate host-plant factors utilized by adult GWSS and associated natural enemies as long-range cues to locate feeding and ovipositional hosts in a complex agricultural landscape.
2. Investigate the effects of continuous deficit irrigation regimes in citrus trees on the population dynamics of GWSS and associated natural enemies.

Objective 1

Olfactometer

An olfactometer system for studying the response of GWSS to host-plant volatiles was built and the airflow dynamics adjusted to equally integrate odor fields from humidity or volatile sources. Bioassays were performed using adult GWSS collected from Bakersfield and Fillmore, CA. However, data analysis showed no conclusive differences among a variety of treatments, suggesting that GWSS may not use olfactory cues during host location. Responses of female *Gonatocerus ashmeadi* to humid air and to potential volatile cues in the excreta of gravid female GWSS were tested in a Y-tube olfactometer, but preliminary analysis indicates no detectable response to these stimuli.

Laboratory choice bioassays

Field-collected adult GWSS were caged with a choice of two potted sweet orange (cv 'Washington navel') plants receiving different nutritional treatments or different water-deficit treatments. GWSS showed no apparent settling or ovipositional preference for plants receiving nutritional treatments of 1:1 or 26:1 nitrate-N: ammonium-N. However, GWSS preferred to settle (average of 62%) and oviposit (71% of egg masses) on surplus-irrigated citrus compared to plants under CDI during the weeklong study. Stressed plants received about 25% of the water applied to the surplus-irrigated plants, which imposed an evapotranspiration rate of about 40% of the control. Xylem-fluid tension was estimated at about 7.3 bars in the CDI-stressed plants compared with 3.53 ± 0.19 bars in the controls. When provided with a choice of a surplus-irrigated plant and one undergoing drought, GWSS began to preferentially settle (about 70%) on surplus-irrigated plants on the eighth day after initiation of drought, at a point when xylem-fluid tension began increasing rapidly ($\geq$ 9.5 bars) beyond the control level.

Laboratory no-choice feeding bioassays

Results from GWSS confined in sachets on citrus receiving the two nutritional treatments revealed no significant differences in excreta production, suggesting similar feeding rates on plants of either treatment. As expected, GWSS produced more excreta on surplus-irrigated plants than on plants under CDI (0.80 ± 0.10 vs. 0.09 ± 0.02 ml per day, respectively). When confined on non-watered citrus, excreta production was similar to the control for eight days, and decreased significantly thereafter. Unexpectedly, a single day of surplus irrigation applied to drought-stressed plants on the 14th day resulted in significantly higher excreta production on these plants than on the control plants for about four days, after which it again fell below the control level.

Objective 2

A study conducted at Agricultural Operations at the University of California, Riverside, from April 2005 to June 2007 in a citrus orchard (cv. 'Valencia') was designed as a 3 x 3 Latin square with three irrigation treatments: 1) trees irrigated at 100% of the crop evapotranspiration (ET_c), 2) a continuous deficit-irrigated treatment maintained at 80% ET_c, and 3) a continuous deficit-irrigated treatment at 60% of ET_c. We monitored the temperature and humidity in the tree canopy, leaf surface temperatures, and pre-dawn trunk water potential. In June 2006 and 2007, all oranges were harvested and immediately taken to a local commercial packing house where oranges were mechanically counted, sized, and color graded. Measurements of fruit sugar solids (°Brix) were also recorded. Populations of GWSS were sampled weekly from April 2005 to Dec 2005 and Feb 2006 to Dec 2006. A 3-min visual inspection of leaves and branches around sample trees was conducted to monitor for GWSS egg masses, nymphs, adults, and natural enemies. GWSS population density was also monitored by collecting beat net samples. Yellow sticky traps were used to monitor insect activity. Repeated measures analyses were performed using measurements of plant condition and insect density data.

Effect of irrigation deficit on microclimate and plant conditions

Higher temperatures inside the tree canopy were recorded during May to September 2005 in the 60% ET_c treatment than in the 100% ET_c treatment. Throughout the study, there were no significant differences in canopy relative humidity among the

treatments. In general, leaf surface temperatures of trees irrigated with 60% ET_c were higher than those of trees irrigated with 80% and 100% ET_c. There was no difference between the 80% and 100% ET_c treatments ($P = 0.23$). Pre-dawn water potential measurements were higher in the 60% ET_c treatment than in the 80% or 100% ET_c treatments recorded among all time periods. There were no differences in water potential between the 80% and 100% ET_c treatments. In 2006, no differences in fruit sugar solid content were detected among the irrigation treatments ($F = 0.91$; df = 2, 134; $P = 0.404$). In 2007, fruit sugar solid contents were higher in trees irrigated at 60% (14.22 ± 0.19 °Brix) and 80% ET_c (14.31 ± 0.17) than at 100% ET_c (13.56 ± 0.15) ($F = 6.63$; df = 2, 134; $P = 0.001$). In 2006, there were no differences in total numbers of harvested fruit and number of fruit per grade category among irrigation treatments. In 2007, the total number of harvested fruit and numbers of fruit across all fruit grade categories in the 60% ET_c treatment were significantly lower than in the 80% and 100% ET_c treatments. There were no significant differences in total number of fruit and number of fruit per grade category between the 80% and 100% ET_c irrigation treatments.

Effect of irrigation deficit on GWSS populations

During the visual inspections in 2005, fewer GWSS adults were found on trees irrigated with 60% of the ET_c than with 80% and 100% ET_c ($F = 4.95$; df = 2, 20; $P = 0.017$). There was no difference in the number of GWSS adults found per tree between the 80% and 100% ET_c treatments ($P = 0.96$). On average (± SEM), 1.1 ± 0.4, 2.4 ± 1.0, and 1.9 ± 0.4 GWSS adults were found per tree at the population peak in mid-July 2005 in the 60%, 80%, and 100% ET_c treatments, respectively. In 2006, up to the peak of GWSS numbers in late-July, fewer adults were found on trees irrigated at 60% of the ET_c than at 80% and 100% ET_c ($F = 7.20$; df = 2, 20; $P = 0.004$). There was no difference in the number of GWSS adults found per tree between 80% and 100% ET_c treatments ($P = 0.78$). In the early-July to early-Oct interval, fewer adult GWSS were found in trees irrigated at 60% of the ET_c than at 80% ET_c ($F = 10.08$; df = 2, 20; $P < 0.001$). The number of adult GWSS was not different in the 100% ET_c treatment vs. those in the 60% ($P = 0.07$) or the 80% ET_c ($P = 0.11$) treatments. On average (± SEM), 5.4 ± 0.7, 13.1 ± 2.8, and 10.8 ± 1.7 adult GWSS were observed in visual counts per tree at the peak period in late-July 2006 in the 60%, 80%, and 100% ET_c treatments, respectively. In 2005 and 2006, less than 1.0 and 2.2 GWSS egg masses were found per sampled tree per week, respectively. In 2005, no differences in the mean number of GWSS egg masses were observed among the irrigation treatments. In 2006, there appeared to be four peaks of GWSS oviposition. The first peak occurred between late-February to early-March. A second peak occurred from late-April to early-June and the third peak occurred between early-July to early-September. A discrete fourth peak occurred between late-September to late-October. Fewer GWSS egg masses were found in the 60% ET_c treatment in comparison to the 80% or 100% ET_c treatments during the second peak oviposition period of 2006 ($F = 12.22$; df = 2, 20; $P < 0.001$).

In 2005, there were no differences among irrigation treatments in the number of GWSS nymphs ($F = 0.77$; df = 2, 20; $P = 0.472$) or adults ($F = 0.48$; df = 2, 20; $P = 0.622$) collected in beat net samples. In 2006, however, significantly more GWSS adults were collected in the 80% than in the 60% ET_c treatment ($F = 7.11$; df = 2, 20; $P = 0.004$). Population numbers in the 100% ET_c treatment were not different from either the 80% ($P = 0.10$) or the 60% ET_c ($P = 0.26$) treatments. Significantly more nymphal GWSS were collected from the 80% ET_c treatment than in the 60% and 100% ET_c treatments ($F = 5.26$; df = 2, 20; $P = 0.014$) between mid-April to early-July. Yellow sticky traps documented the presence of adult GWSS throughout the 117 weekly trapping periods, except for two and one trapping periods in late-June 2005 and early-June 2006, respectively, when no adult GWSS were caught on any of the traps. The following trapping periods showed a steady increase in insect activity to a peak in late July 2005 and 2006, with an average (± SEM) of 11.96 ± 1.16 and 95.22 ± 4.81 adults caught per trap per week, respectively. There were no differences in numbers of GWSS adults per trap per week among the irrigation treatments.

DISCUSSION

When given a choice, adult GWSS appear to select among host plants based on water stress, both for feeding and oviposition. The results indicate that GWSS begin to move off citrus when xylem-fluid tension exceeds 0.7 MPa, but GWSS feed normally on crape myrtle at levels up to 1.2 MPa (Andersen et al. 1992). The discrepancy may due to reduced vigor of the test insects under laboratory conditions. The increased feeding observed in citrus after drought was broken for a day may indicate a reduction in xylem-fluid tension below the level of the control. Although this was not detected in our test, such an extreme post-stress phenomenon was recorded in another citrus species when irrigation resumed after drought (Kaufmann and Levy 1976). This may be further evidence for the hypothesis that GWSS move into citrus directly after irrigation is applied, and warrants further study.

Our measurements of microclimate and plant conditions in the field experiment indicated that water stress increased leaf surface temperatures and trunk water potential. The two irrigation deficit regimes, 60% and 80% ET_c, differentially affected the population dynamics of GWSS in the experimental citrus plots. Severe to moderate water-stressed trees (60% and 80% ET_c) perhaps had increased solute concentrations used for osmotic adjustment (i.e., carbohydrates, amino acids, and organic acids) that might serve as feeding stimulants and primary nutrients of insects (Mattson and Haack 1987). However, increased water potential in more severe water-stress irrigation treatments (60% ET_c) might have been an impediment to GWSS feeding because more energy would be required to extract xylem fluid out of the xylem vessels (Andersen et al. 1992). In contrast, well-watered plants (100% ET_c) had lower mean water potentials that potentially facilitated extraction of xylem

fluid, but more fluid would have to be ingested and filtered to compensate for a more dilute xylem food source. Thus, citrus trees irrigated with 80% ET_c may combine two important plant characteristics for GWSS: 1) a nutrient-concentrated food source and 2) a water potential at acceptable levels for GWSS xylem fluid extraction, at least during periods of low transpirative demand by plants.

CONCLUSIONS

Findings from this study have generated significant new information regarding the host selection behavior of GWSS in California. Trees under severe water stress hosted fewer GWSS than trees maintained under moderate water stress. A more complete understanding of the effect of shorter water stress periods (i.e., regulated deficit irrigation regimes) and the operative host-plant cues that influence GWSS host selection behavior may result in the deployment of strategies to improve control efforts and contribute to limiting the spread of *Xf* induced diseases to susceptible crops.

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COLD STORAGE OF THE ADULT STAGE OF *GONATOCERUS ASHMEADI*: THE IMPACT ON MATERNAL AND PROGENY QUALITY

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Reporting Period: The results reported here are from work conducted January 1, 2007 to October 1, 2007.

ABSTRACT
The effect of storage of adult *G. ashmeadi* at 2, 5 and 10°C on maternal and the unstored progeny fitness qualities was examined. The maternal generation did not survive five d exposure to 2°C and those stored at 10°C survived longer than those held at 5°C. Oviposition of the maternal generation continued for 13 d after storage at 10°C for 10 or 20 d and for eight d for the wasps stored for 30, 40 or 50 d. After storage for 60 d, the parasitoids did not oviposit for the first two d and then there was only a four d oviposition period. Cold storage reduced the fecundity of the parents and their F_1 progeny, but not the F_2 generation. After 20 d storage, the fecundity of the maternal generation decreased by 47% and for 60 d storage, it was reduced 90%. The longevity of F_1 parasitoids was also less than that of F_2 parasitoids. After 30 d storage of the parents, the longevity of the F_1 generation was reduced 49% as compared to a 10 d parental storage period. The F_1 progeny of the stored female parasitoids developed about one d more slowly than that of the F_2 and F_3 generations. Moreover, cold storage caused a reduction in the incidence of parasitism by parental generation, but this effect did not extend to F_1 and F_2 progeny. Emergence of F_1 parasitoids decreased as the length of storage of their parents increased. Further, the parental generation deposited more haploid than diploid eggs after storage for 20 d and after 50 d, production of males was increased by 132%. The sex ratios of the F_2 and F_3 generations did not vary with the storage duration of the ancestral generation.

INTRODUCTION
Cold storage can be a valuable tool in mass-rearing of biological control agents. It provides a steady supply of insects for research, yields flexibility and efficiency in mass production, allows synchronization of a desired developmental stage for releases, and facilitates availability to consumers (Leopold, 1998). Maternal survival and offspring fitness are of great concern when using cold exposure during production or storing of biological control agents. Some reports indicate that the quality of a variety of cold-stored pest and beneficial insects suffer by having reduced emergence, lifespan and/or reproduction (Leopold, 1998).

The egg parasitoid, *Gonatocerus ashmeadi* Girault, is one of the most common natural enemies of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae). It accounts for 80-95% of the observed parasitism of sharpshooter eggs in California. As part of a control program, *G. ashmeadi*, along with two other mymarids, *G. triguttatus* Girault and *G. fasciatus* Girault, has been imported, mass-reared and released in California. However, there are two main problems that reduce the effectiveness of *Gonatocerus* spp. in control programs. One is that the native wasps do not build up rapidly enough to make an impact on the spring generation of *H. vitripennis* in the colder regions of central and northern California (Morse et al., 2005). Another problem is that effective mass production of these parasitoids can not be realized due to a shortage of *H. vitripennis* eggs caused by a reproductive diapause (Hummel et al., 2006), especially in the late winter season. Thus, it would desirable to store large numbers of parasitoids to meet these fluctuating demands.

Methodology for cold storage of *H. vitripennis* and *G. ashmeadi* has been developed to aid the mass rearing of *G. ashmeadi* (Leopold and Chen, 2005; Chen and Leopold, 2007; Chen et al. 2007). We have also examined a number of fitness factors of *G. ashmeadi* and progeny performance following the use of these storage protocols. Using chilling-killed *H. vitripennis* eggs as hosts for rearing *G. ashmeadi*, Chen and Leopold (2007) found that wasp progeny quality was severely reduced when their parents were reared on *H. vitripennis* eggs that had been stored for 60 days before parasitization occurred. Further, by stockpiling immature *G. ashmeadi* within *H. vitripennis* eggs, quality of maternal and first generations also suffered significantly with the length of storage duration (Chen et al. 2007). In this study, we examined the post storage quality of the parental, F_1, F_2 and F_3 generations of *G. ashmeadi* after storing the adult female parents to further understand chilling injury and its expression in this parasitoid.

OBJECTIVES
1. Determine the survival of adult parasitoid following cold storage.
2. Determine the effect of cold storage on fecundity and subsequent development of the progeny.
3. Determine progeny quality by examining some fitness factors including parasitism, emergence and sex ratio.

RESULTS AND CONCLUSIONS

Objective 1. Effect of storage temperature on survival of adult parasitoid.

The survival of adult parasitoids was examined every 10 d after storage in the incubators set at 2, 5, and 10°C with a 8L:16D photoperiod. Figure 1 shows that survivorship of *G. ashmeadi* varied with storage duration when storage was at 5 and 10°C. In addition, because all parasitoids died after 5 d storage at 2°C, their survivorship evaluation was excluded for this study. Temperature during storage significantly affected the survivorship of adult parasitoid (F= 11.01, df =1,108, P = 0.0012). However, under the same temperature, we found no significant difference in survivorship between female and male parasitoids (5°C, F= 2.69, df =1,54, P = 0.107; 10°C, F = 0.96, df =1,54, P= 0.331) although the females slightly outlived the males.

Objective 2. Post storage fecundity of parasitoid and development of progeny.

To determine the effect of cold storage on fecundity of the parental generation and the F_1 and F_2 progeny, 1 mated female parasitoid was introduced into a container having a total of 80 GWSS eggs (<24 h old) on euonymus leaves (*Euonymus japonica* Thumb.) and then held at 24°C and under a16L:8D photoperiod. Mated parasitoids made up six groups of adult females that were stored at 10°C for 10, 20, 30, 40, 50 and 60 d and five groups of F_1 or F_2 offspring obtained from the storage experiments involving the parental generation. Oviposition of *G. ashmeadi* after adult storage is shown in Figure 2. After storage for up to 50 d, the females oviposited on the first day after removal from storage. The duration of storage affected the length of ovipositional period. After storage for 10 and 20 d, oviposition of this parasitoid continued for 13 d, and for 30, 40 and 50 d storage, it lasted for 8 d. When storage was 60 d, oviposition was lacking for 2 d and then the eggs were deposited for only 4 d.

Lifetime fecundity of *G. ashmeadi* following cold storage of adult female parasitoid varied significantly with the length of storage duration (Table 1). After storage for 20 and 60 d, fecundity of the parasitoids was decreased by 47%, and 90%, respectively. The fecundity of the F_1 generation was also significantly affected by the length of storage of their parents. When the parents were stored for ≥ 20 d, the fecundity of their F_1 progeny decreased significantly. However, fecundity of F_2 generation was unaffected by storage duration of grandparental generation. A two-way ANOVA, with storage duration and generation as factors, revealed that lifetime fecundity of *G. ashmeadi* varied significantly with the length of storage of adult parasitoid (F = 9.64, df = 4,114, P < 0.0001) and generation (F = 40.43, df = 2,114, P < 0.0001). There was a significant effect of interaction between storage duration and generation on lifetime fecundity of this parasitoid when storage was ≥20 d (F = 3.32, df = 8,114, P = 0.0019). For 10 d storage, the lifetime fecundity of the parental, F_1 and F_2 generations was unaffected.

Development time of the F_2 and F_3 generations did not vary with the length of storage of parental generation (Figure 3A). However, progeny of F_1 generation developed significantly more slowly than that of F_2 and F_3 generations. There was ca. 1 d difference in progeny development between the F_1 and F_2 generations. Only the storage period for 50 d led to significant difference in development between the F_1 and F_2 and F_3 generations.

Longevity of F_1 and F_2 generations was shown in Figure 3B. The length of storage of the parental parasitoid had a significant influence on the longevity of F_1 parasitoids (F = 4.79, df = 4, 44, P = 0.003), but not on that of F_2 generation (F = 0.12, df = 4,35, P = 0.976). After parental females were stored for 30 d, the longevity of F_1 wasp was shortened by 49% when compared to that of wasps stored for only 10 d.

Objective 3. Effect of cold storage on the quality of maternal generation and progeny.

To determine the incidence of parasitism by the maternal and F_1 and F_2 generations, six excised leaves of the euonymus plant bearing a total of 80 GWSS eggs (< 24 h old) were provided for 1 wasp for 1 d at 24 °C and 16L: 8D photoperiod. Wasps were collected using a procedure described previously (Chen and Leopold, 2007). The GWSS eggs were kept at the same chamber to determine emergence rate and sex ratio of the offspring. Figure 3C shows the data on the incidence of parasitism by the P to the F_2 generation for up to 50 d of storage. Because the parasitoids did not deposit eggs during the first two days after storage for 60 d, they were excluded from this study. Our results showed that post storage parasitism by the parental generation varied significantly with storage duration (F = 7.14, df = 4, 40, P = 0.0002). The incidence of parasitism by the F_1 (F = 0.12, df = 4,45, P = 0.976) and F_2 (F = 0.73, df = 4,45, P = 0.574) generations was not affected by storage duration of the ancestral generation.

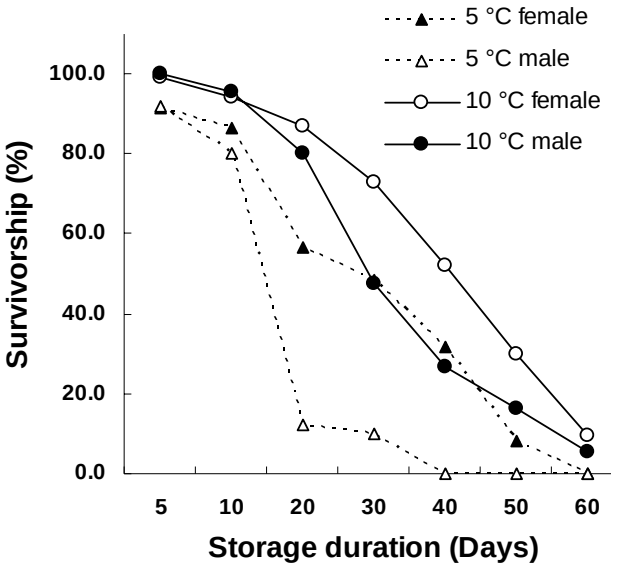

Figure 1. Survivorship of *G. ashmeadi* as a function of the length of adult storage. Each value represents means of 5 separate determinations.

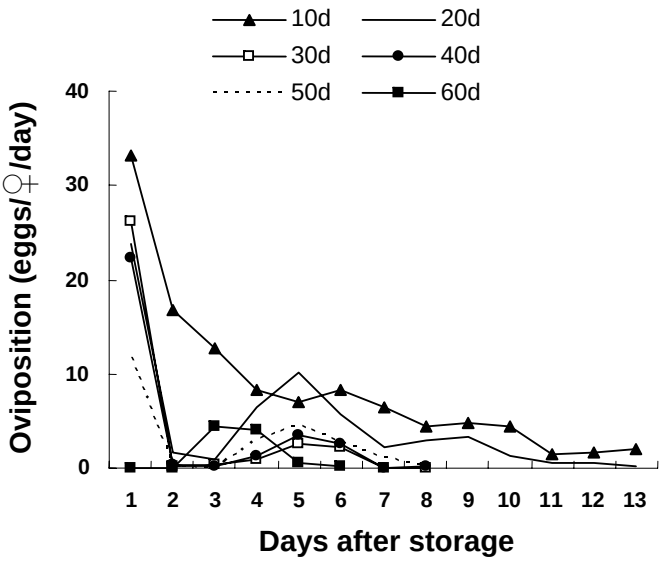

Figure 2. Oviposition of *G. ashmeadi* as a function of the length of adult storage. Each value represents means of 10 separate determinations.

Table 1. Lifetime fecundity of parental, F_1 and F_2 generations following cold storage of the parental generation.

	Parental (P) Storage Duration (Days)								
Gen	**10**	**20**	**30**	**40**	**50**	**60**	***F***	***df***	***P***
P	111.8 ± 23.7Aa	59.8 ± 24.2Cb	32.1± 8.4Cc	30.9 ± 10.2Cc	30.7 ± 20.8Cc	11.4 ±10.6d	16.5	4,34	<0.0001
F_1	106.7 ± 35.2Aa	74.1 ± 15.8Bb	61.7 ± 19.9Bb	61.6 ± 20.4Bb	59.2 ± 32.0Bb	/	5.5	4,43	0.0011
F_2	96.5 ± 15.8Aa	93.9 ± 32.6Aa	96.4 ± 13.1Aa	98.4 ± 29.3Aa	107.5 ± 47.7Aa	/	0.26	4,37	0.902
F	0.40	3.96	35.46	23.68	12.13				
df	2,16	2,23	2,23	2,25	2,27				
P	0.679	0.033	< 0.001	< 0.001	0.0002				

The emergence rate of the F_1 adults was significantly affected by the length of storage duration of parental generation (F = 11.52, df = 4, 38, P < 0.0001) (Figure 3D). However, adult emergence of the F_2 (F = 0.55, df = 4, 45, P = 0. 697) and F_3 (F = 0.08, df = 4, 45, P = 0.988) generations did not vary with storage duration of their ancestral generation

The length of storage duration of maternal generation significantly affected realized sex allocation of the F_1 progeny (F = 2.92, df = 2, 92, P = 0.034). The parental generation females produced more haploid than diploid eggs after storage for ≥20 d (Figure 4). After storage for 50 d, the male proportion of the wasp progeny was increased by 132.5%. Although the female proportion was slightly increased in the F_1 generation after their adult parents were stored for ≥20 d, the sex ratios of F_2 (F = 1.19, df = 4,33, P = 0.334) and F_3 (F = 0.36, df = 4,34, P = 0.834) generations did not vary with storage duration of the ancestral generation.

Chilling adult *G. ashmeadi* females for more than 10 days at 10°C or lower has a deleterious effect on fitness that carries over to the F_1 generation but not to subsequent generations. These observations should be taken into account when producing parasitoids as biocontrol agents for the GWSS.

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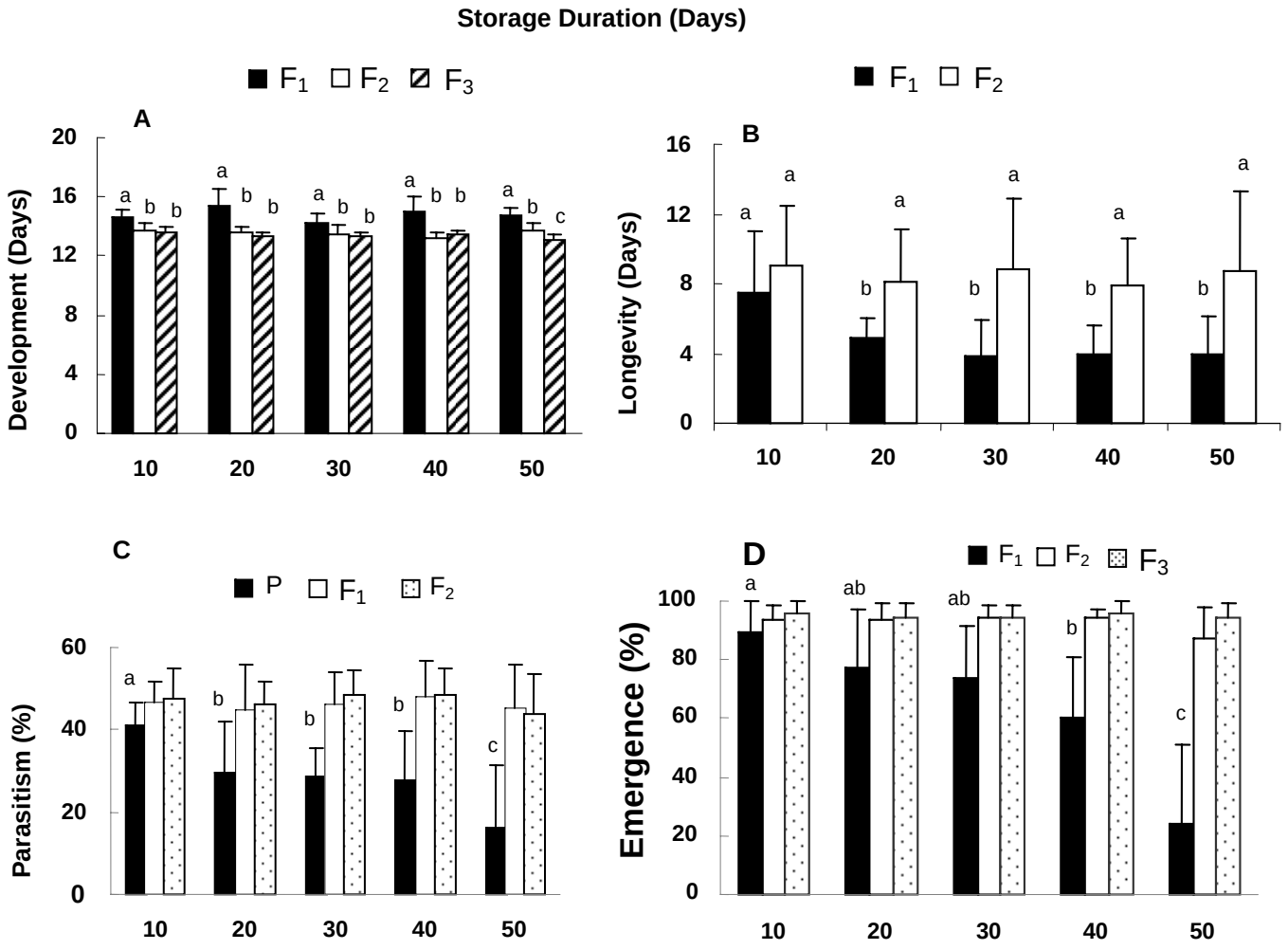

Figure 3. (A) developmental time of progeny of the F_1, F_2 and F_3 generations; (B) F_1 and F_2 longevity as a function of the P generation storage; (C) parasitism by parental, F_1 and F_2 generations; (D) emergence of the F_1, F_2 and F_3 generations as a function of storage of the P generation. Columns with different letters are significantly different from each other.

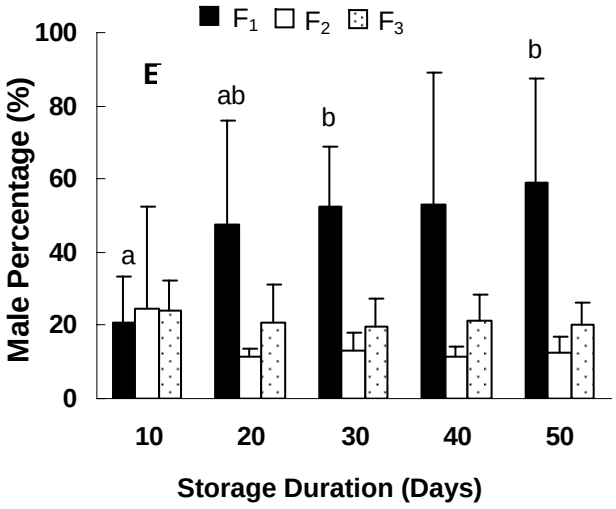

Figure 4. Sex ratios of the F_1, F_2 and F_3 generations of *G. ashmeadi* as a function of the length of storage of the P generation of mated females. Columns with different letters are significantly different from each other.

XYLEM SPECIALISTS COLONIZING TEXAS VINEYARDS: ABUNDANCE AND DISTRIBUTION STUDIED THROUGH GENERALIZED ADDITIVE MODELS

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ABSTRACT

Xylem specialists in the Hemiptera: Auchenorrhyncha feed on xylem fluid and exhibit therefore the potential for transmission of *Xylella fastidiosa (Xf)*, the causal agent in Pierce's disease of grapevine. An increasingly larger survey was initiated in Texas in 2003 and remains ongoing with 45 locations currently monitored. Over 25 insect species belonging in this group were collected from yellow sticky traps. Among these, two leafhoppers and one spittlebug, comprised over 90% of the xylem specialists caught. The glassy-winged sharpshooter *Homalodisca vitripennis*, the most commonly known vector of Pierce's disease of grapevine in the U.S. is the most common and abundant insect captured across the state. Insect abundance varied significantly over seasons, and among locations. Residual populations were found overwintering near vineyards, but also in a variety of habitats including urban and vegetation found near natural sources of water. Generalized additive models which were originally developed to analyze complex population data were used with our Central Texas dataset as a tool to identify changes in the distribution of leafhopper and spittlebug species near vineyards. These models helped understanding insect distribution through time.

INTRODUCTION

Pierce's disease of grapevine is one of the most important limiting factors to grape production in Texas. During the 1990's, the grape growing region of Central Texas witnessed an increase in the incidence and severity of Pierce's disease (Texas Pierce's disease Task Force 2004). A research program was initiated in Texas in 2002 with funding from the U.S. Department of Agriculture. Within this program, researchers are provided an opportunity to study glassy-winged sharpshooters in their native habitat, and their interaction with cultivated vines. 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 all underway.

OBJECTIVES

1. Monitor xylem feeding insect populations in vineyards across Texas. Identify all putative insect vectors of Pierce's disease. Determine the most common vectors requiring population management, make observations and analyze vector distribution, abundance 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. Determine the extent of primary and secondary spread in vineyards.

RESULTS

Insect populations in the Hemiptera have been monitored (mostly) on a bi-weekly fashion in 45 vineyards statewide for over three years. A large database was assembled for analyses using a two-year dataset which included all insect counts per species (from trap data) and a layer of geographical data. Scientists and staff at Fredericksburg and Stephenville worked at organizing the different fields of this database. 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. Relatively new (new to entomology) statistical analyses, generalized additive models (GAMs) were tested using one of our first Central Texas trap dataset. The power of those analytical methods is both impressive and very instructive and useful. This modeling technique is a semi-parametric extension of the more traditional general linear models (GLMs). The statistical models identify non-linear and non-monotonic relationships between the response and the set of explanatory variables. They are a useful tool to identify factors responsible for the distribution of species, and help in understanding changes that occur through time via a model element known as a smooth function, similar in concept to a moving average. A distinctive feature of the data we collected was an over-abundance of zero counts, or zero xylem specialists on one given trap, at a given location, and for a given time period. Using a Poisson based GAM, we were able to better describe particular factors of interest affecting insect populations, and the degree to which factor(s) is/are important to the presence of sharpshooters and disease. Because of the extensive multiyear dataset, this powerful approach will better explain variations in insect populations through time and between locations in a large ecological setting.

Because of its abundance in vineyards, diverse plant host range, high degree of association with *Xf* in Texas and the economic problems it causes in California due to its vector status, the glassy-winged sharpshooter (GWSS) is a high priority target for management. Populations of the GWSS can be very high in Texas depending on where the data are collected. In Central Texas, populations have been consistent both in abundance and time of appearance. Observational and census data indicate that secondary spread of Pierce's disease in vineyards is in play here.

Figure 1 summarizes GWSS census data from traps collected during 2003 – 2006 in Central Texas. Insects were selected from traps on multiple dates during these years and subjected to PCR to detect the presence of *Xf. Xf* is absent from the earliest GWSS and accumulates over the course of the year, ending in nearly 70% prevalence within the insects. Peak bacterial prevalence does not coincide with the population peak in the four years examined. Had primary spread been the main mode of transmittal, one would expect the incoming insects to have a higher load of bacteria. This graph alone is insufficient to defend the point however, and other data are needed.

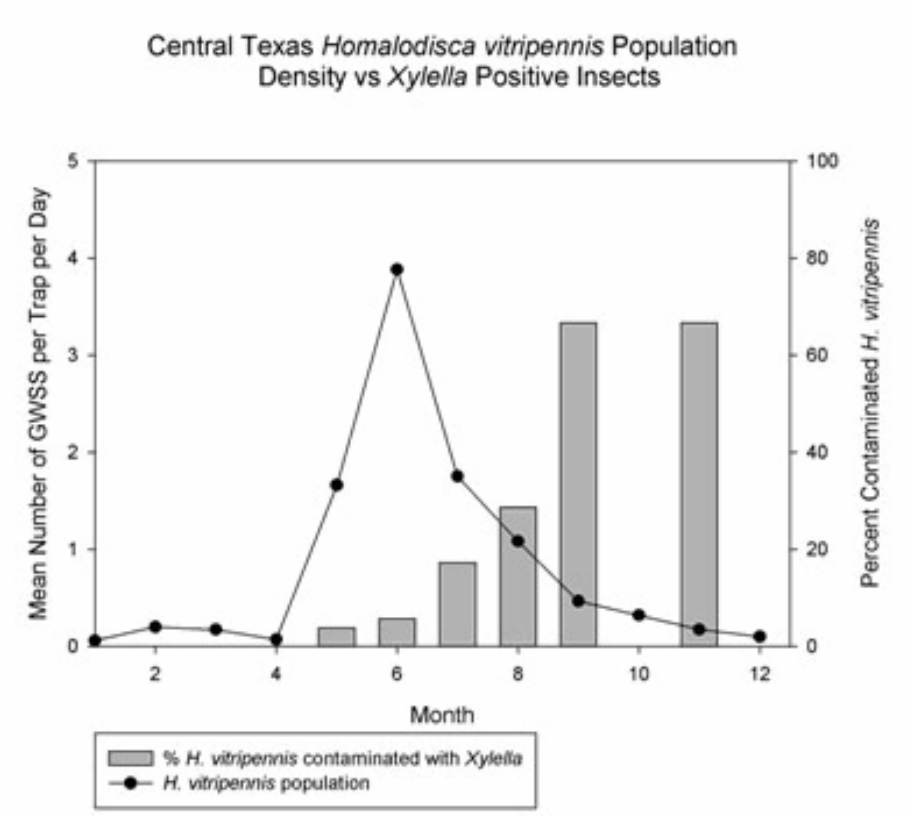

Figure 1. Of the vineyards under census, six have been in the program since 2003. Three of these vineyards are seriously impacted by Pierce's disease, three are not. When the *Xf* prevalence data for GWSS are compared between these vineyards, the following summary in Table 1 results.

Table 1

Vineyard Status	Species	Total Tested	# Positive	# Negative	% Positive	% Negative
Impacted	*H. vitripennis*	490	115	375	23.47%	76.53%
Unimpacted	*H. vitripennis*	404	10	394	2.48%	97.52%

This supports the hypothesis that the increase in bacterial prevalence found within the GWSS arises from within the vineyards and perhaps its vicinity. Other vector species will likely play roles. Data from the second most abundant putative vector species, *Graphocephala versuta*, presents similar results (not shown).

CONCLUSIONS

Over the past months, we have worked on a two-year field database that was built with insect counts (of Central Texas alone) and one layer of geographical data. GAMs are one approach being used to analyze this complex population data. They are more flexible and for this reason are sometimes more suitable than GLMs for some types of analysis. More details can soon

be found in our recently accepted paper at Environmental Entomology. We 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 abundance and made up for over 90% of insects collected. Extensive molecular analyses are being carried out to confirm which of these insects are associated with the grape strain 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. We also are now in the process of building a much larger database that holds more sampling years and sampled locations, along with more geographical and some environmental data to further test this approach.

Secondary spread of Pierce's disease in Central Texas vineyards may be the main mode of transmission. The data are not yet analyzed and experimental detail is needed to confirm this, but should it be the case then management protocols for this type of spread (vector management within the vineyard, roguing infected vines at the earliest opportunity) will be appropriate.

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FUNDING AGENCIES

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

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ABSTRACT
The glassy-winged sharpshooter, (GWSS), *Homalodisca vitripennis* (Germar), as a vector of *Xylella fastidiosa*, is a threat to grapes, almonds, stone fruit and oleander and impacts citrus and nursery crops throughout much of California. It remains an important high-risk quarantine pest for the Napa and Sonoma Valleys and other uninfested areas. Accurate and precise methods for detection of new colony infestations and for monitoring GWSS population dynamics on a temporal and spatial basis are lacking. Due to the unique behavior, biology and ecology of the xylophagous GWSS which is driven by plant xylem chemistry and nutrition, conventional detection and monitoring approaches do not provide the necessary statistical precision needed by the regulatory and producer communities for management decisions. This proposal will address the detection and monitoring needs as well as develop a more strategic approach to management of GWSS.

INTRODUCTION
In previous research we evaluated trap color, size, configuration and height. We compared a large number of commercially available as well as home-made sticky traps in attempts to find a practical and reliable trap to detect and monitor GWSS population dynamics. The conventional two-sided Pherocon AM trap used in CA was tested as the standard for comparisons to other trap types in FL. Traps colored “Safety yellow” placed at 2 m or less from the ground captured the highest numbers of GWSS in these tests. Of all traps tested, we found that a 32.5 cm height x 7.6 cm dia. yellow cylinder (painted mailing tube, referred to as “tube” trap) captured the highest number of GWSS. However, when GWSS trap capture numbers were related to visual counts on host plants, host plant species and nutritional condition were important variables affecting GWSS capture rate. In patches of non-preferred hosts such as peach, there was no significant relationship between trap capture rate and GWSS numbers on trees. GWSS passed through the patches but spent very little time on the trees. In monoculture patches of a preferred host crape myrtle, higher numbers of GWSS were captured with better correlation to GWSS numbers present on trees, but the results were inconclusive due to variation by date and patch type. Moreover, on some sample dates there was an inverse correlation between GWSS plant numbers and trap captures, suggesting that nearby high quality host plants actually reduce trap capture rates.

We have compared the standard Pherocon AM trap configured with two flat surfaces to the same trap configured in the form of a cylinder (somewhat smaller than the tube trap) and to the tube trap. In all tests the mailing tube trap captured significantly more GWSS adults of both sexes with tube capture rates of ca. 5-15 x higher than the other traps. We have tried other embellishments of the tube trap with limited success. We have also attempted to ascertain the “active distance” of operation of the trap with respect to how far away GWSS will respond to the tube trap. These experiments enabled an estimation of the active distance of the tube trap to be at least 8-12m, but these results were inconclusive statistically. In other work we have combined single plants of various species with traps to determine whether combinations of traps with plants will improve trap capture. Again, plant species affected the outcomes. We have also tested larger numbers of traps alone and in combination with preferred crape myrtle and non-preferred peach plants to increase the visual presentation offered to GWSS. Also, we have done mark-recapture studies in large peach and crape myrtle plantings (Northfield et al. 2008b). In these studies, more GWSS were captured by traps in crape myrtle for a longer period of time than on traps in peach or on traps alone. Emigration from the plots was much higher and more rapid in peach than crape myrtle. Finally, the landscape context of the study plots, i.e., whether the plots were isolated by at least 100 m of grassy area or had vegetation within 100 m of the plot, affected GWSS emigration and immigration, as well as within plot trap capture rates. We have concluded that the searching flight, trap and host response behavior of GWSS, while highly visually oriented, is complex and affected by host plants, and driven by a number of habitat variables, landscape structure and unknown innate GWSS behaviors. It appears that single traps will not provide the necessary accuracy in predicting the population dynamics of GWSS for management decision making nor the reliability required for regulatory objectives. However, the tube trap is superior to the standard Pherocon AM trap in detection of GWSS and could easily be implemented for GWSS monitoring in CA. Our objectives are to develop and implement a practical detection and monitoring system for GWSS with requisite statistical precision. At the time of submission of this report we have not received the funds to initiate this project.

OBJECTIVES

General Objective: To determine the most efficient and cost effective trapping system to detect and monitor GWSS population dynamics and the potential to manage GWSS populations.

1a. Evaluate and summarize previous sampling and trapping efforts for GWSS.

1b. Trap configuration and number: Determine potential and optimize the number of traps that are most efficient and cost effective in detecting and estimating GWSS populations.

1c. Effect of host plants in combination with traps: Determine the potential and optimize a combination of GWSS host plants in sentinel plots to detect, estimate and manage GWSS population dynamics.

RESULTS

Objective 1a

Other investigators have addressed various aspects of our objectives in a number of crops with similar mixed results. As part of this project we will review and synthesize previous research into a summary document.

Objective 1b

We have initiated new experiments with only preliminary results at this time.

Objective 1c

While current trap methodology does not enable statistical quantification of GWSS population dynamics at the level needed within crops, traps can be used for investigations that will contribute other important knowledge relevant to improving detection and monitoring for GWSS. Toward that end we have conducted several experiments investigating the landscape level distribution and abundance of GWSS using tube traps. We sampled a 52 trap grid covering 2.59 km^2 of terrain at the North Florida Research and Education Center in Quincy, FL throughout the year for a 3 year period (Northfield et al. 2008a). Traps were placed 229 m apart to detect movement in the landscape, and to correlate GWSS trap catch with different types of vegetation. Tube traps were placed on steel rods in open areas approximately 1 m above ground. Trap catch was recorded and traps were cleaned weekly from 25 January to 6 September in 2001, from 28 February to 10 October in 2002 and from 13 March to 7 August 2003. Years 2001 and 2003 differed from 2002 in rainfall with 2002 receiving much less rain. The location is divided in the middle by a 4 lane highway resulting in east and west sections with 25 and 27 traps, respectively. The east section is characterized by greater irrigated acreage and more diverse vegetation including several plots of crape myrtle, *Lagerstroemia indica*. Weekly trap count distributions were analyzed (kriging) using spherical models in ESRI ArcMap Version 9.2 to view spatial and temporal dynamics. Trap counts indicated differences in abundance and distribution of GWSS across years and between the east and west locations (Figure. 1). To evaluate the response to habitat type over time, traps were categorized into traps along forest edges, and traps away from the forest edges. On the east side of the road there were 13 traps along the forest edge and 12 traps away from the edge. On the west side, there were 14 traps along the forest edge and 13 away from the edge. The proportion of the *H. vitripennis* trap catch that occurred near the forest edge was plotted over time separately for each side of the road to evaluate the seasonal movement from the forest edge to summer hosts. A quadratic equation was fit to the data (Figure 2). We quantified the movement of GWSS populations moving from overwintering in the forest-forest edges out into managed areas during the growing season and back to the forest in the fall (Figure 1) as suggested by Pollard and Kaloostian (1961). Cluster indices, *v* were calculated using SADIE (Spatial Analysis of Distance Indices, version 1.2, Harpenden, UK) red-blue methodologies (Perry et al., 1999). Random permutations in SADIE were then conducted to test for the probability that a randomly distributed population would be more clustered than the data for each date and plot (Perry et al., 1999). GWSS adults were rarely aggregated at the scale tested; indicating that any population clusters had a radius smaller than 229 m (trap grid size). The spatial association tests demonstrated a difference in spatiotemporal stability in the east during the drought in 2002, in comparison to 2001 and 2003. Further details of this study will be published by Northfield et al. (2008a) as will the results from another study using mark-recapture techniques to compare the behavior of GWSS in a preferred and non-preferred host block (Northfield et al. 2008b). This and other research has been synthesized into a model of GWSS biology and behavior that is also submitted for publication (Mizell et al. 2008).

CONCLUSIONS

We are only beginning to understand what will be necessary to develop effective management schemes for GWSS. Clearly, understanding, quantifying and predicting GWSS population dynamics to meet strict regulatory and management objectives will be neither easy nor simple for this unusual insect vector. However, approaching the problem on several levels of resolution will facilitate progress toward implementing useful tactics and strategies to detect and monitor GWSS successfully. We have established that quantitative trapping results will vary greatly depending on patch type (adjacent host plant species, landscape structure, season, climate) and we are now working toward integrating these effects with trap type and spatial distribution to develop functional and useful trapping protocols. This should lead to better methods of disease management.

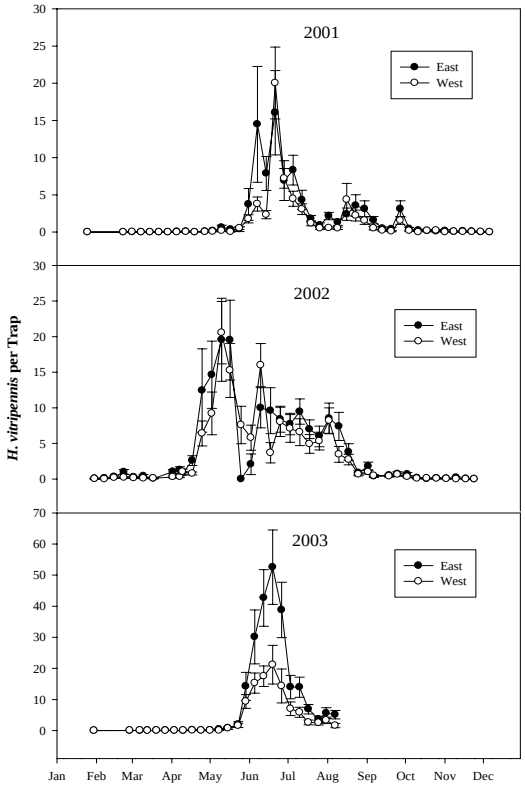

Figure 1. Mean trap catch of GWSS in East and West plots in 2001, 2002 and 2003 on the North Florida Research and Education Center in Quincy, FL.

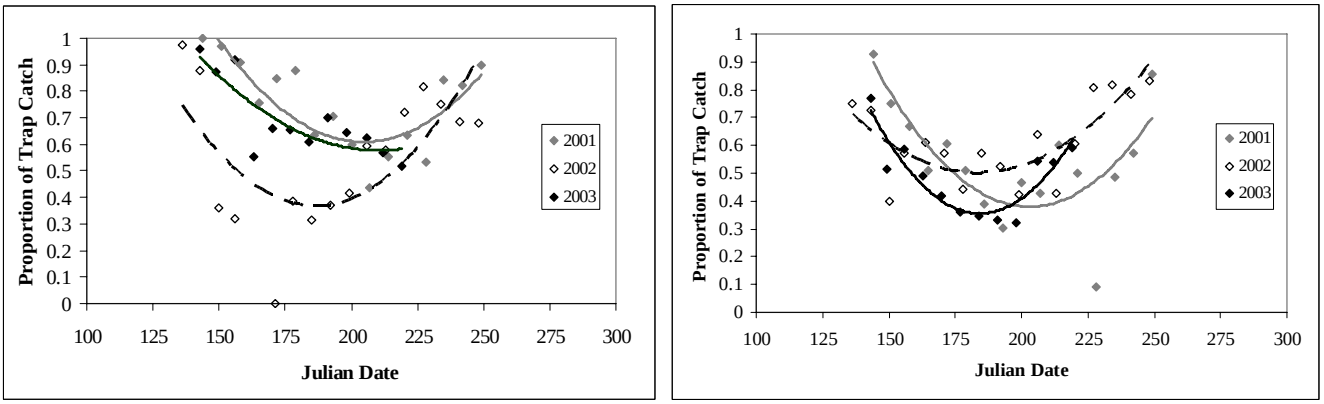

Figure 2. Proportion of total GWSS collected that were found on traps along the forest edge (as opposed to away from the forest edge) on each sample date, in the east (A-left) and west (B-right) plots in 2001, 2002, and 2003. R^2 values for quadratic trendlines in the east are 0.692, 0.451 and 0.686 for 2001, 2002 and 2003, respectively. R^2 values for quadratic trendlines in the west are 0.586, 0.608 and 0.826 for 2001, 2002 and 2003, respectively.

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FUNDING AGENCIES

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

SEASONAL TRANSMISSION OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER FROM GRAPEVINES INFECTED FOR VARIOUS LENGTHS OF TIME

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Reporting Period: The results reported here are from work conducted July 2007 through September 2007.

ABSTRACT
This study was designed to define specific orientation and feeding behaviors by the glassy-winged sharpshooter (GWSS) in association with grapevines that influence vine-to-vine spread of *Xylella fastidiosa (Xf)*, the Pierce's disease bacterium. Thus far, we conducted experiments in which we made hourly observations on the location of individual GWSS adults given access to mature tissue and a terminal growing tip on the same cane. Although GWSS were observed on all tissues, both males and females preferred immature tissues to mature tissues on Cabernet Sauvignon and Chardonnay grapevines, yet sharpshooters moved frequently throughout the day. Immature stems and leaves were consistently the most used tissue, while immature petioles and mature stems and petioles were the least used.

INTRODUCTION
Pierce's disease (PD), a disease of grapes caused by the bacteria, *Xylella fastidiosa* (*Xf*) Wells et al., was described in California in the 1880s during an epidemic in Orange County (Pierce 1882). A second epidemic occurred in Tulare County in the 1930s (Hewitt et al. 1949), and until the mid-1990s, it was considered only a minor problem in vineyards close to riparian areas. In the early 1990s a new vector, the GWSS, *Homalodisca vitripennis* (Germar) (formerly *Homalodisca coagulata* Say), was introduced into the state (Sorenson and Gill 1996), and became associated with a devastating epidemic of PD in the Temecula Valley. Since 1994, at least 1,500 acres of vineyards have been lost to the disease in California; in the Temecula Valley alone, losses have been estimated at $13 million (Wine Institute 2002). The GWSS has different feeding and dispersal capabilities than native insect sharpshooter vectors and these attributes are thought to have contributed to the increased number of PD-infected grapevines in California (Almeida et al. 2005a, Blua et al. 1999, Redak et al. 2004). Like other insect-borne plant pathogen systems, there are two potential types of pathogen spread: primary or secondary spread. Primary spread occurs when the pathogen is obtained by the vector from sources outside the crop and transported and inoculated into the crop. Secondary spread occurs when the vector acquires the pathogen from infected vines in the vineyard, and subsequently inoculates healthy vines within the same vineyard (i.e. vine to vine spread). It is thought that *Xf* spread with native California vectors was the result of primary spread, but that rapid spread by GWSS may be the consequence of primary and secondary spread (Almeida et al. 2005a, Hill 2006). Consequently, the best strategy for managing PD spread by GWSS is to control the vector. This strategy, which is simple in concept, yet difficult and costly to implement over large geographic areas, is exactly the approach of the area-wide management program being implemented by USDA, CDFA, County Agricultural Commissioner's Offices, and the University of California (for example Toscano and Gispert 2005, Stone-Smith et al. 2005). This strategy has been so effective at slowing the spread of PD throughout the state that the "crisis" phase of the PD-GWSS problem appears to have passed. Unknown, of course, is what will happen should the area-wide management program be reduced. Due to this uncertainty, researchers continue to search for alternative solutions, both short and long term. Understanding details of primary and secondary spread of *Xf* by GWSS can assist in the development of alternatives to the area-wide management program. For example, to reduce primary spread, efforts must focus on reducing bacteria-carrying GWSS from entering healthy vineyards, through continued area-wide or local treatment programs outside the vineyard, barriers, trap crops, and/or removal of pathogen sources outsides the vineyard. Reduction of secondary spread can be accomplished by in-field control of GWSS, finding and roguing infected vines in the vineyard (Varela et al. 2001), and/or minimizing acquisition from infected vines and transmission to healthy vines.

The relationship among time of inoculation, location of inoculation, and disease progression in the vine likely plays a role in determining whether disease becomes chronic and when a vine becomes a source plant for additional spread. When another PD vector, the blue-green sharpshooter, *Graphocephala atropunctata*, infected grapevines early in the season, more persistent infections resulted than from later season infection (Purcell 1981). A potential difference between blue-green sharpshooter transmission and GWSS transmission is that the former is known to prefer feeding at the tips of canes (Purcell 1976), whereas the latter has been reported to feed on older plant parts. Almeida et al. (2005b) demonstrated that GWSS could even transmit *Xf* to dormant vines in the field. However acquisitions from dormant vines in the field were negative. Whether these transmissions and acquisitions are important to disease spread depends on GWSS feeding preferences during the winter months when the vines are dormant. Similarly, it is possible that infection at certain times of the season may not become systemic because infection is pruned out at end of year, or environmental conditions limit bacterial spread (Feil and Purcell 2001, Feil et al. 2003, Hill 2006).

OBJECTIVES

While this progress report addresses only the first objective, the objectives of the project are:

1. Document GWSS feeding preference, through the growing season, on established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for 2, 3, or 4 years.
2. Evaluate the acquisition by GWSS, through the growing season, from established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for 2, 3, or 4 years and determine the subsequent transmission from these acquisitions.
3. Determine the relationship between *Xf* inoculation by GWSS at different times of the year and the development of the vine as a source for further acquisition by GWSS.

RESULTS

We placed GWSS adults individually in observation cages fabricated from acetate cylinders (25cm x 17cm diameter) with organdy sleeves attached to the ends. The cage was place over the base of a Cabernet Sauvignon or Chardonnay grape vine cane with the cane terminal looped back into it. The ends of the observation cage were sealed giving each GWSS access to immature and mature stems, petioles, and leaves inside. The grapevines were from a mixed field-grown vineyard at the University of California in Riverside that was covered with 60% shade-cloth to protect them from PD. We made hourly observations from 700 to 1800 hours over three days to determine which tissue each GWSS was on. This experiment was conducted in late August (Experiment 1) and again in mid September 2007 (Experiment 2).

In both experiments, GWSS were found on the cage in 12 to 16% of our observations (Figure 1). When on the cane, GWSS were observed on all tissue types, and although significant differences in tissues used occurred among varietals and genders in both experiments, GWSS did not show a substantial preference for a specific tissue consistently. However, they were found more frequently on immature stems, petioles, and leaves (46-67% of our observations) than mature tissue (21-37%) whether they were enclosed on Cabernet Sauvignon or Chardonnay vines, or were males or females (Figure 1). In general, GWSS were most likely to be found on immature stems and leaves, and least likely to be found on immature petioles and mature stems and petioles (Figure 1). Importantly, averages of 34% and 36% of experimental GWSS across gender in experiments 1 and 2, respectively, switched from one tissue to another each hour on Cabernet Sauvignon. Hourly averages of 30% and 37% of GWSS on Chardonnay switched tissues in experiments 1 and 2, respectively. Averages of 34% and 39% of females switched tissues each hour, and averages of 30% and 34% of males switched tissues each hour in experiments 1 and 2, respectively.

CONCLUSIONS

Vine to vine spread of *Xf* by GWSS has been hypothesized as a critical component of devastating PD epidemics that occurred in Temecula and in the General Beale area of Kern County. A fundamental understanding of this type of spread can lead to strategies insuring that epidemics of these proportions do not occur elsewhere. GWSS landing and feeding behavior, and tissue feeding capacity combine with grapevine phenology, and *Xf* phenology to make vine to vine spread possible. Particularly important is the tendency for GWSS to move frequently in grapevines, as shown in this study, and their characteristic short hopping flights (Turner and Pollard 1959) that would maximize within-vineyard spread of *Xf*. Increased movement by GWSS in search for optimal host tissue would increase the chance of contact with infected and healthy grapevines alike. Our future studies will compare GWSS feeding in no-choice circumstances, and GWSS flight orientation and behavior when confronted with PD-infected and healthy grapevines. We will also examine the relationship between *Xf* inoculation by GWSS at different times of the year and the development of the vine as a source for further acquisition by GWSS.

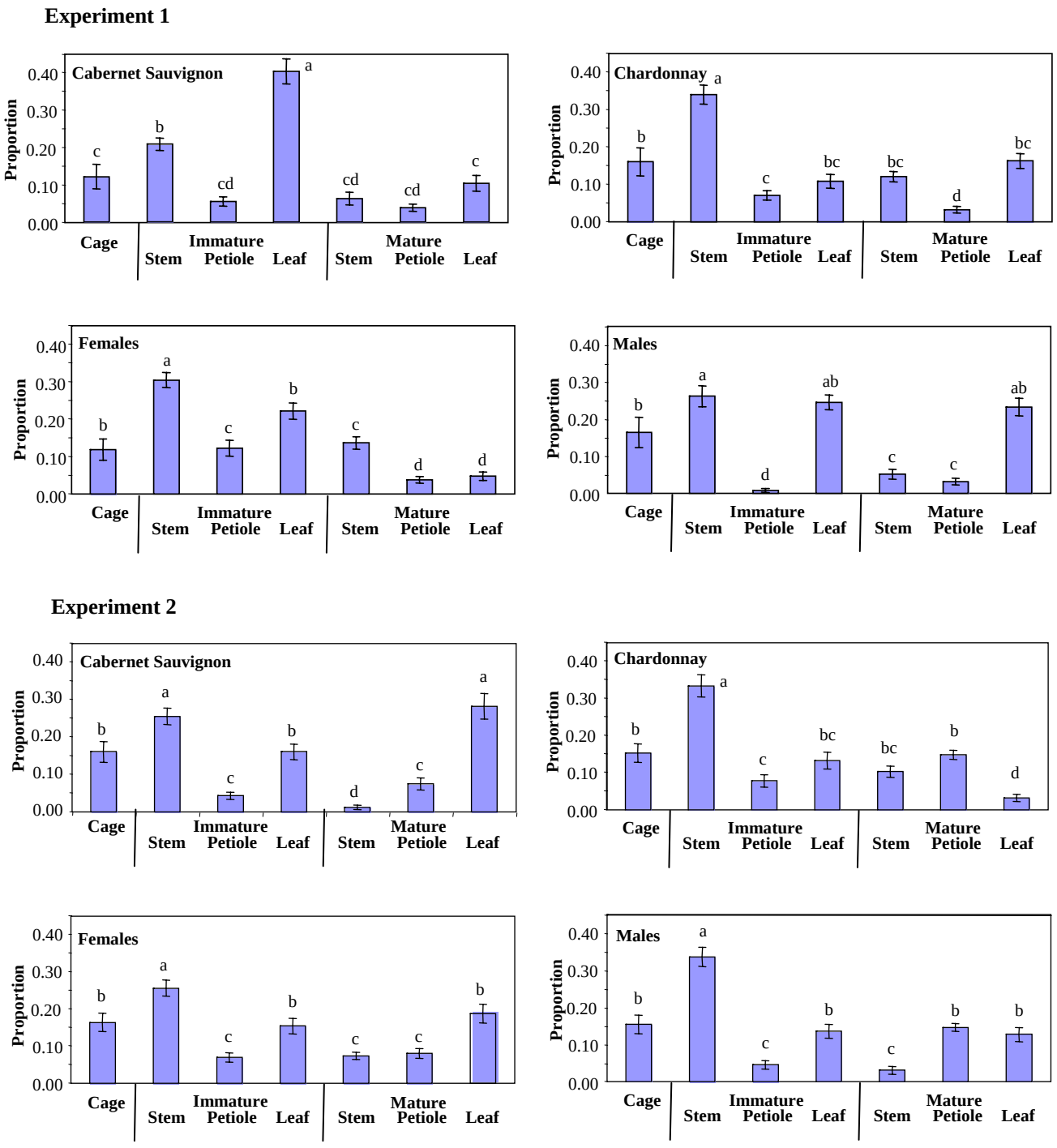

Figure 1. GWSS preference on field-grown Cabernet Sauvignon and Chardonnay grapevines in choice experiments conducted on 29 August 2007 (Experiment 1) and 11 September 2007 (Experiment 2). Bars represent average proportions of GWSS (±SE) observed on various tissue types. Different letters above bars represent statistically significant differences among means at p = 0.05 (ANOVA followed by t-tests for mean separation).

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FUNDING AGENCIES

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IDENTIFYING THE SPECIES OF MYMARIDAE REARED IN ARGENTINA AND MEXICO FOR POTENTIAL INTRODUCTION TO CALIFORNIA AGAINST THE GLASSY-WINGED SHARPSHOOTER AND PREPARING AND SUBMITTING FOR PUBLICATION A PICTORIAL ANNOTATED KEY TO THE *ATER*-GROUP SPECIES OF *GONATOCERUS* – EGG PARASITOIDS OF THE PROCONIINE SHARPSHOOTERS IN THE NEOTROPICAL REGION

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Reporting Period: The results reported here are from work conducted January 1, 2007 to September 30, 2007.

ABSTRACT
At least twenty species of the genus *Gonatocerus* Nees (Mymaridae) were reared and recognized in Argentina and Mexico from eggs of the proconiine sharpshooters (Hemiptera: Cicadellidae: Cicadellinae: Proconiini) in the course of the classical and neoclassical biological control projects against the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (GWSS), in California. During the reporting period, they were identified taxonomically and three of them were described as new species (two from Argentina and one from Mexico). Work is under way to prepare a pictorial, annotated key to more than 60 Neotropical species of the *ater* group of *Gonatocerus*. Results obtained during the last six months of the first year and the first three months of the second year of this two-year project are being reported.

INTRODUCTION
In the new World, eggs of the proconiine sharpshooters, which are known vectors of *Xylella fastidiosa*, are parasitized by various Mymaridae; their natural biological control is mainly due to the beneficial activity of the numerous species of *Gonatocerus*. A key to the Nearctic mymarid egg parasitoids of the proconiine sharpshooters was published recently (Triapitsyn 2006a). Rationale and a more detailed introduction for this project, which will result in publication of an illustrated, annotated key to the Neotropical species of *Gonatocerus*, were given by Triapitsyn (2006b).

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 from eggs of *Homalodisca* and other proconiine sharpshooters (mostly 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. All the available types of the *Gonatocerus* species (70 species), described from the Neotropical region, were located and examined, and digital photographs were taken from them and arranged in plates. All but one (lost) holotypes of the species described by A. A. Ogloblin from Ecuador were remounted into Canada balsam because the original mounting medium was so dark that the specimens were not visible.

Specimen preparation. Due to the enormous volume of the material of *Gonatocerus* from Argentina and Chile (more than 2,000 specimens have already been point-mounted in the course of this project), work on point-and slide-mounting of the specimens, which began in October 2006, will continue until June 2008.

Specimen identification. Morphologically, we recognized at least three more unidentified species among altogether at least 15 species of *Gonatocerus* reared in Argentina by G. Logarzo from eggs of the proconiine sharpshooters. *Gonatocerus* sp. #12, previously misidentified by A. A. Ogloblin as *G. nigriflagellum* (Girault), turned out to be a new species. *Gonatocerus* sp. #1 from Argentina and a similar, yet clearly different species, reared from eggs of *Homalodisca* sp. or *Oncometopia* sp. in Veracruz, Mexico, are also new, undescribed species. *Gonatocerus* sp. #6 from Argentina was described taxonomically in the course of this study (Triapitsyn et al. 2007). Also described was a new species of *Gonatocerus* from Sonora, Mexico, an egg parasitoid of *Homalodisca liturata* Ball (Triapitsyn & Bernal in review). The identities of other species were also figured out, particularly of seemingly the most promising neoclassical biological control agent, *G. tuberculifemur* (Ogloblin) [sp. #7] (Virla et al. 2005), which turned out to be a complex of at least two different species, one of which was described taxonomically as new (Triapitsyn et al. in review). Because *G. tuberculifemur* was originally described from a single, poorly preserved female specimen, G. Logarzo, S. Triapitsyn, and E. Virla made in February 2007 a collecting trip to its type locality in Pucará, at the shore of Lago Lácar in Neuquén Province, Argentina, where *G. tuberculifemur* was collected using sentinel eggs of *Tapajosa rubromarginata* (Signoret) on leaves of a citrus plant. The collected specimens were used to initiate a laboratory colony in Argentina (G. Logarzo) and also for molecular (J. de León) and morphological (S. Triapitsyn) analyses in the USA. The *G. tuberculifemur* complex also includes several morphological forms and molecular clades (de León et al. 2006a,b,c and de León et al. in press), such as *Gonatocerus* sp. #3 from Argentina, whose identity remains to be figured out. *Gonatocerus metanotalis* (Ogloblin) also turned out to be a complex of several molecular clades (de León et al. 2006d), which, however, were not found to differ morphologically. Altogether, more than 2,000 specimens of *Gonatocerus* from South America and Mexico were sorted to morphospecies, compared with the types of the described species, and identified, many as undescribed taxa. The newly collected specimens have been matched with the types of more than 30 described species of *Gonatocerus* from South America, and also more than 30 undescribed species have been recognized, many of which were reared from eggs of the proconiine sharpshooters and thus are of interest to this project as potential neoclassical biological control agents against the GWSS in California.

Progress on Objective 2.

Preparation of the illustrations. High quality digital photographs (arranged in plates, as in Figure. 1) were taken, using an Automontage system, of all the available types of the described *Gonatocerus* spp. from the Neotropical region and also of the new species that will be included in the key, such as an undescribed species from Tamaulipas, Mexico (Figure. 1). Additionally, scanning electron micrographs were taken from some of them to facilitate their recognition and to illustrate some key morphological features.

Preparation of the key. It is under way.

Publications and reports. The project has already resulted in 11 scientific papers and reports that either have been published or submitted for publication to the scientific journals.

CONCLUSIONS

All but one (lost) type specimen of the Neotropical species of *Gonatocerus* were located and examined. High quality digital photographs were taken (and arranged in plates) from the females and, when available, males of all the species of *Gonatocerus* that will be included in the key, including at least ten new, undescribed species. Three of these have already been described. 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 the 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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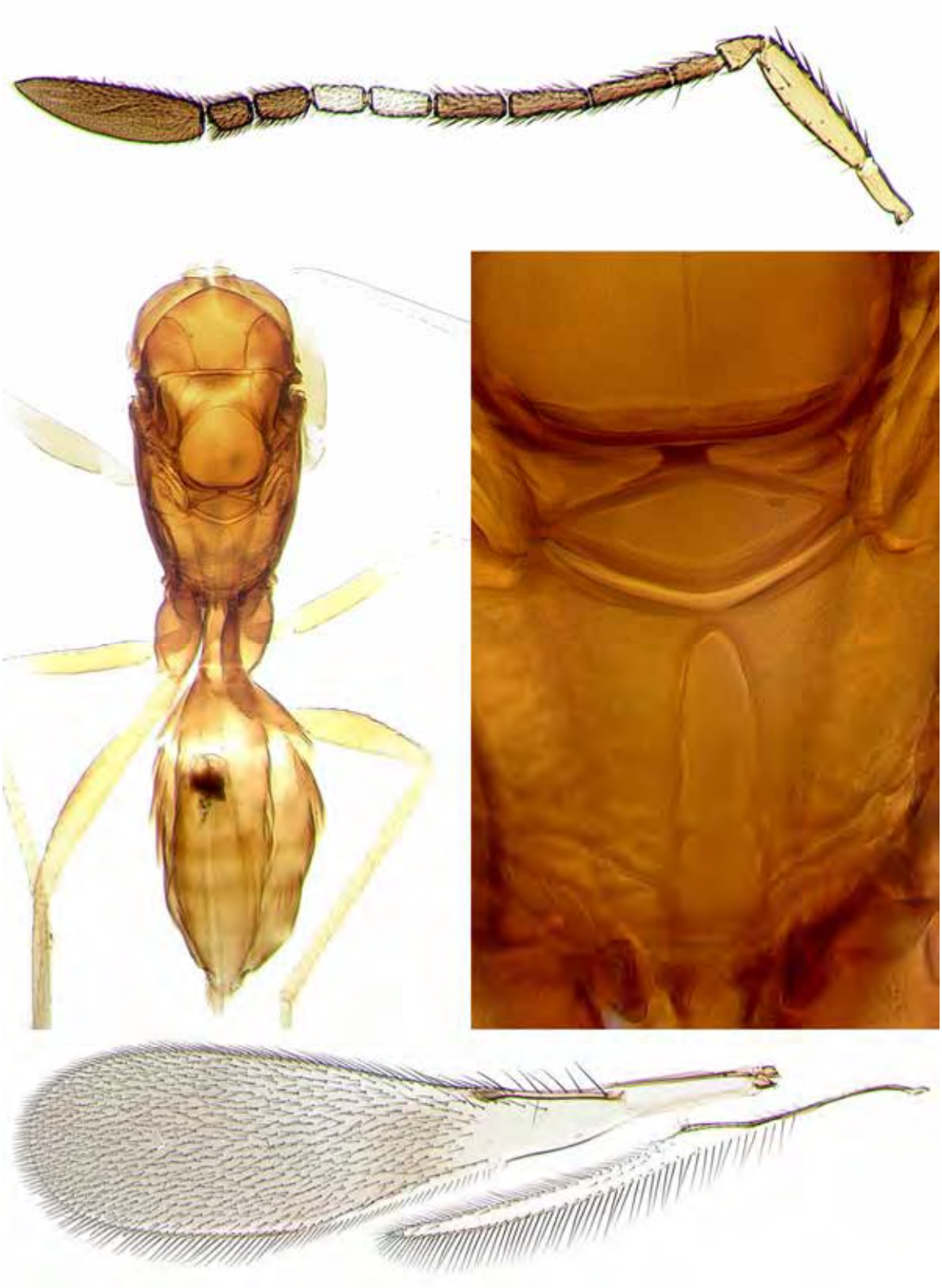

Figure 1. *Gonatocerus* sp. (female) from Tamaulipas, Mexico. Top: antenna; center left: body (without head); center right: dorsellum and propodeum; bottom: wings.

SURVEY OF LEAFHOPPERS ON GRAPES IN THE PIEDMONT AND MOUNTAIN REGIONS OF NORTH CAROLINA

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Reporting Period: The results reported here are from work conducted May 2006 to August 2007.

ABSTRACT

Results of a 2-year study survey of species of leafhoppers found in vinifera vineyards in the Piedmont and Mountain regions of North Carolina are reported. *Graphocephala versuta* and *Agalliota constricta* were the most abundant and ubiquitous leafhoppers. Five species of sharpshooters were found in this survey: *Cuerna costalis, Homalodisca insolita, H. vitripennis, Oncometopia orbona,* and *Paraulacizes irrorata. Homalodisca vitripennis* was found only in one vineyard located in Wake County (Piedmont) in 2007 and this is the first report of this insect in this county.

INTRODUCTION

Vinifera grapes have become economically important in North Carolina (NC). The acreage of vinifera grapes in the Piedmont and Mountains regions of NC doubled from 600 to 1300 acres between 2000 and 2005, respectively; and in 2005 NC ranked 10th in the nation in grape production (MKF Research 2007). However, Pierce's disease (PD) has been increasing rapidly and may be the most important factor limiting the development of the wine industry in NC. In some cases, entire vineyards have been removed and in others, many plants are being removed by the 4th or 5th year. Leafhoppers are vectors of *Xylella fastidiosa* (*Xf*), the bacterium that causes PD. These Hemipterans are found in great numbers in NC; however, the identity of the species found on vinifera grapes, their seasonal abundance and habitats are not well known.

We report a survey of leafhoppers that were collected on four vineyards, two each in the Piedmont (Wake and Alamance Co.) and the Mountains (Polk Co.) of NC. This survey was conducted using yellow sticky traps placed at heights 0.5, 1.0, 1.5 and 2.0 m above the soil in 2006 and at 0.5 and 2 m in 2007. The yellow sticky traps were placed in the vineyards on 10 May and 5 March in 2006 and 2007, respectively. They were replaced every 14 days, 8 November 2006 through 20 September, 2007.

OBJECTIVES

1. Identify the species of the Hemiptera in the families Cicadellidae, Cercopidae, Clastopteridae and Membracidae that are present in vinifera vineyards in North Carolina.
2. Study the seasonal abundance of the most abundant species of leafhoppers present in vineyards.
3. Identify insects that carry *Xf*.

RESULTS

Leafhopper identification is still in progress. We have identified a total of 40 species to date. We have found five large species (length ≥ 13 mm) of sharpshooters (*Oncometopia orbona, Paraulacizes irrorata, Cuerna costalis, Homalodisca insolita* and *H. vitripennis*), 20 medium size species (length between 6 to 13 mm) including cicadellids (*Paraphlepsius irroratus, Graphocephala versuta, G. coccinea, G. hieroglyfica, Scaphytopious frontalis, S. acutus, Japanus hyalinus, Scaphoideus titanus, Gyponana* sp., *Colladonus clitellarius, Agalliota constricta, Stirrelus bicolor, Norvelina seminude, Texananus scultus, Idiononus sp., Mesamia* sp, *Idiocerus sp, Draecucephala antica, D. angulifera, and Penthimia* sp.), 4 membracids (*Atymna* sp., *Spissistilus* sp., *Micrutalis calva, Entylia carinata*), 2 clastopterids (*Clastoptera obtusa* and *C. xanthocephala*.), 1 cercopid (*Prosapia bicincta*) and 7 small species (length < 5 mm) (*Empoasca fabae, Erythroneura vulnerata, E. vitis, E. tricincta, E. ziczac, Illinigina illinoiensis*, and *Graminella* sp.). Many of the small species were previously reported on muscadine grapes (Corrette 1981).

The most abundant leafhopper species collected during 2006 were *G. versuta, A. constricta, P. irroratus, O. orbona.* However, in 2007, *G. versuta, A. constricta, H. insolita and O. orbona* were the most abundant. *Paraphlepsius irroratus* is a carrier of *Xf* (Myers 2005), although its ability to transmit *Xf* has not been established. *Homalodisca insolita* was the most abundant sharpshooter in 2007, but it was found mostly in the lower yellow sticky trap (0.5 m). The glassy winged sharpshooter, *H. vitripennis*, was found only in 2007 in the Wake County vineyard and this is the first report of its presence in this county. Most *H. vitripennis* were collected from the upper yellow sticky traps (2.0 m). The presence of *H. vitripennis* may reflect the unusually warm winter in 2007, because this insect has previously reported only from a vineyard located along the coast of NC, in Currituck Co. and crape myrtle (*Lagerstroemia indica*) in Pender Co.

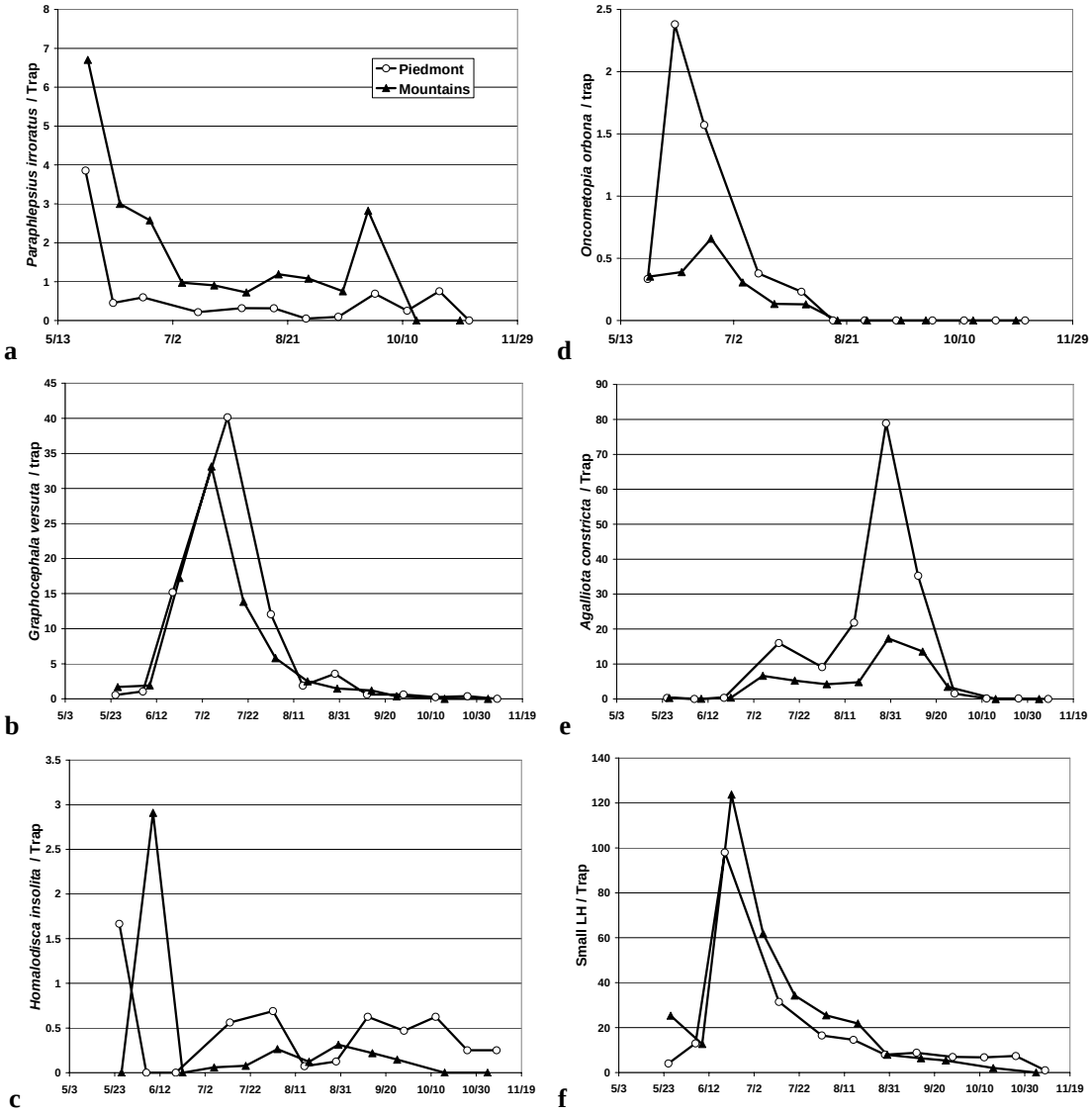

Figure 1. Mean numbers of leafhopper species per yellow sticky trap in the Mountains and Piedmont regions of North Carolina in 2006 (a) *Paraphlepsius irroratus* (b) *Graphocephala versuta*, (c) *Homalodisca insolita*, (d) *Oncomatopia orbona*, (e) *Agalliota constricta*, , and (f) several small species that includes *Empoasca fabae* and *Erythroneura* sp.. Each data point represents the cumulated leafhopper mean numbers for a 2-week period.

CONCLUSIONS

Our data on the leafhopper species present in NC vinifera vineyards provide a framework for identifying which species of leafhoppers are likely to be important in *Xf* transmission. *Graphocephala versuta* and *O. orbona* have been shown to transmit *Xf* in NC (Myers 2005). Previous research has demonstrated that *C. costalis* (Kaloostain, 1962), *H. insolita* (Purcell 1979), and *H. vitripennis* are vectors of *Xf*. The latter species has the ability to disperse long distances, and its presence in Currituck Co. and Wake Co. may reflect the unusually warm winter in 2007. If we continue to experience mild winters due to global warming, *H. vitripennis* may spread further north and west in NC. Other species such as *P. irroratus* may be important in the spread of *Xf*. To identify other potential vectors of *Xf*, specimens of each species have been collected and stored at ~ 1◦ C; they will be subjected to PCR analysis to test for the presence of *Xf* DNA. In 2008 we are planning to continue the survey and study the life history, host plants, and habitat preferences of the most abundant leafhoppers in vinifera vineyards in NC. We will also investigate potential pest management programs for leafhoppers in vinifera grapes.

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Section 2:

Vector Management

IMPORTATION OF PARASITOIDS OF *HOMALODISCA* AND OTHER PROCONIINI GENERA FROM NORTHWESTERN MEXICO FOR BIOLOGICAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

Nine species of Mymaridae and Trichogrammatidae parasitic on eggs of Proconiini sharpshooters were collected in northwestern Mexico in relation to biological control of glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) in California. These included an unidentified (likely new) species of *Gonatocerus* Nees (Mymaridae), an egg parasitoid reared from Proconiini eggs in Sonora state, Mexico. The other species, also reared from Proconiini eggs in Sonora and Sinaloa, included *Gonatocerus atriclavus* Girault, *Gonatocerus morrilli* (Howard), and *Gonatocerus novifasciatus* Girault, and the trichogrammatids *Burksiella* sp(p)., *Paracentrobia* sp., *Pseudoligosita* sp., *Ufens ceratus* Owen, and *U. principalis* Owen. Colonies of *Gonatocerus atriclavus*, *Gonatocerus novifasciatus* and *Pseudoligosita* sp. were successfully established in the quarantine laboratory of the Department of Entomology, University of California, Riverside (UCR), on eggs of the glassy-winged sharpshooter.

INTRODUCTION

Species of Mymaridae and Trichogrammatidae are common parasitoids of Proconiini sharpshooter eggs, such as glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)]. GWSS is native to the southeastern U.S. and northeastern Mexico, and became established in California ca. 1990 (Sorensen and Gill 1996). The economic importance of GWSS stems mostly from its efficiency as a vector of *Xylella fastidiosa*, which is the causal agent of Pierce's disease in grapes, among other important diseases. Substantial research emphasis has been placed thus far on importation biological control of GWSS. To date, two approaches to importation biological control of GWSS in California are being followed. The "classical" approach of reuniting an exotic pest, such as GWSS, with its coevolved natural enemies from the pest's area of origin is being explored via importation of parasitoids from the southeastern U.S. and northeastern Mexico. The "neoclassical" approach of importing non-coevolved natural enemies (i.e. parasitic on closely related host species) against exotic pests is being explored via importation of parasitoids from Minnesota (*Anagrus epos* Girault), southeastern Mexico, and Argentina.

The long-term goal of the activities described herein was to contribute to neoclassical biological control efforts against GWSS, through importation of natural enemies from central- and north-western Mexico. At least six species of Proconiini were known from those areas, though it was doubtful that GWSS occurred there (MacGregor and Gutiérrez 1983, Pacheco Mendivil 1985, Takiya 2006). A neoclassical approach against GWSS was considered promising because efforts to date have not uncovered effective natural enemies in the pest's native range, and because closely related host species occur in central- and north-western Mexico. This report presents the results of activities aimed at surveying and collecting egg parasitoids of Proconiini in the Mexican states of Colima, Jalisco, Nayarit, Sinaloa, and Sonora between July 2006 and June 2007.

OBJECTIVES

1. Import to California via UC Riverside quarantine parasitoids of *Homalodisca* spp. and other Proconiini from the Mexican states of Jalisco, Nayarit, Sinaloa, and Sonora.
2. Systematically document the parasitoid fauna associated with *Homalodisca* spp. and other Proconiini genera in the Mexican states of Jalisco, Nayarit, Sinaloa, and Sonora.

RESULTS

Proconiini collections

Adult specimens of seven Proconiini species were collected and identified from the states of Colima, Jalisco, Sinaloa, and Sonora. These included *Cyrtodisca major* (Signoret), *Homalodisca insolita* (Walker), *Phera centrolineata* (Signoret),

Homalodisca liturata Ball, and three unidentified species of *Oncometopia* Stål. Adult Proconiini were not collected in Nayarit.

Egg masses of Proconiini were shipped to UCR from Sinaloa and Sonora, and these were of *Oncometopia* sp., or *Homalodisca liturata*, respectively. Egg masses were not found in the states of Jalisco, Colima, and Nayarit.

Parasitoids of Proconiini eggs

Mymaridae

Gonatocerus sp.*:* One unidentified species of *Gonatocerus* was reared from eggs of Proconiini collected in Sonora. This is likely a new species of solitary parasitoid, and its taxonomic description will be published separately.

Gonatocerus atriclavus Girault: This species was reared from eggs of Proconiini collected in Sinaloa. *Gonatocerus atriclavus* is being reared under laboratory conditions on GWSS eggs on *Euonymus japonica* leaves. It is a solitary parasitoid, producing one adult per each host egg.

Gonatocerus morrilli (Howard): This species was reared from eggs of Proconiini collected in Sonora. It is a common, solitary egg parasitoid of various Proconiini in the southern U.S., and Mexico**.**

Gonatocerus novifasciatus Girault: This species was reared from eggs of Proconiini collected in Sonora. *Gonatocerus novifasciatus* was being reared under laboratory conditions on GWSS eggs on *E. japonica* leaves, though was discontinued after only males were obtained in the F2 generation. It is a solitary parasitoid.

Trichogrammatidae

Burksiella sp(p).: One or several species of *Burksiella* were reared from eggs of Proconiini collected in Sinaloa, and Sonora. This (or these) species are solitary parasitoids. Specific identifications are pending.

Paracentrobia sp.: This species was collected from eggs of Proconiini collected in Sonora. At least two other congeneres in the Nearctic region are parasitic on Proconiini eggs (Triapitsyn 2003; Tipping et al. 2005). Specific identification is pending.

Pseudoligosita sp.: This species was collected from Proconiini eggs in Sonora. *Pseudoligosita* sp. is being reared under laboratory conditions on GWSS eggs on *E. japonica* leaves. *Pseudoligosita* sp. is a gregarious parasitoid, producing two to four adults per each egg of GWSS. Specific identification is pending.

Ufens ceratus Owen: This species was reared from eggs of Proconiini collected in Sonora. *Ufens ceratus* is a common parasitoid of GWSS eggs in southern California, and of eggs of other proconiine sharpshooters in Mexico and the U.S. (Al-Wahaibi et al. 2005).

Ufens principalis Owen: This species was collected from eggs of Proconiini collected in Sonora. *Ufens principalis* is a common egg parasitoid of *Homalodisca liturata* in southern California, and of GWSS in California (Al-Wahaibi et al. 2005).

CONCLUSIONS

At least seven species of Proconiini were found in the central- and north-western states of Colima, Jalisco, Nayarit, Sinaloa, and Sonora between July 2006 and July 2007: *Cyrtodisca major, Homalodisca insolita, Phera centrolineata, Homalodisca liturata,* and three species of *Oncometopia*. Proconiini egg masses were found and shipped to UCR from Sinaloa and Sonora. These egg masses yielded at least nine species of Mymaridae and Trichogrammatidae parasitoids: *Gonatocerus* sp., *Gonatocerus atriclavus, Gonatocerus morrilli,* and *Gonatocerus novifasciatus*, and *Burksiella* sp(p)., *Paracentrobia* sp., *Pseudoligosita* sp., *Ufens ceratus,* and *Ufens principalis*, respectively. Colonies of *Gonatocerus atriclavus, Gonatocerus novifasciatus* and *Pseudoligosita* sp. were successfully established UCR quarantine on eggs of GWSS.

Discovery of apparently new species of both Proconiini sharpshooters and parasitoids of their eggs, and successful rearing of three parasitoid species by the midpoint of the project's tenure is encouraging because observations and experiences to date will facilitate further searching in Mexico and successful colonization at UCR quarantine. Search efforts in 2007-2008 will focus on Colima, Jalisco, and Nayarit states, where egg masses have to date not been found, and intensive re-sampling of sites and host plants at localities in Sinaloa and Sonora states, where Proconiini and parasitoids have thus far been found. Efforts will continue at UCR quarantine to rear any novel parasitoids received from Mexico, and maintain existing parasitoid cultures.

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EVALUATION OF THE IMPACT OF NEONICOTINOID INSECTICIDES ON THE GLASSY-WINGED SHARPSHOOTER AND ITS EGG PARASITOIDS

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

ABSTRACT
A study was conducted to determine the toxic effects of systemic imidacloprid against the egg stage of the glassy-winged sharpshooter, *Homalodisca vitripennis* (*H. vitripennis*), and its mymarid egg parasitoid *Gonatocerus ashmeadi* (*G. ashmeadi*). During the development of the sharpshooter embryo within the egg, imidacloprid did not have a lethal effect, even at very high concentrations. However, upon emergence from the egg mass, the first instar nymph encountered residues of imidacloprid in the surrounding leaf tissues. There was an excellent dose-response between the imidacloprid concentration and emerging nymph mortality, giving an LC_{50} of 39 ng imidacloprid/cm^2 leaf. In the same way, *G. ashmeadi* did not succumb to toxic levels of imidacloprid during its development within the sharpshooter egg. However, parasitoid adults were sensitive to imidacloprid residues during emergence from the sharpshooter egg. The LC_{50} for parasitoid mortality was 66 ng imidacloprid/cm^2 leaf. In a survey of commercial citrus trees that were treated with imidacloprid, the mean residues of the insecticide within the leaves did not exceed the LC_{50} concentrations for either insect.

INTRODUCTION
In a previous study, imidacloprid was shown to be toxic to sharpshooter nymphs, but only during emergence from the egg (Byrne and Toscano, 2005). During development, the imidacloprid did not penetrate the egg membrane at sufficient levels to cause toxicity. In this study, we are continuing to evaluate the effects of the neonicotinoids against the sharpshooter nymphs and its egg parasitoid, *G. ashmeadi*. Neonicotinoid insecticides are the most widely used insecticides on citrus for the area-wide management of the glassy-winged sharpshooter. Dose-response data are being generated from bioassays to indicate threshold residue levels of insecticides that are lethal to nymphs and parasitoids as they emerge from the sharpshooter egg.

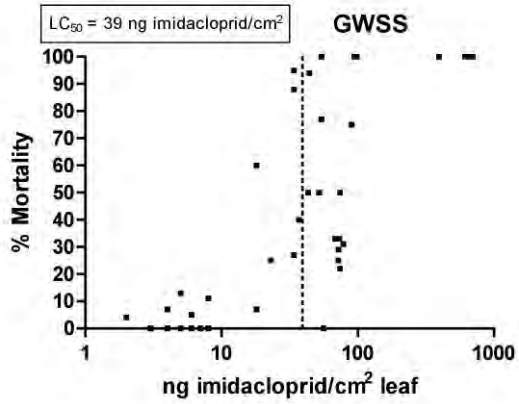
OBJECTIVES
1. Determine the relative toxicities of neonicotinoids (imidacloprid, thiamethoxam and dinotefuran) to the adult and egg stages of the glassy-winged sharpshooter.
2. Determine the impact of neonicotinoids (imidacloprid, thiamethoxam and dinotefuran) on egg parasitoids of the glassy-winged sharpshooter.

RESULTS
The uptake of imidacloprid into excised cotton leaves was variable and ensured a broad range of concentrations to determine its impact against the developing embryo and emerging first instar *H. vitripennis*. Even at the highest concentrations of imidacloprid (almost 1 µg imidacloprid per cm^2 leaf), there was no mortality of the developing embryo. The only mortality that occurred was during the emergence of the first instar from the egg mass. At the highest concentrations, the first instars died as soon as they broke through the egg sac. At lower doses, many of the nymphs emerged half way through the egg sac before succumbing to insecticide. There was an excellent dose-response, resulting in an LC_{50} of 39 ng imidacloprid per cm^2 leaf (Figure 1).

We observed a similar pattern of mortality in bioassays with the parasitoid. As with the sharpshooter nymphs, there was no toxic effect on the developing parasitoid within the confines of the sharpshooter egg. However, once the insect began to emerge, it encountered insecticide and there was a dose-response between the concentration of imidacloprid and mortality. The LC_{50} for the adult *G. ashmeadi* was 66 ng imidacloprid per cm^2 leaf, indicating its greater tolerance to the insecticide (Figure 1).

Imidacloprid concentrations were measured in the xylem and leaf tissues of citrus trees treated with 2.34 l ha^{-1} of 240 g imidacloprid liter^{-1} SC (Figure 2). There was a steady increase in xylem fluid levels of imidacloprid up to 40 days after the application. At this time, the titers remained steady, and only declined after 110 days. Residues of imidacloprid within leaves were measured at 60 days after the initial treatment when the imidacloprid concentration within the xylem system had stabilized (Figure 2). The level of variation between the four trees was not significant ($F_{3,40} = 0.77$, $P = 0.52$). The apparent differences between the imidacloprid concentrations in leaves sampled from the north and south sides of the trees were also not significant ($F_{1,40} = 2.30$, $P = 0.14$). The combined leaf residue data for the four trees was used to generate a frequency

distribution curve (Figure 3), which showed more clearly that the residues present within most of the leaves were lower than the LC_{50} values for the two insects.



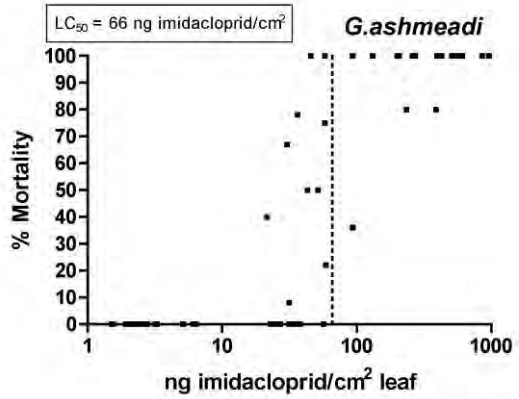


Figure 1. Dose response for GWSS nymphs and *G. ashmeadi* adults as they emerge from sharpshooter eggs that developed on imidacloprid-treated leaves. Data for the GWSS is copied from Byrne and Toscano (2005), and is included to show comparative toxicity of imidacloprid to emerging nymphs and parasitoid adults.

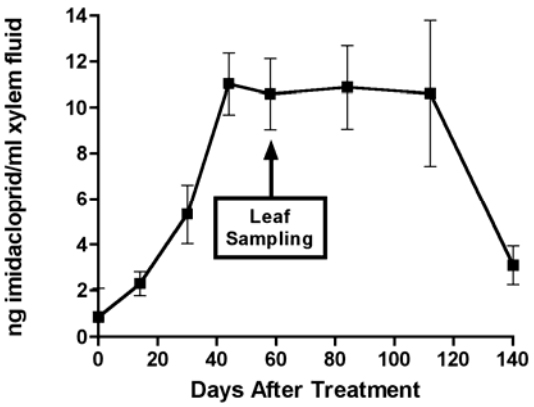


Figure 2. Temporal profiles of imidacloprid concentrations within the xylem fluid of citrus trees treated with 240 g l^{-1} imidacloprid. Each point represents the mean (± SEM) imidacloprid concentration in extracts from 12 trees. The arrow indicates the day on which leaves were sampled from four of the trees to determine the leaf concentrations of imidacloprid.

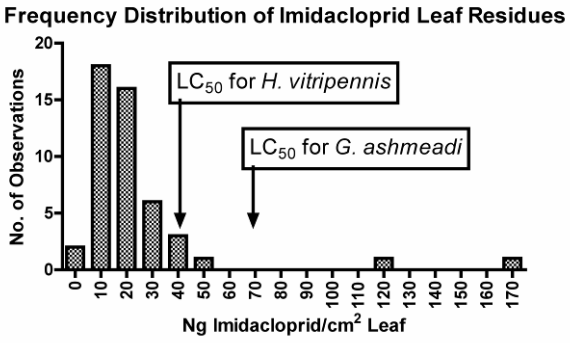


Figure 3. Frequency distribution of imidacloprid concentrations in citrus leaves sampled from trees treated with 240 g^{-1} l imidacloprid. The arrows indicate the LC_{50} values for *H. vitripennis* and *G. ashmeadi*.

CONCLUSIONS

In this study, we have provided further evidence that imidacloprid residues within leaves do not penetrate through the chorion of the sharpshooter egg. There was, however, a toxic effect on the emerging first instar nymph, which was most dramatic at higher insecticide concentrations within the leaves. Imidacloprid residues within the leaves had a similar effect against parasitoids. All parasitoids developed fully to the adult stage, and only upon emergence did they encounter insecticide residues. As with the emerging sharpshooter nymphs, there was a dose response between the levels of parasitoid mortality and imidacloprid concentrations present within the leaves.

In the treated citrus trees (Figures 2 and 3), measurements of imidacloprid were taken from leaves when the insecticide levels within the xylem system had stabilized. The distribution of imidacloprid levels within the leaves (Figure 3) clearly showed that the residues present would not have a dramatic effect on the emergence of either the sharpshooter nymph or the parasitoid. However, nymph populations have been shown to decline on treated citrus when concentrations of imidacloprid

were as low as 3 ng/ml xylem fluid (Castle et al., 2005). Therefore, the greater impact of the insecticide treatment would occur on the sharpshooter nymphs when they began to feed on the xylem fluid since the imidacloprid concentrations were above threshold levels necessary to induce mortality (Figure 2).

G. ashmeadi is an important parasitoid of the glassy-winged sharpshooter. The success of this parasitoid against the sharpshooter has highlighted the importance of biological control in the management of the sharpshooter (Triapitsyn et al., 1998). There is much interest in enhancing biological control through the search and release of additional egg parasitoids, both *Gonatocerus* species and others (Hoddle and Triapitsyn, 2003, 2004; Morgan et al., 2000; Morse and Stouthamer, 2005). The success of these efforts could depend in part upon the impacts that insecticide treatments have on their survival. One of the primary objectives of the release program is to enhance the impact of biological control during the early part of the year when the first generation is developing. However, it is at this time that the first systemic treatments of imidacloprid are applied. It is important, therefore, that the interaction between the two management systems is fully understood, so that the benefits of both systems can be maximized. Our data show that there is great potential for the integration of the two systems – imidacloprid has no toxic effect on the sharpshooter embryo during its development within the egg, ensuring that the parasitoid will be able to develop to the adult stage. The greater tolerance of the parasitoid to the insecticide will further ensure that the leaf residues of insecticide will have a lesser impact on its survival during emergence.

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PROGRESS ON RESOLVING THE *GONATOCERUS TUBERCULIFEMUR* COMPLEX: NEITHER COI NOR ITS2 SEQUENCE DATA ALONE CAN DISCRIMINATE ALL THE SPECIES WITHIN THE COMPLEX, WHEREAS, ISSR-PCR DNA FINGERPRINTING CAN

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Reporting Period: The results reported here are from work conducted fiscal year 2006 to 2007.

ABSTRACT

We utilized two molecular methods to aid in resolving the *Gonatocerus tuberculifemur* (*G. tuberculifemur*) complex, potential glassy-winged sharpshooter (GWSS) biological control candidate agents from South America. The two methods used were DNA sequencing of both the mitochondrial cytochrome oxidase subunit I gene (COI) and the ribosomal internal transcribed spacer region 2 (ITS2) and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting. COI sequence variation, as shown previously, was able to discriminate *G. tuberculifemur* individuals belonging to clades 1 and 2, but *G. tuberculifemur* individuals from clade 2 were not discriminated from the *G. tuberculifemur* specimens collected from the type locality (Pucará) or *G*. sp 3. *G. tuberculifemur* individuals emerging from *Hortensia similis* (*H. similis*) (Cicadellini leafhopper) formed a new clade (Y). On the other hand, ITS2 rDNA sequence data could not discriminate *G. tuberculifemur* individuals belonging to clades 1 and 2. However, ITS2 was able to discriminate *G*. sp 3 and *G. tuberculifemur* specimens from the type locality (Pucará), forming two new clades, Z and X, respectively. Specimens emerging from *H. similis* were also discriminated by ITS2 analysis. Interestingly, the separation of all of the species or strains within the *G. tuberculifemur* complex was accomplished by ISSR-PCR DNA fingerprinting. No single gene (COI and ITS2) sequenced was able to discriminate all of the species within the *G. tuberculifemur* complex. Based on the current data, it appears that there could be four species within the *G. tuberculifemur complex*. 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

The GWSS, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) that invaded California (CA) USA have their origins in Texas USA (de León et al. 2004), an observation that was later confirmed by Smith (2005). GWSS is a serious economic pest that poses a serious threat to the wine and table industry, therefore, a biological control program is in place to try to control this devastating pest (CDFA 2003). Currently, our research is focused on developing a neoclassical biological control program against GWSS in CA (de León et al. 2006a,b,c,d, 2007, Triapitsyn et al. 2007a,b). Egg parasitoids of closely related hosts belonging to the sharpshooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to CA (Jones 2001, Logarzo et al. 2003, 2004, 2005). Prospective egg parasitoid candidate agents were identified by S. Triapitsyn (UC-Riverside) among several *Gonatocerus* Nees species (Hymenoptera: Mymaridae) reared from *T. rubomarginata* (Logarzo et al. 2005, Virla et al. 2005), including specimens emerging from a different host tribe (Cicadellini). Among the species identified was *Gonatocerus tuberculifemur* (Ogloblin), one of the most promising biological control agents from South America because of its unique climate match to CA and not to the southeastern U.S., as it would not be predicted to migrate to the southeastern U.S. and attack non-target native leafhoppers. This is very important because it can reduce the risk factors of releasing this egg parasitoid in CA. However, it appears that *G. tuberculifemur* is part of a species complex involving several species [e. g., *G. tuberculifemur* clades 1 and 2 (de León et al. 2006a,d, 2007, Triapitsyn et al. 2007b), *G. tuberculifemur* collected from the type locality (Pucará, Neuquen Province, Argentina), *G. tuberculifemur*-like individuals emerging from a different host tribe (e. g., *Hortensia similis*), and *G*. sp 3 (de León et al. 2006b)].

OBJECTIVES

The aim of the present study was to continue resolving the *G. tuberculifemur* complex. Two molecular methods, ISSR-PCR DNA fingerprinting and DNA sequencing of both the mitochondrial cytochrome oxidase subunit I gene (COI) and the ribosomal internal transcribed spacer region 2 (ITS2) were utilized and compared. Included in the analyses were *G. tuberculifemur* individuals belonging to both 'clade 1' and 'clade 2' (de León et al. 2006a,d, 2007), *G*. sp 3 (de León et al. 2006b), *G. tuberculifemur*-like individuals emerging from *H. similis* (Cicadellini leafhopper); and *G. tuberculifemur* individuals collected from the type locality (Pucará, Neuquén Province, Argentina). Included in the analyses to provide phylogenetic support were several species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus* (de León et al. 2007, Triapitsyn 2006).

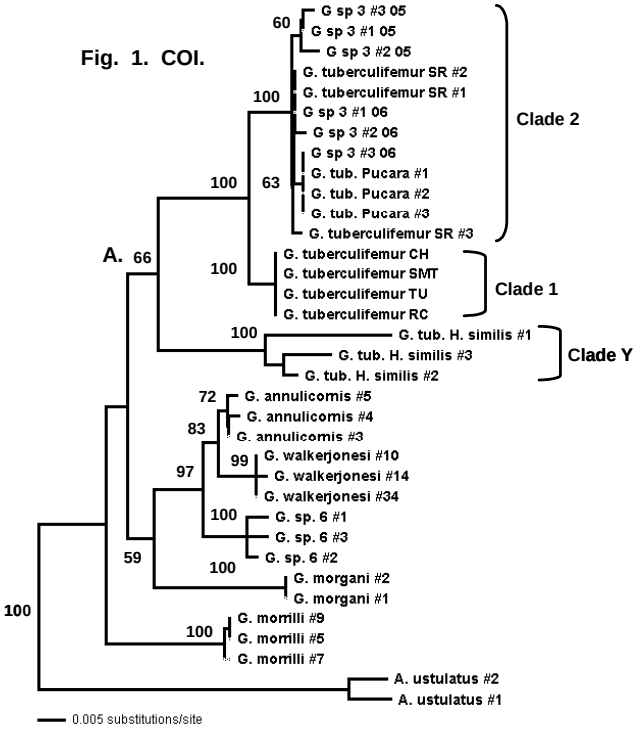
RESULTS AND CONCLUSIONS

Phylogenetic analysis of individuals belonging to the* G. tuberculifemur *complex inferred from COI sequence data

Ten *Gonatocerus* species (or strains) were included in this study. A total of 33 ingroup specimens were analyzed and two specimens from *Anagrus ustulatus* (Haliday) (also a mymarid species) were included as an outgroup. Many species formed their own taxonomic unit or distinct clade, confirming the species boundaries shown recently, including the two clades (1 and 2) of *G. tuberculifemur* (de León et al. 2006c,d, 2007, Triapitsyn 2006) (Figure 1). In addition, a new clade (Y) was observed in *G. tuberculifemur* individuals emerging from the host *H. similis*, suggesting that clade Y individuals are a valid species. However, COI sequence variation was unable to discriminate *G. tuberculifemur* clade 2 individuals (San Rafael) from those of *G*. sp 3, as shown previously (de León et al. 2006b), and *G. tuberculifemur* (G. tub Pucará) individuals collected from the type locality (Pucará). The current data also suggests that individuals from the *G. tuberculifemur* complex (main clade 'A') are related or could belong to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. Very strong bootstrap support (100%) was seen within the clades (1, 2, and Y) of the *G. tuberculifemur* complex.

Phylogenetic analysis of individuals belonging to the* G. tuberculifemur *complex inferred from ITS2 sequence data

The results of this analysis are shown on Figure 2. Again, the species boundaries of many of these species utilizing the ITS2 rDNA fragment were confirmed (de León et al. 2006c), with very strong support (96-100%). Clade Y (G. tub *H. similis*) was again observed based on ITS2 phylogenetic analysis, confirming the results of the COI analysis. ITS2 sequence variation was able to discriminate individuals from both *G*. sp 3 (clade Z) and G. tub Pucará (clade X). However, ITS2 was unable to discriminate individuals of *G. tuberculifemur* belonging to clades 1 and 2. This is an interesting observation because COI sequence data and ISSR-PCR DNA fingerprinting were both able to discriminate these two clades (de León et al. 2006d, 2007); in addition, preliminary cross-mating data suggest that individuals from the two clades of *G. tuberculifemur* are reproductively incompatible (de León et al. 2006a). Furthermore, morphological analyses uncovered some differences between the two clades [Triapitysn et al. 2007 (submitted)]. Taken together, all of these data highly suggest that the individuals of *G. tuberculifemur* belonging to the two clades are valid species.



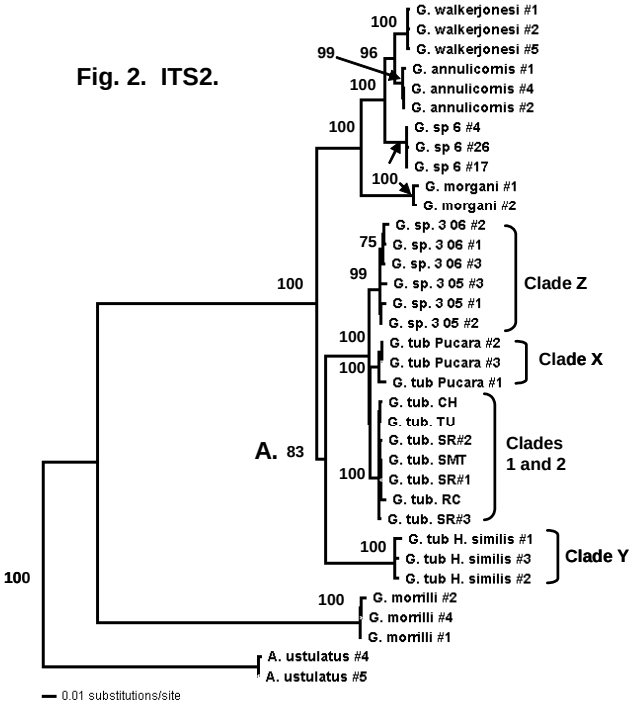


Figure 1 (COI) and **Figure 2** (ITS2) show phylograms of egg parasitoid species belonging to the *G. tuberculifemur* complex inferred from COI and ITS2 sequence data, respectively. Analyses were performed with the alignment program ClustalX and the neighbor-joining distance trees utilizing the uncorrected 'p' genetic distance were reconstructed with the phylogenetic program PAUP 4.0b10 for Macintosh (PPC). The trees display bootstrap values, as percentage of 1000 replications. Collections of *G. tuberculifemur* (G. tub) were from: San Rafael (SR) (Mendoza Province), these individuals belong to 'clade 2' (de León et al. 2006d, 2007). RC, Rio Colorado (Rio Negro); SMT, San Miguel de Tucumán (Tucumán); TU, Tunuyán (Mendoza); and CH, Chile; these individuals belong to 'clade 1' (de León et al. 2006d, 2007). *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)] (de León et al. 2006b). *G. tuberculifemur*-like individuals emerged from *H. similis* (Cicadellini leafhopper) (G. tub *H. similis*); the rest emerged from *T. rubromarginata*. *G. tuberculifemur* individuals from Pucará (G. tub Pucará) are from the type locality. Main clade 'A' are individuals from the *G. tuberculifemur* complex.

ISSR-PCR DNA fingerprinting of individuals belonging to the G. tuberculifemur complex

The results of this experiment are shown on Figure 3. ISSR-PCR uncovered fixed banding pattern differences in all of the species or strains belonging to the *G. tuberculifemur* complex: G. tub Pucará (clade X); G. tub *H. similis* (clade Y); results for *G*. sp 3 (clade Z) are shown elsewhere (de León et al. 2006b); and *G. tuberculifemur* clades 1 and 2, shown previously (de León et al. 2006d, 2007). Not sharing bands among species is usually an indication of reproductive isolation. Cross-mating studies and morphological analyses are in progress to confirm the current molecular results.

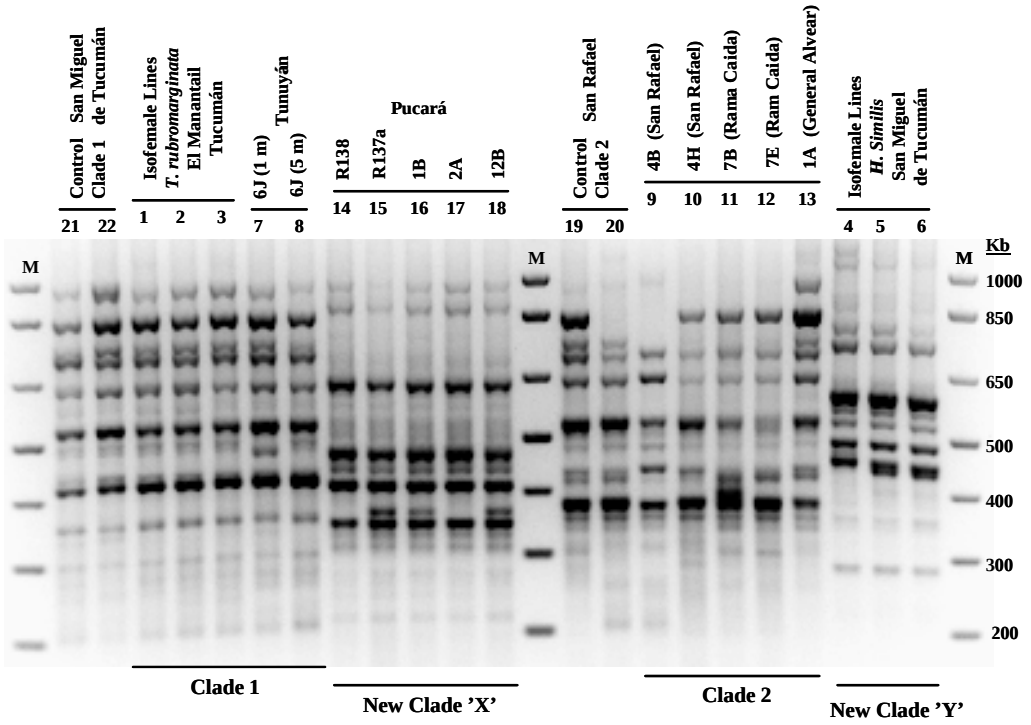


Figure 3. ISSR-PCR DNA fingerprinting of various species within the *G. tuberculifemur* complex. Reactions were performed with total genomic DNA from 2-5 separate individuals and a 5'-anchored ISSR primer (de León et al. 2004, 2007). Specimens from San Miguel de Tucumán and El Manantial are from Tucumán Province; Pucará individuals are from Neuquén Province (type locality); and San Rafael, Rama Caida, and General Alvear individuals are from Mendoza Province. *G*. sp. 3 specimens are not included [see de León et al. 2006b]. *G. tuberculifemur*-like individuals emerged from *H. similis*, a different host tribe (Cicadellini); the rest emerged from *T. rubromarginata* (Proconiini host tribe). **M**, 1.0-Kb Plus DNA Ladder.

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DEVELOPMENT OF MOLECULAR MARKERS TOWARD GLASSY-WINGED SHARPSHOOTER (GWSS) EGG PARASITOIDS TO AID IN BOTH IDENTIFYING KEY PREDATORS OF THE GWSS EGG STAGE AND IN TAXONOMIC IDENTIFICATIONS

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Reporting Period: The results reported here are from work conducted fiscal year 2006 to 2007.

ABSTRACT

To aid in identifying key predators of the glassy-winged sharpshooter (GWSS) egg stage, we developed 'one-step' molecular diagnostic markers specific toward the internal transcribed spacer 2 region (ITS2) of five primary GWSS egg parasitoid species belonging to the genus *Gonatocerus* Nees. In addition, these markers will also serve to aid taxonomic identifications, especially before the egg parasitoids emerge from GWSS egg masses. The molecular markers were designed toward the following species: *G. triguttatus* (Gtri-F/R); *G. walkerjonesi* (wjca-F2/R); *G. morrilli* (gmtx-F/R); *G. ashmeadi* (Gash-F/R); and *G. morgani* (Gmorg-F/R). Cross-reactivity assays with 21 *Gonatocerus* species, each with specific assay conditions, demonstrated that the developed markers were highly specific toward the species that they were designed for, as cross-reactivity was not observed with non-target species. One of the markers (Gtri-F/R) was tested in predator gut content assays. Positive reactions were observed in gut assays in which lacewings were allowed to feed on parasitized (*G. triguttatus*) GWSS eggs. In addition, positive banding was also seen with GWSS parasitized eggs alone, that is before the egg parasitoids emerged from the GWSS egg masses. The current results confirm the specificity and utility of the various one-step molecular diagnostic markers. In addition to the predation studies, these markers should be useful to the biological control program.

INTRODUCTION

The glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] (Hemiptera: Cicadellidae) was reported in California (CA) USA in 1989 (Sorenson and Gill 1996) and since then it has spread throughout CA (Blua et al. 2001), posing a serious threat to the wine and table grape industry. The GWSS that invaded CA have their origins in Texas USA (de León et al. 2004), an observation that was later confirmed by Smith (2005). A biological control program has been put into place to try to control this devastating pest (CDFA 2003). To effectively control GWSS, an area-wide pest management approach is required. However, limited knowledge is available pertaining to the GWSS generalist predator complex. At present, the two most common methods used to study predation by gut content analysis are immunoassasys (ELISAs) (Hagler 1998; Fournier et al. 2006, 2007) that detect prey-specific proteins and polymerase chain reaction (PCR)-based markers targeting prey DNA (Agustí et al. 2003; Greenstone and Shufran 2003; de León et al. 2006; Fournier et al. 2006, 2007). Furthermore, some prey specific ELISAs are also life-stage specific, for example, toward the egg stage of GWSS (Hagler et al. 1991, 1993; Fournier et al. 2006). Combing this type of ELISA with a PCR-based analysis could provide a more accurate definition of a predation event (Fournier et al. 2007).

OBJECTIVES

Our research is in the initial stages. We plan to identify key predators of the GWSS egg stage [e.g., targeted (effective) biological control] and GWSS parasitoid complex [e.g., non-targeted (interference) biological control]. We will also identify and quantify egg parasitism for several of the key native and exotic GWSS parasitoid species. Another objective of our research is to aid in taxonomic identifications, a request from the CDFA (D. Morgan). Our first immediate goal is to develop 'one-step' molecular diagnostic markers toward the internal transcribed spacer region 2 (ITS2) of several Gonatocerus species (5): *G. triguttatus* (Girault), *G. walkerjonesi* S. Triapitsyn; *G. morrilli* (Howard); *G. ashmeadi* (Girault); and *G. morgani* S. Triapitsyn (Hymenoptera: Mymaridae). Our second immediate goal is to begin to test the utility of the developed markers by examining the gut contents of predators (lacewings) feeding on parasitized (*G. triguttatus*) GWSS egg masses. Along with these DNA markers, future studies will involve using an established GWSS-egg specific ELISA (Hagler et al. 2003; Fournier et al. 2006).

RESULTS AND CONCLUSIONS

Development and specificity of the 'one-step' species-specific molecular markers directed toward the ITS2 rDNA fragments

ITS2 rDNA sequences from several *Gonatocerus* Nees species (21) from both North and South America were aligned with the program DNAStar (DNAStar, Inc; Madison, WI) using the ClustalW algorithm (Higgins et al. 1994). Five primer sets were designed that discriminated the species of interest from each other and from the rest of the *Gonatocerus* species,

including the most important species present in California. To determine the specificity of the five molecular diagnostic markers, we tested specific amplification assay conditions and screened a total of 21 *Gonatocerus* GWSS egg parasitoid species for cross-reactivity. Figure 1: **A**, Gtri-F/R (*G. triguttatus*); **B**, wjca-F2/R (*G. walkerjonesi*); **C**, gmtx-F/R (*G. morrilli*); **D**, Gash-F/R (*G. ashmeadi*); and **E**, Gmorg-F/R (*G. morgani*) shows that all of the developed markers amplified DNA fragments of the correct size, and all were highly specific as cross-reactivity with the 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* (Triapitsyn 2006). Markers for *G. morrilli* and *G. walkerjonesi* have recently been reported (de León and Morgan 2007), but the current study expands on those results.

Fig. 1.

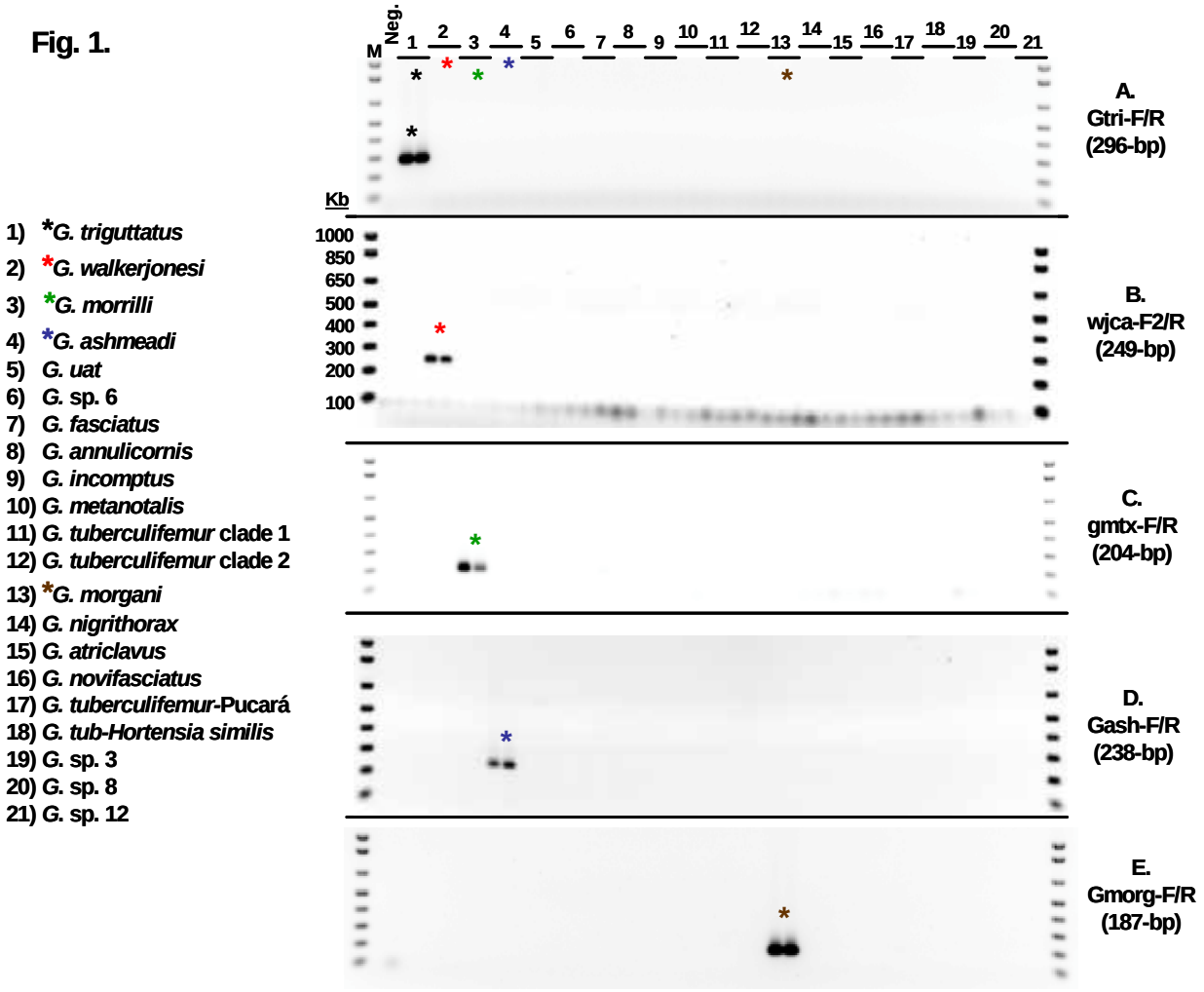

Figure 1. Specificity assays using the developed 'one-step' species-specific ITS2 diagnostic markers. A) Gri-F/R (*G. triguttatus*) (Expected size: 296-bp); *B) wjca-F2/R (*G. walkerjonesi*) (249-bp); *C) gmtx-F/R (*G. morrilli*) (204-bp); D) Gash-F/R (*G. ashmeadi*) (238-bp); and E) Gmorg-F/R (*G. morgani*) (187-bp). Total genomic DNA per *Gonatocerus* species (21) was used in amplification assays to test for cross-reactivity of the markers, each with specific assay conditions. Neg., negative control (no template DNA). M, 1.0-Kb Plus DNA Ladder. *Previously reported with 16 *Gonatocerus* species (de León and Morgan 2007).

Detection of parasitized GWSS eggs in predator gut contents

For this particular experiment, lacewings were allowed to feed (4 h) on GWSS egg masses parasitized by *G. triguttatus*, a primary egg parasitoid of GWSS native to the southeastern U.S. (Texas) and northeastern Mexico and imported into CA as a biological control agent of GWSS (Triapitsyn 2006). Figure 2A demonstrates that the Gtri-F/R markers were able to produce a positive band using parasitized (*G. triguttatus*) GWSS egg masses alone, that is, before the egg parasitoids emerged from the GWSS egg masses. Furthermore, a positive reaction was generated in the gut contents of lacewings that were allowed to feed on parasitized (*G. triguttatus*) GWSS egg masses. These reactions were highly specific, as the controls produced negative banding with this specific marker. The controls included: 1) GWSS egg alone, 2) starved lacewings alone, and 3) lacewings fed on non-parasitized GWSS eggs.

These same samples were also assayed with the GWSS-specific COI marker (HcCOI-F/R) [life-stage independent (de León et al. 2006)] (Figure 2B). As expected, positive reactions were generated with the lacewings that fed on non-parasitized GWSS eggs. Positive reactions were also produced with the parasitized (*G. triguttatus*) GWSS eggs alone. These results confirm that both the GWSS egg DNA and the egg parasitoid DNA can be detected using our specific molecular markers. The detection of the GWSS egg DNA probably depends on the age of the parasitized egg, in other words, as the egg parasitoid embryo is developing with time, the less likely it will be to detect GWSS remains or DNA. Slight detection of GWSS DNA was detected in lacewings allowed to feed on parasitized GWSS eggs. As previously shown, the GWSS-specific marker (HcCOI-F/R) is highly specific (de León et al. 2006).

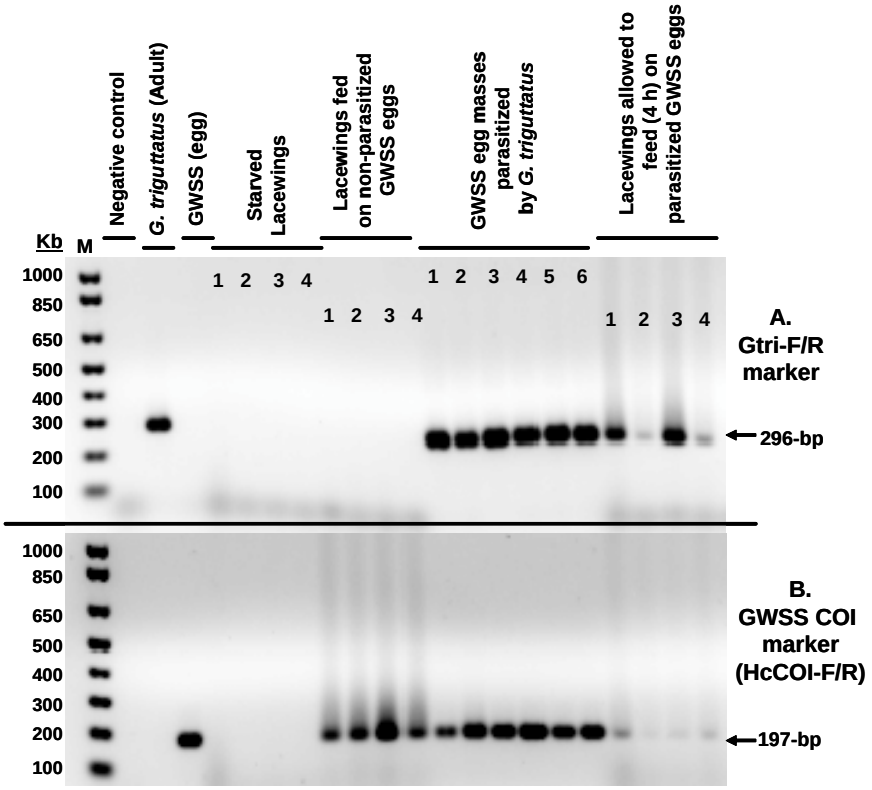

Figure 2. Detection of parasitized (*G. triguttatus*) GWSS eggs in predator (lacewings) gut contents. Diagnostic amplification assays were performed with A) *G. triguttatus* markers (Gtri-F/R) (296-bp) and B) GWSS mitochondrial cytochrome oxidase subunit I gene (COI) markers (HcCOI-F/R) (197-bp); this marker was developed by de León et al. (2006). The test predators included were third-instar green lacewings, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). Experimental conditions on shown above the gel. M, 1.0-Kb Plus DNA Ladder.

Our proposed research addresses every 'high priority' research recommendation for biological control research set forth by the National Academies, National Research Council review of the GWSS/Pierce's disease research effort. The committee strongly recommended that biological control efforts (predators and parasitoids) focus on the establishment of protocols for the effective selection of natural enemies, the development of strategies that will increase the success of biological control agents, and the rigorous evaluation of the effectiveness of natural enemies.

The National Research Council has identified the lack of information on GWSS natural enemies as a critical weakness with a strong recommendation to develop more information on this topic (NRC 2004). The experiments in this study are designed to advance our ability to precisely identify the key GWSS parasitoids and predators and identify the non-target impact of the GWSS generalist predator complex.

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Additional Note: We acknowledge Marissa González and Lisa A. Ledezma (current address: USDA, APHIS, PPQ, CPHST-Edinburg, TX) for their excellent technical assistance and Joseph Patt for providing parasitized GWSS egg masses and assistance in feeding.

EVALUATION OF MOLECULAR MARKERS FOR DISCRIMINATING *GONATOCERUS MORRILLI*: A BIOLOGICAL CONTROL AGENT IMPORTED FROM THE ORIGIN OF THE GLASSY WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted fiscal year 2006 to 2007.

ABSTRACT

We examined the utility of molecular markers for discriminating between two very closely related species, *Gonatocerus morrilli* (*G. morrilli*) (Howard), imported from Texas and *Gonatocerus walkerjonesi* S. Triapitsyn (*G. walkerjonesi*), native to California, to determine whether post-release *G. morrilli* specimens could be detected and discriminated in the field. We started by analyzing post-release specimens collected in 2002 and 2003. Amplification size of the internal transcribed spacer region (ITS2) demonstrated that all of the specimens were of the *G. walkerjonesi* ITS2 genotype. ISSR-PCR DNA fingerprinting experiments of specimens from the original *G. morrilli* 'release' colony showed that the DNA banding patterns were superimposable to that of the native *G. walkerjonesi*, confirming a colony contamination. A new *G. morrilli* colony was initiated in the spring of 2005, and we continued to survey random post-release specimens from the 2004-2006 collections. As expected, from 2004 and most of 2005, only the *G. walkerjonesi* ITS2 genotype was detected. In the fall of 2005 and in the spring and fall of 2006, we detected the *G. morrilli* ITS2 genotype at sites where the new colony was previously released. Analyses with two newly developed 'one-step' species-specific ITS2 diagnostic markers were in agreement with the results of the markers described above, demonstrating their usefulness in aiding the biological control program. *G. morrilli* is now one of the most recovered imported natural enemies in certain regions of California.

INTRODUCTION

In the last 20 years, the glassy-winged sharpshooter (GWSS) has established and spread in southern California, where it poses a serious threat to the wine and table grape industry. Recently, we demonstrated that the GWSS that invaded California is of Texas USA origin (de León et al. 2004a), an observation that was later confirmed by Smith (2005). Pinpointing the origin of a pest and examining the role that natural enemies play in that region can aid in designing biological control strategies (Scheffer and Grissell 2003, Roderick 2004). Natural enemies have usually co-evolved with the target pest in the area of origin and therefore have highly specialized host-finding abilities that may increase the potential success of a biological control program (Scheffer and Grissell 2003, Roderick 2004, Brown 2004, Narang et al. 1993).

Initially, populations of *G. morrilli* from Texas and California were identified as a single species (Phillips et al. 2001). The California population of *G. morrilli* was identified as such because it was similar in several morphological features, including the antennae, coloration, and the wings, and also because it keyed to *G. morrilli* in Huber's key (Huber 1988, Triapitsyn 2006). Populations of *G. morrilli* imported from Texas had supposedly been released in California since 2001. It had therefore been difficult to distinguish between the native and imported natural enemies to determine their establishment. Molecular studies by de León et al. (2004a,b; 2006) strongly suggested that geographic populations of *G. morrilli* could be cryptic species. Molecular diagnostic markers were developed that distinguished the native and imported natural enemies from California and Texas. The two types of molecular markers were: 1) size differences in the internal transcribed spacer region (ITS2) fragment and 2) different inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting banding patterns (de León et al. 2004a). These studies prompted a closer morphological analysis of the populations of *G. morrilli* (Triapitsyn 2006). Based on these observations combined with molecular and hybridization studies (de León et al. 2004a, 2006), the population of *G. morrilli* from California was determined to be a new species, *G. walkerjonesi* S. Triapitsyn (Triapitsyn 2006). *G. walkerjonesi* and *G. morrilli* belong to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. The development and use of molecular diagnostic markers is very important for reasons discussed extensively in the literature (Powell and Walton 1989, Narang et al. 1993, Karp and Edwards 1997, Unruh and Woolley 1999, MacDonald and Loxdale 2004).

OBJECTIVE

The objective of the current study was to evaluate the utility of various types of molecular markers in detecting and discriminating *G. morrilli* populations released in California against GWSS. Two previously developed molecular markers were tested: ISSR-PCR DNA fingerprinting and amplification size of the ITS2 (de León et al. 2004a, 2006). In addition, two newly developed 'one-step' species-specific primer sets targeting the ITS2 fragment of each parasitoid species were also evaluated (de León and Morgan 2007). Randomly chosen post-release specimens from collections made by the California Department of Food and Agriculture (CDFA) from 2002-2006 from several counties in southern California were analyzed.

RESULTS AND CONCLUSIONS

The utility of the ITS2 size fragment in discriminating* G. morrilli *and evaluating post-release specimens

Releases and post-release collections were made by the CDFA from several locations in southern California, a total of 329 specimens were analyzed in 19 total locations (data not shown) (see Table in current poster presentation). The expected ITS2 fragment sizes were: *G. morrilli* (TX) = 1063-1067 bp and *G. walkerjonesi* (CA) = 851-853 bp (Figure 1). Based on the size (851-853 bp) of the ITS2 fragments, all the individuals from the representative San Juan Capistrano, CA location corresponded to the *G. walkerjonesi* ITS2 genotype. Analyses of the remaining specimens (total of 280) from the various locations showed the same trend, that is, 100% of the individuals were of the *G. walkerjonesi* ITS2 genotype and none were of the *G. morrilli* ITS2 genotype.

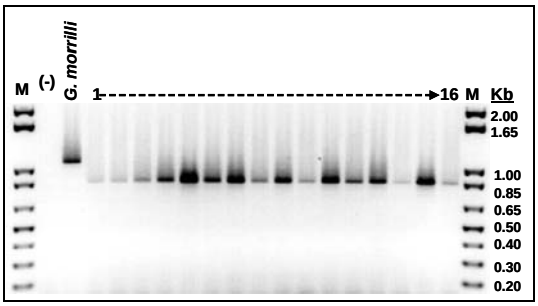

Figure 1. Representative example of the utility of the size of the ITS2 rDNA fragment. The ITS2 size was used as a molecular tool for discriminating and evaluating post-release specimens of *G. morrilli.* The ITS2 rDNA fragment was amplified with standard primers from 16 individuals from randomly chosen post-release specimens collected in San Juan Capistrano, CA. The size of the expected ITS2 amplification products are as follows: *G. morrilli* = 1063-1067 bp and *G. walkerjonesi* = 851-853 bp. The *G. morrilli* (TX) is included as a control. (-), negative control (no template DNA). **M**: 1.0 Kb Plus DNA Ladder.

Molecular analysis of the original* G. morrilli *'release' colony by ISSR-PCR DNA fingerprinting

Since egg parasitoids recovered from the 2002-2003 collections were only of the *G. walkerjonesi* ITS2 genotype, a possibility existed that the released *G. morrilli* did not establish or the released colony was contaminated. ISSR-PCR DNA fingerprinting of several individuals per colony were performed to answer this question. As controls, *G. morrilli* and *G. walkerjonesi* were included. The ISSR-PCR DNA banding pattern of the original *G. morrilli* 'release' colony was superimposable to that of the native California *G. walkerjonesi* species, demonstrating that the individuals from the 'release' colony were not *G. morrilli* (Figure 2). These results were in accordance with those seen with the post-release specimens from the 2002 and 2003 collections based on ITS2 fragment size (Figure 1).

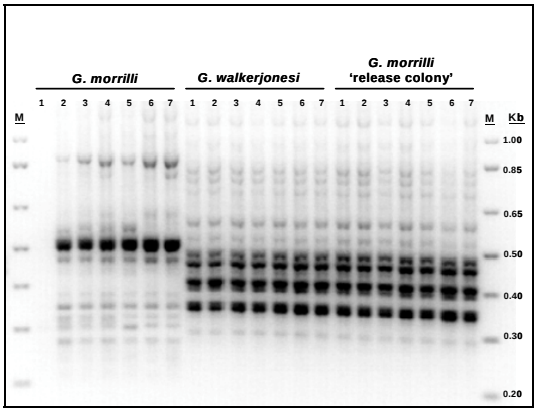

Figure 2. Evaluation of the original *G. morrilli* colony that was used for releases in California. ISSR-PCR DNA fingerprinting was utilized as a molecular tool to evaluate the *G. morrilli* 'release' colony. *G. morrilli* and *G. walkerjonesi* were included as controls. **M:** 1.0 Kb Plus DNA Ladder.

Evaluation of post-release specimens from the 2004-2006 collections by ITS2 size fragment
After realizing that the original *G. morrilli* 'release' colony used for previous releases were *G. walkerjonesi*, J. de León (ARS-Weslaco) sent the CDFA *G. morrilli* from Texas, the origin of GWSS. The CDFA began releases with the new *G. morrilli* colony in the summer of 2005. We continued surveying *G. morrilli* specimens from post-release collections from 2004 through 2006 by amplification of the ITS2 fragment. For the whole year of 2004 and most of 2005, we only detected the *G. walkerjonesi* ITS2 genotype in most locations. However, in three locations where CDFA made releases from the new *G. morrilli* release colony, this species was detected in the fall of 2005 and in the spring and fall of 2006 (data not shown) (see Table on current poster).

Development, specificity, and utility of the 'one-step' species-specific ITS2 markers for* G. morrilli *and* G. walkerjonesi, *respectively
To determine the specificity of the two molecular diagnostic markers, we tested specific amplification assay conditions and screened a total of 16 *Gonatocerus* Nees GWSS egg parasitoid species for cross-reactivity. Figures 3A (gmtx; *G. morrilli*) and 3B (wjca; *G. walkerjonesi*) show that the markers were specific. Cross-reactivity with the 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* (Triapitsyn 2006).

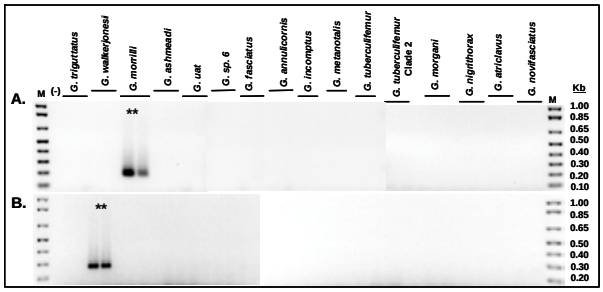

Figure 3. Specificity assays using the 'one-step' species-specific ITS2 diagnostic markers: A) gmtx (*G. morrilli*) and B) wjca (*G. walkerjonesi*). Genomic DNA from two individuals per *Gonatocerus* species (16) was used in amplification reactions to test for cross-reactivity of the two diagnostic markers with specific assays conditions. The species are listed on the figure. Expected fragment sizes: gmtx = 204 bp and wjca = 249 bp. **(-)**, negative control (no template DNA). **M**, 1.0 Kb Plus DNA Ladder.

Molecular analysis of the original* G. morrilli *'release' colony used before the summer of 2005 with the ITS2 species-specific markers
To test the utility of the newly developed 'one-step' diagnostic markers, we analyzed the same individuals from the previous *G. morrilli* release colony (those shown in Figure 2). Amplification with the 'gmtx' marker showed positive banding in only the control *G. morrilli* (TX) species (Figure 4A) but not in the control *G. walkerjonesi* species. Amplification with this marker of the *G. morrilli* 'release' colony also produced negative banding, confirming that the original release colony was not *G. morrilli*. Analysis of the same colony with the 'wjca' marker showed negative banding in the control *G. morrilli* species and positive banding in both the control *G. walkerjonesi* species and in the original 'release' colony (Figure 4B), confirming the results of ISSR-PCR DNA fingerprinting experiment and unambiguously confirming that the original 'release' colony was contaminated with the native species (*G. walkerjonesi*) from California.

The utility of the 'one-step' ITS2 species-specific markers in discriminating and evaluating post-release* G. morrilli *specimens in California
To confirm the usefulness of these diagnostic markers, we randomly screened post-release specimens of *G. morrilli*, including the specimens that tested positive by utilizing the amplification size of the ITS2 fragment. Amplification with the 'gmtx' marker produced positive banding in the three locations (Figure 5A, Lanes e, g, and h) that tested previously positive, whereas amplification with the 'wjca' marker tested positive only with the *G. walkerjonesi* specimens (Figure 5B, Lanes a-d and f), confirming the results inferred by amplification of the ITS2 region. In early 2007, the new *G. morrilli* release colony was tested with the 'gmtx" marker and the results showed that the colony was indeed *G. morrilli*, confirming the purity of the colony (results not shown).

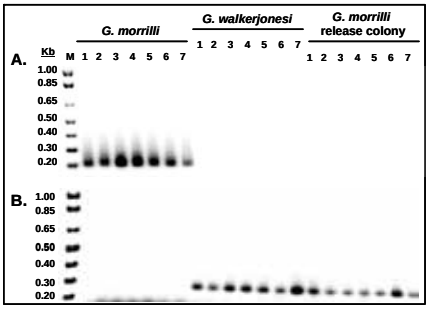

Figure 4. Evaluation of the original *G. morrilli* 'release' colony by the 'one-step' ITS2 markers: A) gmtx and B) wjca. *G. morrilli* and *G. walkerjonesi* were included as controls. Seven individuals per colony were included. **M**, 1.0 Kb Plus DNA Ladder.

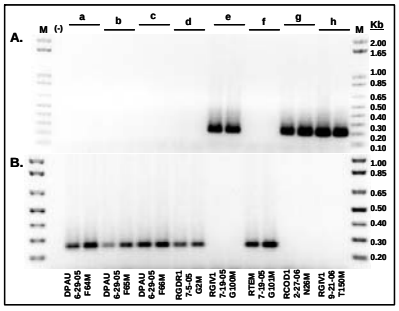

Figure 5. Representative example of the utility of the 'one-step' species-specific markers: A) gmtx and B) wjca were used for detecting and discriminating post-release random specimens in California. Two individuals per collection site were included. **M**, 1.0 Kb Plus DNA Ladder.

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RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF PIERCE'S DISEASE

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

ABSTRACT

We initiated a collaborative biotechnological effort to develop an RNA interference (RNAi) strategy to target and control sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease. We have constructed cDNAs corresponding to specific genes of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and for two genes, actin and SAR1, have generated dsRNAs *in vitro*. We will evaluate these dsRNAs for their ability to induce RNAi effects against GWSS using cells as well as whole leafhoppers. We have established GWSS cells in culture (with help from Drs. George Kamita and Bruce Hammock) and have established reproducing colonies of the GWSS insects (with help from Dr. R. Almeida) in the UC Davis Contained Research Facility. Initial experiments have shown that we can deliver and express genes in GWSS cells.

INTRODUCTION

Pierce's disease (PD), caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf*), is an important threat to the California grape industry (http://www.aphis.usda.gov/lpa/pubs/fsheet_faq_notice/fs_phglassy.html and http://orsted.nap.edu/openbook.php?record_id=11060&page=21). Although PD has been recognized in California for at least a century, recent events have shown the destructive potential of this disease in the California grape industry. For example, an epidemic of PD devastated commercial grapes in the Temecula region of Southern California beginning in 1997 (Blua et al., 1999). This epidemic was found to be associated with the introduction into California of the GWSS, *Homalodisca vitripennis*, an invasive sharpshooter leafhopper known to be indigenous to parts of the Southeastern United States (Blua et al., 1999). The GWSS is a large, robust leafhopper with a broad host range including many native, ornamental and crop plants. One of the preferred hosts in Southern California and other areas is citrus (Adlerz 1980; Blua et al., 1999). The combination of this new PD vector species, its wide host range, abundance of host plants, its affiliation for citrus as a host for reproduction, and its ability for long-distance dispersal (Blua and Morgan, 2003) has raised concerns that PD and GWSS are important threats to the California grape industry beyond the Temecula region.

In addition to being transmitted by *Homalodisca vitripennis*, *Xf* is transmitted to plants by several other species of xylem-feeding leafhoppers (see Redak et al., 2004). When sharpshooter vectors acquire *Xf* from the xylem of *Xf*-infected plants, bacterial cells form a biofilm and attach in a polar fashion in the foregut (Newman et al., 2004). There, *Xf* cells multiply within the leafhopper foregut, establishing essentially a lifelong infection within the leafhopper vector. It is interesting to note that as opposed to phloem-feeding hemipterans, xylem feeders must ingest much greater volumes of plant sap. This is because xylem sap is much less nutrient rich than is phloem sap, and thus greater volumes are required to yield necessary amounts of nutrients (Milanez et al., 2003; Redak et al. 2004). However, because such large volumes are ingested, this offers the potential to deliver toxic molecules to leafhoppers, even if they are produced in low concentration in xylem sap. This is an important component of our strategy.

The complex interactions between host plants, leafhopper vectors and *Xf* make controlling PD in grapes difficult. Genetic resistance to *Xf* or its leafhopper vectors is not yet generally or universally effective in commercial grape production. However, several new strategies are being investigated for developing new resistance approaches. Some of these include several biotechnological approaches that may directly affect *Xf* or its leafhopper vectors. Here we propose a new approach, one based on RNA interference (RNAi) directed towards *Homalodisca vitripennis*.

OBJECTIVES

1. To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis.*
2. To generate transgenic plants capable of expressing and delivering *Homalodisca vitripennis* deleterious RNAi molecules within their xylem.
3. To evaluate transgenic plants for their ability to generate RNAs capable of inducing RNAi vs. *Homalodisca vitripennis*.

RESULTS

Objective 1 - To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis*

For this effort we will utilize *in vitro* and *in vivo* delivery systems. We will assess RNAi effects in cultured *Homalodisca vitripennis* cells as well as in whole leafhoppers. Drs. George Kamita and Bruce Hammock, UC Davis, Entomology, generously supplied these cells (see Kamita et al., 2005, GWSS cell line Z15) and have greatly assisted us in learning how to maintain and manipulate them. We have also established reproducing colonies of GWSS in the UC Davis Contained Research Facility and will perform experiments using cultured cells as well as whole insects. *Homalodisca vitripennis* insects were collected from southern California and donated to us by Dr. R. Almeida (UC Berkeley). The GWSS were transferred into the CRF facility at UC Davis and have been maintained there for more than two and a half months. So far, we have been able to establish four colonies, but also to rear new generations of GWSS. The young colonies appear to be parasitoid free.

Choice of dsRNA inducers

Fourteen *Homalodisca vitripennis* nucleotide sequences, derived from EST based nucleotide sequences available in GenBank and translatable in putative proteins, were used to design gene specific primers and to generate cDNAs from GWSS cell line Z15. Corresponding sequences were amplified by RT-PCR (Figure 1). Two of the above mentioned sequences, corresponding to the vitellogenin and rhodopsin genes, could not be amplified from the *Homalodisca vitripennis* cell line mRNA pool. This may be because these genes are expressed only in whole insects. cDNAs of three actin mRNAs and SAR1 mRNA expressed in the *Homalodisca vitripennis* cell line were cloned in pGMTeasy vector in both orientations downstream of the T7 RNA polymerase promoter, and sequenced. The vectors were directly used for T7 RNA polymerase-mediated *in vitro* transcription to generate specific dsRNAs (Ambion, dsRNA MaxiScript; see Figure 2). These dsRNA will be delivered via transfection into GWSS cells, and via injection into *Homalodisca vitripennis* whole insects.

Cell transfection system

GWSS line Z15 cells were transfected with a plasmid expressing GFP under the control of an inducible insect promoter (kindly provided by Dr. Shou-wei Ding, UC Riverside). "DOTAP," "FuGene HD" transfection reagents from Roche and "Cellfectin" transfection reagent from Invitrogen were compared for their ability to assist in transfecting GWSS cells. Manufacturer protocols were followed in all the transfection experiments. The maximum transfection efficiency (equal to 5%) was obtained using the Cellfectin transfection system (Figure 3).

Realtime RT-PCR primers/ probe sets were designed and tested using real time RT-PCR assays of GWSS cell derived RNA. The resulting amplifications plots were specific (Figure 4). This system will be used to measure the amount of SAR1 and actin mRNAs in transfected cells and whole *Homalodisca vitripennis* insects, following RNAi delivery. Since the assays will be performed using only one gene per experiment, the second gene will be used as an endogenous control.

We are attempting to develop a microscopic means to assess RNAi effects in GWSS cells also, and complement our real time RT-PCR efforts. This is based on actin development/staining assays. Figure 5 shows light microscopic, fluorescent analysis of actin when GWSS cells are grown on glass cover slips.

CONCLUSIONS

During the first three months of this project, we were able to successfully rear *Homalodisca vitripennis* insects, to amplify and clone a pool of GWSS specific gene sequences, to produce dsRNAs for two genes and to develop different assays to test the outcome of RNAi experiments in *Homalodisca vitripennis* cell lines and whole insects. We believe that we are in an excellent position to move on to experiments within objective 1, and evaluate GWSS cells and whole insects for RNAi effects.

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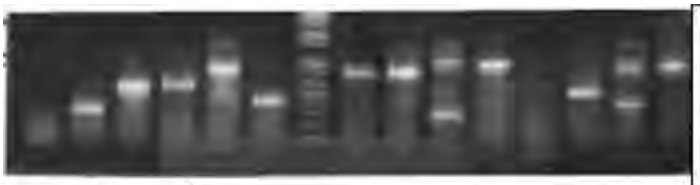


Fig. 1. 1.5% agarose gel: one tube RT-PCR products using gene specific primers designed based on GenBank available sequences.From left to right, products represent vitellogenin, Histone 3, SAR 1, RAB1 1, Kinase creceptor, Ubiquitin conjugatine enzyme, 1 Kb marker, tropomyosin, Micotchondrial porin, Delta 9 saturase 1, Fructose 1,6 biphosphate aldolase, rhodopsin, ferritin, Arginine kinase, Actin.

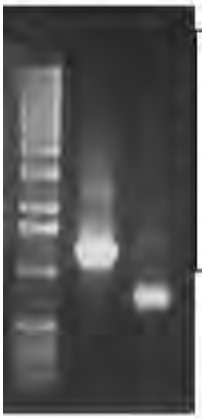
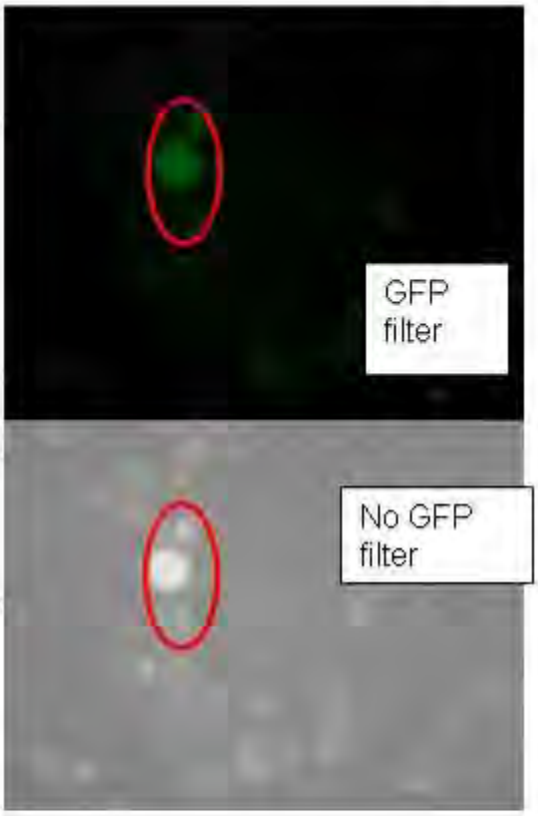


Fig. 2. In vitro transcrtipts were annealed to dsRNA and analyzed by agarose gel electrophoresis. Lane 1 = markers, 2 = actin dsRNA and 3 = SAR1 dsRNA.

Fig. 3. GWSS cells at right were electroporated with a GFP-expressing plasmis. Top shows fluorescent cells, bottom shows light visualization. Maximum efficiency so far is 5%.



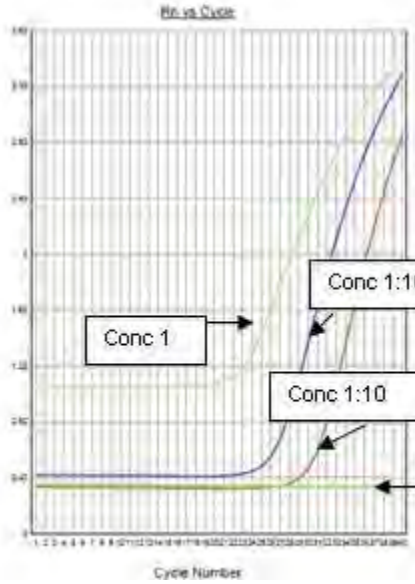


Fig. 4. Real time PCR using SYBRGreen and Actin primers.

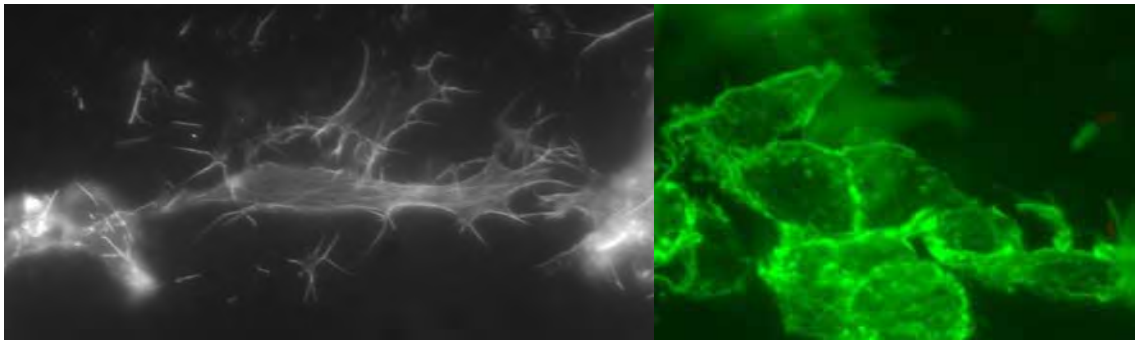


Figure 5. Image of GWSS cells grown on a glass cover and stained with phalloidin. Using transmitted light, actin filaments are white. However when examined with a UV filter, actin filaments are green. Fibroblast like cell is visible in the middle.

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

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

ABSTRACT
Results from exposing four to eight glassy-winged sharpshooter (GWSS) eggs to one female parasitoid for one h in a 'complex experimental system' showed that parasitism of eggs one-five days of age ranged from 81-97% for *Gonatocerus ashmeadi* (*G. ashmeadi*), whereas, parasitism by *Gonatocerus tuberculifemur* (*G. tuberculifemur*) ranged from 18-66%. Eggs five days of age were less suitable for *G. tuberculifemur* development (resulting in 18% parasitism). When *G. ashmeadi* and *G. tuberculifemur* were presented simultaneously with one GWSS egg mass for one h (complex system) or 15 min (simple system), parasitism by *G. ashmeadi* was consistently 12-54% higher than *G. tuberculifemur* for all egg ages. There was no significant difference in overall percentage parasitism of GWSS eggs between vials containing *G. ashmeadi* only and vials containing both *G. ashmeadi* and *G. tuberculifemur* for both simple and complex systems. This may be attributable to conspecific presence increasing non-ovipositional behavior such as aggressive chasing (up to 1.3% of time) and antennating conspecifics (up to 1.3% of time), thereby reducing time available for oviposition. This may suggest that the addition of *G. tuberculifemur* to the resident parasitoid guild in California may not enhance biological control of GWSS. Finally, in experiments where 50 GWSS eggs were exposed simultaneously to one female *G. ashmeadi* and *G. tuberculifemur* for 24 h or five days, parasitism by *G. ashmeadi* was 44-53% higher than *G. tuberculifemur* for both exposure times. Results from all completed experiments assessing the effect of environmental complexity, egg age, and competition indicate that *G. tuberculifemur* is an inferior competitor for GWSS egg masses when *G. ashmeadi* is present and that the introduction of *G. tuberculifemur* into California may not benefit GWSS biological control unless this species can efficiently exploit an unknown niche where competition with *G. ashmeadi* is likely to be low or non-existent.

INTRODUCTION
G. tuberculifemur is a sharpshooter parasitoid from Argentina that has been regularly imported into the UCR I & Q facility since September 2002 and reared on GWSS egg masses. There is substantial uncertainty about the safety of releasing this agent and whether it 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 in the home range of this pest. The purpose of this work is to ascertain in Quarantine whether this neoclassical biological control agent from Argentina can outperform the dominant GWSS parasitoid in California, *G. ashmeadi*. These data will help guide the decision to release the Argentinean parasitoid from quarantine for liberation and establishment in California.

OBJECTIVES
This research project has five objectives:
1. Ascertain oviposition preferences of *G. ashmeadi* and *G. tuberculifemur* for GWSS egg masses of different ages.
2. Determine the competitiveness of these two parasitoid species simultaneously foraging for GWSS egg masses in complex and simple environments.
3. 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.

To date, we have completed Objectives 1-3. Three experiments were conducted to investigate egg age preferences and competitive ability of *G. ashmeadi* and *G. tuberculifemur*. These involved complex and simple systems and an experiment with long host exposure times. Objectives 4 & 5 will be completed in year 2.

RESULTS
Egg age preferences and competitive ability
Complex system:
One mated female *G. ashmeadi* and *G. tuberculifemur* (~24-36 h) was presented simultaneously to one GWSS egg mass (~four-eight eggs) camouflaged amongst four other similar sized lemon leaves in a double ventilated vial. This 'complex system' was replicated 15 times for GWSS eggs aged one, three and five days of age. After 60 minutes exposure to foraging parasitoids, leaves with egg masses were placed into individual Petri dishes, labeled and held at 27°C for emergence of parasitoids and GWSS nymphs. The number of emerged and unemerged males and females of each parasitoid species was

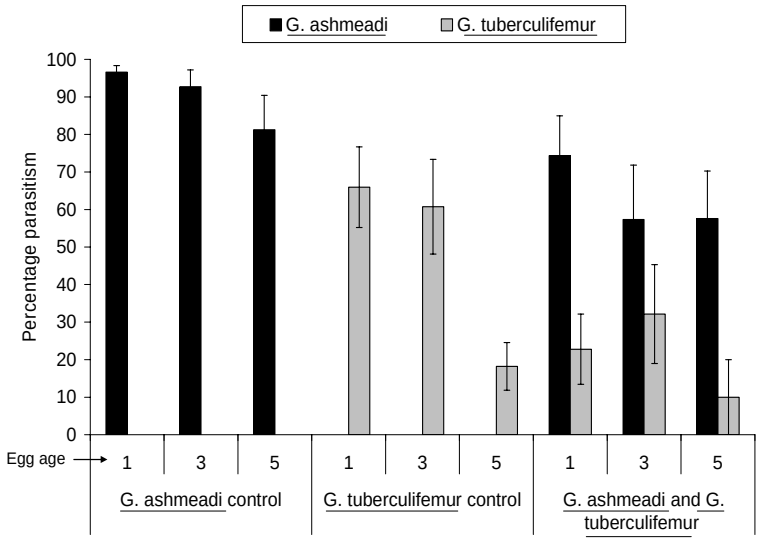

Figure 1. Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses aged one, three, and five days of age were exposed to parasitoids either alone or in competition with each other.

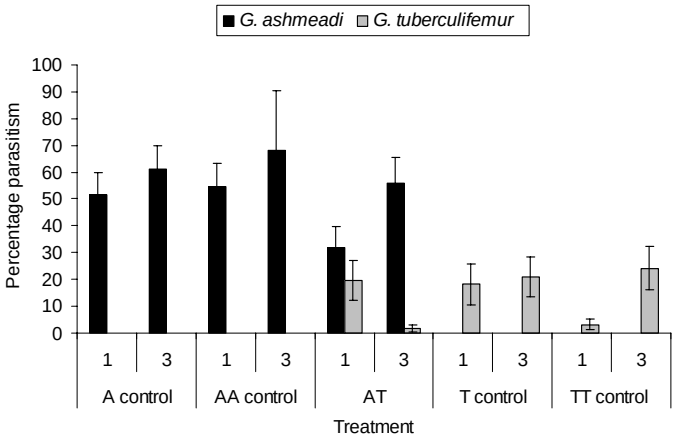

Figure 2. Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses aged one and three days of age were exposed to parasitoids either alone or with intraspecific or interspecific competition (A – control vial containing one female *G. ashmeadi*; AA = control vial containing two female *G. ashmeadi*; AT = one female *G. ashmeadi* and *G. tuberculifemur*, TT = two female *G. tuberculifemur*; T = one female *G. tuberculifemur*).

recorded. Fifteen control vials containing one female parasitoid were set up for each species. Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* was calculated as the percentage of total eggs.

Figure 1 shows percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses one, three or five days of age were exposed to three different treatments: (i) *G. ashmeadi* control vials consisting of one female *G. ashmeadi*, (ii) *G. tuberculifemur* control vials consisting of one female *G. tuberculifemur*, and (iii) vials containing one female of both *G. ashmeadi* and *G. tuberculifemur*. Results from the *G. tuberculifemur* control vials show that 60-66% of eggs one and three days of age were successfully parasitized by *G. tuberculifemur* (Figure 1). Eggs five days of age were less suitable for *G. tuberculifemur* development and resulted in just 18% parasitism. In contrast, results from the *G. ashmeadi* control vials showed that *G. ashmeadi* parasitism ranged from 81-97% and there was no significant difference in parasitism between egg ages (Figure 1). This result for *G. ashmeadi* is similar to that observed by Irvin & Hoddle (2005a). The higher rates of parasitism and larger host age range demonstrated by *G. ashmeadi* may indicate that this species may be more competitive than *G. tuberculifemur* and may outcompete it in the field for GWSS egg masses.

Results from vials containing one GWSS egg mass exposed simultaneously to one *G. ashmeadi* and *G. tuberculifemur* in a 'complex experimental system' for one h showed that parasitism by *G. ashmeadi* was consistently 25-51% higher than *G. tuberculifemur* for all three egg ages (Figure 1).

Simple system

One mated female *G. ashmeadi* and *G. tuberculifemur* (~24-36 h) was presented simultaneously to one GWSS egg mass (~four-eight eggs) on a single leaf in a double ventilated vial. This 'simple system' was replicated 15 times for GWSS eggs aged one and three days of age. Egg masses were not camouflaged amongst four other similar sized leaves. Exposure time was 15 mins and each minute the behavior [searching container (SC), searching leaf (SL), searching egg mass (SE), oviposition (O), resting (R), grooming (G), aggressive chasing (C), antennating conspecific (AC), searching egg mass from top side of leaf (SETS), ovipositing from top side of leaf (OTS), feeding (F)] of each female was recorded. Fifteen replicates of two types of control vials were also set up for each species. These contained either one female parasitoid or two female parasitoids of the same species.

Results from vials containing one GWSS egg mass exposed simultaneously to one *G. ashmeadi* and *G. tuberculifemur* in a 'simple experimental system' for 15 min showed that parasitism by *G. ashmeadi* was 12-54% higher than *G. tuberculifemur* for both egg ages (Figure 2).

Long exposure time

Approximately 50 GWSS eggs (one-two days of age) were placed in a double ventilated vial cage and exposed to one mated

female and male (24-48 h old) for either 24 h or five days. Approximately 20 replicates were set up for each exposure time. The number of male and female *G. ashmeadi* and *G. tuberculifemur* offspring were recorded for each vial. Figure 3 shows that parasitism by *G. ashmeadi* was 44-53% higher than *G. tuberculifemur* for both exposure times.

Results described in Figures 1-3 suggest that *G. ashmeadi* is superior to *G. tuberculifemur* under complex and simple experimental conditions with short and long exposure times. Results from competition experiments where both parasitoids are presented simultaneously to host eggs demonstrated that *G. ashmeadi* should outcompete *G. tuberculifemur* in the field, thereby possibly preventing widespread establishment and proliferation, of *G. tuberculifemur* in California. This result is similar to Irvin & Hoddle (2005b) who showed that *G. ashmeadi* was superior to *G. triguttatus* and *G. fasciatus* in laboratory studies investigating egg age preference, GWSS parasitism rates and adult parasitoid longevity. *Neither G. triguttatus nor G. fasciatus have performed well following mass releases in California where G. ashmeadi is present, which suggests that the results of these competitive lab experiments may accurately predict field performance. Despite substantial effort and significant cost being dedicated for several years to the mass rearing and distribution of these two poorly performing parasitoids.*

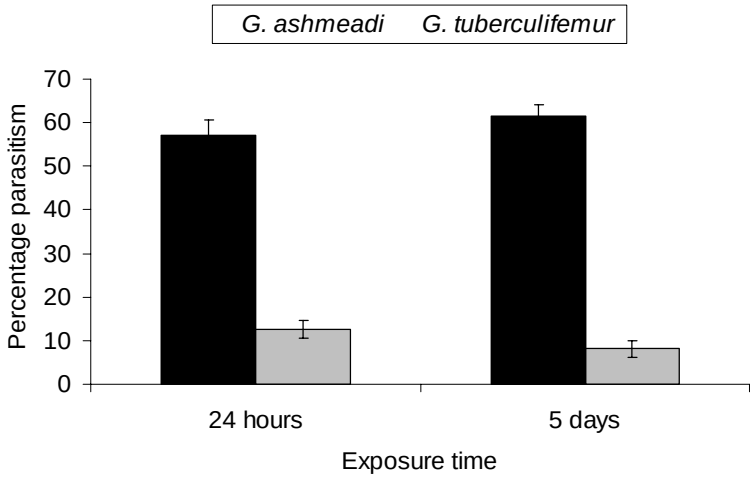

Figure 3. The mean percentage of *G. ashmeadi* and *G. tuberculifemur* offspring emerging when 50 GWSS eggs were exposed simultaneously to one mated female *G. ashmeadi* and *G. tuberculifemur* for 24 h *or* 5 days.

There was no significant difference in overall parasitism of GWSS eggs between vials containing *G. ashmeadi* only (A) and vials containing both *G. ashmeadi* and *G. tuberculifemur* (AT) for the complex system (Figure 4). This may be attributable to host availability since one female *G. ashmeadi* already reached maximum percentage parasitism (86-90%) and a small proportion of GWSS eggs are always lost through desiccation and superparasitism. For the simple system, parasitism remained at 55-60% regardless of whether vials contained one *G. ashmeadi* (A), two *G. ashmeadi* (AA), or one *G. ashmeadi* and *G. tuberculifemur* (AT) (Figure 5). This may be attributed to conspecific presence increasing non-ovipositional behavior such aggressive chasing and antennating conspecifics, thereby reducing time available for oviposition.

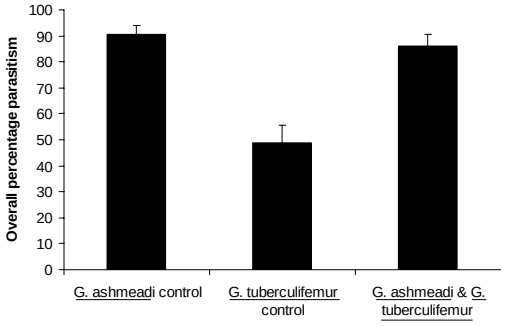

Figure 4. Overall percentage parasitism of GWSS egg masses exposed to three treatments in a 'complex experimental system' for 1 h.

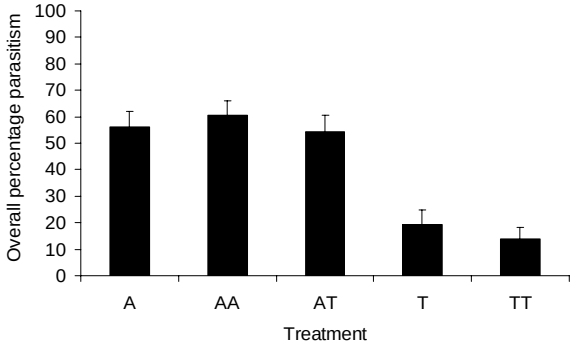

Figure 5. Overall percentage parasitism of GWSS egg masses exposed to five treatments in a 'simple experimental system' for 15 min (see Figure 2 for treatment legend).

Table 1 shows that female *G. ashmeadi* and *G. tuberculifemur* allocated up to 1.3% of time to each aggressive behavior when they were presented with a conspecific. *These results may suggest that the introduction of G. tuberculifemur in California may not benefit biological control of GWSS because no additive or synergistic effect on parasitism of GWSS eggs is observed in the laboratory.* Alternatively, the parasitism rates observed in treatments A, AA and AT (55-60%) may indicate that 60% is the maximum mean parasitism obtainable in this experimental design since GWSS eggs were exposed for 15 min, and there were always a small proportion of replicates that resulted in no parasitism which reduced overall parasitism estimates.

Table 1: The percentage of time allocated to eleven behaviors when *G. ashmeadi* and *G. tuberculifemur* were exposed to one GWSS egg mass for 15 min in five experimental treatments (see Section 3.1.2 and Figure 2 for behavior and treatment legends).

Behavior	Treatment					
			AT			
	A	AA	A	T	T	TT
SC	18.96 ± 4.26	21.14 ± 2.93	19.13 ± 3.36	38.92 ± 6.49	42.88 ± 6.22	39.44 ± 4.22
SL	8.12 ± 2.17	18.02 ± 2.22	16.12 ± 3.57	18.06 ± 4.30	8.00 ± 1.98	18.00 ± 2.69
SE	11.04 ± 1.73	8.43 ± 1.23	9.89 ± 1.63	7.74 ± 1.54	6.66 ± 1.91	6.77 ± 1.22
O	42.08 ± 5.56	32.5 ± 2.58	37.41 ± 4.61	22.79 ± 4.84	29.11 ± 6.17	14.00 ± 2.84
R	10.83 ± 4.22	10.1 ± 2.67	8.81 ± 3.21	5.59 ± 2.56	7.55 ± 3.07	11.00 ± 2.87
G	8.33 ± 3.16	4.37 ± 1.18	4.08 ± 1.89	3.87 ± 1.41	5.77 ± 2.01	8.33 ± 1.54
C	0 ± 0	0.10 ± 0.10	1.29 ± 0.65	0.21 ± 0.21	0 ± 0	0.33 ± 0.33
AC	0 ± 0	1.14 ± 0.38	0.64 ± 0.35	1.29 ± 0.48	0 ± 0	0.88 ± 0.29
SETS	0 ± 0	1.14 ± 0.68	0.86 ± 0.51	0.43 ± 0.29	0 ± 0	0.22 ± 0.15
OTS	0 ± 0	2.29 ± 1.43	1.29 ± 1.09	0.86 ± 0.67	0 ± 0	0 ± 0
F	0.62 ± 0.45	0.73 ± 0.36	0.43 ± 0.29	0.21 ± 0.22	0 ± 0	1.00 ± 0.52

Functional response

The functional response (Type I, II, or III) is a measure of how many hosts a parasitoid can process in a given exposure time. Handling time and area of search are the two most significant quantifiable factors that affect the shape of the functional response curve. Figure 6 shows that both *Gonatocerus* species demonstrate a type II functional response curve (number of hosts attacked per unit time decreases as host density increases), and that *G. ashmeadi* outperformed *G. tuberculifemur* at all host densities greater than five eggs.

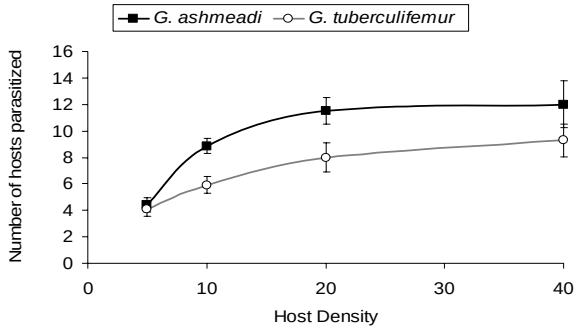

Figure 6. The relationship between host density and the number of GWSS eggs parasitized by a single female *G. ashmeadi* or *G. tuberculifemur* during a one hour exposure period at 26°C.

CONCLUSIONS

Preliminary results of studies completed thus far suggest that the potential impact of releasing *G. tuberculifemur* in California on the biological control of GWSS may not out-weigh the cost of mass rearing and releasing *G. tuberculifemur* in California. When time and labor costs for large-scale colony maintenance, long-term concerns about non-target impacts in California, disruption of existing levels of control, and potential invasion by *G. tuberculifemur* back into the southeast USA where GWSS originated are all considered there appears to be no quantifiable benefit to releasing *G. tuberculifemur* in California for the biological control of GWSS.

The experiments addressing the five objectives outlined in this report will provide important biological data on the neoclassical biological control agent, *G. tuberculifemur*, while in this parasitoid is still in quarantine. Work presented in this report demonstrates that *G. tuberculifemur* may be inferior to *G. ashmeadi*, and this would suggest no advantage to releasing this neoclassical agent from quarantine. A decision not to release based on these assessments would negate potential long-term concerns about non-target impacts in California (i.e., against *H. liturata*, the smoketree sharpshooter), possible infiltration of the home range of GWSS, and interference and reduction of current levels of biological control achieved with the resident natural enemy guild of old association parasitoids.

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Irvin, N. A., Hoddle, M. S. (2005b). The competitive ability of three mymarid egg parasitoids (*Gonatocerus* spp.) for glassy-winged sharpshooter (*Homalodisca coagulata*) eggs. Biological Control 34: 204-214.

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

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Reporting Period: The results reported here are from work conducted October 2006 to September 2007.

ABSTRACT

What appear to be eight or nine different *Anagrus* species were obtained from 18 collection sites for morphological and molecular examination. Confirming our hypothesis that there might be cryptic species hidden in this complex, an *Anagrus* species in Mexico, two species in Colorado, and one in the state of Washington were found to be genetically different from the Minnesota strain of *A. epos*. Genetic and morphological analyses are nearly complete and we are preparing a manuscript on this work (Triapitsyn et al. 2008). Rearing *A. epos* on GWSS eggs for field release has been problematic and to help solve this, several studies were undertaken (Krugner et al. 2007, 2008) which compared alternative rearing hosts (i.e. eggs of various leafhopper species) and investigated the basic biology of *A. epos*. Limited field releases have been made during summer 2006 and 2007 (due to rearing difficulties) but to date, *A. epos* has not been recovered. Based on this work, we have started rearing aster leafhopper as an alternative *A. epos* host to allow greater numbers of parasitoids to be released and field sleeve cage releases are planned for next year.

INTRODUCTION

Anagrus epos is a common and seemingly widespread egg parasitoid of leafhoppers (Cicadellidae) in North America. Location records for this species 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. and *Erasmoenura* 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 UC Riverside Quarantine facility on eggs of the glassy-winged sharpshooter.

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 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.

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 - Objective 1. Examination of Male and Female *A. epos* Complex Populations for Unique Morphological Characters.

Proposed research is complete and we were able to collect and examine specimens from a larger number of locations that we anticipated. Line drawings are complete and a manuscript will be finished and submitted soon.

Table 1 summarizes the specimens we had available in several collections or that were collected to date and the results of genetic examination. We were fortunate that a number of the specimens Dr. Triapitsyn had collected earlier (a number as far back as 1994) were preserved in very good condition allowing genetic study followed by slide-mounting for morphological examination. In addition, due to a number of productive trips by Dr. Triapitsyn and the assistance from several cooperators (special thanks are due Dr. Larry Wright, Washington State University, Prosser and Dr. Kent Daane, UC Berkeley) we have filled in all major "holes" in the *A. epos* species complex.

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 have also been completed. Genetic examination is complete (results are discussed below) as is morphological examination and Dr. Triapitsyn has prepared line drawings of all relevant specimens. We are currently discussing what journal would be most appropriate for a combined taxonomic/ genetic presentation of our data in which a number of new species will be described.

Table 1. Summary of specimens collected for morphological (Objective 1) and genetic (Objective 2) research.

Collection	Genus	Species	Collection site
1	*Anagrus*	*epos*	UCR culture, originally collected near Glyndon, Clay Co., MN, 2004
2	*Anagrus*	*sp.*	Campo Experimental INIFAP, Sonora, Mexico, 1994
3	*Anagrus*	*sp.* (same as 2)	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
8-10, 12	*Anagrus*	*tretiakovae*	Albuquerque, Bernalillo Co., NM, 2005 (ex. *Erythroneura triapitsyni* eggs)
11	*Anagrus*	*erythroneurae*	Temecula, Riverside Co., CA, 2006
13	*Anagrus*	*tretiakovae*	Pavich vineyard, Harquahala Valley, Maricopa Co., AZ, 1994 (ex. *Erasmoneura variabilis* eggs)
14	*Anagrus*	*new species*	Grand Junction, Mesa Co., CO, 2006 (ex. *Erasmoneura vulnerata* eggs)
15	*Anagrus*	sp. 1 near *A. daanei*	Palisade, Mesa Co., CO, 2006 (ex. *Erythroneura ziczac* eggs)
16	*Anagrus*	sp. 2 near *A. daanei*	WSU-Prosser Research Center, Prosser, Benton Co., WA, 2006
17	*Anagrus*	*new species*	Grand Junction, Mesa Co., CO, 2007 (ex. *Erasmoneura vulnerata* eggs)
18	*Anagrus*	sp. 1 near *A. daanei*	Palisade, Mesa Co., CO, 2007 (ex. *Erythroneura anfracta* eggs)

In September 2007, Dr. Triapitsyn made another trip to Colorado to collect *Anagrus* spp., ship them alive under permit to UCR's Quarantine Facility, and determine in Quarantine if they would attack GWSS eggs. Collections were made on 4 September 2007 and were sent to Quarantine the same day. Numerous parasitoids emerged 7-9 September and were exposed in two or three replicates each, to fresh GWSS eggs on *Euonymus japonica* leaves at 22-23°C. By 26 September, GWSS nymphs had started to emerge and it was clear there were no signs of parasitism. Collection #17 from Grand Junction, CO was determined by Dr. Triapitsyn to be a new *Anagrus* species, obtained from eggs of *Erasmoneura vulnerata* (Fitch) on wine grapes. A manuscript describing this species is in preparation based on the 2006 specimens. A second *Anagrus* species tested in Quarantine were specimens from Collection #18 from Palisade, CO which were determined to be *Anagrus* sp. 1 nr. *daanei* Triapitsyn from eggs of *Erythroneura anfracta* Beamer on Virginia creeper. Both of these *Anagrus* species have

shorter ovipositors compared with *A. epos* and we speculate this might be a reason why they failed to successfully parasitize GWSS eggs.

Progress - Objective 2. Molecular Characterization of Mitochondrial and Ribosomal DNA of *A. epos* Populations.
Genetic analyses have confirmed our hypothesis that there are (at least three) cryptic species hidden within specimens that 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*. Without genetic examination, this situation would have remained hidden. Using the *A. epos* (Minnesota) strain that is kept in culture on GWSS eggs in the Department of Entomology, UC Riverside, we first determined the DNA sequence of several indicator gene regions. We optimized PCR conditions so that other "*A. epos*" strains could be compared by amplifying and sequencing the chosen gene regions. Methods were tested and adapted from previous *Scirtothrips* research (Rugman-Jones et al. 2006) for use on *Anagrus* allowing us to extract DNA from whole wasps in such a way that after DNA extraction, the remainder of the wasp could be used for morphological studies. This allowed a direct link between the DNA characters and the morphological characters for all the individuals that have been studied.

Members of the genus *Anagrus* are very small and lack definitive species-specific morphological characters. The 28sD2 region of ribosomal RNA has been shown to be well conserved within a species but is different between species. The work here illustrates the preponderance of species that would morphologically be classified as *Anagrus epos*, but are different species (Collections 2/3 and 14/17 each different from the MN "*A. epos*" and each other) whereas Collections 15/18 and 16 morphologically resemble *A. daanei*.

Progress - Objective 3. Mating Compatibility Studies Between *A. epos* strains.
Because genetic studies clearly suggest there is a cryptic species in Mexico and one in Colorado different from the Minnesota strain of *A. epos*, as well as a species in Colorado similar to *A. daanei*, we decided mating studies would not be productive. Instead, we shipped the two Colorado species to UCR's Quarantine facility to allow parasitoids to emerge and determine if they would parasitize GWSS eggs. As mentioned above, neither appeared able to do so.
Our original plan was to conduct mating studies between closely related strains of "*A. epos*" to confirm our genetic results. However, we have run into unexpected problems rearing *A. epos* for field release and evaluation (see Objective 4 below). In addition, our genetic studies have identified what are clearly 23 different species in the "*A. epos*" complex and this, along with their collection from different areas of North America (one in Mexico, one in Colorado, both distant from Minnesota) suggests mating studies are not the highest priority at this time. We decided instead that it was most productive to determine if one or both of the Colorado species would attack and survive in GWSS eggs. For this purpose, Dr. Triapitsyn traveled to Colorado in early September 2007 (when parasitoid levels were high) to collect and ship (via permit) parasitized leafhopper eggs to UCR's Quarantine facility. In Quarantine, emerging wasps were exposed to GWSS eggs to determine if parasitization would occur (it did not).

In addition, we have shifted resources from mating studies to research on the basic biology of the Minnesota *A. epos* strain so that we can improve insectary rearing to allow greater numbers of parasitoids to be released in the field (see Objective 4).

Progress - Objective 4. Field Release and Evaluation of the "Minnesota strain" of *A. epos*.
Completing this objective has been compromised by the difficulty in rearing the Minnesota strain of *A. epos* on GWSS eggs during the winter, allowing us to build the colony to moderate numbers in spring for release. To solve this problem, Ph.D. student Rodrigo Krugner undertook a study examining other leafhopper species that might be used to rear the parasitoid (Krugner et al. 2007). Based on the results of that work, we have started to rear the aster leafhopper, *Macrosteles severini*, as an alternative leafhopper egg host.

We have spent much of the past two years studying the basic biology of the Minnesota strain of *A. epos* and working with CDFA on how to mass rear this species for field evaluation. CDFA and UCR alternated bi-weekly monitoring of endemic and released egg parasitoids at each of 6 lemon study sites, three in the coastal region and three in interior southern California.

The CDFA currently has two colonies of *A. epos*, one in Riverside County and one in Kern County. A total of 59 releases were made in 2006 and a further 10 releases have been made in 2007 in Kern, Tulare, Riverside, and Orange Counties. Over 9,000 parasitoids have been released to date but no recoveries have been made. This may be due to poor survival of *A. epos* in the field, but may also be due to the difficulty in maintaining field-collected specimens sufficiently long to allow emergence of *A. epos* from egg masses.

CONCLUSIONS
See the Abstract.

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FUNDING AGENCIES

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

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ABSTRACT

The uptake of dinotefuran (applied as Venom 70SG) and imidacloprid (applied as Admire Pro) was compared in vineyards located in Napa Valley, Temecula Valley, and Coachella Valley. Our study clearly showed the interaction between the insecticide chemistry and the local soil and climatic conditions. In Napa, imidacloprid uptake was poor. Better uptake of the more soluble product dinotefuran was achieved, and this may provide growers in that region with an effective pest management tool for the protection of their vines against Pierce's disease (PD) vectors. In terms of the concentration of active ingredient in the xylem fluid, dinotefuran uptake was also superior in Coachella and Temecula vineyards. However, imidacloprid remains an excellent chemical for the protection of Temecula vines against glassy-winged sharpshooter (GWSS) and PD.

INTRODUCTION

Neonicotinoid insecticides play a major role in the management of the glassy-winged sharpshooter. The focus of this project has been to identify the factors that influence the successful deployment of these insecticides within different cropping systems affected by the GWSS. Soil type and irrigation play a major role, and their effects vary depending on the chemical properties and water solubility of the insecticides, as well as agronomic practices used in the production of table and wine grapes. In this project, we are studying the uptake and persistence of the neonicotinoids imidacloprid (Admire Pro) and dinotefuran (Venom) in three viticulture regions of California – Coachella Valley (table grapes), South Coast (wine grapes at Temecula Valley) and North Coast (wine grapes at Napa Valley) regions. These regions represent the extremes in terms of climate, soil type and irrigation practices, and are therefore good study sites for comparing the efficacy of the two most important neonicotinoids used for sharpshooter management. In terms of water solubility, imidacloprid and dinotefuran represent the two extremes within the neonicotinoid insecticide class. Dinotefuran has 80-fold greater solubility in water than imidacloprid, and this may make it a more viable option for North Coast growers looking for an effective soil-applied neonicotinoid insecticide under reduced irrigation. In contrast, imidacloprid may be the preferred product in Coachella where excessive use of irrigation water may compromise the use of highly soluble chemicals in favor of less soluble products.

OBJECTIVES

1. Determine regional differences in the uptake and persistence of imidacloprid and dinotefuran in grapevines in order to maximize protection of vineyards.
2. Evaluate generic formulations of imidacloprid in grapevines.
3. Develop an ELISA for the detection and quantification of dinotefuran residues within plant tissues.

RESULTS

Trials to evaluate the uptake of imidacloprid and dinotefuran in vineyards located in three viticulture regions of California have been completed (Figure 1). In Coachella and Temecula Valley vineyards, two rates of Venom were evaluated – the full label rate (6 oz/acre) and half the label rate (3 oz/acre) - and the half label rate of Admire Pro. We used the half label rate of Admire Pro (7 fl oz/acre) because this rate achieved the desired 10 ppb threshold concentration in the xylem necessary to kill a sharpshooter (Byrne and Toscano, 2006). In Napa, we examined the same rates of Venom used in Coachella and Temecula, but used the full label rate of Admire Pro. In an earlier study, both the half and full label rates resulted in poor uptake (Weber et al., 2005), so we chose the maximum rate for this study.

The uptake of imidacloprid in at the Napa vineyard was poor, corroborating our earlier findings (Weber et al., 2005). Of the 144 samples taken, only one vine recorded a value above the 10 ppb threshold. Most vines were below the detection limit of

the ELISA (4 ppb). The uptake of imidacloprid in Coachella and Temecula was better, although levels were consistently better in Temecula. Levels in Coachella were marginally lower then the recommended threshold throughout most of the assessment. While we expected better concentrations, it is likely that the irrigation regime at this vineyard was not conducive to providing the desired levels of uptake. In Temecula, imidacloprid moved into vines quickly. There was a period, however, when levels dropped below the required threshold. This dip in concentration occurred when irrigation water was reduced. During the period when weekly irrigation was practiced, the uptake rose well above the threshold levels and the vines were well protected from PD.

Dinotefuran was detected in vines at each vineyard location, and the concentrations in the xylem were highest in vines treated at the full label rate. In Napa, dinotefuran levels rose quickly, suggesting that the uptake occurred at the time the treatments were made (the only time water was used in this vineyard). The rate of uptake in Coachella and Temecula was also rapid but more sustained, again suggesting the influence of the irrigation at these sites. In Temecula, the dip associated with imidacloprid uptake was evident at the lower rate of dinotefuran, but not at the high rate.

The effect of the greater solubility of dinotefuran compared with imidacloprid is reflected in the higher levels of dinotefuran at the three sites. But this also results in a more rapid decline.

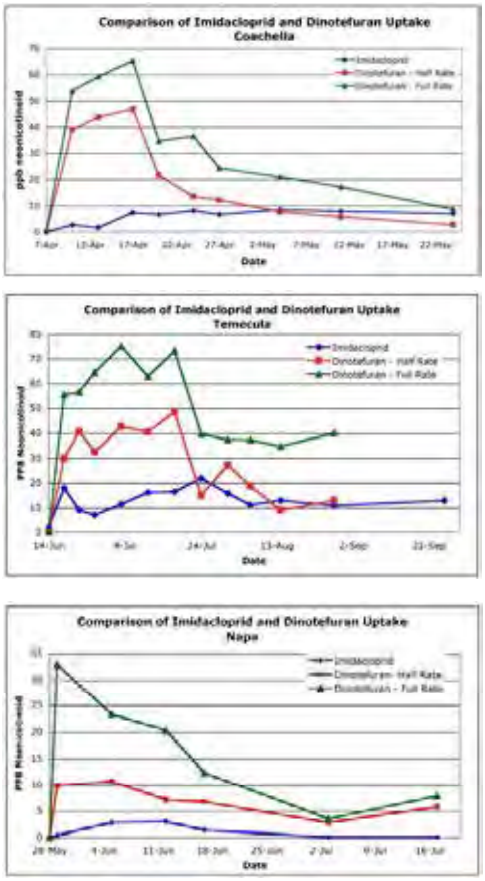

Figure 1. Uptake of imidacloprid and dinotefuran in vines at three vineyard locations in California. Dinotefuran was applied as Venom 70SG and imidacloprid was applied as Admire Pro. The full rate of Venom was 6 oz/acre. Both full and half label rates were compared at each location. Imidacloprid was applied at half label rate in Coachella and Temecula, while the full label rate was used at the Napa vineyard. The final dinotefuran sample set for Temecula has yet to be analyzed. Each point on the graphs represents the mean for at least 12 vines.

CONCLUSIONS

Dinotefuran proved better than imidacloprid in terms of uptake in the Napa vineyard. In this region, imidacloprid uptake is compromised by the heavy clay soils (which bind the insecticide tightly, making it unavailable for uptake through the roots) and the lack of irrigation (a consequence of the local climatic conditions). Although we observed rapid uptake at the full label rate of Venom, the concentrations began to decline after the first week. The spike in uptake occurred at the time of the application and suggests that irrigation at the time of the application will need to be carefully controlled if optimal delivery of the product is to be achieved.

It is clear from our data that the uptake of dinotefuran is superior to that of imidacloprid at all sites. But the question remains whether the levels attained are actually high enough to provide effective pest management. This is an area of research that will need to be investigated.

Imidacloprid remains the neonicotinoid of choice for Temecula vineyards. The uptake at half the label rate provides good protection to vines, both in terms of rapid uptake and persistence during the growing season. In vineyards with a heavier soil, growers might be advised to use a higher rate of application, particularly if there are periods when irrigation water is withheld. At these times, the imidacloprid is likely to bind to the clay particles, making it more difficult for uptake to occur in the reduced water environment around the roots.

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FUNDING AGENCIES

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

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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, builds 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. Temecula and Coachella Valley GWSS populations have decreased since the treatment programs were initiated. With the exception of a little upsurge in 2007 Temecula GWSS populations; overall GWSS numbers in both valleys have declined substantially relative to the pre-action levels due to insecticide applications.

INTRODUCTION

The GWSS vectors a bacterium that causes PD. This insect and bacterium are a severe threat to California's 833,644 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 over $100 million of revenue for the economy of the area. GWSS/PD caused a 40% 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. Populations of this insect in Coachella Valley increased the danger of PD occurrence in this area, as has occurred in similar situations in the Temecula and San Joaquin Valleys. In July 2002, the occurrence of *Xf*, the PD bacterium, was found in 13 vines from two adjacent vineyards in the southeastern part of Coachella Valley. With this discovery, and the increasing GWSS populations, there was and is a real need to continue an area-wide GWSS/PD management program, to prevent an economic disaster to the work forces and associated 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 in that area. 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 950 acres of citrus in Riverside County were treated for the GWSS in June through September, 2007, through a cooperative agreement with USDA-APHIS and the Riverside County Agricultural Commissioner's Office for the area wide management of the GWSS in the Coachella and Temecula valleys. The cost of 2007 Riverside County GWSS insecticide treatments was approximately $126,000.

OBJECTIVES

1. Delineate the areas to be targeted for follow-up treatments to suppress GWSS populations in the Temecula and Coachella Valleys for 2007.
2. Determine the impact of the 2006 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 participation of growers, pest management consultants, and citrus and vineyard managers. 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, UC Riverside, UC Cooperative Extension, and grower consultants. Representatives of these agencies meet periodically 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 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 traps (7 x 9 inches) were used help determine GWSS population densities and dispersal/movement within groves and into vineyards. A total of 1,397 GWSS yellow sticky traps are monitored weekly. Based on trap counts and visual inspection, approximately 885 and 70 acres of citrus were treated in Coachella and Temecula, respectively, for GWSS control in 2007. In Temecula and Coachella Valley, treatments for GWSS in citrus were initiated when at least two GWSS adults were found at the same trap location for two consecutive weeks. In Temecula Valley only the citrus where the GWSS was found were treated. In Coachella Valley all citrus located within a 1.0 mile radius from the trap find were treated as a preventive measure to protect surrounding groves and vineyards. The decision to treat a greater area around 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 during the fourth year after GWSS area-wide programs were initiated. One hundred percent of the 885 acres of Coachella Valley citrus were treated with a single application of Alias (imidacloprid) at 36 ounces per acre. In Temecula Valley 40 acres of citrus were treated with Lorsban (chlorpyrifos) at the rate of 7 pints per acre followed by an application of AdmirePro (imidacloprid) at 14 ounces per acre. In the remaining 30 acres in the Temecula area where PyGanic was used to manage GWSS in organic groves, a follow-up treatment of PyGanic was applied a month after the first application for two consecutive months. PyGanic (5% pyrethrum) was applied at the rate of 7 pints per acre. Treatment threshold numbers of GWSS were not trapped until July (Figures 1, 2 & 3). Therefore, 2007 Coachella and Temecula GWSS insecticide applications were applied in late July and early August. Application of imidacloprid in citrus this late into the season is not ideal for GWSS control.

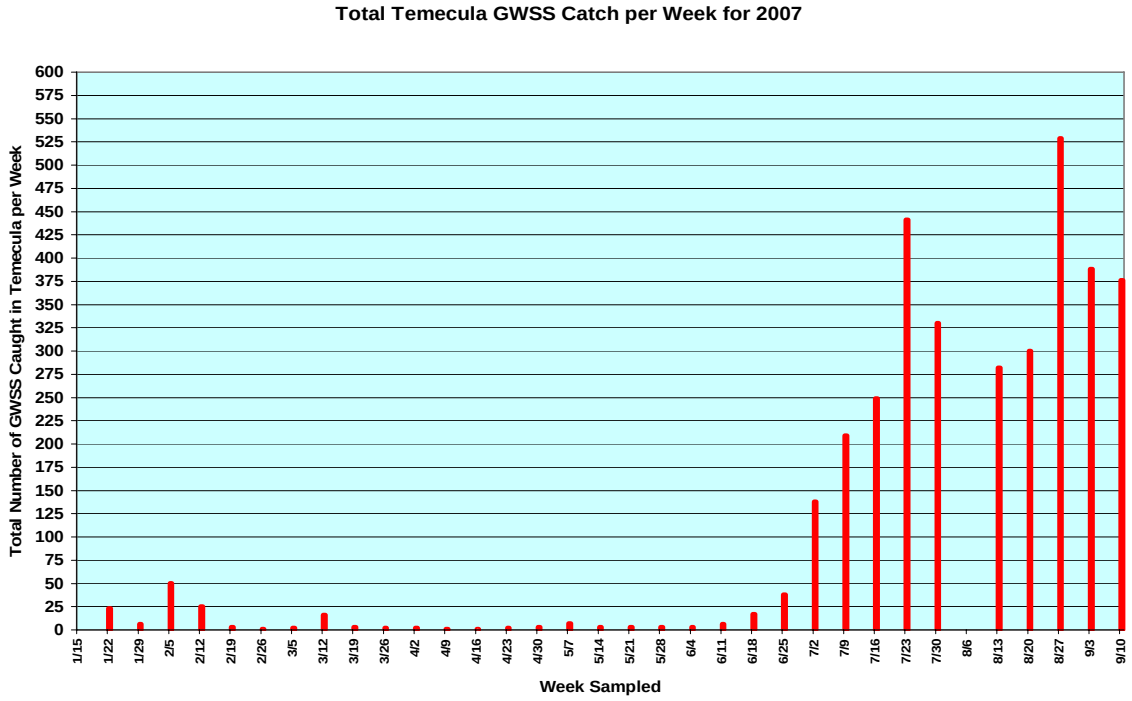

Figure 1. In 2007, high numbers of adult glassy-winged sharpshooters were caught on the yellow sticky traps in Temecula, with populations peaking in August and reaching a total of 530 trapped the week of August 27.

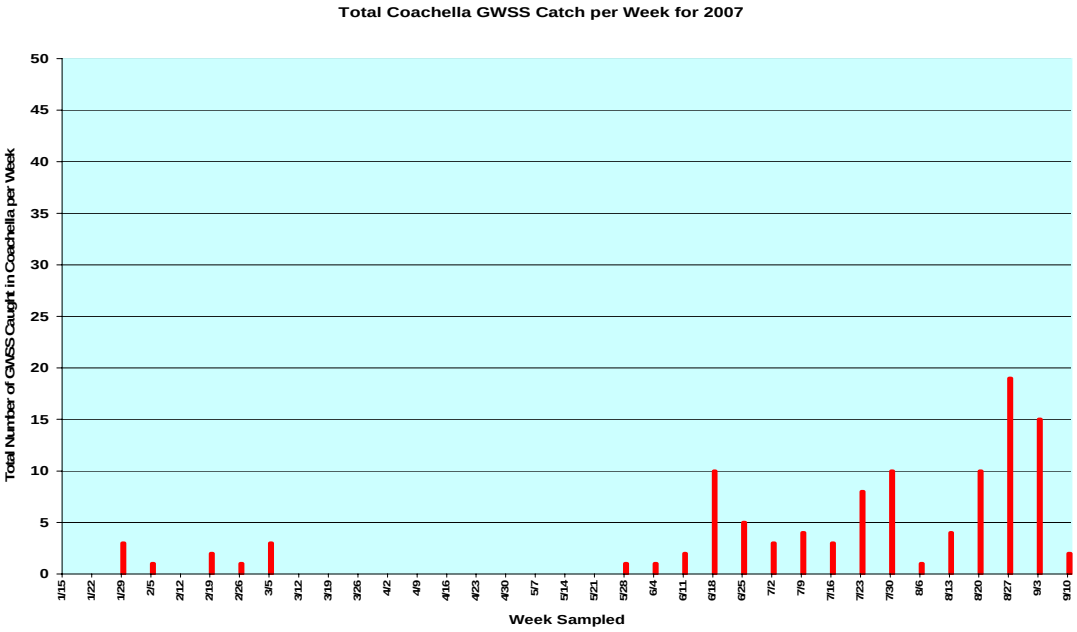

Figure 2. Glassy-winged sharpshooter populations in Coachella Valley peaked August 27 with a high of 19 trapped.

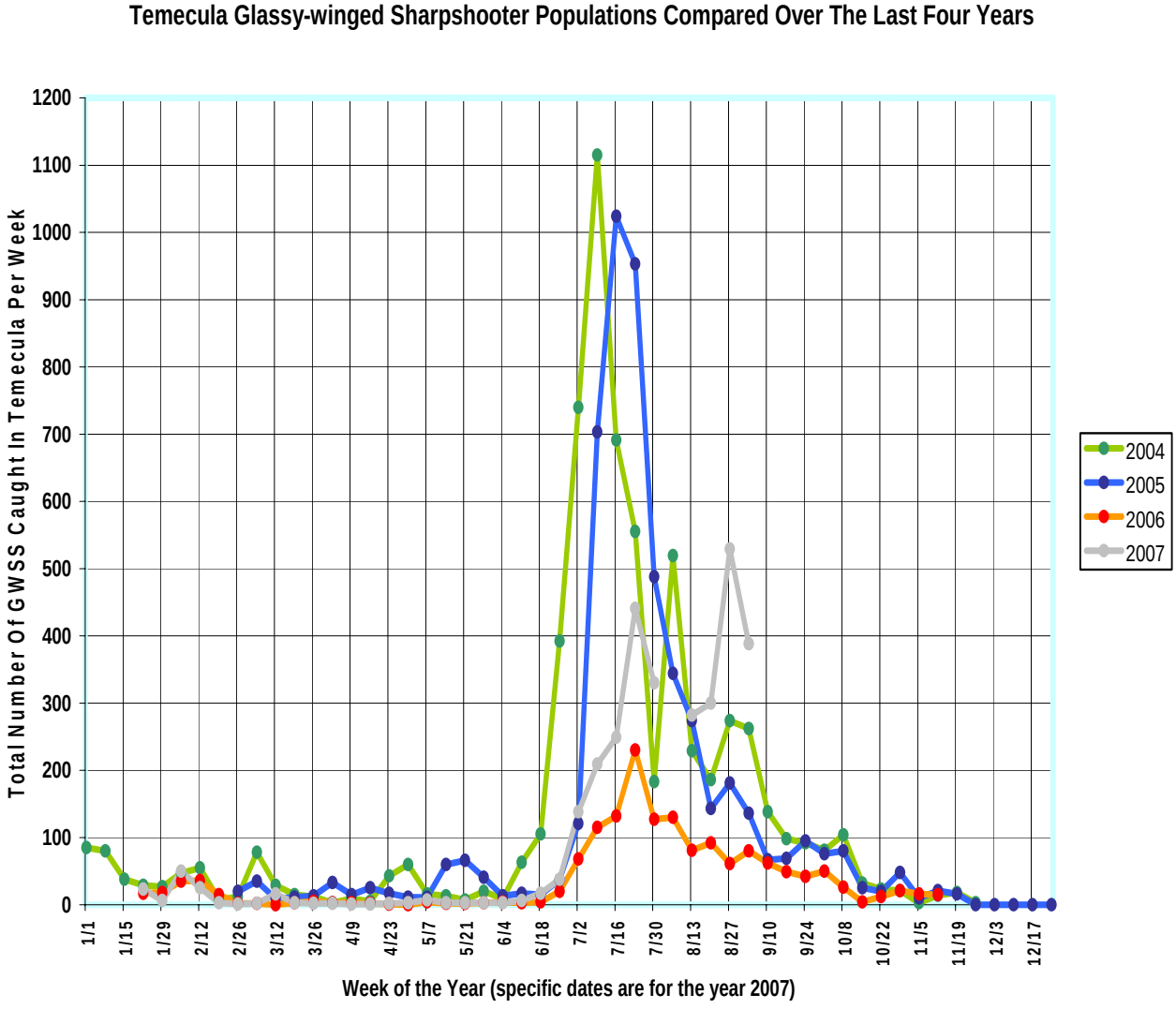

Figure 3. Temecula glassy-winged sharpshooter populations compared over the last four years

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Additional note: We would like to especially thank Ben Drake of Drake Enterprises for his input, and grape and citrus growers, managers and pest control advisors for their needed cooperation to make the Riverside County GWSS area-wide management program successful.

Section 3:

Pathogen Biology and Ecology

BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

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ABSTRACT
The Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is the causal agent of many economically important plant diseases. This bacterium attaches to, multiplies in, and is inoculated from the foregut of sharpshooter leafhoppers, which feed on plant xylem sap. Little is known about the specific interactions between *Xf* cells and its vectors' foregut. Constituents of the foregut include mucopolysaccharides, chitin, proteins and other components. We have developed an in vitro assay to study the attachment of *Xf* to carbohydrates using nitrocellulose membranes coated with different polysaccharides. We found that *Xf* cells attach to polysaccharides that contain a chain of glucose or *N*-acetylglucosamine. These results suggest that the attachment of *Xf* cells to surfaces may be carbohydrate mediated. *Xf* cells treated with either protease K or pronase showed less attachment to the polysaccharides tested. Competition experiments with different sugars and lectins suggest that attachment of *Xf* to vector's foregut is carbohydrate mediated and that proteins of *Xf* may work as lectins that have affinity to sugars, especially glucose and *N*-acetylglucosamine. In order to identify carbohydrate binding proteins in *Xf*, we have studied the attachment of 15 different mutants to foregut extracts and different polysaccharides blotted in nitrocellulose membranes. Only hemagglutinin-like protein mutants exhibited less attachment. Insect transmission tests for the different mutants were also done; we found a correlation between the in vitro attachment assays and vector transmission to plants of the mutants tested.

INTRODUCTION
Sharpshooter transmission of *Xf*
Transmission of *Xylella fastidiosa (Xf)* by sharpshooters is unique in terms of vector-pathogen relationships. Firstly, the interactions seem to be group-specific when compared to other systems, i.e. virtually all xylem sap-sucking insects can (and those tested do) transmit different strains of *Xf* (Almeida et al. 2005). Such lack of specificity suggests that there is a general mechanism for pathogen attachment, multiplication and detachment. The lack of transstadial transmission and latent period indicates that the *Xf* inoculum is limited to the cuticle of the foregut of vectors, which is shed with each nymphal molt. Purcell et al. (1979) and Brlansky et al. (1983) showed the presence of *Xf* in the cibarium and precibarium of vectors, corroborating the assumption that cells should be present in the foregut of infective individuals. Newman et al. (2003), using cells with a GFP-*Xf* cells, demonstrated the presence of cells in the precibarium of infective vectors. The direct association of the precibarium as the source of inoculum, however, has only been recently demonstrated. Newman et al. (2004) showed that poorly-transmitted *Xf* mutants did not colonize the precibarium of vectors; and Almeida and Purcell (2006) showed that insects transmitting *Xf* had the bacterium in their precibarium, whereas non-transmitting individuals did not. The later work indicated that *Xf* must attach to the precibarium of vectors to be inoculated into plants.

Molecular interactions between *Xf* cells and the foregut of vectors
Xf cells probably have complex interactions with the foregut of vectors, as other xylem-limited bacteria such as *Leifsonia xyli* are acquired but not transmitted by insects (Barbehenn and Purcell 1993). However, little is known about the specific interactions between *Xf* and the foregut of vectors. On the other hand, the insect's cuticle structure is reasonably well understood. The cuticle lining of the foregut of sharpshooters, to our knowledge, has not been studied, but is assumed to be similar to the cuticle of insects in general. Considering the components of cuticle, we hypothesize that *Xf*'s attachment to cuticle could be mediated by carbohydrates. Those could be mucopolysaccharides, glycoproteins, chitin or other polysaccharides and lipopolysaccharides. Thus, our approach was to initially determine the general type of interaction between *Xf* and components likely to be present on the surface it attaches to in insects.

OBJECTIVES
1. Determine the nature of molecular interaction between sharpshooter vectors and *Xf*.
2. Develop an *in vitro* assay to study attachment of *Xf* to sharpshooter foregut and polysaccharides.
3. Identifying *Xf* proteins involved the transmission process.

RESULTS
I- Characterization of *Xf*-vector interface
An in vitro assay was developed to study the attachment of *Xf* cells to polysaccharides. In this method, pieces of nitrocellulose membrane were soaked for one hour in different polysaccharide solutions (concentration 1%), which were

washed and incubated with buffer or *Xf* cells. Detection of cells was done with Ponceau S or amido black dyes. Both of them are protein specific dyes, but amido black is more stable and quantifiable.

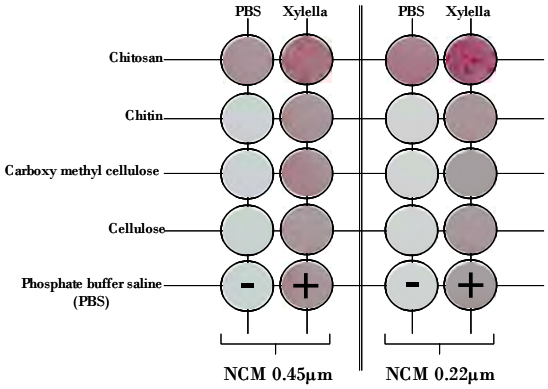

Figure 1. Attachment of *Xf* cells to different polysaccharides. Attached cells were detected by protein specific staining using Ponceau S. (darker spots indicate presence of *Xf*). The PBS columns are a buffer control. Note that chitosan stained unspecifically, but *Xf* cells were still detectable. Some of these polysaccharides are expected to be similar to those present in the foregut cuticle of vectors.

Figure 2 shows results obtained from tests performed with wheat germ agglutinin (WGA) as a lectin and amido black as protein staining dye. In this experiment attachment of WGA to polysaccharides was used as a positive control and to measure the binding of the *Xf* cells to polysaccharides. *Xf* cells showed an attachment profile similar to that of the lectin WGA. In contrast, bovine serum albumin (BSA) negative controls did not attach to any polysaccharide except chitosan, with which we have consistently found background problems. This experiment showed that the attachment to polysaccharides is specific and confirms that *Xf* cells have outer membrane molecules which act as lectins and attach to polysaccharides.

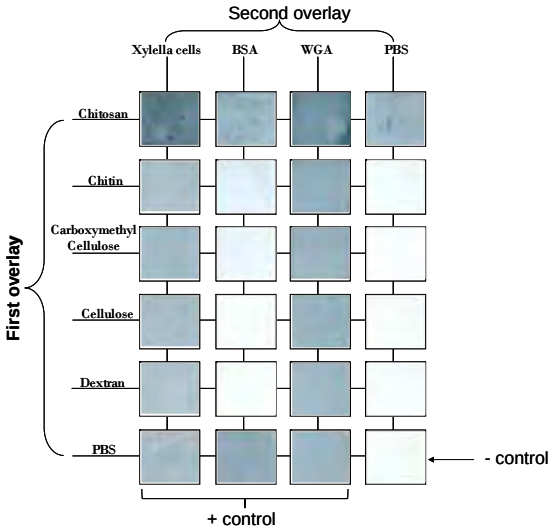

Figure 2. *Xf* attachment to polysaccharides. Acronym: *PBS: Phosphate buffer saline.*

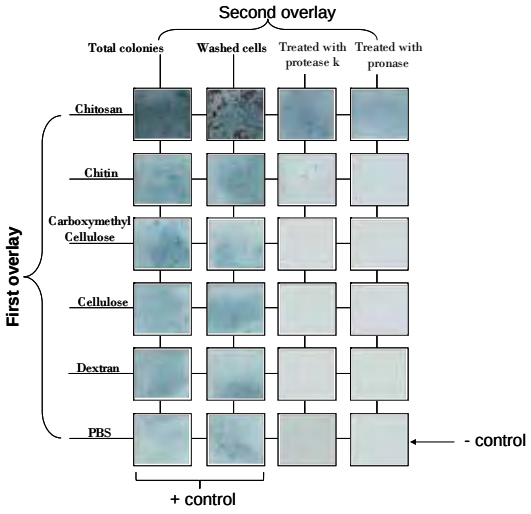

Figure 3. Effect of protease treatment on *Xf* attachment to polysaccharides.

We also studied the effect of treatment of *Xf* cells with proteases on its attachment to polysaccharides (Figure 3). The aim of the experiment was to determine if secreted and membrane proteins play a role in attachment. Washed cells showed similar attachment to polysaccharides as the control. On the other hand proteinase treated cells attached less to the polysaccharides. These results suggest that *Xf* secreted proteins may play a minor role in attachment and that membrane proteins have an important role in this process.

II- Identification of *Xf* membrane proteins associated with attachment to polysaccharides *in vitro*

In order to identify carbohydrate binding proteins in *Xf*, we used several mutants including those of cell-cell signaling and hemagglutinin-like proteins, pili and gum mutants. Hemagglutinin genes affect the virulence of *Xf* in grapevines. The mutants *hxfA*- and *hxfB*- showed hypervirulence in plants; and interestingly attached less to glass than the wild type (Guilhabert and Kirkpatrick 2005). The series of *rpf* cell-cell signaling system mutants have different attachment to glass, aggregation and biofilm formation phenotypes (Newman 2004, S. Chatterjee *personal communication*). In addition, we also used new mutants that were produced by the Lindow lab, which targeted *Xf*'s intracellular cell-cell signaling system; in our study those mutants were labeled mut1 and mut2 (the difference is the mutation site in the gene). Both mut1 and mut2 attached to glass and formed more cell aggregates than the wild type. We also used another *hxfB* mutant, produced by

homologous recombination (Feil *et al*. 2007). Pili and fimbriae mutants were thought to be important in the attachment to foregut and the insect transmission and were included in the study. Bacterial gum production plays a role in virulence in plant like host xylem vessel blockage (Roper et al 2007). As the role of gum in the interactions with vector is unknown, we included two gum mutants in our study. *In vitro* attachment assays were performed to test these mutants in their capacity to bind to foregut extracts and polysaccharides. In these assays, foregut extracts, foregut proteins (extracted by ultra sonication and acetone precipitation), wing extracts, crab shell chitin, PBS, ovalbumine and BSA were dotted in strips of nitrocellulose membrane and *Xf* cells added as an internal control in each strip. After blocking with 6% non fatty milk in PBS (0.1M), strips were incubated with PBS containing *Xf* mutants. The interaction buffer had 0.01M PMSF as anti-protease to protect cells from degradation. Immunological detection of attached *Xf* cells was performed using purified IgGs polyclonal antibodies against *Xf* whole cells.

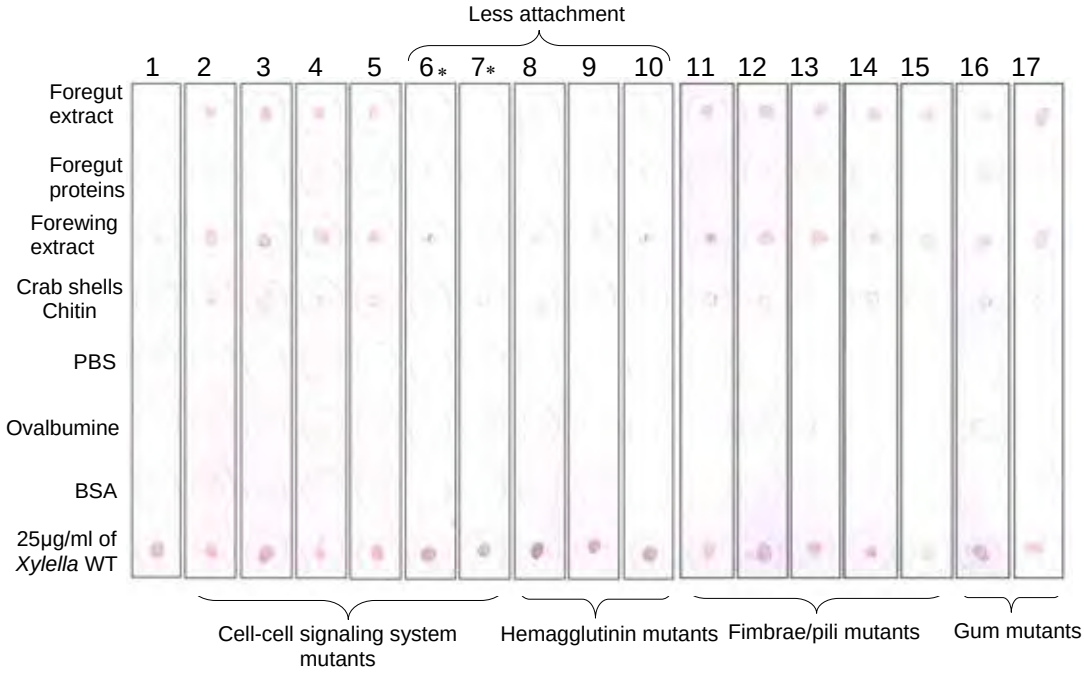

Figure 4. Attachment of *Xf* mutants to foregut extracts and polysaccharides *in vitro*. 1: PBS, 2: WT, 3: mut1, 4: mut2, 5: rpfC, 6: rpfF, 7: rpfF/rpfC, 8: hxfA, 9: hxfB, 10: hxfB (Feil), 11: fimA, 12: fimF, 13: pilB, 14: fimA, (6E11), 15: fimA/pilO (DM12), 16: GumD, 17: GumH. Strips were incubated with 2ml of 50µg/ml of *Xf* cells. Immunodetection performed with polyclonal antibodies against whole *Xf* cell followed with anti-rabbit IgG (Whole molecule) - Alkaline phosphatase antibodies. Alkaline phosphatase activity was detected by BCIP/NBT substrate.

Results obtained from this experiment show that hemagglutinin-like protein mutants are affected in the attachment to polysaccharides and foregut extracts. In addition, only the mutant *rpfF* and the double mutant *rpfF/rpfC* were also affected in attachment to the compounds tested. In studies on the gene expression of *HxfA* in the *rpfF* mutant, Lindow`s group found that *hxfA* was expressed less in the mutant in comparison to the wild type (Nian et al 2006). These results suggest an important role for hemagglutinin in *Xf* attachment to the foregut of sharpshooters. Also the low expression of hemagglutinin in the *rpfF*- mutant could explain the low percentage of its insect transmission (5% in the comparison with wild type) (Newman et al. 2004).

III- Sugar affinity

Competition experiments with different sugars were carried out in order to determine the affinity of the carbohydrate binding proteins in *Xf*. Results obtained showed that the presence of glucose, N-acetylglucosamine (GlcNAc), chitobiose ($[GlcNAc]_2$), and chitotriose($[GlcNAc]_3$) reduced the attachment of *Xf* to foregut extracts. Mannose also had an effect in reducing the attachment but no effect was observed for galactose. This affinity suggests the presence of chitin binding proteins (ChBPs) in the *Xf* cell membrane. These ChBPs could play an important role in the attachment to foregut and the efficient transmission of *Xf* to plants.

VI- Vector transmission of *Xf* mutants to plants

We conducted several experiments to determine the transmission efficiency of *Xf* mutants to plants in relation to the wild type. In the first one, we studied the transmission and retention of *rpfB*- mutant. We observed that *rpfB*- mutants were

transmitted similarly to the wild type, but that cells were not observed in the foregut of vectors by scanning electron microscopy. Instead, a complex matrix was detected (Almeida and Lindow, unpublished data). We hypothesized that *rpfB*- could be lost by vectors over time, and that transmission efficiency would be reduced with sequential transfers of infective vectors to new plants. This was confirmed with transmission experiments. In another experiment, we looked at the transmission of fimbrial adhesin mutants (*fimA*-, *pilB*- and a *fimA-/pilQ* double mutant). Transmission experiments showed that these appendages are not essential for vector transmission. The mutants *fimA*- and *pilB*- were transmitted less efficiently than the wild type, and the *fimA-/pilO*- double mutant was not transmitted, but we were not able to determine if that is due to the *Xf*-vector interaction or low pathogen population in plants that may have affected acquisition efficiency. S. Lindow, a cooperator in this project, performed work with an *rpfC*- mutant. *rpf* genes are associated with *Xf*'s cell-cell signaling and have been shown to affect pathogenicity and transmission to plants. We have transmission data for three *rpf* mutants (*F, C, B*). *rpfF*- is poorly transmissible, *rpfC*- has transmission rates slightly lower than the wild type and *rpfB*- is poorly retained by vectors. For a few of these mutants it is difficult to determine if they were not transmitted with similar rates as the wild type because i) they were not acquired as well, ii) they did not attach to vector's cuticle, iii) acquisition and retention occurred, but inoculation events did not generate infections, or iv) those successful infections did not multiply/move within plants and were not detected. Nevertheless, the results show that fimbrial adhesins are not essential for *Xf* transmission and that different genes are likely important for cell attachment, indicating that this is a complex biological process. As a side note, our results also demonstrated one more time how inefficient glassy-winged sharpshooter is in transmitting *Xf* to plants when compared with Blue-green Sharpshooter (data not shown). We are currently conducting tests with other mutants of interest identified by our in vitro work. In addition, gene expression data of several mutants and their analysis in relation to transmission biology indicate that hemagglutinin expression is strongly correlated with vector transmission of *Xf* to plants. On the other hand we showed that, fimbrial adhesions are not implicated in insect transmission. Taken together, our results suggest that hemagglutinin-like proteins are important for efficient vector transmission of *Xf*. Due to the biological properties of hemagglutinin, much of our current work is focused on glycobiology.

CONCLUSIONS

The goal of this project was to generate information on how *Xf* interacts with leafhopper vectors at the molecular level. Although this has been assumed to be a complex association, so far it has remained a 'black box.' We have started to dissect this system. We demonstrated that fimbrial adhesins, which were previously considered as essential for transmission, are not required for it. In addition, our biochemical characterization of these interactions indicated that proteins are associated with attachment to vector's cuticle and tentatively identified hemagglutinins as important for *Xf* transmission. We are now testing if that [hemagglutinin] gene (two copies in the Temecula genome) is essential for transmission. In summary, we went from a 'black box' to testable hypotheses and the identification of genes that may be important for *Xf* vector transmission. The determination of how *Xf* interacts with vectors will open new venues to control disease spread, as understanding how pathogen and vector interact may lead to strategies to block the transmission of *Xf* to plants.

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EVOLUTION OF *XYLELLA FASTIDIOSA* AVIRULENCE

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

ABSTRACT
The main objective is to quantitatively and qualitatively determine how and when *Xylella fastidiosa (Xf)* loses pathogenicity and potentially transmissibility, after serial passages *in vitro*. We will replicate *Xf in vitro* for several generations, creating parallel populations that are not pathogenic and maybe not transmissible by insects. We will study host plant colonization by and insect transmission to grape of these populations. Once phenotypes of interest are identified (e.g. reduced pathogenicity or transmissibility), we will compare these *Xf* populations with the original isolate and search for differences. Tools to identify phenotypical differences will include pathogenesis and insect transmission assays, molecular differences will be identified with genomic and proteomic approaches. We will also be able to quantify the rate of genetic change in these populations, providing a molecular calibration data for researchers interested in *Xf* evolution, diversity and ecology.

INTRODUCTION
Much has been learned in the last few years regarding the biology of *Xylella fastidiosa (Xf)*. In addition, recently reported research has demonstrated, under laboratory conditions, that proof-of-concept approaches to disease control may be successful. Hopkins (2005) tested the capability of weakly virulent and avirulent *Xf* isolates to control infections of pathogenic isolates of the same pathogen. Biological control of plant pathogens with avirulent strains is not a new idea in phytopathology, in fact, it has been successfully used in many occasions. However, it had never been tested for *Xf*. Hopkins' work demonstrated that this approach has great potential to control Pierce's disease (PD) under field conditions.

This project explores the idea developed by Hopkins (2005) that avirulent isolates of *Xf* can control PD symptom progression in grapevines. If such strategy is ever to be used to control PD, understanding how it works will be of paramount importance. To achieve such objective, we propose to conduct research with a pathogenic isolate that has been sequenced, develop avirulent descendent populations of such isolate, and study their ability to reduce disease progression in plants challenged with the original pathogenic isolate. We will also take advantage of the fact that *Xf* can be stored in -80^{o}C to keep samples of the pathogen as it becomes avirulent. This will allow us to retrospectively study when and how an isolate lost pathogenicity, and what is the impact of such change on *Xf*'s biology.

OBJECTIVES
1. Generation of in vitro evolved populations
2. Phenotypical and molecular characterization of populations
3. To test avirulent populations as PD biological control agents

RESULTS
This work is ongoing. So far we have sequentially transferred 10 parallel populations of *Xf* on solid media for 50 weeks (estimated 1,150 generations) and have those stored in a -80^{o}C freezer for phenotypical characterization.

CONCLUSIONS
This work is ongoing (objective 1) and the characterization of populations and testing of avirulent isolates as biological control agents will be performed in 2008

REFERENCES
Hopkins, D.L. 2005. Biological control of Pierce's disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. Plant Disease 89: 1348-1352.

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

MODELING SHARPSHOOTER TRANSMISSION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted September 2006 to September 2007.

ABSTRACT
The dynamics of vectored diseases are governed by the interplay of a variety of biotic and abiotic factors. We studied some of these factors that are expected to be important to the epidemiology of Pierce's disease (PD) in California vineyards. We conducted a series of greenhouse transmission experiments to quantify how sharpshooter species, sharpshooter number, plant inoculation access period, and temperature affect transmission of *Xylella fastidiosa* (*Xf*) to grapes and PD symptom development. For the blue-green sharpshooter (BGSS), vector number and inoculation period had similar strong effects on infection rate. Interestingly, larger numbers and longer inoculation periods increased the onset of PD symptoms. However, while the number of glassy-winged sharpshooters (GWSS) increased infection rate, inoculation access period did not. In a final experiment looking at the relationship between temperature and transmission efficiency, high temperature (30^{o}C) resulted in low BGSS survival yet marginally higher infection rate. Moreover, *Xf* population in vines was positively related to the number of vectors that tested positive for *Xf* using realtime PCR at the time of plant inoculation. These results suggest that high sharpshooter numbers may not only increase transmission rate but also decrease incubation period – presumably because of a larger inoculum. We expect that high vector densities and temperatures will increase the rate of disease cycling, which is particularly relevant to disease prevalence in this system.

INTRODUCTION
Pierce's disease (PD) epidemiology is complicated by the potential for several insects to vector *Xylella fastidiosa (Xf)* (Severin 1949), by having many alternative reservoirs of the pathogen (Wistrom and Purcell 2005), by *Xf* strain- or grape cultivar-specific differences in pathogenicity or resistance, and by seasonal acquisition and vine recovery (Hill and Hashim 2006). These factors likely contribute to variability in disease spread, hampering efforts to quantitatively describe disease dynamics in this system.

An understanding of transmission biology is critical to predicting PD epidemiology (Almeida et al. 2005). Fortunately experiments on vector transmission have been a major focus of PD research for over 50 years – contributing a vast amount of biological data on important vectors and their ability to transmit *Xf* (Severin 1949, Purcell 1981, Hill and Purcell 1995, 1997, Almeida et al. 2005). None-the-less little effort has been made to synthesize this work into a quantitative framework. To date the only quantitative description of *Xf* transmission is that of Purcell (1981). This statistical model predicts the probability a plant becomes infected (**P**) as a function of vector number (***n***), inoculation access period (***t***), vector infectivity (***i***), and inoculation efficiency (***E***): $\mathbf{P_{nt} = 1\text{-}e^{-niEt}}$. Two aspects of this model are important to note. First, any environmental (e.g., temperature, humidity) or ecological factors (e.g., vector species, pathogen virulence, plant abundance) that affect vector or pathogen abundance, vector feeding behavior, or efficiency will influence transmission. Second, the model assumes that vector number and inoculation access period are algebraically equivalent. In other words, 10 vectors for one day should result in the same infection rate as one vector for 10 days.

Our goal was to test and further refine this model via experimental estimation of the parameters explicitly included in this model as well as certain other ecological or environmental factors which may influence transmission of *Xf* to grapes. To this end, we first tested the assumption that vector number and inoculation period have equivalent effects on transmission rate. We also tested for effects of ambient temperature on transmission efficiency, as sharpshooter survival, growth, and feeding rate depend on temperature (Johnson et al. 2006). These two experiments were conducted with two important vectors of *Xf*, the BGSS; *Graphocephala atropunctata* and the GWSS; *Homalodisca vitripennis* to compare species-specific differences. Finally, in order to better understand how these factors ultimately contribute to disease dynamics, we tracked symptom development and infection level in vines after insect transmission occurred.

OBJECTIVES
1. Decouple estimated effects of sharpshooter number and inoculation period on transmission.
2. Quantify effects of temperature on transmission efficiency.
3. Link transmission to disease progression in the plant.

MATERIALS AND METHODS

In the fall of 2006 we conducted two greenhouse transmission experiments that varied independently sharpshooter species, number, and inoculation access period (IAP). In the first experiment we confined *Xf*-free BGSS adults on infected (STL strain) grapevines for a four day AAP. We then caged one, two, or four of these BGSS on a healthy grape seedling (var. Cabernet Sauvignon) for half, one, two, or four days. There were at least nine replicate plants for each of the 12 bug number-IAP treatment combinations. Beginning one month later we visually inspected plants one to two times per week to determine when they first showed PD symptoms. After three months, seedling infection status was determined by culturing (Hill and Purcell 1995). We compared the onset of symptoms among treatments using a Cox proportional hazards survival analysis with bug number and IAP as continuous variates. We followed up significant effects with a logistic regression of IAP and bug number versus plant infection status.

Approximately one month later, we repeated this experiment with GWSS. *Xf*-free GWSS adults from a Riverside, CA colony were placed on *Xf*-infected (Temecula strain) grapevines for four days (AAP). Afterwards one, two, or four bugs were transferred to a healthy grape seedling for half, one, two, or four days. There were at least five replicate plants for each treatment combination. We did not inspect plants for PD symptoms, but after three months they were all cultured for the presence of *Xf*. We used logistic regression to quantify the effect of bug number and IAP on plant infection status.

In the summer of 2007 we conducted a third transmission experiment that varied BGSS number and ambient temperature. We caged BGSS adults on known *Xf*-infected source plants (STL strain) for four days (AAP), after which we caged either one or four of these BGSS on healthy grape cuttings. These insects and vines were then placed in one of three temperature controlled rooms at UC Berkeley's Jane Gray Research Greenhouse that were set to approximately 20, 25, or 30°C during the day and 20°C at night. The rooms received natural light filtered through a shade cloth, plus grow lights on a 12:12 (L:D) photoperiod. There were at least 10 replicates of each treatment. After two days (IAP) we removed bugs and froze them. Three, six, and nine weeks later we sampled randomly two petioles from each plant, and at 10 weeks we sampled the stem at the top of where the sleeve cage was. We quantified *Xf* populations in stems and petioles using realtime PCR (qPCR; Francis et al. 2006), and qPCR was used on each sharpshooter to estimate the total *Xf* inoculum potentially introduced into each vine. We analyzed effects of bug number and temperature on disease status using logistic regression. For all positive plants, we used multiple regressions to quantify the effects of temperature, number of positive bugs, and total inoculum on stem infection level.

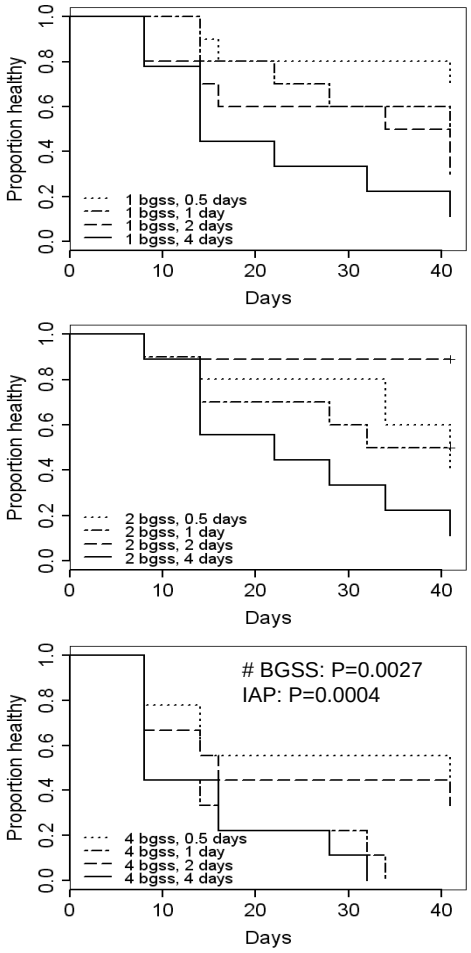


Figure 1. PD symptom onset as a function of BGSS number and inoculation period. Day 0 equates to approximately one month post inoculation.

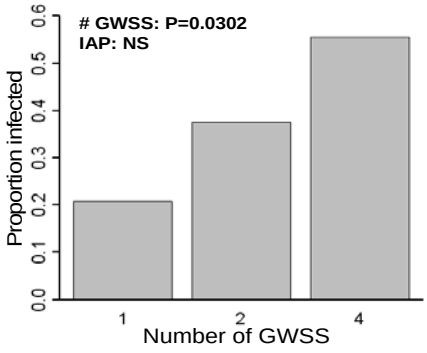


Figure 2. Infection rate increased with number of GWSS, while inoculation access period had no effect.

RESULTS

For BGSS, both vector number and IAP were strongly related to infection rate. IAP had a slightly stronger effect (regression coefficient = 0.5718, SE=0.1857) than did bug number (0.3963, SE=0.1785), though not significantly (P=0.4730). Both variables also increased the onset of first PD symptoms (Figure 1). Conversely, GWSS was not only less efficient over-all (36% infection vs. 64% for BGSS), but there were substantially different effects of bug number and IAP. Number of GWSS strongly increased vine infection rate, while IAP did not (Figure 2).

Temperatures in the third experiment averaged 24.3, 21.4, and 17.4°C. Higher BGSS number increased infection rate and temperature had a marginally significant effect (Figure 3). The proportion of plants infected was the highest in the four BGSS-high temperature treatment, despite substantially higher sharpshooter mortality at this temperature (45% at 24.3, 5% at 21.4, and 20% at 17.4°C). Few petioles tested positive at weeks three and six, but several were positive by week nine – with

generally increasing trends in infection level through time. *Xf* population in the stems (excluding uninfected vines) was not significantly affected by temperature or total *Xf* from bugs, but there was a positive effect of the number of sharpshooters that tested positive for *Xf* from qPCR analyses (Figure 4).

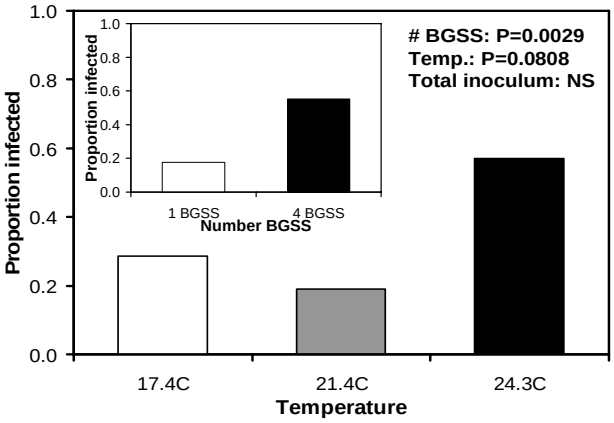


Figure 3. Infection rate as a function of temperature and BGSS number (inset).

DISCUSSION

Our first goal was to quantify the independent contributions of vector abundance and inoculation period on transmission rate, which is necessary to begin to parameterize models such as that of Purcell (1981). BGSS is clearly a more efficient vector of *Xf* than is GWSS, though the mechanism is not known. It may relate to feeding site preference or probing behavior. Regardless, our results suggest that sharpshooter species may differ in the relative strengths with which vector number and inoculation period contribute to transmission. Vector number and inoculation period appear to have equivalent effects on BGSS transmission, whereas IAP was weakly related to GWSS transmission (Figures 1, 2). This result suggests that GWSS inoculation, if it occurs, occurs soon after encountering the healthy plant (e.g., within 1 day) – a conclusion that is inconsistent with previous transmission experiments (Almeida and Purcell 2003).

In conjunction with these two experiments we are compiling the results of prior BGSS transmission experiments to conduct a meta-analysis on the role vector number, acquisition period, and inoculation period play in determining transmission of this efficient *Xf* vector. This work will provide more statistical power to precisely evaluate the fit of Purcell's (1981) transmission model.

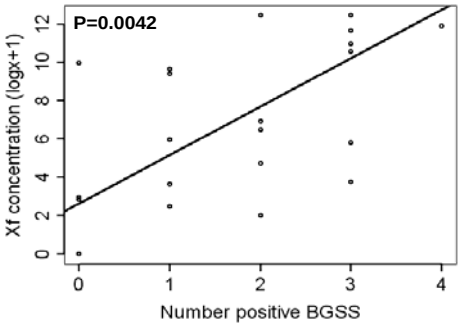


Figure 4. *Xf* population in the stems increased as a function of the number of BGSS identified as positive by qPCR

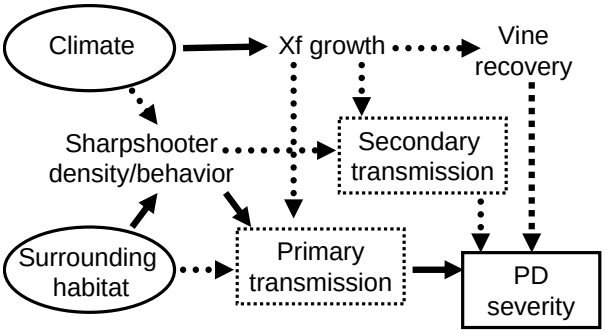
The second set of results we collected relate to the effect of temperature on sharpshooter transmission efficiency. A study with GWSS indicated that its survivorship and feeding rates are an increasing function of temperature up to a point (maximum approximately at 30°C), above which mortality greatly increases and feeding shuts down (Johnson et al. 2006). Therefore, if transmission is related to feeding rate, sharpshooter transmission efficiency is expected to also be an increasing function of temperature. In our experiment, BGSS transmission was higher at the warmest temperature (mean=24.3°C) despite substantially higher sharpshooter mortality at this temperature (Figure 3). These results are consistent with the hypothesis that stressed sharpshooters may adjust feeding behavior, thereby affecting transmission rates. Testing this hypothesis would require estimates of BGSS feeding rates, movement on the plant, and probing behavior over a range of temperatures. We are currently conducting a similar experiment with GWSS to determine how temperature affects its transmission efficiency.

The third set of results we collected relate to the link between transmission and disease progression in the plant. In the first experiment grape seedlings with more BGSS or with longer inoculation periods had shorter incubation periods. We attribute this result to the higher sharpshooter loads favoring the introduction of larger *Xf* inoculum. In the last experiment we measured infectivity of individual bugs using realtime PCR. This provided us with two related metrics of potential *Xf* inoculum for each replicate – the number of positive insects that tested positive, and the total estimated *Xf* population among insects on a given plant. Quantitative measurements of *Xf* populations in insects were highly variable and therefore a poor predictor of the infection level in the plant. However, the number of positive insects was positively related to plant infection level (Figure 4). Together these results support the prediction that high vector loads increase PD onset because of larger initial inoculum. Based on previous research showing that temperature affects *Xf* growth (Feil and Purcell 2001), we expected to see higher *Xf* populations in infected vines at higher temperature. The weak affect of temperature on plant infection levels is probably the result of relatively low sample sizes of infected plants in all but the highest temperature, high bug number treatment.

These experiments quantitatively describe the effects of sharpshooter abundance, inoculation period, sharpshooter species, and temperature on transmission of *Xf* to grapes. The experiments also describe the consequence of these factors on disease progression in the plant. Collectively these results, along with other field and lab experiments, allow us to begin to link the transmission biology of *Xf* to the epidemiology of PD. Our ultimate goal is to begin to describe quantitatively how these

environmental and ecological factors that affect transmission interact with other elements of PD epidemiology to predict disease dynamics in different regions of California (Figure 5). We expect that high vector loads and warmer temperatures will increase the rate of disease cycling by shortening the time required for *Xf* populations in the plant to build up to the level where efficient re-acquisition can occur. The ultimate consequence of this would be to increase the likelihood of secondary spread, especially by the GWSS, and therefore increased PD severity.

Figure 5. Conceptual model for how climate and surrounding habitat drive PD severity in CA vineyards. Established links are denoted by solid arrows. Dashed arrows denote hypothesized effects that require more study.



FUNDING AGENCIES
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FEEDING BEHAVIORS OF THE GLASSY-WINGED SHARPSHOOTER THAT CONTROL INOCULATION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 1, 2006 to May 30, 2007.

ABSTRACT
In the final eight months of this grant, we completed remaining studies and emphasized writing results for several publications. Overall, all objectives of this grant were successfully completed. The work identified the electrical penetration graph (EPG) waveforms responsible for both ingestion and egestion by the glassy-winged sharpshooter (GWSS), and provided evidence that the mechanism of inoculation of *Xylella fastidiosa* (*Xf*) is a combination of egestion and salivation. Objective 1 successfully showed that ingestion is represented by the EPG waveform C, which nearly always occurs in some type of xylem cell on susceptible grape. Earliest events of waveform C (usually short in duration) often occur in non-functional, primary xylem, whereas later events (longer in duration) always occur in lignified, presumably functional, secondary xylem. GWSS adults virtually never ingest from non-xylem cells while on susceptible grape. Objective 2 showed that the B1s waveform is correlated with muscular fluttering of the precibarial valve, and that the B1w waveform represents salivation. These waveforms occur throughout the pathway phase of feeding, thus occur in all cell types that are penetrated. During Objective 3 work, several experiments with both GWSS and smoke tree sharpshooter also identified for the first time the sharpshooter X-wave, a waveform family that definitively represents xylem penetration by the stylets. The X-wave incorporates the waveforms B1w, B1s, proto-C, and C. X-ray images of GWSS feeding taken at the Argonne National Lab are also discussed. Taken together, our findings support that the B1s and proto-C of the X-wave represent egestion (expulsion) of fluids from the precibarium into xylem, and likely represent the instant that *Xf* cells are inoculated.

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 *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 (now termed egestion) 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, egestion 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

This year, we were able to resume use of lab-reared insects very kindly provided by D. Morgan, CDFA.

Objective 1 – Correlation of ingestion with EPG Waveforms

Study a: Ingestion-waveform correlations and cell types in which ingestion occurs

Prior to this grant, we correlated the C waveform (Figure 1b) with ingestion via observation of particle movement in artificial diets (Joost et al. 2005). Last year's electromyographic (EMG) study of cibarial muscle potentials by former post-doc S. Dugravot showed that C waveform was also correlated with both cibarial dilator activity and excretory droplet production. Two to four cibarial pumps produced a single droplet. This year, additional analysis of the data showed that the fine structure of the C waveform is correlated with directional flow of fluid through the foregut. Thus, whether fluid is flowing into (rise portion of each plateau) or out of (falling portion) the cibarium can be judged by voltage changes alone. A paper of these findings is *in press* (Dugravot et al. 2008).

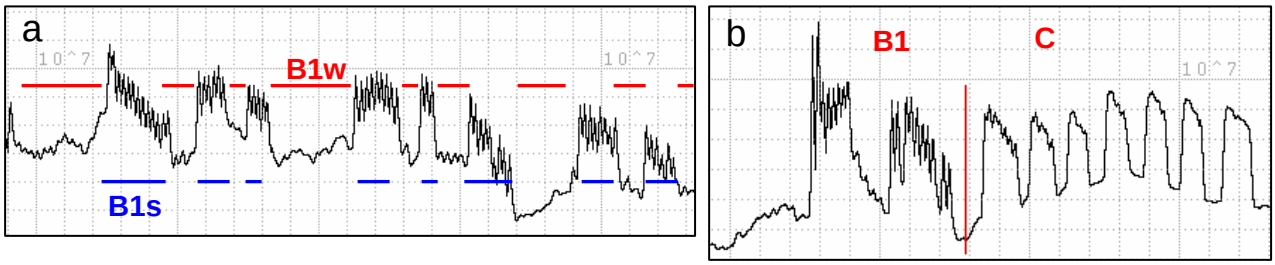

Figure 1. Examples of the main EPG waveforms studied in this project, in the order in which they occur during stylet penetration. **a.** The B1 waveform, composed of B1w (wave) and B1s (spikelets). This waveform occurs frequently throughout pathway and interruption phases. **b.** The C waveform, following a length of B1. C waveform is the landmark waveform for ingestion phase.

Last year we also performed a study at the Argonne National Lab. EPG waveforms were recorded from wired GWSS that were subjected to high-energy X-ray imaging during feeding. This allowed the cibarial muscle movements to be viewed via movements of two sets of tracheae that apparently aerate different functional groups of the cibarial muscles. Video images and waveforms were further analyzed during this year. Results to date confirm and expand upon the EMG study. The two sets of muscle groups move at different times during feeding. Major, coordinated convulsions of both muscle groups are correlated with the rise phase of the C waveform, and therefore with the muscle potentials and cibarial pumping previously recorded. However, very small contractions of the major cibarial muscle group were correlated with a new waveform, proto-C. We believe these small, C-like plateaus represent test pulls of the cibarial pump. In addition, atypical C waveform shapes, hypothesized to correlate with ingestion from non-xylem cells, were actually caused by unusual cibarial muscle contractions. In fact, C waveform fine structure was entirely correlated with cibarial muscle movements alone.

Last year, we reported results of a third project to determine which ingestion events were performed in xylem, and whether xylem ingestion can be identified by waveform appearance alone. Further analysis this year found that waveform C always occurred in xylem, but any of several xylem cell types. Very early, especially short-duration, C events occurred in primary proto-xylem cells or small, unlignified secondary xylem cells. In contrast, later, longer-duration, C events occurred in large, lignified secondary xylem cells. The thesis for this work was completed this year (Holmes 2007) and a manuscript is in preparation.

Study b: Recognizing ingestion from waveforms alone

Results from Study a, altogether, support that waveform C represents ingestion (i.e. cibarial pumping), but its fine structure is not correlated with ingestion tissue type, as we hypothesized. Instead, waveform C predominantly occurs in xylem, and the type of xylem cell is correlated with the C event's order and/or event duration.

Objective 2 – Correlation of egestion with EPG Waveforms

Study a: Correlate B1 waveform with muscle movements and fluid flow in and out of stylets

A second EMG study was also performed last year, and results were analyzed this year. Muscle potentials were recorded from the precibarial valve muscles, which were hypothesized to control egestion. Results conclusively showed that the valve's muscle potentials occurred only during pathway and were temporally correlated with B1 spikelet bursts (B1s)

(Figure 1a). Muscle potentials also strongly resembled waveform B1s (data not shown) (Backus & Dugravot 2008). Thus, B1s represents voluntary valve fluttering, and this occurs only during pathway phase, not during ingestion. The directionality of fluid flow (described in Objective 1 results, above) further supports that fluid moves up and down within (and possible out of) the precibarium during B1s.

Study b: Determine in which plant cells B1 occurs

A combination of the precibarial EMG study and Holmes sheath histology study, above, show that B1 occurs throughout pathway, in all cell types penetrated by the stylets. Work described below shows that B1 also 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, egestion, or both) during which bacteria are expelled

Last year's report detailed findings by two previous post-docs, P.H. Joost and S. Dugravot, that green fluorescent protein (GFP)-expressing *Xf* were seen embedded in salivary sheaths in artificial diet on which putatively inoculative GWSS fed. Yet, only eight out of a combination of nearly 81 EPG-recorded probes revealed GFP, as confirmed by confocal microscopy. Problems with our insects acquiring the GFP-*Xf* had been the cause. This year, a third post-doc, B. Reardon, attempted this diet-inoculation project again. He found two out of 30 confirmed salivary sheaths with GFP-*Xf* (Figure 2). We suspect that the rarity of GFP-*Xf* in diet was due to lack of acceptance of our diet. Nonetheless, these findings strongly support that *Xf* is inoculated during salivation.

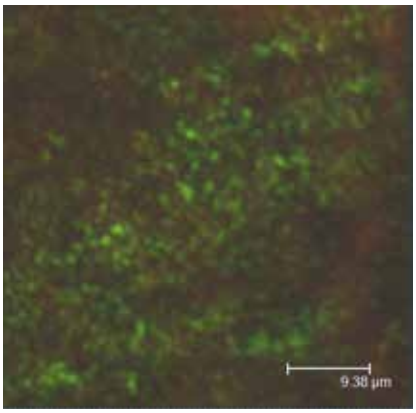

Figure 2. Confocal laser scanning micrograph of green GFP-*Xf* inside the lumen of a GWSS salivary sheath in artificial diet.

During completion of the Holmes (2007) thesis project in the last year, fine-structure analysis of the interruptions (waveform N) between trial ingestion (C) events was performed. We found that each interruption consistently was composed of B1w, B1s, and proto-C waveforms (Figure 3). At the same time, further analysis of the Argonne X-ray data suggested (although the data are not yet fully analyzed) that the smaller of the cibarial muscle groups twitches slightly but rhythmically during B1w, during both pathway and interruption phases. This supports that "micro-ingestion" of a small amount of fluid occurs, probably into only the precibarium, where it can be tasted by the precibarial chemosensilla. In addition, salivary sheath tips were always in a xylem cell during interruption and trial ingestion events. The first "interruption" (actually at the end of pathway, before the first C event) marked the first stylet penetration of the ingestion cell; this is the definition of a specialized EPG waveform family called the "X-wave" that is seen in many vectors species. Thus, we have now discovered the sharpshooter X-wave (Figure 3), and theorize that it is the *Xylella* inoculation behavior.

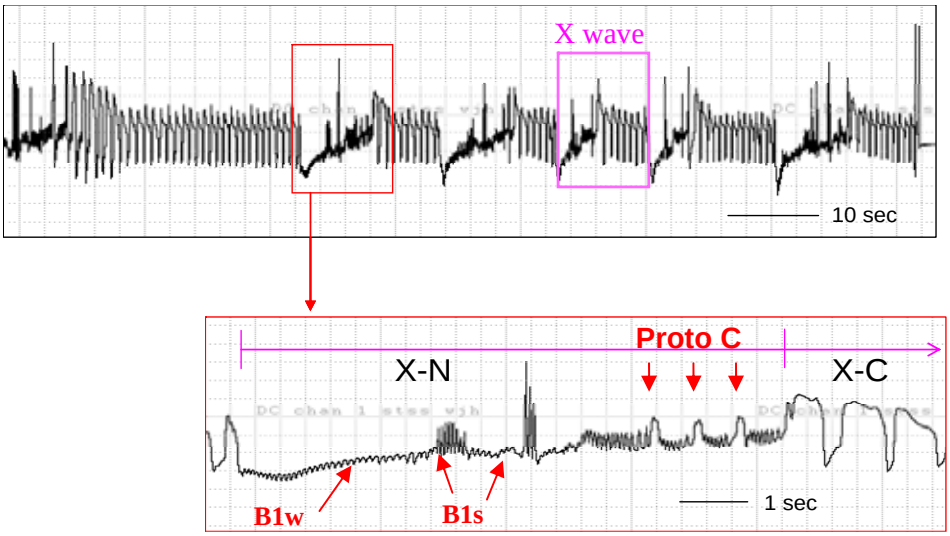

Figure 3. A repetitive series of sharpshooter X-waves (one in pink box). Each includes interruption and trial ingestion events. The red box, expanded below, shows components of the X-wave.

The behaviors of the X-wave apparently function in tasting the fluid in the xylem cell to determine its acceptability, and testing the strength of the mechanical seal of the stylets. B1w represents watery salivation (Joost et al. 2005), perhaps simultaneous with micro-ingestion into the precibarium for the purpose of tasting partially-digested xylem fluid. If so, the insect would be micro-ingesting a mixture of plant fluid and its own saliva. B1s is precibarial valve fluttering, which probably further aids movement of fluids back and forth across the sensilla, during tasting. When tasting is nearly complete, proto C may represent small contractions of the large cibarial muscle group, for "mini-ingestion" into the cibarium. Perhaps when these muscles release, some fluid can be pushed back into the precibarium, then leak past the valve and into the stylets. This would be egestion of a mixture of plant fluid and saliva.

Thus, there is good evidence that the component waveforms of the X-wave represent all behaviors necessary for *Xf* inoculation via a mechanism of combined salivation and egestion. We are in the process of conclusively testing this hypothesis with *Xf* inoculation studies underway.

Study b: Determine into which plant cells bacteria are expelled

In Backus 2007, we describe a plant inoculation experiment using EPG-identified probes that artificially terminated stylet penetration after 3-6 min of ingestion phase (3 to 8 X waves). This work complemented the diet inoculation study described above. PCR evidence from that study showed that a single GWSS probe of this type could inoculate enough *Xf* to kill the test plant (3 months later), in 100% of 36 plants tested. This indirectly supports that *Xf* is inoculated into the xylem during X waves, at the start of ingestion. Thus, duration of ingestion probably does not directly relate to inoculation. This experiment is the first time that GWSS has been shown to exhibit 100% vector efficiency per individual insect, let alone from a single probe. This project will be repeated with histology of salivary sheaths in the coming year, using a new method we have developed to retain the fluorescence of GFP-*Xf* through paraffin sectioning and process for confocal microscopy (see Backus & Labavitch 2007).

CONCLUSIONS

We have succeeded in meeting all objectives of this grant proposal. The accumulated evidence supports that the EPG waveform C represents ingestion from xylem, and the B1s and proto-C waveforms represent egestion of a mixture of plant fluid and saliva. Our findings will help solve the PD/GWSS problem by providing: 1) insights into the mechanism of *Xf* transmission (acquisition and inoculation); 2) 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 Penetration Index); 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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FUNDING AGENCIES

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BETA-1,4 GLUCANASE IN GLASSY-WINGED SHARPSHOOTER SALIVA AND ITS POSSIBLE ROLE IN INFECTION AND MOVEMENT OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 1, 2006 to September 30, 2007.

ABSTRACT

The purpose of this project is to determine whether β-1,4 glucanase (EGase), the major enzymatic protein in the watery saliva of glassy-winged sharpshooter (GWSS), co-localizes via immunocytochemistry with the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated into a plant by this vector. If it does, then this suggests that watery, enzymatic saliva of the vector is a carrier of the bacteria during inoculation, and that therefore the saliva might somehow aid in this process. This year, we: 1) completed and tested the development of a method to retain fluorescence of *Xf* cells that are transformed to express green fluorescent protein (GFP) through the paraffin-sectioning process, 2) dissected an additional 1500 pairs of salivary glands for protein extraction, 3) extracted and purified EGase, and 4) contracted with a commercial company to produce antibodies to it. Work in the coming year will complete the objectives by using secondary antibodies to the EGase and confocal laser scanning microscopy to co-localize the EGase with GFP *Xf*.

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. It has been hypothesized that *Xf* can cause Pierce's disease (PD) only if bacteria can 'break out' of the initial, imprisoning inoculation xylem vessel(s) to produce a systemically increasing population (Hopkins 1989). Thus, *Xf* lateral movement through adjacent vessels is one determinant of initial infection success. Recent evidence by Labavitch and colleagues (Roper et al. 2007) studying *Xf* movement through stems supports the idea that pit membranes limit bacterial movement. A sufficient quantity of cell wall-degrading enzymes can break down parts of the primary cell wall network of the pit membrane, allowing bacteria to pass. Mature *Xf* populations produce the enzymes polygalacturonase (PG) and β-1,4 glucanase (EGase, often identified as cellulase in the literature) (Roper et al. 2007). Thus, in later stages of infection, those enzymes may function to facilitate systemic movement. However, fewer than 200 cells are typically inoculated by sharpshooters (Hill and Purcell 1995). It seems to us, therefore, that these few pioneer bacteria first inoculated by a vector are unlikely at first to produce a sufficient titer of enzymes to digest through the pit membrane.

It is routine to histologically image sheath saliva in fed-upon plants (e.g. Leopold et al, 2003, Backus et al 2005). However, no researcher has ever *directly* visualized watery saliva in plants, due to its fluid and dispersive nature. Yet Backus and colleagues have defined electrical penetration graph (EPG) waveforms that represent salivation and ingestion (Joost et al. 2006; Dugravot et al. 2008). Histology of salivary sheaths in probed plant tissues, correlated with EPG waveforms, revealed the cell types into which saliva is injected (Backus et al. 2005). Watery saliva is mixed with and spreads out from the salivary sheath, in all plant cells penetrated by the stylets, including xylem. Labavitch and colleagues recently have found very high activity of cell wall polymer-degrading enzymes, especially EGase, in GWSS salivary gland fractions (unpublished data). Thus, cell wall-degrading salivary enzymes are injected into xylem along with very few bacterial cells during *Xf* inoculation by GWSS. This finding 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

Objective 1 – Purify and characterize β-1,4-glucanase and develop antibodies

Study a: Enzyme purification and characterization

Over 1500 paired salivary glands were successfully dissected by Backus and colleagues from wild GWSS field-collected on ornamental shrubs in Bakersfield, CA, during June and July, 2007. Glands were frozen in extraction buffer at -20 ^{o}C and hand-carried from Fresno to Davis. Protein extraction, purification, and assaying of EGase were performed in the lab of Labavitch by Greve in August to September, 2007, and purified EGase was delivered to Antibodies, Inc., in Davis, CA. Polyclonal antibody serum will be raised in guinea pig by late November, then purified in the Labavitch lab, for later Objective 2 work by Kingston planned for January 2008. At present, enzyme characterization is scheduled for spring, 2008.

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 with the following steps:

1) Allow one group of GWSS to acquire *Xf* expressing green fluorescent protein (hereafter, GFP-*Xf) (*Study a) and another (control) group to remain non-inoculative (Study b), then…
2) EPG-record a single, standardized probe consisting of pathway followed by ingestion lasting no more than 3 – 6 min, as described in Backus (2006), then…
3) Excise, histologically prepare, and section the fed-upon grape tissue, using methods that retain fluorescence of GFP, then…
4) Probe the sectioned tissue with primary antibody to EGase (from Objective 1) then secondary, fluorescently conjugated antibody, and finally…
5) Use confocal laser scanning microscopy (CLSM) to simultaneously locate and image autofluorescent salivary sheaths and cell walls, GFP-*Xf*, and fluorescently-stained EGase/watery saliva.

Time permitting, these studies may also 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, Backus and Shugart made further progress developing each of the individual protocols to be combined in the larger test. In particular, we completed development of an all-new method for retaining the fluorescence of GFP *Xf* throughout the entire procedure for classical histology fixation, embedding in paraffin, sectioning and examination. An example of GFP-*Xf* in grapevine is shown in Figure 1. Such a procedure has never been accomplished before, by any research group using GFP, anywhere.

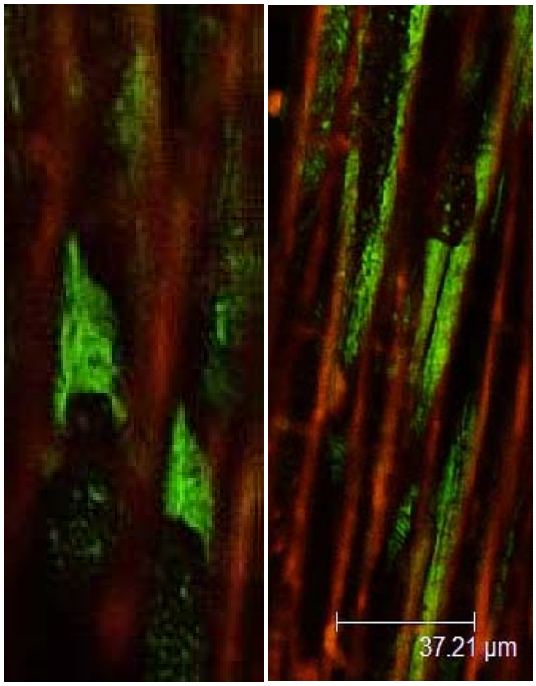

Figure 1. Green *Xf* revealed in paraffin-embedded and longitudinally-sectioned stems from mechanically inoculated grape, at the site of inoculation. **Left:** High magnification view of two xylem vessel elements completely occluded with green *Xf*. **Right:** Lower magnification view of multiple vessels with bacterial biofilm.

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. In addition, differences in vector efficiency among GWSS populations, or numerous other vector species, could be related to salivary enzyme composition. Testing of other vector species (e.g. Brazilian vectors of Citrus Variegated Chlorosis, or vectors of Oleander and Almond Leaf Scorches) could aid understanding of the epidemiology of all *Xylella* diseases.

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THE ROLES THAT DIFFERENT PILI CLASSES IN *XYLELLA FASTIDIOSA* PLAY IN COLONIZATION OF GRAPEVINES AND PIERCE'S DISEASE PATHOGENESIS: CHEMOSENSORY CLUSTER CONTROLLING TWITCHING MOTILITY

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Reporting Period: The results reported here are from work conducted December 2006 to September 2007.

ABSTRACT

We provide evidence that twitching motility in *Xylella fastidiosa* (*Xf*) is controlled by a signal transduction pathway (pilG-chpC cluster), which is highly similar to chemosensory systems controlling flagella rotation in several bacteria including *Pseudomonas aeruginosa*. The gene pilL is shown to be essential for twitching motility as three different insertional mutations in this gene resulted in a twitching defective phenotype. We have also identified a cheY homolog, cheY2, in this pathway and show it is required for wildtype twitching motility. The cheY2 mutant was complemented by expression of the wildtype gene cloned in pBBRMC5. It was demonstrated that cheY2 is also involved in biofilm formation as the mutant produced a reduced biofilm. Transmission electron microscopy revealed type IV (and type I) pili are present in the cheY2 mutant and its complement. In silico analysis of CheY2 predicts its role in a chemosensory transduction signal cascade. In addition we identified a gene near the pilG-chpC cluster, tonB3, that is also related to twitching motility. We also report the recent advances in the production of monoclonal antibodies against *Xf* pili.

INTRODUCTION

Twitching motility (TM) functions in host colonization of many gram-negative bacteria. *Xylella fastidiosa* (*Xf*) has both type I pili and type IV pili, and exhibits twitching motility and biofilm formation (Meng et al, 2005). We have identified several genes in *Xf* associated with pili development and their associated phenotypes (ie. fimT, pilB, pilQ, pilR, pilX and pilY1) (Li et al, 2007). In P. aeruginosa (Pa) TM is regulated by a chemosensory system that involves two gene clusters pilG-K and chpA-E, that are analogous to bacterial chemotaxis systems that control swimming motility in response to environmental stimuli (Whitchuch et al, 2004). Chemosensory systems are composed of sensory receptors networking with components of cytoplasmic phosphorylation and dephosphorilation cascades (Mariconda et al, 2006). Here we describe three new genes in *Xf* that are associated with expression of TM. Two of them, cheY2 and pilL, are predicted to be part of a putative chemosensory system that controls TM in *Xf*; the third is tonB3 that may be responsible for the transport of pili subunits outside of the cell. CheY2 (ORF XP1757) was previously annotated as unnamed and part of a two-component regulatory system. In this report we predict by deduced protein structure that CheY2 most likely functions as a phosphoacceptor.

We also report our progress on the production of monoclonal antibodies against *Xf* pili that we propose to use for development of diagnostic tests and eventually in the development of novel controls for Pierce's Disease.

OBJECTIVES

1. Identify the putative TM chemosensory cluster and phenotype-associated genes.
2. Characterize three additional genes that are likely to be involved in the chemosensory control of TM and related functions.
3. Develop monoclonal antibodies against *Xf* pili.

RESULTS

Screening and sequence analysis of twitching mutants. We previously generated a library of TM mutants through transposon (EZ::TN Transposome system) mutagenesis of the *Xf* Temecula genome (Li et al., 2007). Sequence analysis identified three mutants (2A5, 17A8 and TM25), that contain transposon insertions in the ORF XP0874 (gene pilL) (Figure 2A). The mutants showed a smooth colony margin i.e. a complete lack of peripheral fringe in PW medium (Figure 1). We also identified another mutant (TM26) which has transposon insertion in the region immediately downstream of pilL, ORF XP0869 (predicted gene tonB) (Figure 1B; Figure 2A). A third mutant (3E10) resulted from a transposon insertion in the ORF XP1757 was annotated as part of a two-component regulatory system we designate as cheY2 (Figure 1; Figure 2B).

Sequence analysis of the *Xf* putative chemosensory cluster and related genes. The pilL mutant resulted from insertions in different regions of the ORF XP0874. This 5178 bp ORF encodes a 1726 aa multidomain protein that belongs to the family of CheA-like histidine kinases. The predicted protein is believed to result from a fusion of PilL (the Tfp sensor histidine kinase response regulator) and ChpA (Tfp chemotaxis-related protein kinase). CheA-like proteins contain a CheY docking domain, an autocatalytic histidine kinase domain that is required for ATP binding (HATPase) and a CheW-like domain that

is involved in mediating CheA interactions with CheW (ChpC in *Xf*). *Xf* PilL/ChpA is a predicted fusion protein that possesses four histidine-containing phosphotransfer domains in its N-terminal - HPt (Figure 2B). Upstream of pilL resides a tonB homolog (XP0869); a 900 bp ORF divergently transcribed, encoding a predicted 300 aa protein annotated as a TonB- dependent receptor. TonB interacts with outer membrane receptor proteins in the process to transduce cytoplasmic membrane energy. The TonB system is responsible for energizing transport events at the outer membrane using the proton-motive force of the cytoplasmic membrane (Postle, 2007). *Xf* contains thirteen TonB-dependent receptor copies in its genome; the first is ORF XP0008 and the last is the ORFXP2172. We named the ORF XP0869 tonB3, as it is the third tonB gene from the beginning of genome. Upstream of pilL resides pilG (XP0871), a 417 bp ORF that encodes a predicted 139 aa CheY-like protein. Downstream is gene is pilI (XP0872) a 531 bp ORF that is predicted to encode a 177 aa CheW-like protein that forms a complex with CheA (PilL/ChpA in *Xf*) that together functions as an allosteric enzyme (Fan, 2006). In P.a, the gene downstream of pilG is pilH (Figure 2. A). In *Xf* however, pilH (XP1715) comprises a 387pb ORF encoding a 129 aa protein located 8.4Mb downstream from the main putative chemosensory cluster.

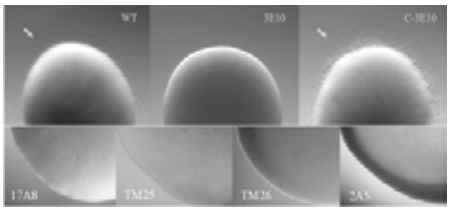


Figure 1. Colony morphologies of Wild-type *Xf* and the mutants grown on modified PW agar for 4 days.

The last gene upstream of pilL is pilJ (XP0873) an ORF comprised of 2043 bp that encodes a putative 681 aa protein predicted to be a methyl accepting chemotaxis protein (MCPs) that is known to be coupled via the adaptor protein CheW (ChpC in *Xf*) to the multidomain histidine protein kinase CheA. Downtrean of pilL are the genes chpB (XP0875 – 1170 bp) and chpC (XP0876 - 471bp) (Figure 2). The protein ChpB (390 aa,) is homologous to the CheB methylesterases, that in chemotaxis systems function to demethylate the sensory MCPs and ChpC, a CheW homolog that is believe to couple other MCPs with ChpA in P.a. *Xf* does not have chpD or chpE or their homologs.

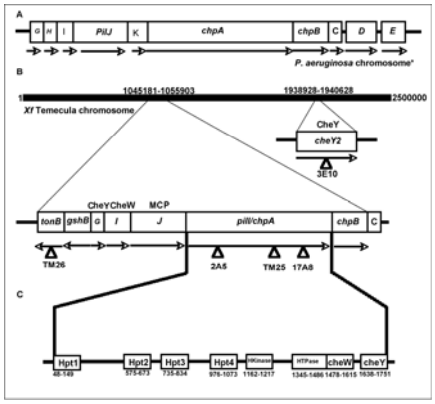
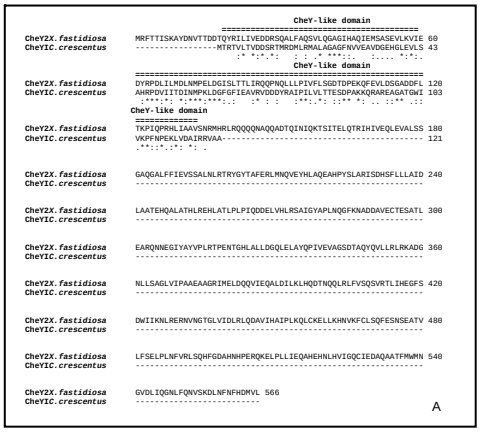


Figure 2. Organization of *pilG-chpC* cluster in *Xf* and *Pa.* (a) *Pa.* PilG-K and ChpA-E clusters (b) *Xf* chromosome and the organization of the chemosensory cluster and its domains. Open arrows denote genes disrupted in this study.

Sequence analysis of CheY2. Mutant 3E10 carries a transposon insertion in ORF XP1757. This 1701 bp ORF is predicted to encode a cytoplasmatic 567 aa protein, that also belongs to the CheY family. It has a CheY-like receiver domain and an N-terminus comprised of amino acids 20 to 133 (Figure 3). We named this protein CheY2, with PiLG being the first CheY-like protein in this chemosensory system. CheY2 showed high identity with homologs in strains of *Xf*; 98% identity with *Xf* Ann-1 (EAO33015), 97% identity with *Xf* Dixon (ZP_00650939) and 96% identity with *Xf* 9a5c (NP_297691). In all strains the gene was annotated as part of a two component response regulator. CheY2, CheY and CheY-like proteins exhibited low identities with CheY and CheY-like proteins in E. coli (20%), P.a (21%) and 31% identity to CheYI of Caulobacter crescentus. The alignment of *Xf* CheY2 and C. crescentus CheYI is shown in the Figure 3A. We used 3D modeling to examine the putative structure of *Xf* CheY2. *E. coli* CheY is a 129-residue protein, arranged in an alpha/beta parallel motif with five-stranded parallel β-sheets surrounded by five α-helices. The β-strands and α-helices alternate along the sequence and are connected by loops (Sola et al, 2000) where the central β-sheet plays the major role. Based on sequence similarity with other response regulators and CheY1 from C. crescentus, the conserved backbone β-α-loop was predicted (β2-α1-loop) for CheY2 (Figure 3B). This putative conformational structure of CheY2 is predicted to function as the part the twitching chemosensory system of *Xf*.



Agregation, growth and biofilm formation. Complementation analysis of the CheY2 mutant, 3E10, was performed by cloning the gene into plasmid pBBRMC5 and then electroporating it into competent cells of the mutant. No significant differences in growth rates between the mutant and its complement were found when compared to *Xf* Temecula wild-type (not shown).

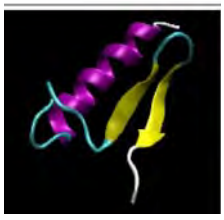


Figure 3. Similary and modeling of CheY2. (a). sequence aligments of CheY2 (*Xf*) and CheY1 (*C. crescentus*) used for structural modeling. (b). Ribbon structures of the two β-sheets and the α-helix predicted for CheY2.

Differences in the dynamic formation and dispersal of bacterial aggregates and cell movement were monitored by light microscopy and in microfluid chambers. The CheY2 mutant showed aggregation patterns different from wildtype, which shows mostly small transient aggregates. The CheY2 mutant forms large clumps of transient aggregates that move slower than wildtype aggregates. The development of biofilms

by mutant, complement and wildtype in PD2 cultured in glass flasks with continuous agitation are shown in the Figure 4. The CheY2 mutant forms less biofilm than wildtype. The biofilm formed by the complemented mutant was visibly more pronounced as compared to the wildtype or mutant (Figure 4). Furthermore, it was noted that the biofilms formed by both 3E10 and C-3E10 were more easily removed from the glass surfaces by swirling in distilled water than the wild-type (data not shown).

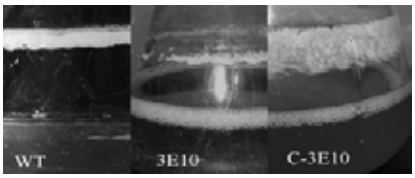


Figure 4. Biofilm formation of *Xf*. Wild-type, 3E10 and C-3E10 cells. Following 10 days of growth in culture with agitation.

Type I and IV Pili were observed on the CheY2 mutant and complement (Figure 5). We predict the lack of twitching phenotype in the mutant is due to absence of a functional CheY2 protein, responsible for initiating the signal transduction cascade associated with pili retraction.

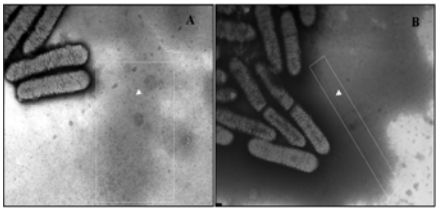


Figure 5. Electron micrographs of *Xf* cells. Transmission electron microscopy micrographs of 3E10 (a) and C-3E10 (b) cells negatively stained. White arrows inside box type IV pili.

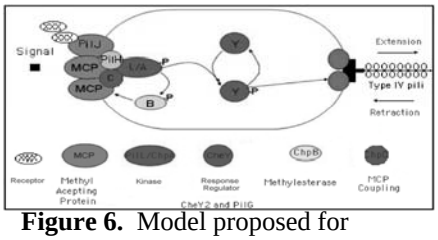


Figure 6. Model proposed for signal transduction pathway of *Xf* chemosensory system for twitching motility.

Twitching motility chemosensory model. We propose a model for TM chemosensory in *Xf* (Figure 6); receptors at the cell surface detect changes in the concentrations of attractants and generate shifts in the level of phosphorylation of diffusible signaling proteins CheY2 and PilG. Phosphorylated CheY2 and PilG modulate the extension and retraction of Tfp, thus affecting twitching behavior of the cell. The entire signal transduction pathway includes chemotaxis proteins: PilL/ChpA is a kinase that phosphorylates the response regulators CheY2 and PilG and also the methylesterase ChpB; ChpC is an adaptor protein coupling PilL/ChpA to MCPs (PilJ); ChpB, a methylesterase, mediates adaptation to a constant attractant concentration by adjusting the methylation level of receptors.

Production of monoclonal antibodies against *Xf*. Monoclonal antibodies (MAb's) will be used for further characterization of *Xf* pili. In addition, they may be useful in inhibition of migration and colonization of *Xf* in vitro and in planta, and in development of a field-applicable diagnostic biodetection sensor with enhanced sensitivity and speed of detection. We have thus proceeded to produce MAb's toward various surface proteins of *Xf*. Thus far, mice have been immunized with whole live cells of *Xf*, and they demonstrate excellent immunogenic reactions with the blood serum (both by ELISA and by immunocytochemistry—see Figure 7). We are scheduled to screen for MAb producing cell lines during October-November 2007.

CONCLUSIONS

We provide evidence that the *Xf* proteins, PilL/ChpA and CheY2 are part of a complex chemosensory system that controls type IV pilus-mediated motility in *Xf*. This predicted chemosensory system contains many modules that are similar to the chp system of P. aeurginosa (PilG-ChpC). The CheY mutant possesses Tfp but does not twitch again suggesting its role in the signal transduction pathways that control TM. Indeed, time-lapse video microscopy suggests that the CheY2 has lower rate of cellular movements and abnormal frequency of cellular reversals during twitching motility. The in silico study of CheY2 predict the conformational structure would confer the capacity to function as a phosphoacceptor in the cascade of signal trasnduction in TM leading ultimately to the movement of extension and retraction of the Tfp. We also have observed reduced biofilm formation by the mutant and an increase in the biofilm formation following complementation. This outcome may result from an overexpression of CheY2 possibly interfering not only with cell movement and direction but also cellular attachment. Within the vicinity of pilG-chpC cluster we have identified another gene tonB3, that also affects the TM in *Xf* (Figure 2B); it may be involved in some aspect of the transport and secretion of pili subunits or of a component required for their formation. TonB is known to provide energy to efflux systems for export molecules out of the cell (14). Further research is required to understand the role of *Xf* tonB3 in TM. As our knowledge of *Xf* Tfp production, regulation and functioning advances, the coordinate regulation of Tfp as a virulence factor is being elucidated. An understanding of how this regulation occurs is central to determining how to counter movement and plant colonization. The targeting of genes

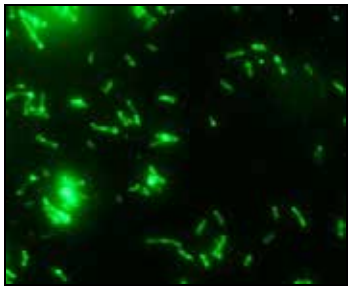


Figure 7. Immunocytochemical detection of *Xf* surface antigens with mice blood serum. Cell surfaces and, in many instances, a 'bright' polar spot (= type I pilus domain) are detected.

required for pathogenicity including colonization may be an effective strategy. The examination of the role of other genes from pilG-chpC cluster and their relation to TM is a crucial step in this process. The production of monoclonal antibodies against *Xf* pili will certainly help contribute to this step as well as to the development of diagnostic tools and eventually for the development of novel controls for Pierce's Disease.

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SEASONAL BEHAVIOR OF *XYLELLA FASTIDIOSA* CAUSING ALMOND LEAF SCORCH DISEASE UNDER FIELD CONDITIONS AND DETECTION OF THE BACTERIA BY MEANS OF ARRAY-PCR

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ABSTRACT

Diseases caused by *Xylella fastidiosa* (*Xf*) have re-emerged as a serious threat to several economically important crops, such as grape and almond, in the San Joaquin Valley of California. Knowledge of the bacterial behavior in plant hosts under field condition is important for disease control. This research characterized populations of *Xf* almond leaf scorch disease (ALSD) strains in almond orchards. In 2006, two almond orchards were selected based on known history of ALSD. One ALSD tree and one non-ALSD tree were identified from each orchard. The branch pattern of each tree was mapped. Samples were taken every month from each scaffold at both distal and proximal positions. Presence of leaf scorch symptoms were monitored, bacteria were isolated from different regions of the trees, and genotypes were determined by PCR analysis of both conserved housekeeping genes and the hypervariable *psp*B locus. We reported the population dynamics of ALSD bacteria and the pattern of leaf scorching symptoms in 2006, and continued the study in 2007. In 2007, the earliest occurrence of leaf scorching symptoms was in July, almost one month later than that in 2006. In both 2006 and 2007, PCR detected *Xf* in plant tissue one month ahead of symptom development. PCR was slightly more sensitive than cultivation method for early bacterial detection. However, uneven bacterial distribution and random sampling errors may contribute to the differences among the assays. Correlation between cultivation and PCR detection was over 90%. Analyses of tandem repeat numbers (TRNs) at the *psp*B locus showed that three TRN genotypes existed in the same almond tree, although one genotype predominated. To reduce PCR error that result from large volume sample processing, we developed an array-PCR protocol using primers from seven housekeeping genes. This array-PCR significantly improved detection accuracy.

INTRODUCTION

Xylella fastidiosa (*Xf*) is the causal agent for several economically important diseases including Pierce's disease of grapevine (PD) and almond leaf scorch disease (ALSD) in the San Joaquin Valley of California. The bacterium has received extensive laboratory characterization in the past few years, leading to the completion of genome sequencing of six *Xf* strains (9a5c, Temecula-1, Dixon, Ann-1, M12, and M23). Among them, three (Dixon, M12 and M23) are ALSD strains. More research in genomics, molecular genetics and phenotypic characterizations are underway. However, knowledge of bacterial behavior under field condition is limited. This information is important not only for the bacterial biology research, but also for effective disease management programs.

In this project, we characterized the population of *Xf* ALSD strains in almond orchards in Fresno County of California. In 2006, two almond orchards were selected based on the previously known history of ALSD. One ALSD tree and one non-ALSD tree were identified from each orchard. The branch pattern of each tree was mapped. Samples were taken every month from each scaffold at both distal and proximal positions. Presence of leaf scorch symptoms were monitored, bacteria were isolated from different sections of the trees, and genotypes were determined by PCR using primers designed from 16S rDNA sequence directly from freeze-dried pulverized tissue (FDPT). The population dynamics of ALSD bacteria in 2006 was reported previously (Chen et al., 2006). We continued to study disease development in these same trees in 2007. In addition to the use of FDPT as PCR template, petiole sap mixtures (PSM) derived from isolation experiment (Chen et al., 2005, 2007) were also used.

To reduce PCR errors that result from large volume sample processing, the concept of multiple PCR was employed. Using the 96-well microplate format, we developed an array-PCR for *Xf* detection. In this protocol, multiple primer sets were designed based on different housekeeping genes identified from the genome sequence of strains Temecula-1 (causing PD) and Dixon (causing ALSD). By placing each well with a different primer sets along with appropriate controls, a row of 12 wells was used for simultaneous PCR for every sample. Comparing to the previous one-well-one-sample PCR, the 12-well-one-sample PCR was designed to tolerate occasional failures in PCR amplification. Results from multiple primer sets allow a conclusion on a sample detection to be drawn with high confidence.

Since sequences of housekeeping genes are highly conserved, they are reliable in detecting *Xf* at the species level. Multiple alignment sequence analyses can identify single nucleotide polymorphisms in housekeeping gene that differentiate A- and G-genotype ALSD strains. However, DNA polymorphisms in housekeeping genes are not sensitive enough to detect intra-pathotype variation. To further increase detection sensitivity in *Xf* population, the *psp*B locus was used. The *psp*B gene encodes a serine protease and contains a hypervariable tandem repeat region. Specific primer set flanking the tandem repeat

region was designed. Tandem repeat number (TRN) from each strain could be determined from PCR amplicons and used as a parameter to describe the bacterial population.

OBJECTIVES

1. To study the seasonal leaf scorch symptom expression patterns in the orchards and its association with pathogen detection.
2. To study the distribution and variation of *Xf* in almond trees under the field conditions.
3. To develop a PCR–array protocol for better detection accuracy of *Xf*.

RESULTS

ALSD symptom expression in 2007 was one month later than that of 2006 (Figure 1). However, the patterns of symptom appearance in both years were similar, i.e. the disease severity showed a pattern of gradual increase. In contrast, patterns of the rates of bacterial isolation, PSM-PCR and FDPT-PCR showed a much higher level of fluctuation during the growing season, although the trend of overall increases were still be seen (Figure 1). Isolation of *Xf* from different sections of the same almond petiole showed difference in bacterial titer. The inconsistent detection rate in both culture and isolated methods was at least in part related to the uneven distribution of *Xf* cells *in planta*.

A total of 312 samples were collected from both almond orchards in 2007, 46 % of the samples showed symptoms, 37 % were culture positive, 35% were SM-PCR positive, and 50 % were FDPT-PCR positive. The correlation between ALSD symptom appearance and bacterial detection was from 80% to 100% as of September 2007. Similar to 2006, PCR detection of *Xf* in 2007 was also one month earlier than symptom expression. PCR was slightly more sensitive than cultivation method for early bacterial detection. However, uneven bacterial distribution and random sampling errors might contribute to the differences among the assays.

Results of array PCR on 12 FDPT samples are tabulated in Table 1. Primers used in this study were checked by BLAST analyses using each primer set as query against the current GenBank database and they were specific to *Xf*. As expected, 10 out of the 12 samples had 1- 3 unsuccessful PCR amplifications that could lead to false negative conclusions, should there be a one-well-one-sample PCR was performed. We considered these variations as random experimental errors. The use of array-PCR minimized such errors.

Observation of TRN variation in the two Fresno almond orchards are shown in Table 2. Contrasting to the limited variations in the conserved genomic loci, at least three TRN genotypes, ranging from 12 to 14, were found in both orchards. TRNs in *psp*B seemed to decrease as the growing season proceeded.

CONCLUSIONS

1. The occurrence of ALSD symptoms is heavily influenced by environmental factors, as shown by the one month delay of ALSD symptoms, as well as *Xf* detection, in 2007. However, we do not know what exactly those environmental factors are.
2. ALSD symptom expression is related to the increase of *Xf* titer. Regardless of the variation in symptom expression, *Xf* cells were detected consistently one month ahead of symptom expression.
3. Because of the uneven distribution of *Xf* cells in the affected almond tree, there exists a large margin of sampling error for pathogen detection. Such sampling errors could lead to false negative conclusion in disease diagnosis. Array-PCR significantly improved detection accuracy.
4. An almond tree can harbor multiple *psp*B TRN genotypes. The role of these genotypic variations in ALSD development remains to be studied in the future.

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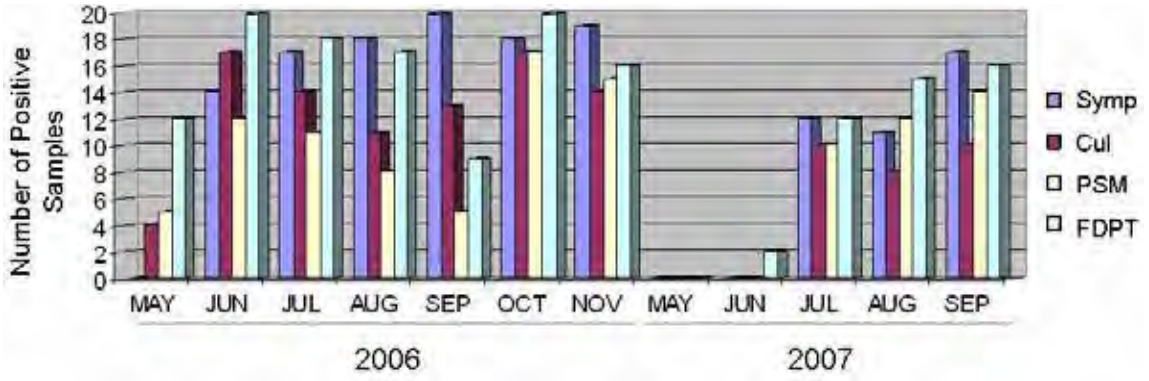

Figure 1. Temporal development of almond leaf scorch disease from May 2006 to September 2007. SYMP-leaf scorch symptom observed; Cul-cultivation of *Xf* on PW-G medium; PSM-petiole sap mixture as template for PCR; and FDPT-freeze-dried pulverized tissue as template from PCR.

Table 1. A summary of array-PCR on the detection of almond leaf scorch disease samples.

		Genomic loci									
NO	SAMPLES	GYR	MDH	DAS	GLT	T-D	16SG	16SA	PspB	ACON	ICD
1	MC-asym	+	+	+	+	+	+	+	-	+	+
2	MC-symp	+	+	+	+	+	+	+	+	+	+
3	MC-asym	+	+	+	+	+	+	+	+	+	+
4	MC-symp	+	+	+	+	+	+	+	-	+	+
5	MC-asym	+	+	+	-	+	+	-	-	+	+
6	MC-asym	-	+	+	+	+	-	-	+	+	+
7	MC-symp	+	+	+	+	+	+	-	+	+	+
8	MC-asym	+	+	+	+	+	+	-	+	+	+
9	MC-symp	+	-	+	+	+	+	-	+	+	+
10	FS-asym	+	-	+	+	+	+	-	-	+	+
11	FS-symp	+	+	+	+	+	+	-	+	+	+
12	FS-symp	+	+	+	+	+	+	-	+	+	+
13	Teme DNA	+	+	+	+	+	+	+	+	+	+
14	Teme Cells	+	+	+	+	+	+	+	+	+	+
15	No DNA	-	-	-	-	-	-	-	-	-	-

Table 2. Variation of tandem repeat numbers in *psp*B locus observed from two locations in Fresno County.

Location	Month	Number of tandem repeats
MC	May	13
	June	14
	July	14
	August	13
	September	13
	October	13
	November	12
FS	May	14
	June	14
	July	14
	August	14
	September	13
	October	12
	November	12

CHARACTERIZATION OF REGULATORY PATHWAYS IN *XYLELLA FASTIDIOSA*: GENES AND PHENOTYPES CONTROLLED BY GacA

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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. One such global regulatory gene, *gacA*, controls various physiological processes and pathogenicity factors in many gram negative bacteria, including biofilm formation in *P. syringae* pv. *tomato* (*Pst*) DC3000. Cloned *gacA* of *Xf* was found to restore the hypersensitive response and pathogenicity in *gacA* mutants of *Pst* and *E. amylovora* (*Ea*). An *Xf gacA* mutant (DAC1984) had significantly reduced abilities to adhere to a glass surface, form biofilm, and incite disease symptoms on grapevines compared to the parent A05. cDNA microarray analysis identified seven genes positively regulated by GacA, including genes encoding putative outer membrane proteins XadA and Hsf, and 20 negatively-regulated genes including *gumC* and *cvaC*, predicted to encode an antibacterial toxin. These results suggest that GacA of *Xf* regulates many factors, which contribute to attachment and biofilm formation, as well as some physiological processes that may enhance the adaptation and tolerance of *Xf* to environmental stresses and the competition within the host xylem.

INTRODUCTION
The xylem-limited, insect-transmitted bacterium *Xylella fastidiosa* (*Xf*) causes Pierce's disease in grapes through cell aggregation and vascular clogging. Pathogenic bacteria use gene regulatory mechanisms to rapidly respond to and survive in changing environments (Storz and Hengge-Aronis, 2000). Inside the plant's xylem, *Xf* is exposed to a range of variable stress factors, such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates (Alves et al., 2004). To ensure survival, *Xf* may respond to these stress situations via specific regulatory mechanisms involving specific regulatory genes. We have previously reported the role in virulence of the regulatory gene, *algU*, and identified a number of genes regulated by AlgU through microarray analysis of an *algU* mutant (Shi et al, 2007). Among other regulators identified in pathogenic and environmental bacteria, the GacS and GacA regulators are involved in sensing environmental cues and signals (Heeb and Haas, 2001). In this system, hypothetically, GacS is a putative sensor kinase that perceives a signal or environmental cues, and GacA is a response regulator, which functions as the transcriptional activator of one or more genes. Genes controlled by GacA include those regulating pathogenicity factors, quorum sensing, and toxins, and also genes involved in motility, biofilm formation, extracellular polysaccharides (EPS) in a wide range of pathogenic bacteria including *P. syringae, E. carotovora* and *P. aeruginosa* (Chatterjee, 2003; Cui, 2001; Parkins, 2001). The high similarity between *gacA* of *Xf* (designed as $gacA_{Xf}$) and *gacA* of *P. syringae* (designed as $gacA_{DC3000}$) suggests that, like $gacA_{DC3000}$, $gacA_{Xf}$ may regulate pathogenicity of *Xf* by acting as a global regulator during infection and the process of disease development. Interestingly, while a *gacA* homolog was identified in *Xf*, a *gacS* homolog was not found, which suggests that there may be specific regulatory roles for *gacA* in *Xf* (Simpson et al., 2000). However, the role of *gacA* and its regulation in *Xf* is unknown. In this study, we cloned and characterized $gacA_{Xf}$ and analyzed the effect of a *gacA* deletion of *Xf* (DAC1984). We also performed whole-genome microarray analysis of gene expression in the mutant in comparison with the parent and identified genes whose expression *in vitro* is controlled by GacA. Functional studies of global regulatory genes in *Xf* should identify the specific regulatory pathways and roles of specific virulence factors in disease development, which in turn, could reveal the pathogenicity mechanisms of the bacteria in responding to, adapting, and surviving in the stress environment of xylem.

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

RESULTS
Effects of $gacA_{xf}$ on the hypersensitive response (HR) and pathogenicity
An alignment of the predicted amino acid sequences of GacA protein from *Xf* and *Pst* DC3000 showed that the sequences are 211aa in length and are 43% identical and 69% similar overall (data now shown). In the HR and pathogenicity test experiments, *Pst*AC81 and *Pst*AC812 elicited typical HR in tobacco (*Nicotiana tabacum* L. cv. Samsun), whereas water control, *Pst*AC811 and *Pst*AC813 did not (Figure 1, left, A), confirming that it is $gacA_{xf}$ that restores the elicitation of HR in

tobacco. *Ea*EC19 and *Ea*EC192 produced disease symptoms in African violet leaves. In contrast, *Ea*EC191 and*Ea*EC193 failed to produce disease symptom (Figure 1 Left, B), suggesting that $gacA_{xf}$ restores the ability of *Ea*EC192 to cause disease in African violet leaves. This demonstrated that $gacA_{xf}$ can complement *gacA* deficiencies of *P. syringae* and *E. amylovora* in the hypersensitive response and pathogenicity.

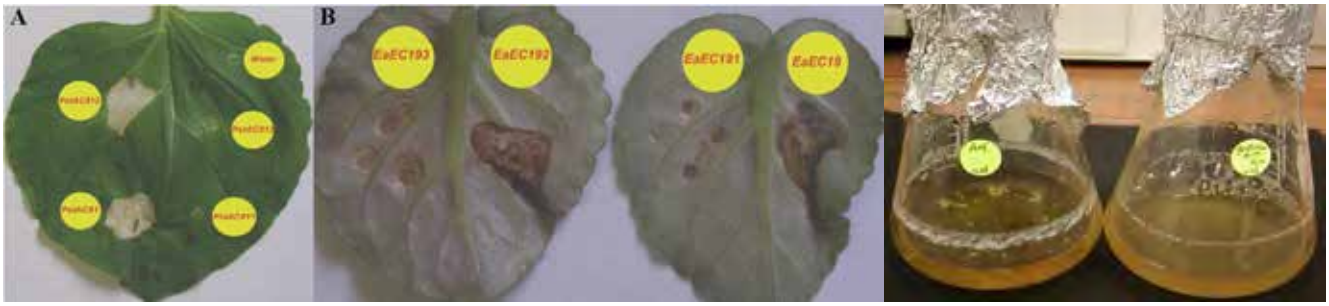

Figure 1. Left. Complementation of *gacA* function by $gacA_{xf}$ in *gacA*-deficient mutants of *P. syringae* pv. *tomato* DC3000 (*Pst*) and *E. amylovora* (*Ea*). **A.** Effect of $gacA_{xf}$ in *Pst*AC811 on the elicitation of the hypersensitive response (HR) in tobacco leaf (*Nicotiana tabacum* L. cv. Samsun). Leaf panels were infiltrated with bacterial cells. Site 1, *P. syringae* pv. tomato DC3000 (*Pst*AC81) (1×10^7 CFU/ml); site 2, DC3000 $gacA_{DC3000}^-$ (*Pst*AC811) (1×10^7); site 3, $gacA_{DC3000}^-$ carrying $gacA_{xf}$ (*Pst*AC812) (5×10^7); site 4, $gacA_{DC3000}^-$ carrying pCPP47 (*Pst*AC813) (5×10^7); and site 5, water. **B.** Disease symptoms caused by *E. amylovora* and its $gacA^-$ mutant in Africa violet. Site 1, *E. amylovora* (*Ea*EC19); site 2, *E. amylovora* $GacA^-$ mutant (*Ea*EC191); site 3, *E. amylovora* $gacA^-$ mutant carrying $gacA_{xf}$ (*Ea*EC192) (5×10^7); site 4, *E. amylovora* $GacA^-$ mutant carrying pCPP47 (*Ea*EC193) (5×10^7). **Right**. The ability of DAC1984 to adhere to a glass surface was reduced.

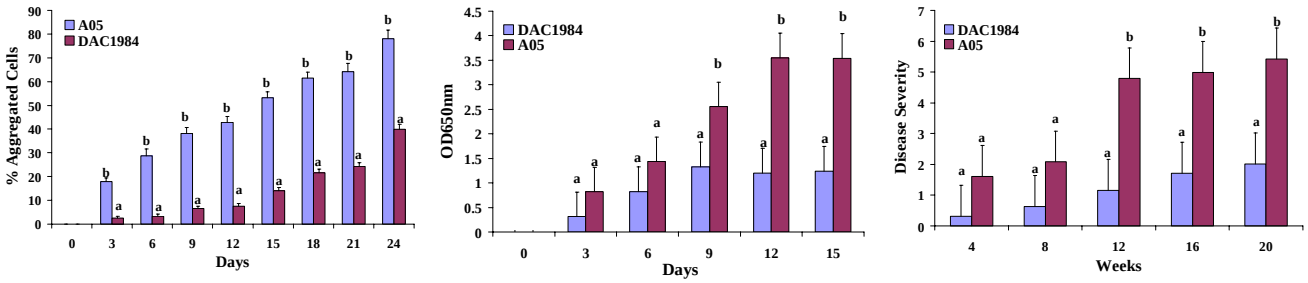

Figure 2. Cell-to-cell aggregation, biofilm formation and virulence of *Xf* DAC1984 and A05. **Left.** Quantitative assessment of *Xf* A05 or DAC1984 cell-to-cell aggregation. Student's *t* test, $p < 0.05$. **Middle**. DAC1984 had reduced ability to form biofilm. Student's *t* test, $p < 0.05$. **Right**: Pierce's disease progression of grapevines inoculated with *Xf* A05 and its *gacA*-deficent derivative DAC1984. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20 weeks after inoculation. Student's *t* test, $p < 0.05$. The data are an average of 10 independent replications.

Characterization of a Δ*gacA::Gm* mutant

The replacement of the *gacA* ORF by a gentamycin cassette (Gm) in the genome of the Δ*gacA::Gm* mutant (DAC1984) was confirmed by electrophoresis (data not shown). Sequence analysis indicated that Gm physically replaced most of the *gacA* ORF, from 25 bp downstream from the ATG start codon to 35 bp upstream of the TGA terminal codon of the *gacA* ORF. After streaking five to eight times on PD3 Gelrite medium with 10 µg/ml gentamycin, DAC1984 still grew well and was indistinguishable from the parent, indicating that the mutant is genetically stable. RT-PCR analysis showed that there was no expression of *gacA* within DAC1984 cells, but strong expression was detected in wild-type A05 cells (data not shown). *In vitro* growth curves of *Xf* A05 and DAC1984 over 21 days were similar (data not shown), indicating that mutation of the *gacA* gene did not affect the growth pattern of *Xf* in culture. As is typical of *Xf*, cells of A05 attached to the surface of the flasks and formed wide rings, but while DAC1984 cells attached to the surface formed rather light rings (Figure 1, Right). An optical density assay was used to quantify the effect of DAC1984 on cell-to-cell aggregation and showed that the percentage of aggregated cells of DAC1984 was significantly lower than that of A05 (Figure 2, Left). The ability of

DAC1984 to form biofilm was investigated further by a crystal violet staining method. *Xf* A05 formed more biofilm in PD3 broth than did DAC1984 (Figure 2, Middle).

Virulence assay

Grapevines inoculated with the *Xf* DAC1984 mutant developed less severe disease than did those inoculated with the wild type strain 12-20 weeks after inoculation (Figure 2). Grapevines inoculated with DAC1984 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 600nm with that of positive control *Xf* concentrations (data not shown). The cell populations of the DAC1984 were less than that of the wild-type, indicating that *gacA* affects the growth and possibly the movement of *Xf* inside the xylem resulting in reduced pathogenicity.

Table 1. Genes differentially expressed in *Xf* Δ*gacA::Gm* mutant (DAC1984) *in vitro*

ORF	Gene[c]	Description	Mutant /Wild type	Signfig.[b]	The expression in mutant
PD0264	*oprO*	porin O precursor.	0.24	*	lower
PD1926		pilus assembly fimbrial protein	0.12	*	lower
PD1294	*actP*	acetate permea-cotranscribed with the acs gene	0.337	*	lower
PD2039		Oxidoreductase-Putative multicopper oxidases	2.75	*	higher
PD1688	*bioI*	cytochrome P450-like enzyme	2.45	*	higher
PD1703		Conserved Domains: Lysophospholipase	6.31	*	higher
PD1702		Conserved Domains: LIP, Secretory lipase	5.29	*	higher
PD0215	*cvaC*	colicin V precursor-antibacterial polypeptides toxin	3.63	*	higher
PD0216	*cvaC*[d]	colicin V precursor-antibacterial polypeptides toxin	0.51	*	lower
PD0731	*XadA*	outer membrane protein-Autotransporter adhesin	8.93	*	lower
PD0744	*hsf*	surface protein-Autotransporter adhesin	3.24	*	lower
PD1395	*gumC*	protein involved in exopolysaccharide biosynthesis	3.17	*	higher
PD0243		Conserved Domains-Membrane-fusion protein	3.01	*	higher
PD0244		Conserved Domains-acriflavin resistance protein	2.7	*	higher
PD0956		V8-like Glu-specific endopeptidase	4.77	*	higher
PD1299		Conserved Domains-polyvinylalcohol dehydrogenase	6.18	*	higher
PD1295		putative membrane protein-unknown function	0.20	*	lower
PD0521		unknown	3.24	*	higher
PD0657		unknown	3.28	*	higher
PD0743		unknown	6.62	*	higher
PD0955		unknown	2.67	*	higher
PD0911		phage-related proteins, unknown function	6.04	*	higher
PD0912		phage-related proteins, unknown function	8.41	*	higher
PD0917		phage-related proteins, unknown function	3.94	*	higher
PD0924		phage-related proteins, unknown function	6.39	*	higher
PD0925		phage-related proteins, unknown function	9.12	*	higher
PD0930		phage-related proteins, unknown function	3.96	*	higher

[a] Hybridization signal intensity obtained with mutant was divided by that from wild-type in order 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. Student's *t*-test, $p < 0.001$. [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 *in vitro*

RNA was prepared from DAC1984 and wild type A05, 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. Twenty seven genes were differentially expressed in DAC1984 compared to A05 (Table 1). Differential expression of *hsf, gumC, cvaC* (PD0216), fimbrial protein (PD1926), and PD1295 were validated by RT-PCR (data not shown). Genes involved in surface structures and attachment components, such as fimbrial proteins, PD1926, *Hsf*, and *XadA*, were positively regulated, and *GumC* was negatively regulated by GacA in *Xf*. *hsf* (PD0744) has a high similarity to the *hsf* adhesin gene of *Haemophilus influenza* (St Geme et al., 1996), and *XadA* encodes a putative afimbrial outer membrane protein adhesion (Simpson et al., 2000). The expression of *hsf* and *XadA* were decreased in DAC1984, likely contributing to a reduced ability to adhere to xylem cell walls. GumC may be responsible for gum

polymerization or secretion through the membrane of *Xf* (Vojnov et al., 1998; de Pieri et al., 2004). *gumC* was negatively regulated by GacA *in vitro*, but is likely to be expressed *in planta*. Genes involved in cell transport and physiological metabolism, such as *oprO* and *actP*, were positively regulated, and an oxidoreductase and *bioI* were negatively regulated by GacA in *Xf*. Culture-independent analysis of bacterial populations inside plants in relation to *Xf* suggest that bacterial endophytic population are much more diverse than previously realized (Araujo et al., 2002; Cooksey and Borneman, 2005). Colicin V is an antibacterial polypeptide toxin produced by *E. coli*, which acts against closely related sensitive bacteria (Havarstein et al., 1994). *cva*C may play a role for *Xf* in competing with indigenous microbes to survive in the poor nutriment xylem environment.

CONCLUSIONS

Attachment and biofilm formation should be key mechanisms by which *Xf* persists in plants. It is hypothesized that there are different steps for attachment to xylem vessels and biofilm formation (de Souza et al., 2004). Previously, we showed that mutation of the regulatory gene *algU* resulted in decreased attachment, decreased biofilm formation, and decreased virulence (Shi et al., 2007). Similar results were obtained here with deletion of *gacA*. However, of the many genes found to be regulated by these two regulatory genes, only four were regulated by both. PD0216, encoding a putative antibacterial toxin, and PD1295, encoding a putative membrane protein, were positively regulated by both AlgU and GacA. However, PD1926, encoding a putative pilus assembly protein, was negatively regulated by AlgU but positively regulated by GacA. Also, PD0521, of unknown function, was positively regulated by AlgU but negatively regulated by GacA. Several select candidate pathogenicity genes that were regulated by AlgU and by GacA are being mutated, and the effects of the mutations on phenotype and virulence are being 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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UNDERSTANDING CONTROL OF *XYLELLA FASTIDIOSA* CELL AGGREGATION: IMPORTANCE IN COLONIZATION AND BIOFILM DEVELOPMENT IN GRAPEVINE AND SHARPSHOOTER FOREGUT

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ABSTRACT

Our investigation focused on aggregation and 'autoaggregation'of *Xylella fastidiosa* (*Xf*) cells. This study is particularly directed toward elucidating how *Xf* cells aggregate to cause PD symptoms. We have documented that both type IV and type I pili are important in the process. Furthermore, it was discerned that surface properties of WT cells change from being more hydrophobic to more hydrophilic as the cells age, and that cell surface charge does not prevent aggregation. In addition, all cells with pili were effectively transmitted by BGSS while the double mutant, *fimX/pilO*, was apparently not transmitted which may not be surprising since no pili are present on these mutants to adhere the cells to the insect foregut regions.

INTRODUCTION

The ability of *Xylella fastidiosa* (*Xf*) to cause symptoms of Pierce's disease is generally assumed to be related to its colonization of grapevine xylem where the pathogen forms biofilms and cell aggregates. From a disease standpoint such aggregates and biofilms are important for several reasons, including possible direct blockage of sap flow through xylem vessels or indirect blockage through initiation of tylose formation. Cell aggregates may also facilitate pathogen spread from vessel element to vessel element via enzyme digested pit membranes (Newman et al., 2004) — individual cells likely lack sufficient 'enzymatic power' to breach pit membranes, but a compact aggregate of cells would be much more effective in this regard. Furthermore, enzyme production may not be expressed in individual cells, but be regulated in aggregates associated with quorum sensing. From the standpoint of the pathogen, cell aggregates and biofilms likely facilitate nutrient adsorption, protection from environmental stresses, and phytochemicals.

Determining how *Xylella fastidiosa* is able to inhabit the xylem environment and block the transpiration stream through the production of biofilms and bacterial cell masses would be informative toward facilitating development of novel control approaches. Furthermore, insight into the selective acquisition, retention, and transmission of *Xf* by leafhopper vectors represents a priority area of interest. Earlier, we demonstrated several unique and important features of *Xf* biology not previously recognized, including the observation that the bacteria posses functional type IV pili that allow the cells to migrate via twitching motility upstream in grape xylem elements (http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/; Meng et al., 2005), that they posses type I pili that function in adhering the cells to xylem (De La Fuente et al., 2007a; 2007b; Li et al., 2007), and more recently that at some as yet undefined time or condition individual bacteria that are separated by relatively great distances 'autoaggregate' into large masses. In our *in vitro* studies, this occurred after six or more days of growth (initiated from only a few cells) in PD2 media (http://www.nysaes.cornell.edu/pp/faculty/hoch/agg/). Aside from a slow population build up of cells in xylem vessels at or near sites of sap flow constrictions (pits, element end-wall openings) which we consider cell aggregates, it is possible that many individual cells normally distributed throughout xylem elements are able to quickly autoaggregate into large cell masses contributing to vessel blockage. This phenomenon may explain, in part, why PD symptom development (reddening and drying of leaf margins) often occurs within a short time span—from overnight to a few days.

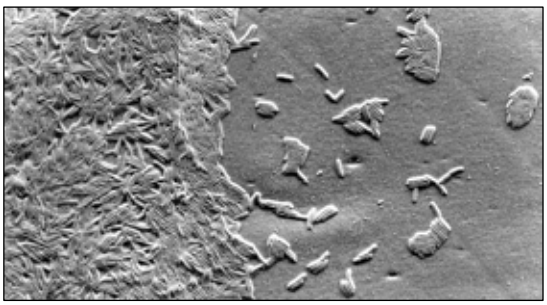

Figure 1. SEM of frozen-hydrated *Xf* colony edge exhibiting a peripheral fringe of twitch-migrating cells in 'rafts.'

OBJECTIVES

Our overall goal is to understand the relationship of *Xf* cells within the confines of the xylem environment. This project centers on the development and importance of bacterial cell aggregates and biofilms, and their involvement in expression of Pierce's disease. Recent observations in our lab revealed that *Xf* cells 'autoaggregate' as the cell population matures. That observation has led us to examine the biological and genetic mechanism associated with this phenomenon by generating aggregation and autoaggregation-defective mutants. Mutants thus obtained will be examined for their activities within microfabricated 'artificial' xylem vessels (which provide superior observation opportunities) as well as in *bona fide* xylem vessels, for disease development, and for vector transmission.

Specific objectives are to:

1. Identify genes associated with aggregation and autoaggregation of *Xf* cells.
2. Assess spatially and temporally aggregation and autoaggregation activities as they occur *in planta* and in microfluidic 'artificial' xylem vessels.
3. Assess selected aggregation and autoaggregation-defective mutants *in planta* for disease development and movement within the plant.
4. Assess aggregation mutants generated in Objective 1, and related attachment mutants already in hand, for acquisition, retention, and transmission by sharpshooter vectors.

RESULTS

Mutant generation. In this period we screened for mutants with deficiencies in the ability to form tight 'rafts at colony peripheries (Figure 1). A number of interesting and potentially useful mutants have been identified as a result of our mutational screenings; however, to date we have not identified colonies with 'raftless' peripheries, viz., individual cells not closely associated 'side-by-side.' The mutation and screening process is continuing. We are encouraged about the prospects of identifying such mutants, in part because the screening procedure is straight forward and relatively quick. In addition to screening for raftless mutants, we have identified and isolated a number of cell lines that are deficient in biofilm formation and in the formation of large aggregates—all of which are being further characterized.

Table 1. *Xylella fastidiosa* Temecula mutants referenced in this report. Gene, ORF, and Gene Product designations are according to the recently revised annotation for *Xylella* (http://www.xylella.lncc.br/)

Gene	Single Mutation	ORF	Gene Product	Observed Characteristics *in vitro*
pilB	1A2	XP2038	Type IV fimbrial assembly protein PilB	No twitching motility.
cheY2	3E10	XP1759	two-component system, regulatory protein	Very poor twitching motility.
fimX	6E11	XP0065	Fimbrial adhesin protein	Twitching motility. Colony 'fringe' wider than wild type.
pilY1	TM14	XP0026	Type IV pilus assembly protein, tip-associated adhesin PilY	Twitching motility. Colony margin smooth to crenulate.
pilL	TM25	XP0876	PilL/ChpA fusion protein: pili sensor histidine kinase-response regulator / chemotaxis-related protein kinase	Reduced twitching motility
	Double Mutation			
fimX, *pilO*	DM12	XP0065 XP1782	Fimbrial adhesin protein Fimbrial assembly protein PilO	No twitching motility. Colony 'fringe' deficient.

Influence of pilus type on autoaggregation. Using microfluidic chambers (Meng et al., 2005) coupled with time-lapse microscopy, we observed a process previously referred by our group as "autoaggregation." During this process, dispersed WT *Xf* cells formed compact, aggregates over 3-10 hours (Figure 2a). This autoaggregation process occurs after cells were grown in PD2 broth medium within microfluidic chambers for 7-11 days. We investigated the influence of both type I and type IV pili in this process by using a series of mutants defective in biosynthetic and structural genes (Meng et al., 2005, Li et al., 2007) (Table 1). *pilY1* mutants (deficient in encoding for a putative tip adhesin protein of type IV pili) exhibited similar aggregation processes as the WT cells. Mutant cells lacking type I pili [*fimX* (previously designated *fimA*)] also formed cell aggregates, but the process differed from the WT in that the cells repeatedly aggregated and dispersed, but eventually formed relatively stable, albeit smaller aggregates (Figure 2b). Adherence among *fimX* cells appeared reduced, probably due to the lack of strong attachment conferred by type I pili (De La Fuente et al., 2007a; 2007b). Mutants deficient in type IV pili (*pilB*) did not form spherical aggregates; instead, loose lace-like cell-cell associations were formed (Figure 2c). These looser cell

aggregates were generally non-adherent to the chamber walls. Mutants deficient for both type I and IV pili (*fimX*, *pilO*) did not form cell aggregates, and as a result generally remained separated from each other (Figure 2d).

Table 2. Aggregation assay.

Gene	Single Mutation	Observation
---	WT	Aggregation observed immediately
pilB	1A2	Aggregation observed immediately
pilO	TM1	Aggregation observed immediately
fimX	6E11	Aggregation observed immediately
pilY1	TM14	Aggregates did not form, or formed very slowly; aggregation was not as pronounced
pilY1	TM23	Aggregation observed immediately; larger clumps
hecA	Lindow	Aggregation observed immediately; larger clumps
xadA	Lindow	No aggregation observed
	Double Mutation	
fimX, pilO	DM12	Aggregation observed immediately

Toward the pursuit of assessing aggregation, we noted that WT cells removed from PW agar plates and suspended in water or buffer aggregated shortly thereafter, within 1-2 minutes. We thus explored this approach as a means of screening mutant cells for deficiencies in aggregation (Table 2). Most notable were the observation that *pilY1* and *xadA* mutants remained in a homogenous suspension and that after 1 hour the *pilY1* mutant suspension appeared as if it may have been starting to clump. The *hecA* mutant exhibited clumps that were noticeably larger than in the wild-type. All other mutants and the WT clumped within seconds.

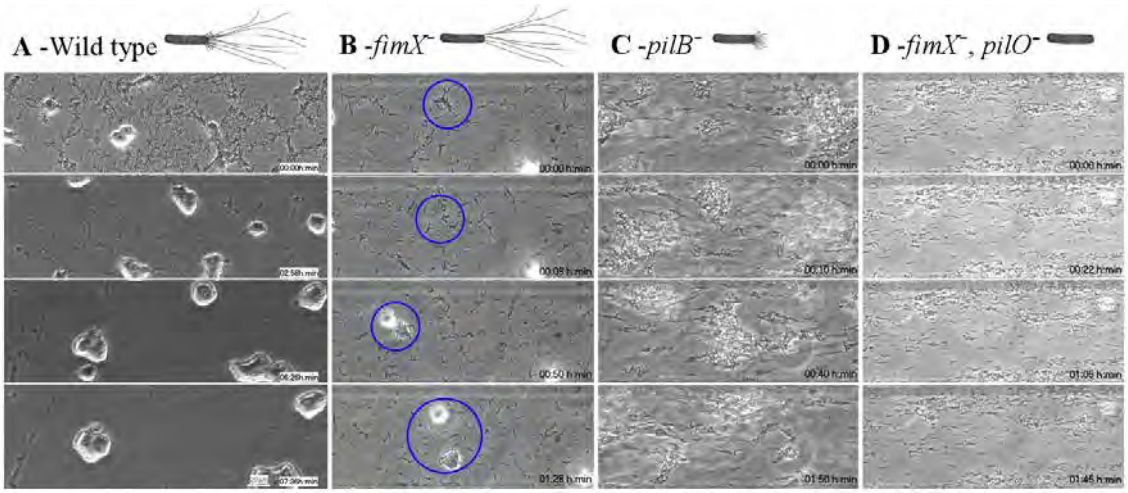

Figure 2. Autoaggregation of *Xf*. A) wild-type *Xf* densely dispersed cells merged into small aggregates within hours. B-D: mutants defective in type I and/or type IV pili genes.

Cell surface physical properties related to autoaggregation: To ascertain that autoaggregation occurred as a result of pilus or other cell surface adhesin characteristics, we investigated other factors that may be involved—namely, cell surface hydrophobicity and surface charge.

- *Surface hydrophobicity*. The BATH (Bacterial Adhesion to Hydrocarbons) technique (Rosenberg et al., 1980) was used to measure the adhesion of *Xf* cells to three hydrocarbons (n-hexadecane, n-octane and p-xylene). WT cells were grown in flasks on PD2, harvested, washed, and subjected to phase separation with each of the hydrocarbons. The affinity for the organic phase (measured as a reduction in the optical density of the aqueous phase) is an indication of the hydrophobicity of the cells. Three-day old cell aggregates were more hydrophobic than 8-days old cells (Figure 3). The hydrophobic properties of 'young' cells may help on the process of cells aggregation, since they are immersed in a hydrophilic environment (PD2 medium/xylem sap). The increase in hydrophilic properties

of older cells may be a consequence of exopolysaccharide, since these molecules are generally hydrophilic (Sutherland, 2001; Schär-Zammaretti and Ubbnik, 2003).

- *Surface charge*. The net charge at the surface of a particle affects the distribution of ions that surrounds it. Zeta potential is one means of assessing surface charge and is defined as the potential of the most outer stable ion layer surrounding a particle. When a particle moves, ions within this boundary layer move with it, but ions beyond this boundary layer do not. WT *Xf* cells from PD2 broth culture were washed with PBS (pH 7.4), and their zeta potential was determined using a Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). No differences were noted for the zeta potential between two or seven day old cells (Figure 4). The average zeta potential for two-day old cells was -16.0 mV, and -9.60 mV for seven-day old cells. In general, particles with zeta potentials more negative than -30 mV are considered stable. Zeta potential values for WT *Xf* cells indicate that these bacterial cells are 'non-stable' and, thus the force is not strong enough to prevent cell aggregation. These results indicate that *Xf* cells from both time points have adequate zeta potential to form aggregates.

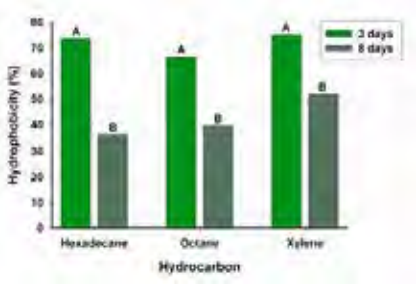

Figure 3. Surface hydrophobicity of *Xf* WT cells was measured by adhesion to hydrocarbons (hexadecane, octane, and xylene).

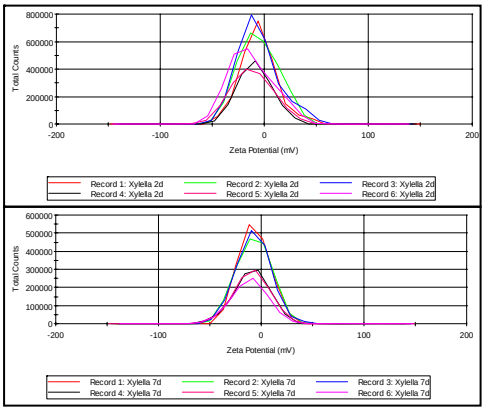

Figure 4. Zeta potential of WT *Xf* cells grown for 2 days (upper) and 7 days (lower).

Assessment of acquisition, retention, and transmission by sharpshooter vectors

For this objective we supplied our collaborator, Rodrigo Almeida *fimX*, *pilB*, and *fimX/pilO* (double mutant) mutants with which he inoculated greenhouse confined plants, and subsequently assessed transmission efficiencies by sharpshooters. In brief, both WT and the *fimX* mutant expressed severe symptoms in needle inoculated plants, *pilB* less severe symptoms, and *fimX/pilO* very mild symptoms, all consistent with our previous observations. However, both WT and the *fimX* mutant were efficiently transmitted by BGSS followed by severe symptom development. *pilB* mutant cells were also efficiently transmitted (which we would expect due the presence of type I pili), but it did not move in the infected plants. *fimX/pilO* were apparently not transmitted and this may not be surprising since no pili are present on these mutants.

CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili and fimbriae have in *Xf* attachment, aggregation, and biofilm formation. We have demonstrated that microfluidic devices can effectively serve as 'artificial xylem vessels' to gain valuable information about the biology of *Xf*, and to infer roles for these phenomena *in planta*. In this report we show that autoaggregation in *Xf*, a phenomenon that could explain the rapid development of symptoms in grapevines affected by PD, is reliant upon the presence of pili.

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

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ABSTRACT
Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Here, we report our studies on the putative autotransporter protein PD0528 of *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease of grapevines. The passenger domain of PD0528 contains six tandem repeats of a 50-60 amino acid motif that is found only in *Xf* species. To determine the role of this passenger domain in *Xf* virulence, we have begun a detailed characterization of PD0528. We have generated a mutation in PD0528 by homologous recombination and shown that the PD0528 protein is not present in the outer membrane of this mutant strain. We have also constructed a transcriptional fusion between the PD0528 regulatory region and the luciferase gene (*luc*), which has allowed us to identify bases important for PD0528 expression. Finally, we have expressed PD0528 in the *Escherichia coli* strain UT5600 and discovered that the resulting *E. coli* strain exhibits increased autoaggregation and biofilm formation.

INTRODUCTION
Xf is a fastidious, Gram-negative bacterium, which is the causative agent of numerous plant diseases relevant to the California agricultural economy. Diseases caused by *Xf* strains include Pierce's disease of grapevine (PD), citrus variegated chlorosis (CVC), almond leaf scorch (ALS), and oleander leaf scorch (OLS) (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.

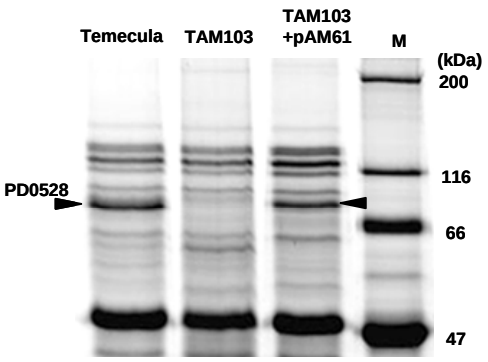
In Gram-negative bacteria, secretion occurs through one of six major secretion pathways, numbered I to VI (Henderson *et al.* 2004, Pugsley *et al*. 2004, Pukatzki *et al.* 2006). These pathways are highly conserved and exhibit functionally distinct mechanisms of protein secretion. One of the simplest secretion mechanisms is exhibited by the AT-1 autotransporters, a subcategory of Type V secretion systems (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. During the period under review, we have been conducting a detailed characterization of the putative autotransporter protein, PD0528. The passenger domain of PD0528 contains six tandem repeats of a 50-60 amino acid motif. 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.

OBJECTIVES
The primary goal of this project is to 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.
1. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence.
2. 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
Characterization of TAM103, a strain containing a PD0528 null mutation:
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* cell physiology and virulence, we generated a null mutation in the PD0528 gene by inserting a chloramphenicol cassette into a *Nhe I* site within the PD0528 open reading frame. Introduction of this construct into the *Xf* chromosome resulted in the PD0528::Cm^R mutant strain, TAM103. Comparison of the membrane profiles of a wild-type *Xf* Temecula strain and the isogenic PD0528::Cm^R mutant revealed that the band corresponding to the PD0528 protein is missing in the outer membrane of TAM103 (Figure 1).

The next step was to perform complementation analysis. For this experiment, we generated the plasmid pAM61, which carries the wild-type PD0528 gene and is a derivative of the plasmid pBBR1MCS-5 (Kovach *et al.* 1995). Introduction of pAM61 into the PD0528::Cm^R mutant resulted in the strain TAM103/pAM61. We then compared the membrane protein profile of TAM103/pAM61 to the wild-type strain and the TAM103 mutant. As shown in Figure 1, the band that is missing in TAM103 is present in both the wild-type Temecula strain and TAM103/pAM61. Thus, the PD0528 gene carried on the pAM61 plasmid can complement the PD0528 defect in TAM103 and restore the presence of PD0528 in the *Xf* outer membrane.

Figure 1: Membrane protein profile of the PD0528 deletion mutant. Outer membrane proteins were isolated from *Xf*-PD Temecula, TAM103, and TAM103/pAM61. The outer membrane proteins were isolated using a sucrose gradient, separated on an 8% SDS-PAGE gel and then stained with Sypro Ruby. The sizes of the molecular weight standards (lane M) are indicated on the right. The position of the missing outer membrane protein in the PD0528 mutant is indicated by the arrow. The identification of the band indicated by the arrow as the PD0528 protein was confirmed by MALDI-TOF mass spectrometry.



Once we had generated the PD0528 null mutation and confirmed that PD0528 is not present on the *Xf* cell surface, we began to investigate the impact of this mutation on *Xf* cell physiology. We discovered that the strain containing the PD0528 null mutation (TAM103) has the same growth phenotype as wild-type *in vitro*. Specifically, like wild-type, growth of TAM103 in liquid culture requires ~7-10 days and TAM103 is not able to form a confluent lawn on solid media. Moreover, a variable level of biofilm formation and autoaggregation was observed for TAM103, but not the wild-type strain. Based on these comparisons, the absence of PD0528 in the outer membrane may have an impact on *Xf* cell physiology *in vitro*. Experiments are currently underway to determine if the absence of PD0528 impacts the ability of *Xf* to cause PD in grapevines.

Our inability to detect a definitive phenotype for the PD0528 null mutation might be due to genetic redundancy. The species specific 50-60 amino acid motif found in the PD0528 passenger domain is also observed in two other *Xf*-PD proteins. PD1379, which is an AT-1 autotransporter protein, contains three copies of this motif in its passenger domain. Four copies of this motif are also found in PD0794. Therefore, it is possible that the loss of PD0528 is compensated for by either PD1379 or PD0794. To test this hypothesis, we have inserted a gentamicin cassette into the PD1379 gene and have introduced this construct into both the wild-type strain and into TAM103. The creation of the PD0528/PD1379 double mutant should provide insight into the role of the *Xf* specific 50-60 amino acid motif in *Xf* cell physiology and virulence.

Identifying the sequences important for PD0528 regulation:

In many bacterial pathogens, the production of key virulence proteins is tightly regulated at the transcriptional level, so that the proteins are only produced under certain environmental conditions. Therefore, in order to understand how *Xf* survives in and interacts with its hosts, it is important to discover how different environmental conditions impact gene expression. However, rapid progress in this area is affected by the lack of the genetic and molecular tools necessary to investigate how environmental signals affect *Xf* transcription. During the past year, we have developed a system for examining transcription in *Xf* using the firefly luciferase (*luc*) gene as a reporter gene.

To establish the usefulness of the *luc* gene for studying transcriptional regulation in *Xf*, we constructed a transcriptional fusion between the PD0528 regulatory region and the *luc* gene. The *PD0528-luc* fusion was inserted into the plasmid pBBR1MCS-5, which carries a gentimicin resistance marker. As a negative control, we also constructed a similar plasmid that carries a *promoterless-luc* gene. The resulting plasmids were then introduced into both *Escherichia coli* strain DH5α and *Xf*-PD and the relative activity of the *PD0528-luc* fusion was determined by comparing the amount of luminescence produced by the four strains. As shown in Figure 2A, high levels of luciferase were produced in the *Xf*-PD strain containing the *PD0528-luc* fusion. In contrast, the *PD0528-luc* fusion was not expressed in *E. coli.* A simple interpretation of this result is that PD0528 expression involves regulatory elements and/or regulatory proteins, which are only present in *Xf*-PD. To test this hypothesis, we first mapped the PD0528 transcriptional start site by 5' RACE analysis. We then generated mutations in the regulatory region upstream of this start site in the *PD0528-luc* fusion. Our first approach was to generate a series of deletions that removed portions of the PD0528 regulatory region. Our second approach was to introduce base changes into the region immediately upstream of the PD0528 transcription site. We then examined the impact of these mutations on PD0528 transcription by measuring luciferase activity. The results for some of these mutations are presented in Figure 2B.

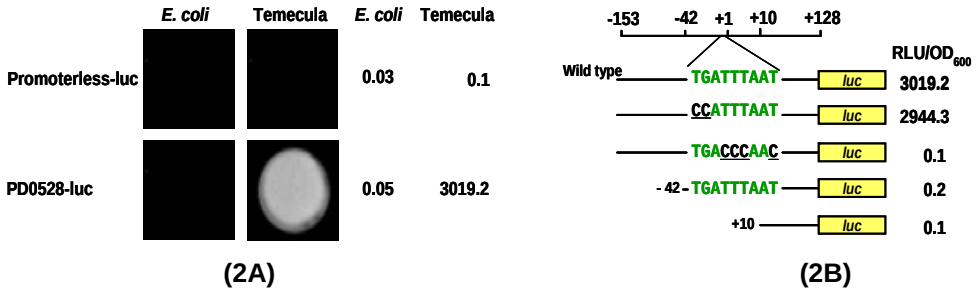


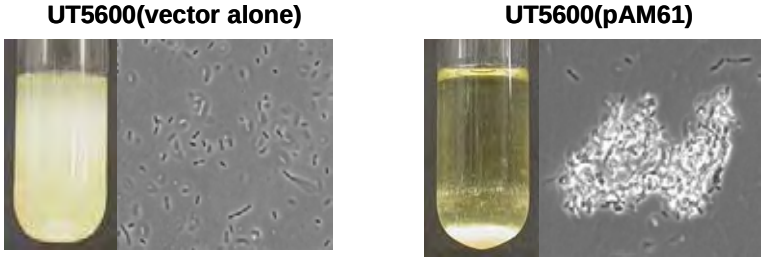
Figure 2: Examination of PD0528 regulation using a *PD0528-luc* fusion. For the luminescence analysis, 1mM D-luciferin was added to cell extracts. Luminescence (RLU) was measured by a Turner TD-2020 luminometer and standardized using OD600. The values are given as RLU/OD600.

We were able to draw two conclusions from these initial experiments. First, deletion analysis indicates that there is a regulatory element in the region between -153 and -42 upstream of the PD0528 transcription start site. Removal of this element is sufficient to eliminate PD0528 transcription. In addition, introduction of three base changes in the -10 region also severely impacts PD0528 promoter activity. It is possible that the two regulatory elements work together as part of the RNA polymerase recognition sequence. However, it is also possible that one of these regulatory elements interacts with a transcriptional activator protein. We are currently generating additional mutations to refine the mapping of these two regulatory elements and to distinguish between these two possibilities.

Expressing the PD0528 autotransporter protein in a heterologous system:

Another method for determining the role of the PD0528 passenger domain in *Xf* cellular physiology and virulence is to express the protein in a heterologous system. This strategy has successfully been used to generate *E. coli* strains that display the passenger domain of heterologous autotransporter proteins on their cell surface. These recombinant strains have been employed for binding assays, for developing antibody specificity tests, and for exposing antigenic determinants for vaccine development (Yang *et al.* 2004). In addition, these strains have provided important insights into the biological properties of the heterologous protein. For example, when expressed in *E. coli*, the *Neisseria meningitis* NadA protein is exported to the surface and assembled in oligomers anchored to the outer membrane. The resulting recombinant *E. coli* strain is able to adhere and invade epithelial cells (Capecchi *et al*. 2005), suggesting that NadA may also play an important role in the attachment of *N. meningitis* to epithelial cells. Therefore, given the success in other systems, we predict that our use of a similar strategy will uncover important information concerning the role of PD0528 in *Xf* virulence.

Figure 3. Heterologous expression studies reveal a possible role for PD0528 in autoaggregatation.



Although we would eventually like to introduce PD0528 into endophytic bacteria, we decided to test the feasibility of this approach using *E. coli* in our initial studies. Specifically, we introduced the plasmid pAM61, which carries the gene encoding PD0528 into the *E. coli* strain UT5600. UT5600, which has been successfully used for the heterologous expression of other autotransporter proteins, is deficient in the outer membrane proteases OmpT and OmpP. As shown in Figure 3, the presence of the PD0528 gene in *E. coli* (UT5600/pAM61) results in an increase in the autoaggregation of the *E. coli* cells. In addition, UT5600/pAM61 exhibits increased biofilm formation when compared to the UT5600 strain. Finally, membrane profiles have established that the PD0528 protein has been localized to the *E. coli* outer membrane. Although more experiments need to be done to establish conclusively that the PD0528 protein is responsible for the new physiological properties of the UT5600/pAM61 strain, these initial experiments are extremely promising and suggest that expression of the

Xf autotransporter proteins in a heterologous system is a powerful approach for uncovering the role of these proteins in *Xf* cell physiology and virulence.

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FUNDING AGENCY

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HIERARCHICAL ANALYSIS AND DIVERSITY STUDIES OF *XYLELLA FASTIDIOSA* POPULATIONS IN CALIFORNIA BY MULTI-LOCUS SIMPLE SEQUENCE REPEAT MARKERS

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ABSTRACT
Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease (PD) in grapevine. Using 18 simple sequence repeat (SSR) markers, we assessed variation within and between populations of *Xf* isolated from grapevine in California. Eighty-three *Xf* isolates from 15 populations present in four regions of California were evaluated for sequence variation. Average genetic diversity was substantial (H = 0.807, I=0.5385) across the 18 loci examined. Diversity within populations varied from 0.227±0.058 (population *Sangiacom*) to 0.583±0.63 (population *Temecula*) but was not dependent upon the grape cultivar serving as host. Analysis of Molecular Variance (AMOVA) indicated no hierarchical population structure, as 98% of the variation was attributed to within population diversity. Higher order variation was minor; diversity among populations or among regions each accounted for only 1% of the variation observed. Principal component analysis (PCA) indicated that only ~53% of the variation was explained by the first three components, further suggesting a lack of hierarchical population structure in *Xf* infecting grape in California. Collectively, these results indicate high levels of gene flow between populations and further suggest that strong selection may dominate other population genetic forces responsible for structuring *Xf* populations resident in Californian grapes. We also initiated similar studies for almond specific *Xf* strains to better understand host species specific diversity patterns and population structure, if any, for almond *Xf* strains. While acquiring and preparing infected almond samples for further SSR analysis, we observed that seasonal variation plays an important role in extent of *Xf* infection.

INTRODUCTION
California has one of the most productive agricultural ecosystems in the United States. Yet, limited genetic variation among crop cultivars and monoculture practices may impose directional selection on pathogen populations. Host plant resistance is a critical component of integrated crop management. Changes in pathogen population structure or virulence can lead to host resistance breakdown. Therefore, understanding pathogen genetic diversity is critical for development of effective disease control strategies. An important aspect of plant pathogen population genetics is the extent to which populations are subdivided, either geographically or by host species/cultivar. The goal of this project is to analyze *Xylella fastidiosa* (*Xf*) haplotypes generated by SSR genotyping within a hierarchically structured (individual plants, individual vineyards, and distinct growing regions) sampling strategy to understand pathogen population dynamics in grapevine hosts at various levels. Previously, we reported development of multilocus simple sequence repeat (SSR) markers for *Xf* population genetic analysis. This marker system appears to be sensitive in detection and powerful in discriminating *Xf* genotypes. This marker system also provides high throughput capability for a large scale sampling and analyses.

OBJECTIVES
1. Analyze *Xf* strain variation and diversity specific to individual host grapes, including hierarchical analysis of population subdivision to understand population differentiation in host-specific pathogen populations.
2. Study the effect of host cultivar on structure of pathogen populations.
3. Evaluate pathogen population dynamics in almond-specific *Xf* strains from three almond populations.

RESULTS AND DISCUSSION

Objective 1.

We analyzed genetic diversity and geographic population structure of *Xf* in Californian vineyards (Napa, Sonoma, Kern and Riverside counties). Results based on multi-locus SSR marker systems and hierarchical sampling showed that all 18 SSR primers were able to discriminate among *Xf* strains (N=83), revealing diversity with a mean H value of 0.609 across all loci. Diversity ranged from a low (H=0.093) for the loci GSSR6 and ASSR 19 to a high of H= 0.818 for locus OSSR 11. The average genotypic richness (no. of polymorphic loci) was high (67%) across populations. However variation was not as evenly distributed, ranging from a low of 38.9% for population Clos du Bois to a high of 88.9% for populations Monticello and Temecula.

The hierarchical datasets allowed partitioning of genetic differentiation among regions, within a region among populations, and within population among individuals. Analysis of Molecular Variance (AMOVA) results revealed 1% diversity among different regions and 1% among populations within a region. Nearly all of the diversity (98%) was represented by individual strains within populations (Table 1). Lack of clear genetic structure was also evidenced by Principal Component Analysis (PCA), where 53.13% of variation was explained by first three components across the hierarchical structure. The conclusion from the current 18 SSR loci analysis doesn't agree with the analysis based on 6 SSR loci reported earlier (Lin et al., 2005). The discrepancy may be due to the fact that when only a few loci were used, each locus contributes significant weight to the overall genetic variation, which could overestimate genetic variation. Absence of genetic differentiation observed among grape *Xf* populations in the present study is supported by the observations of Schuenzel et al (2005), in which no genetic differentiation among northern and southern California populations of *Xf* from grapes was observed. We hypothesize that the reasons for this may include relatively recent spread of the pathogen and the geographic range of insects vectoring the pathogen. The values for overall gene flow (Nm =2.2272) supported lack of population differentiation. Sonoma region showed least gene flow (Table 2) compared to highest for Temecula region.

Objective 2

In this study, observed genetic variation of *Xf* strains was not related to or dependent on the different grape cultivars from which the strains were collected. Preliminary cultivar effect results indicated that the type of grape cultivar did not play any role in grouping *Xf* populations from various counties. We observed that *Xf* isolated from Chardonnay grown in Sonoma county grouped with other *Xf* strains isolated from other grape varieties grown within Sonoma county rather than grouping with *Xf* isolated from Chardonnay grown in Napa or Temecula counties (Figure 1). Although this is the first report to this effect in grape, similar results were observed for *Xf* isolated from citrus; e.g., different sweet orange varieties did not affect population structure of *Xf* in Brazil (Coletta-Filho and Machado (2002). However, further research of this effect in grape is necessary before concluding that grape cultivar does not affect *Xf* population structure.

Objective 3

Using the same SSR marker system, we extended our investigation to almond leaf scorch (ALS) disease in California's San Joaquin Valley. The seasonal collection and detection studies (Figure 2) showed that *Xf* populations were low in early season (March and April), when *Xf* is less easily detected with PCR. *Xf* populations quickly increased with increased vector activity in late spring/early summer. Successful *Xf* isolation/culture and PCR detection were comparable after July through October. To increase the power of our analysis, we are analyzing the results from 13 additional SSR markers this year (in addition to the five SSR markers used in previous years) from 73 *Xf* strains of almond. Allelic types and allelic frequencies of haplotypes among and within populations are being analyzed to better understand the genetic structure and population dynamics of *Xf* strains in almond.

CONCLUSIONS

Hierarchical sampling and multilocus genetic marker analysis provided informative details of genetic diversity, population structure, and the evolutionary process of selection/adaptation of grape *Xf* strains in grapevine growing regions. Our preliminary conclusions about the lack of a grape cultivar effect on *Xf* population dynamics may be an important addition to the current knowledge of this pathogen in grape. Information from population differentiation within grape specific *Xf* strains facilitates better understanding PD epidemiology and the development of an effective disease management strategy.

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FUNDING AGENCIES
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Table 1. Summary of AMOVA analysis with population differentiation parameters.

Source	df	SS	MS	Est. Var.	%	Stat	Value	Prob
Among Regions	8	17.898	2.237	0.013	1%	PhiRT	0.006	0.001
Among Pops/Regions	6	12.909	2.151	0.012	1%	PhiPR	0.006	0.002
Within Pops	68	141.227	2.077	2.077	98%	PhiPT	0.012	0.001
Total	**82**	**172.033**	**6.466**	**2.102**				

Table 2. Population structure parameters of *Xf* strains from four regions of California**.**

Region name	Diversity (H)	Effective # alleles (A_E)	Migration index (Nm)	Private alleles	# of significant LD (p=0.05)
Sonoma	0.4414 ± 0.23	2.08 ± 0.82	0.6050	0.06-0.33 Ave=0.18	0 - 223 Ave=71.3
Napa	0.4005 ± 0.22	1.95 ± 0.88	1.3891	0.06-0.50 Ave=0.39	24-74 Ave=41.2
Kern	0.4789 ± 0.29	2.50 ± 1.30	3.5660	0.22-0.28 Ave=0.25	89-129 Ave= 109
Temecula	0.5422 ± 0.22	2.55 ± 0.88	2000	0.6	180
Total	**0.5347 ± 0.24**	**2.62 ± 1.09**	**2.2271**	**0.36**	100.3

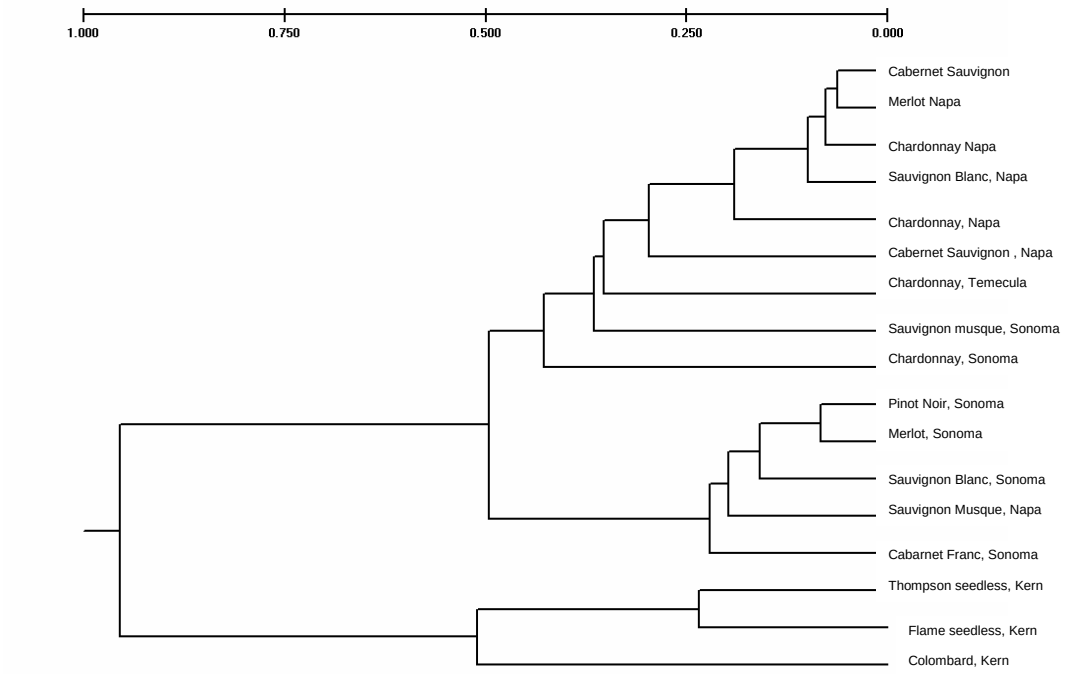

Figure 1. Dendrogram representing grouping of *Xf* strains from four regions according to grape cultivar.

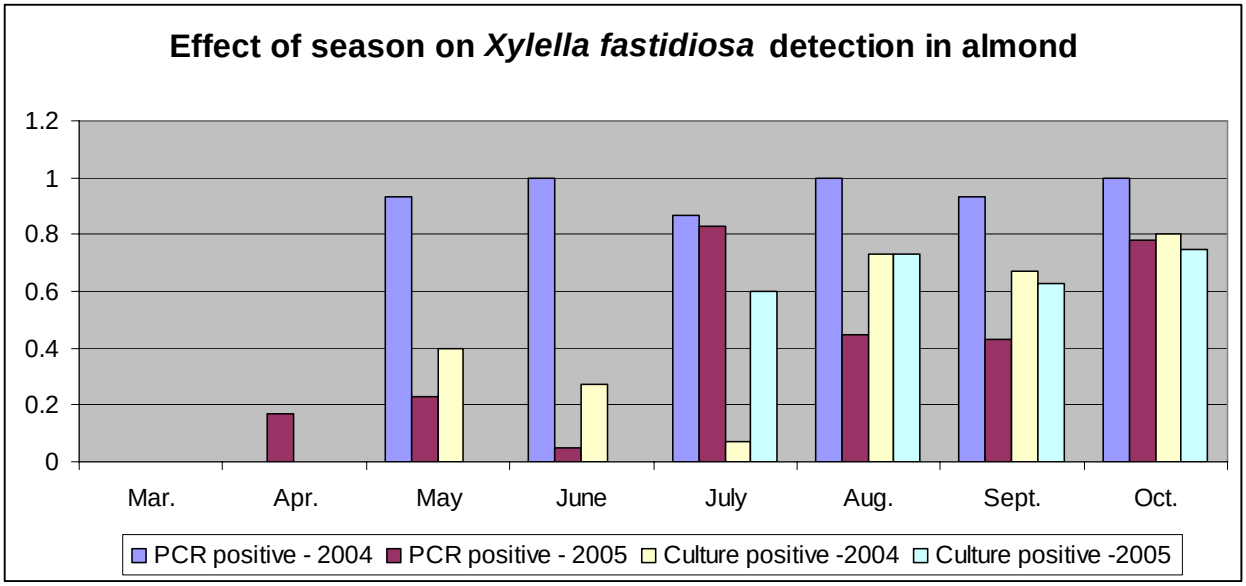

Figure 2. Effect of seasonal variation on PCR and culture based detection levels of *Xylella* infection.

ASSESSMENT OF THE PROCESS OF MOVEMENT OF *XYLELLA FASTIDIOSA* WITHIN SUSCEPTIBLE AND RESISTANT GRAPE VARIETIES

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ABSTRACT

In an effort to better understand the colonization of grapevines by the pathogen *Xylella fastidiosa (Xf)*, and to develop a method of screening for resistant plant genotypes, we are investigating the spatial distribution of cells of *Xf* within susceptible and resistant grape varieties and to examine the spatial segregation of mixtures of *Xf* cells within the xylem vessel systems of different grape varieties. A single *Xf* strain or an equal mixture of two different isogenic *Xf* strains, were co-inoculated into different varieties and their movement was followed closely by culturing and epifluorescence microscopy, with time and distance from the point of inoculation. We followed simultaneously the movement and population size of a gfp-marked strain of *Xf* (KLN59.3) in Cabernet Sauvignon, Roucaneuf and Tampa grape varieties. Very low population sizes of *Xf* and infrequent occurrence in xylem vessels in the stem were seen in the resistant varieties. The percentage of infected vessels in a stem cross section (as determined by microscopy) and bacterial populations were very strongly and directly related indicating that the low bacteria population detected in the resistant genotypes is due to a low number of infected vessels that each were colonized to similar levels as in susceptible varieties, rather than poor growth in a many vessels. In contrast, similarly high percentages of vessels in petioles of susceptible and resistant plants were colonized, and similar population sizes were attained, suggesting that *Xf* is unrestricted in movement within the petiole. These results suggest that resistance to Pierces disease is not due to inhibitory compounds that circulate through the xylem or to host defenses since they might be expected to operate similarly in these two tissues. Resistance to movement thus appears to be due to structural differences in the vessels of the resistant varieties and is associated with a limitation of the number of vessels into which *Xf* can spread and thus grow. We have produced a cyan-marked strain of *Xf* that is being used in mixed inoculation studies with the gfp-marked strain to better define the process of movement through the plant.

INTRODUCTION

Nearly all studies of *Xylella fastidiosa* (*Xf*) colonization of grapes have focused on the petioles, with little examination of *Xf* movement and distribution of in the stems. Importantly, the work from the Walker lab has noted that the mechanism of resistance to *Xf* is localized within the stem xylem and not fully functional or absent in the xylem of petioles and leaf blades. This was based on the observation that there was little difference in the colonization of the petioles and leaf blades, as opposed to the stems. They speculate that a more constitutive resistance mechanism is present in the stem xylem based on nutritional or structural differences between resistant and susceptible types. Our study was designed to examine differences in the colonization process of the stem of different grape genotypes to identify resistance mechanisms.

Before initiating studies of the segregation of differentially marked strains of *Xf* in various grape varieties, we explored the process of colonization of *Xf* in stems of Cabernet Sauvignon to establish control data and optimize sampling schemes for the strain mixtures. We set out to determine how quickly *Xf* moves within stems throughout the plant, the fraction of the xylem vessels colonized as a function of time and distance from the point of inoculation, and the relative likelihood of finding *Xf* in xylem vessels as compared to tracheal elements. We specifically considered the longitudinal movement of *Xf* in the xylem vessels in the internodal stem locations and the rate at which segregation of the two strains occurs. A more detailed examination of the movement of *Xf*, using epifluorescence microscopy, was performed on stem sections at various locations and times after inoculation with *Xf* strains harboring gfp marker genes.

We followed simultaneously the movement and population size of a gfp-marked strain of *Xf* (KLN59.3) in Cabernet Sauvignon, Roucaneuf and Tampa grape varieties. Plants were inoculated with the gfp-marked strain to directly compare the movement and growth of *Xf* in resistant and susceptible grape genotypes. We then correlated the percentage of infected vessels in a stem cross section (as determined by microscopy) and bacterial populations. A strong correlation would indicate that the low bacteria population detected in the resistant genotypes is due to a low number of infected vessels.

OBJECTIVES

1. Study the process of movement of *Xf* cells between xylem vessels and through plants 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

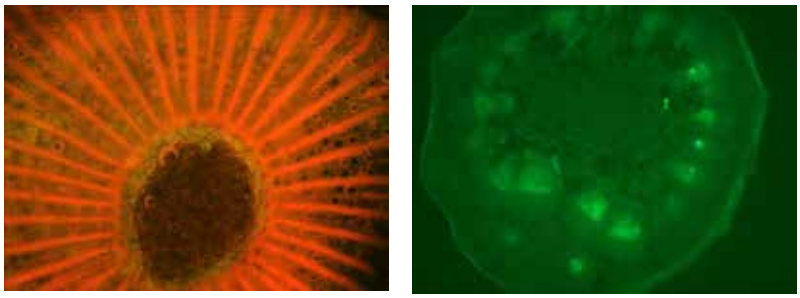
2. Determine if bottlenecks in movement of cells of *Xf* from xylem vessel to xylem vessel is more extreme in resistant plants than in susceptible plants and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *Xf*.

RESULTS

We initiated our investigation by co-inoculating Cabernet Sauvignon stems with a mixture containing an equal amount of the wild-type and a gfp-marked (KLN59.3) *Xf* strain. This was designed specifically so that the temporal and spatial patterns of segregation of the two strains could be tracked and correlated to resistance characteristics of the plant variety. The population size of the gfp-marked strain of *Xf* was somewhat smaller at a given location and time after inoculation than the wild-type strain. It was known that this strain caused disease symptoms slightly slower than the wild-type strain, and this difference thus appears to be due to a slower growth in the plant. Given that future experiments will emphasize the spatial segregation of this gfp-marked strain and a similar cfp-marked strain which is expected to have a similar growth rate as the gfp-marked strain we do not expect that this lower growth compared to the wild-type strain will complicate our measurements of ratios of these two strains in ongoing experiments. The results of these initial experiments has provided us the needed information on the speed with which these bacterial move through the plant and the rapidity with which segregation is occurring; such results have enabled us to develop more detailed experiments where intensive sampling will provide the needed information on the rapidity with which bottlenecks occur in *Xf* populations during the movement process. We also recently co-inoculated the gfp-marked strain with a strain (SC1) that we have recently constructed that harbors a constitutively-expressed cyan fluorescent protein (CFP) driven by a kan promoter and located in the same insertion site in the chromosome as the GFP reporter gene is located in KLN59.3. (Figure 1). These two strains thus should exhibit identical behavior in plants but be differentiated by their different colors of fluorescence emission. On-going studies involve inoculation of this combination of two marked strain into both resistant and tolerance genotypes.

Figure 1. cyan-marked cells of *Xf*

Susceptible Cabernet Sauvignon and resistant varieties including Tampa and Roucaneuf were inoculated with the gfp-marked *Xf* strain and examined by sequential culturing and epifuorescence microscopy. Roucaneuf is a complex hybrid that includes V. c*inerea* and V. *berlandieri* and has been described as "fully-resistant" in field conditions to PD (A.F.Krinvanek at all. 2004). Tampa also is a PD resistant genotype. Microscopy did not reveal any obvious differences in the stem and petiole anatomy of resistant and susceptible varieties (Figures 2 to 7). We followed population growth by culturing and also visually by microscopy of numerous cross sections of both stems and petioles. Culture sampling was done at weeks 2, 3, 4, 6, and 11 following inoculation. A total of six plants at each time point, two from each resistant genotype and two from the susceptible genotype were evaluated. Each plant was sampled at the petiole near the point of inoculation and at six internodal locations 10, 20, 30, 60, 80, and 120 cm away. The sample sites were examined the same day by epifluorescence microscopy of numerous sections near the site of culturing. An average of nine sections was prepared for each stem location and photos were taken from each sample.



Figures 2-3. Roucaneuf stem section (left) and petiole section (right) inoculated with *Xf* Gfp. Week 11 post inoculation at 30 cm from point of inoculation.

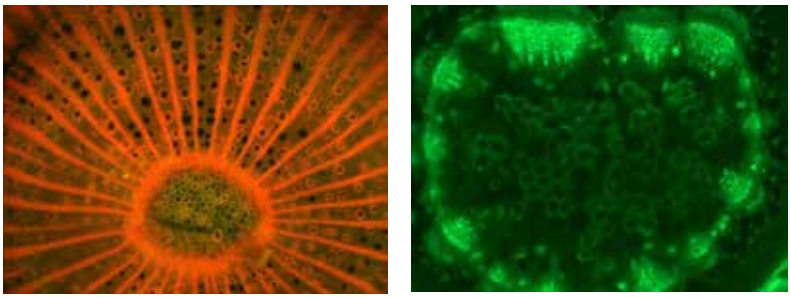
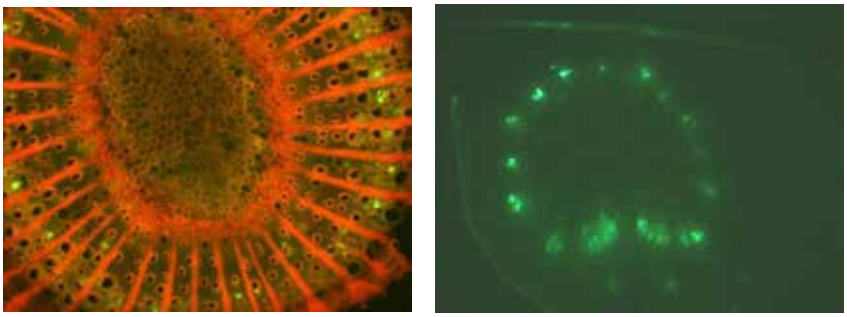


Figure 4-5. Tampa stem section (left) and petiole section (right) inoculated with *Xf* Gfp. Week 11 post inoculation at 30 cm from point of inoculation.



Figures 6-7. Cabernet Sauvignon stem section (left) and petiole section (right) inoculated with *Xf Gfp*. Week 11 post inoculation at 30 cm from point of inoculation.

The proportion of infested vessels in five microscopy stem cross sections per genotype assessed per sampling point for each plant genotype at 6 and 11 weeks post-inoculation, in different internodes locations. The percentage was calculated counting an average of five cross sections at four different locations from the point of inoculation. The vessels were counted positive if any presence of gfp-marked cells were noted**.** It was clear that very few of the stem vessels at sites away from the point of inoculation of Roucaneuf and Tampa were colonized by any cells of *Xf* compared to that of Cabernet (Figures 2-7). This correlates well with the higher viable population sizes of *Xf* in Cabernet compared to that of Roucaneuf and Tampa. This procedure allowed us to compare the *Xf* populations in each genotype to determine cell viability as a function of time and distance from the point of inoculation (Figure 8). The direct relationship between population size of *Xf* and the proportion of vessels colonized indicates that that the low bacteria population detected in the resistant genotypes is due to a low number of infected vessels that each were colonized to similar levels as in susceptible varieties, rather than poor growth in a many vessels.

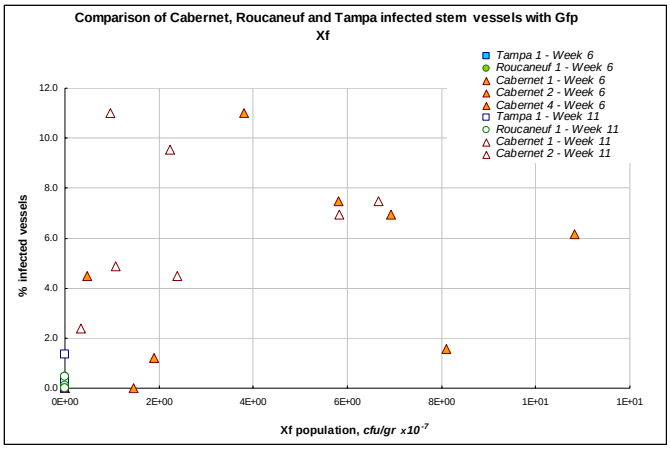


Figure 8. Direct relationship between increasing incidence of xylem colonization and population size in several grape varieties.

It was also clear from visualization of cells of *Xf* in petioles of Cabernet and Roucaneuf and Tampa that petioles of these plants were all equally well colonized by the gfp-tagged cells of *Xf* (Figures 2 to 7). This is in contrast with the stems of these two varieties where very few vessels of Roucaneuf were colonized but a large percentage of vessels of Cabernet were colonized (Figures 2 to 7). It was evident that there was no significant difference in bacteria population between the resistant and susceptible genotypes in the petioles (Table 1).

Table 1. *Xf* cells in petioles of different grape varieties (Log cells/g)

Week	Roucaneuf	Tampa	Cabernet Sauvignon
3	7.77	4.86	7.6
4	7.71	5.55	7.43
6	7.18	4.79	6.26
11		5.59	8.46

The proportion of total stem xylem vessels that are colonized by *Xf* appears to be similar to that of xylem vessels in the petiole. That is, between 6-11 % of the total stem xylem vessels were colonized whereas 12-15% of the petioles of the same plant were colonized in Cabernet Sauvignon. We also observed that population sizes of *Xf* determined by plating, reached higher values in the petioles in the same amount of time after inoculation.

It seems clear that the numbers of *Xf* in stems of resistant varieties such as Roucaneuf are low and apparently spatial variable. Thus, at a given sampling time, not all one cm stem segments include detectable cells of *Xf*. Since *Xf* was frequently detected in petioles, even some distance from the point of inoculation, it appears that *Xf* follows a sinuous path up the vessels in the stem, never colonizing a large number of vessels, but when it enters the petiole it can multiply to high numbers (Table1). In contrast, the population in the Cabernet Sauvignon remained stable and *Xf* moved much further from the POI, reaching a distance of 120 cm from the inoculation point after 11 weeks compared the 30 cm reached in Roucaneuf and 80 cm in Tampa in the same amount of time.

To compare the spatial segregation between vessels within the grapevine stems ninety individual Roucaneuf grapevines were stem inoculated as described above, with an equal mixture of *Xf* strains Temecula and rpfF- mutant KLN61. In Roucaneuf bacterial growth was notably lower in both distance and time compared to susceptible Cabernet. Most importantly the incidence of recovery of viable cells decreased greatly with time (Figure 9). While *Xf* could be recovered from a high proportion of plants within the first 5 to 8 weeks after inoculation, the proportion dropped precipitously by weeks 11 and 16. This suggests that cells in such resistant plants are not growing, and are dieing after they colonize a vessel but can not move to new ones. This is being investigated using propidium iodide staining to visualize dead cells.

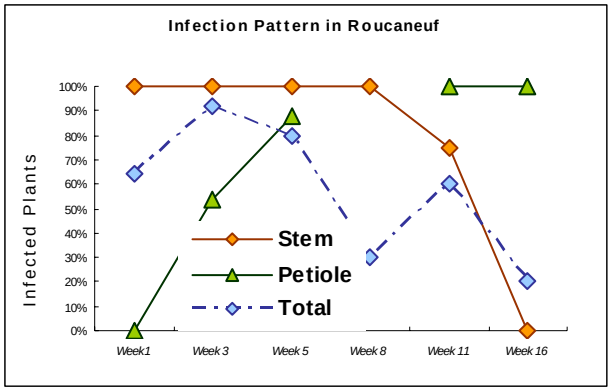


Figure 9. Incidence of isolation of *Xf* from various parts of Roucaneuf grape at various times after inoculation showing a reduced recovery from both stems and petioles after 8 weeks. This is in contrast with continued high recovery in Cabernet (data not shown).

CONCLUSIONS

These results all suggest that structural differences in the stems of grape account for resistance to Pierce's disease. These results suggest that resistance to Pierces disease is not due to inhibitory compounds that circulate through the xylem or to host defenses since they might be expected to operate similarly in these two tissues. Resistance to movement thus appears to be due to structural differences in the vessels of the resistant varieties and is associated with a limitation of the number of vessels into which *Xf* can spread and thus in which they can grow.

FUNDING AGENCIES

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

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

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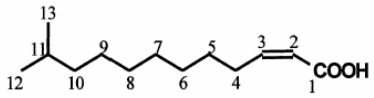
Reporting Period: Most results reported here are from work conducted October 2006 to October 2007.

ABSTRACT
Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that changes its gene expression in cells as they reach high numbers in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. We have investigated both the role of DSF-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* mutant 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 DSF-degrading strains greatly reduced the incidence and severity of disease in grape. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Studies are underway to determine if DSF produced by rootstocks can move to scions and confer disease control.

INTRODUCTION
Xylella fastidiosa (Xf) colonizes the internal tissues of grape and other hosts, forming a biofilm inside the plant. Given that there is a very strong correlation between the number of xylem vessels heavily colonized by *Xf* and disease symptoms in grape (9, 24, 28) we thus think of Pierce's disease as a result of uncontrolled proliferation of *Xf* in the plant, thereby causing disruption in water flow. Such an endophyte presumably acquires most of its nourishment from the dilute solutes present in the xylem sap. A key determinant of success for an endophyte such as *Xf* is the ability to move within the plant since its success would be dependent upon moving from one xylem vessel to another, presumably though pits, where it could intercept increasing amounts of xylem sap; the population size in the plant would be proportional to the number of xylem vessels into which it had moved. *Xf* possess traits such as endoglucanases and polygalacturonases that allow it to degrade pit membranes and hence move within the plant. Work by Roper et al. (29) has shown that polygalacturonases are required for movement in the plant, presumably by contributing to pit membrane degradation; other enzymes such as endoglucanases also probably contribute. While occasional paths that transit significant distances within the plant occasionally occur (34) enabling passive movement of *Xf*, it appears that *Xf* cells introduced into a given vessel are usually constrained by the anatomy of the vessel and must actively move by altering the plant (19,20).



We have found that the virulence of *Xf* is strongly regulated in a cell density-dependent fashion by a process similar to quorum sensing (the *rpf* locus) which involves a small signal molecule. Numerous species of bacteria communicate using small molecules, such as *N*-acyl homoserine lactones, small peptides, butyrolactone derivatives or fatty acids, as signals. 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 greatly reduced when the ability to produce signaling compounds is disrupted by mutation. *Xf* and *Xanthomonas campestris* pv. *campestris* (*Xcc*) are very closely related pathogens. *Xcc* makes a farnesoic acid derivative, cis-11-methyl-2-dodecenoic acid called diffusible signal factor (DSF). We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but probably slightly different than the DSF of *Xcc*. In striking contrast to that of *Xcc*, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DSF-deficient mutants of *Xf* are consistent with the role of

this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf*. Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow too excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *Xf* we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF mediated cell-cell signaling in *Xf*. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the extent to which other endophytes can modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, obtained genetic transformation of grape and other hosts of *Xf* to express DSF, and explored other means to alter DSF abundance in plants to achieve 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 Pierce's disease 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

Understanding the pathway of cell-cell signaling mediated by DSF

Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. In many pathogenic bacteria, intracellular signaling couples extracellular cell-cell signaling to regulate different cellular processes. The Rpf signaling component RpfG encodes a response regulator with domain which modulates the level of cyclic di-GMP. In many pathogenic bacteria, it has been reported that di-Cyclic GMP influence many virulence traits such as biofilm formation, motility, adhesion etc. In *Xf* we have identified an ORF (PD0279), which encodes a GGDEF domain protein predicted to be involved in the synthesis of di-cyclic GMP. *Xf* mutants in the GGDEF domain proteins were produced and initial analysis revealed that they are also altered in *in vitro* attachment and biofilm formation, much like the other cell-cell signaling mutants-*rpfF and rpfC*. Comparative transcription analysis of the GGDEF domain protein mutant revealed that both RpfF and RpfC are involved in its regulation. Interestingly, transcription analysis revealed that the GGDEF protein (and indirectly, di-Cyclic GMP) is involved in regulation of cell-cell signaling in *Xf* (Table 1). Mutants in this regulator highly over-express rpfF and hence express very high levels of DSF (Table 1). Mutants in the GGDEF protein, like rpfC mutants which over-produce DSF are severely reduced in their ability to incite Pierce's disease to grape (Figure 1). This is consistent with our other results that show that DSF over-production attenuates virulence of *Xf* (Figure 1). We thus will be testing the DSF overproducing GGDEF mutant strain for PD control.

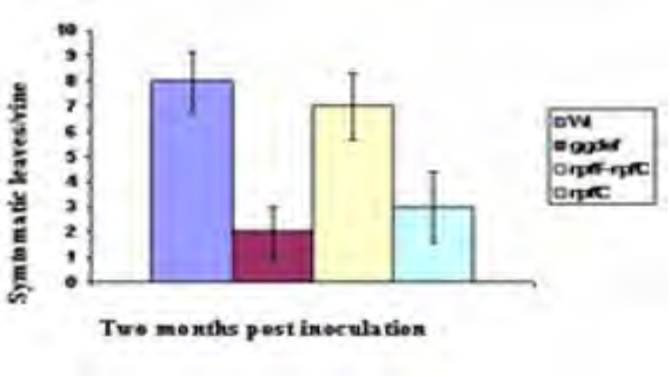


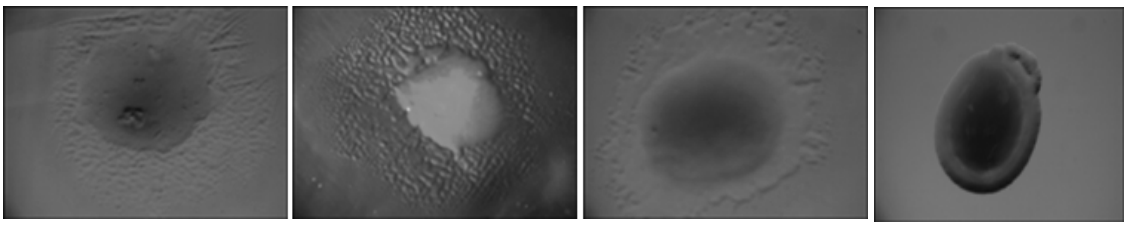
Figure 1. Severity of Pierce's disease of grapevines inoculated with DSF overproducing strains of *Xf* (Wild type, *rpfC* and *ggdef* mutants).

Table 1. Transcriptional activity of genes in the rpf cluster of *Xf* as well as other genes contributing to virulence in *rpfF*, *rpfC* and GGDEF mutant backgrounds. ***** Relative quantification of RNA calibrated with the wild type *Xf* RNA as 1, and normalized to the 16S ribosomal.

	* Fold change in expression		
Gene Name	GGDEF mutant	*rpfF* mutant	*rpfC* mutant
rpfG	6.2	0.60	2.01
rpfE	3.65	0.33	1.6
tolC	1.78	6.53	3.80
rpfB	1.5	0.82	3.27
PD0279 (GGDEF domain protein)	n.d	6	3
pglA	3.0	5.83	6.80
rpfC	5.38	4.0	n.d
rpfF	10	n.d[1]	8.0

DSF negatively regulates twitching motility in *Xf*

Since *rpfF* mutants of *Xf* are hyper virulent and move more rapidly in the xylem vessel, we investigated whether DSF overproduction can influence twitching motility in *Xf*. Assay for the twitching motility (1) revealed that the *rpfF* mutant which does not produce any DSF, showed an increased colony fringe indicative of high twitching motility (Figure 2). However an *rpfC* mutant which is a DSF over-producing strain of *Xf* showed reduced colony fringe. Interestingly the GGDEF domain protein mutant, which also greatly over-produces DSF and is deficient in di-cyclic GMP synthesis and also altered in *rpfF* expression was severely reduced in twitching motility (Figure 2). This suggests that DSF regulates adhesion and motility at least partially via its indirect regulation of di-cyclic GMP levels. Combined with our other studies of those genes that are regulated by DSF accumulation in *Xf* as determined from microarray and quantitative RT-PCR studies, we have developed a model for DSF-mediated gene regulation in *Xf* (Figure 3).



Wild type ***rpfF*** ***rpfC*** ***ggdef***

Figure 2. colonies of different *Xf* strains were spotted on cellophane placed on modified PWG media with low BSA. Twitching motility is indicated by the formation of a peripheral fringe.

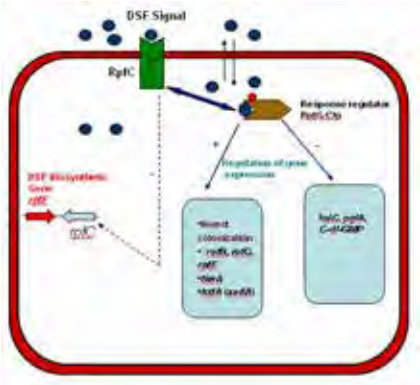


Figure 3. A proposed model for DSF-mediated cell-cell signaling regulation in *Xylella fastidiosa*. *rpfF* encodes the DSF synthase. RpfC encodes a hybrid two component sensor which can sense DSF. DSF levels negatively regulate the expression of *rpfF* by feed back which acts as a negative feed back. Expression of other virulence associated functions is regulated by a putative intracellular DSF sensor/response regulator (like-RpfG/Clp). DSF can diffuse inside the cell and is sensed by this putative intracellular DSF sensor/response regulator, which acts as positive regulator of functions associated with attachment-biofilm formation and other components of rpf regulon. The intracellular DSF sensor/response regulator, in the presence of RpfC, can negatively regulate the expression of other virulence associated genes like *pglA*, *tolC* and GGDEF domain encoding gene.

Production of DSF in transgenic plants for disease control.
We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (Figure 4). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on tobacco (Figure 5).

Figure 4. Symptoms caused by *Xf* on SR1 Tobacco.

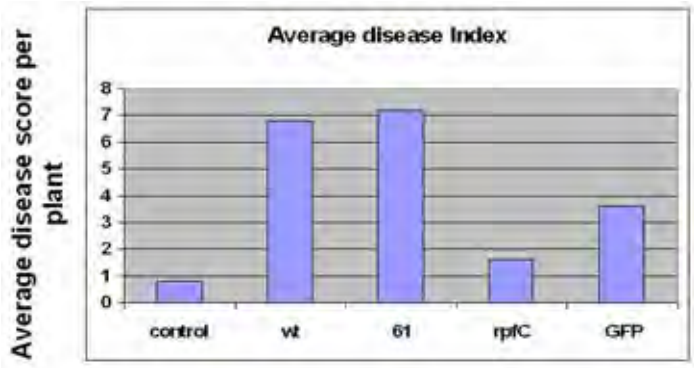


Figure 5 Disease caused by WT *Xf*, an rpfF- mutant (61), an rpfC- mutant, and a gfp-marked *XF* strain.

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility. The first transformed plants have been tested for DSF 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. Large numbers of clonal *rpfF*-expressing grapes have now been produced and inoculated with *Xf* to test for susceptibility to Pierce's disease. The *rpfF*-expressing grape are MUCH less susceptible to Pierce's disease. (Figure 6). 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 DSF-overproducing strains of *Xf*.

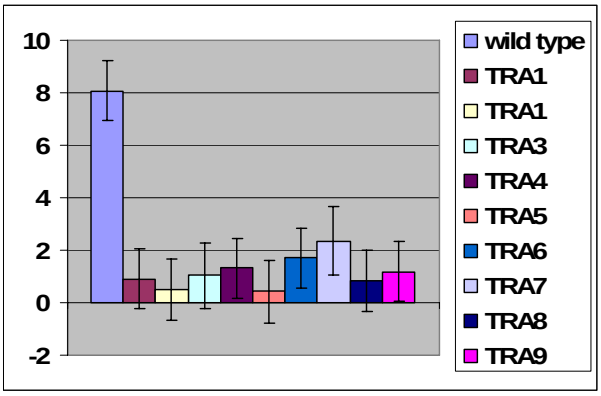


Figure 6. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

We are thus very excited about the prospects of enhancing DSF 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.

Enhancing the DSF levels in plants
Given that fatty acid synthesis in plants occurs primarily in the chloroplast and that DSF is presumably a fatty acid derivative, we have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. The *Arabidopsis* ribulose bisphosphate carboxylase small subunit 78 amino acid leader peptide and mature N-terminal which is sufficient to target the protein to the chloroplast, has been fused with the RpfF protein of both *Xylella* and *Xcc*. Assay of DSF in transgenic SRI tobacco plants-where the RpfF is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared

to the plants in which the RpfF is expressed in the cytosol. Transcription analysis of the chloroplast targeted *rpfF* transformed plants indicates high level expression of the rpfF gene (Figure 7). We have generated seeds from the transgenic SRI tobacco plants and we are conducting pathogenicity assay with *Xf*.

Figure 7. DSF extracted from transgenic tobacco SR1 plants expressing the chloroplast targeted RpfF, compare to nontransgenic SR1 and native DSF extracted from *Xcc*. The DSF is spotted at the right hand side on a filter disc and the *Xcc* DSF bioindicator is streaked on the left side of the spot. The green GFP fluorescence is indicative of DSF production.

We have also initiated transformation of grapes with the improved chloroplast targeted *rpfF* constructs- with the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic Arabidopsis plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants can complement the virulence of the nonpathogenic *rpfF* mutant of *Xcc* (Table 2). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more suseptable to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels.

Table 2. Disease severity from topical application of bacteria varying in DSF production to Arabidopsis. Bacteria were inoculated on different Arabidopsis genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

***Arabidopsis* Genotype**	***Xcc* strains**	
	Wild type	*rpfF*⁻
Col (wild type)	++++	-
rpfF transformed	++++	+
rpfF and *rpfB* transformed	++++	++

To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the rpfF of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (Figure 8). In addition, a large number of greenhouse experiments are underway in which we have introduced purified DSF into plants, have sprayed purified DSF onto plants, and have applied DSF as a soil drench to plants. The results of these experiments will soon be available as further disease development occurs. Preliminary results indicate that direct introduction of purified DSF into plants reduced Pierce's disease by over 50%.

Figure 8. Grafted tobacco plants into which *Xf* has been inoculated. A normal SR1 tobacco scion is grafted onto transgenic DSF-producing tobacco. The graft point is noted with blue tape, and *Xf* has been inoculated above the graft union. The plant is as yet asymptomatic.

Producing DSF in bacterial endophytes. We have previously been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (Table 3). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain (Figure 9). This DSF-producing endophyte has been co-inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process.

Table 3. Production of DSF by *E. coli* and *Erwinia herbicola* harboring cloned *rpfF* genes from *Xf* and *Xcc*.

Strains	Relative DSF production (Units)
Xcc	100
Xf Temecula	4-5
E. coli DH10B (ptrp*XccrpfF*)	3000
E. coli DH10B (ptrp*XfrpfF*)	100
E. herbicola (ptrp*XccrpfF*)	6000
E. herbicola (ptrp*XfrpfF*)	200

Figure 9. Endophytic strains of *R. etili* producing *Xcc and Xf* DSF. The GFP florescence produced by an *Xcc* DSF biosensor which is sprayed on the plates, is indicative of DSF production.

Further support for the role of carAB, required for production of carbamoyl phosphate (required for pyrimydine biosynthesis in bacteria) in the ability of bacteria to degrade DSF and to control disease was obtained. The *carAB* genes from *Pseudomonas* strain G (which is a highly efficient degrader of DSF and capable of biological control of Pierce's disease) were cloned and used to restore CarAB function in a *carAB* mutant of strain G and restored its ability to reduce disease caused by *Xf*. (Figure 10). These results suggest that it should be possible to enhance biocontrol by DSF-degraders by over-expressing CarAB.

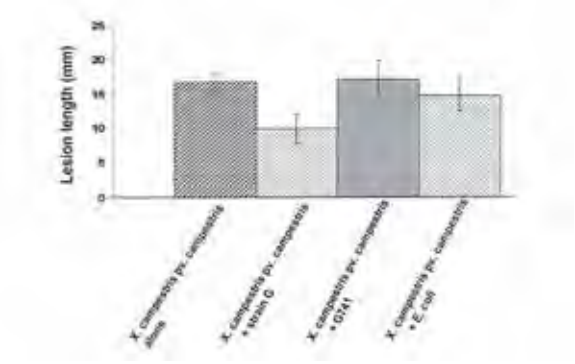


Figure 10. Severity of Pierce's disease of grape co-inoculated with *Xf* strain STL and DSF degrading *Pseudomonas* strain G, *CarAB* mutant G741 of *Pseudomonas* strain G, or with mutant G741 complemented with pSC4 when measured 3 months after inoculation. The vertical bars represent the standard error of the mean of the number of symptomatic leaves per vine.

CONCLUSIONS

Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, topical application of such bacteria to plants with surfactants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated, more work is needed to determine how to achieve this in the most practical means.

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INITIAL GENETIC ANALYSIS OF *XYLELLA FASTIDIOSA* IN TEXAS

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Reporting Period: The results reported here are from work conducted winter 2006 to summer 2007.

ABSTRACT

Xylella fastidiosa (*Xf*) is the causative agent of Pierce's disease of grape. *Xf* genetics has not been evaluated in Texas despite growing financial risk to the US grape industry, a Texas population of the insect vector now spreading in California, and evidence that the bacterium is ubiquitous to southern states. Using sequences of conserved *gyrB* and *mopB* genes, we have established at least two strains in Texas, grape strain and ragweed strain, corresponding genetically with subspecies *piercei* and *multiplex*, respectively. The grape strain in Texas is found in *Vitis vinifera* varieties, hybrid vines and in wild *Vitis* near vineyards; whereas, the ragweed strain in Texas is found in annuals, shrubs and trees near vineyards or other areas. RFLP and QRT PCR techniques were used to differentiate grape and ragweed strains with greater efficiency than sequencing and are practical for screening numerous *Xf* isolates for clade identity.

INTRODUCTION

The bacterium, *Xylella fastidiosa* (Wells) (*Xf*) resides in the xylem tissue of many plant species and is moved within plant communities through insect transmission. While many plants are not impacted by *Xf* infection, colonization of the xylem vessels of several economically important plants including citrus, coffee, grape, almond, oleander and oak result in disease (Purcell 1997). Symptoms of *Xf* infection include leaf chlorosis, leaf scorch, crop loss and plant death and are most severe in non-native crops (Purcell 1997). *Vitis vinifera* grapevine varieties (Cabernet Sauvignon, Chardonnay, etc.) are not native to the Americas and are susceptible to *Xf* infections or Pierce's disease (PD) which causes the rapid decline and mortality of vines. PD occurs throughout the US and has consistently occurred along the Texas Gulf coast (Kamas et al. 2004) and intermittently in the California grape growing regions for more than one hundred years (Purcell 1997). In the 1990s, a PD epidemic in southern California's Temecula region resulted from the introduction and subsequent range expansion of the glassy-winged sharpshooter (GWSS), (*Homalodisca vitripennis*) (de Leon et al. 2004) creating an enormous PD risk to the California grape industry.

Investigation of disease ecology and epidemiology in Texas has scientific advantages over simply investigating PD in California where it poses the greatest financial threat. Genetic evidence used to develop a phylogeny of GWSS has revealed that populations of this insect pest introduced into California originated from Texas (de Leon et al. 2004). The temperate Gulf climate of Texas and large numbers of *Xf* positive native plants (Buzombo et al. 2006) suggest that the bacterium has a long evolutionary history in the region. Therefore, genetic variability among *Xf* strains in Texas is critical to understanding the natural history of the disease in the US. Additionally, PD is the major limiting factor in the Texas Hill Country, a major wine grape production area in Texas. Despite the importance of understanding the genetics of *Xf* in this region, there are no published reports on *Xf* diversity within the state of Texas.

Xf strains demonstrate variable host ranges with some strains causing disease symptoms only in a specific plant species and others in multiple species (Almeida and Purcell 2003). *Xf* strains can be separated into clades, the most valuable first step in determining strain diversity. Current genetic analysis suggests five main clades: 1. isolates causing citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS), 2. isolates causing PD, 3. isolates causing oleander leaf scorch (OLS), 4. isolates causing Dixon-like almond leaf scorch (ALS), and 5. isolates infecting hardwood (angiosperm) trees including oak, maple, and mulberry (Chen et al. 2002, Hendson et al. 2001, Meinhardt et al. 2003, Rodrigues et al. 2003, Schaad et al. 2004). Several techniques have been used in determining these clades genetically including REP-PCR and RAPD-PCR, (Chen et al. 2002, Hendson et al. 2001, Pooler et al. 1995, Rodrigues et al. 2003) and CHEF analysis (Hendson et al. 2001). Recently, three subspecies of *Xf* have been named and these subspecies are consistent with three of identified clades (excluding ALS and OLS) (Schaad et al. 2004). The PD strain has been labeled "subsp. *piercei*"; the hardwood group, peach and plum group "subsp. *multiplex*" and CVC strain group is "subsp. *pauca*. As these three clades have clear genetic differences and are differentially pathogenic we consider these to be distinct strains

Most genetic diversity studies have included sequencing a particular DNA fragment to determine phylogenetic relationships between isolates including the 16S-23S rDNA intergenic spacer region, 16S rDNA gene (Hendson et al. 2001) and the gyrase B (*gyrB*) gene (Rodrigues et al. 2003). Another gene useful for phylogenetic analysis is the *mopB* gene. The mopB protein is an outer membrane protein of bacteria with a fairly conserved sequence within the OmpA family of proteins (Fjellbirkeland et al. 2000).

OBJECTIVES

1. Analyze a preliminary collection of *Xf* isolates from Texas vineyards (grapevines and surrounding vegetation) as an initial screen of genetic diversity. Using *mopB* and *gryB* sequences, determine whether *Xf* isolates belonged to the grape clade (subsp. *piercei*), the hardwood mulberry clade (subsp. *multiplex*), OLS or ALS groups.
2. Determine if restriction fragment length polymorphism (RFLP) digestion and quantitative real-time polymerase chain reaction (QRT PCR) analyses of *gyrB* were consistent with gene sequence data.

RESULTS

Sequences of the *gyrB* and *mopB* genes were conserved within two strain groupings. One group of isolates showed perfect alignment with the PD strain 'Temecula' using BLAST and was designated the 'grape' strain. Another strain group aligned perfectly with the 'multiplex Dixon' strain and was designated the 'ragweed' strain. There was no genetic variability between 'grape' strains or 'Texas ragweed' strains for these two conserved genes and no evidence of OLS, CVC, or Coffee strains in this analysis of 14 isolates. Grape strain was identified from a variety of grapevines including: mustang grape (*Vitis mustangensis*), SO4 rootstock (*V. cinerea var. helleri* x *V. riparia*), Black Spanish/Lenoir (*V. aestivalis* hybrid with *V. cinerea* and *V. vinifera* parentage) and several European grape varietals (*V. vinifera*) (Table 1). The ragweed strain was identified from weed and woody species including: redspike Mexican hat flower (*Ratibida columnifera*), western ragweed (*Ambrosia psilostachya*), giant ragweed (*Ambrosia trifida var. texana*), annual sunflower (*Helianthus annuus*), sea myrtle (*Baccharis halimifolia*), cedar elm (*Ulmus crassifolia*) and heartleaf ampelopsis (*Ampelopsis cordata*) (Table 1).

Restriction digests of the *gry*B PCR amplicons indicated differential banding patterns consistent with predicted digestion patterns given by ChromasPro. Digestion of the *gyrB* PCR amplicons (408 bp) with the restriction enzyme BsrD1 showed a single cut (at bp 174) resulting in two bands for grape strain DNA. Ragweed DNA had no cuts for BsrD1 and one band for ragweed strains (data not shown). The enzyme Taq1 showed the opposite reaction. Taq1 cleaved grape strain *gyrB* amplicons, but did digest ragweed PCR products (Table 1).

QRT-PCR of the internal *gyr*B gene of these isolates showed Tm differences between grape and ragweed strains that were statistically distinct with grape strain DNA melting approximately 0.4-0.5^{o}C lower than DNA from ragweed strains ($P<0.0001$). Tukey's mean separation test showed strains could be placed into grape or ragweed categories based on Tm. Five strains showed variability in at least one to two runs such that they could not be placed statistically into one category (Table 1 on next page).

CONCLUSIONS

This initial investigation into the genetic variability of *Xf* strains in Texas indicates that there are two main strains in and near central Texas vineyards, subsp. *piercei* and subsp. *multiplex*. The strain isolated from grapevines in Texas is genetically identical to the Temecula PD strain in California. The grape strain was isolated from multiple European *V. vinifera* varieties, from hybrid *Vitis* species and rarely from wild *Vitis* species. Our preliminary data suggests that native *Vitis* species around the vineyard can harbor the grape strain and therefore are potential reservoirs for novel PD infections. With our current selection of plants in and around vineyards it is not possible to determine if the *Xf* isolated from wild and hybrid grapevines originated from infected vineyards or if the *Xf* isolated now in vineyards came from nearby wild or hybrid grapevines. We plan to analyze wild vines many kilometers from established vineyards for the presence and identity of *Xf* strains to help answer this question.

The ragweed strain (subsp. *multiplex*) is present in numerous weed species surrounding Texas vineyards. This strain is common in a large diversity of plants from annuals such as giant ragweed, annual sunflower, and redspike Mexican hat to perennial shrubs (sea myrtle) and woody trees (cedar elm). Interestingly, the ragweed strain was also found in peppervine (*Ampelopsis cordata*) a member of the Vitaceace, but not in the genus *Vitis*. Future work is planned to isolate and characterize Texas isolates from symptomatic oleander and stone fruit trees. Previous work using a multigenic approach indicate that *Xf* clades split 15,000 years ago and despite low variability within all subspecies, the subsp. *multiplex* shows the greatest variability, presumably because of a large host range (Schuenzel et al. 2005). Likewise, the California PD strain (subsp. *piercei*) has the lowest genetic variability and is speculated to have the narrowest host specificity (Schuenzel et al. 2005).

RFLP digestion of PCR amplicons was useful for distinguishing an isolate as either grape or ragweed. RFLP requires standard PCR and a 5-hour digestion, faster than the cloning methods used for sequencing. Even faster still was the comparison of the Tm between grape and ragweed strains after QRT PCR reactions (Bextine, data not shown). The QRT

PCR process allows for Tm to be measured for 96 samples in 1 hour allowing for a quick initial screen of multiple strains. QRT PCR occasionally resulted in samples not falling into either grape or ragweed categories with statistical certainty but was very useful for an initial screen of clade identity. QRT PCR requires lower DNA concentrations so strains could be identified directly from insect gut or sap. Although clearly advantageous for its speed we suggest QRT PCR be combined with RFLP for increased accuracy and that both techniques include controls of strains with known sequence identity.

Table 1. List of *Xf* isolates and strain designation as G = 'grape' (subsp. *piercei*) or R = 'ragweed' (subsp. *multiplex*) based on gyrB and mopB sequences, RFLP and QRT PCR analysis.

Isolate #	Host plant	Scientific name, host plant	Texas county	*gyrB* **	*mopB* **	RFLP	QRT PCR
VAL VAL 048	Mustang grape	*Vitis mustangensis*	Val Verde	G EU026151	G EU019700	G	G*
LLA FAL 634	SO4 rootstock	*Vitis cinerea var. helleri* x *Vitis riparia*	Llano	G EU026150	G EU019703	G	G*
BLA TEX 001	Cabernet Sauvignon	*Vitis vinifera*	Blanco	G EU026149	G EU19705	G	G
TRA FLA 300	Torringa Nacional	*Vitis vinifera*	Travis	G EU026148	G EU019702	G	G
VAL VAL 036	Black Spanish	*Vitis aestivalis* hybrid	Val Verde	G EU026147	G EU019701	G	G
GIL BEC 631	Wine grape	*Vitis vinifera*	Gillespie	G EU026146	G EU019704	G	G
HAR UHD 001	Sea myrtle	*Baccharis halimifolia*	Harris	R EU026138	R EU019694	R	R
TRA FLA 420	Redspike Mexican hat	*Ratibida columnifera*	Travis	R EU026139	R	R	R
LLA FAL 749	Western ragweed	*Ambrosia psilostachya*	Llano	R EU026142	R EU019696	R	R
LLA FAL 752	Giant ragweed	*Ambrosia trifida* var. *texana*	Llano	R EU026140	R EU019695	R	R
GIL BEC 626B	Giant ragweed	*Ambrosia trifida* var. *texana*	Gillespie	R EU026141	R EU019699	R	R
LLA FAL 650	Annual sunflower	*Helianthus annuus*	Llano	R EU026143	R EU019698	R	R*
LLA FAL 651	Heartleaf ampelopsis	*Ampelopsis cordata*	Llano	R EU026144	R EU019697	R	R*
UVA TAM 241	Cedar elm	*Ulmus crassifolia*	Uvalde	R EU026145	R EU019693	R	R*

* indicates that on at least one of two runs the strain indicated could not be categorized statistically as grape or ragweed (ANOVA, $p < 0.05$)

** indicates NCBI accession number after strain designation

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

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ABSTRACT

Xylella fastidiosa (*Xf*) is a Gram-negative, endophytic bacterium, which is the causative agent of Pierce's disease of grapevine (PD). Current approaches to understanding the progression of PD are limited by the lack of genetic and molecular tools that can be used to perform complementation analysis, an important step for establishing the importance of a particular gene in *Xf* virulence. To overcome this problem, we have developed integration vectors, which will allow researchers to introduce genes into two different nonessential regions of the *Xf* chromosome. The first set of vectors, the pAX1 series, contains an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between two divergently transcribed genes PD0702 and PD0703. Each vector in pAX1 series carries a different antibiotic resistance marker: pAX1Cm (chloramphenicol), pAX1Km (kanamycin), and pAX1Gm (gentamicin). We have introduced all three vectors into *Xf* and established that the antibiotic-containing cassette is integrated into the desired location in the *Xf* chromosome. We have also shown that the insertion of the antibiotic cassette between PD0702 and PD0703 does not alter the growth properties of *Xf in vitro*. Finally, we have cloned a wild-type copy of the *Xf* catalase gene into the MCS of pAX1Cm and introduced the resulting construct into a catalase-defective *Xf* mutant. The complemented strain showed a similar level of resistance to hydrogen peroxide as the wild type strain establishing the usefulness of our insertion vector for complementation analysis in *Xf*. Recently, we have constructed an additional set of vectors, the pAX2 series, which results in the insertion of the antibiotic resistant cassettes into the intergenic region between PD1160 and PD1161. Experiments are currently underway to test the usefulness of the pAX2 vectors for complementation analysis in *Xf*.

INTRODUCTION

The causative agent of Pierce's disease (PD) is the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). *Xf* is highly specialized and is capable of multiplying in both the foregut of xylem-feeding insects, such as the glassy-winged sharpshooter (GWSS) and in the xylem system of susceptible host plants (Hopkins and Purcell 2002). 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. As with many bacterial pathogens, the construction of directed gene deletion mutants has been crucial for identifying possible roles for specific *Xf* genes in the development of PD. 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 the phenotypes of these mutants is that the disrupted gene is required for the normal development of PD, it is also possible that the gene disruption is affecting the expression of neighboring genes through its impact on operon structure or 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 for conclusively establishing that a specific gene is responsible for a specific physiological and virulence function is to perform complementation analysis. Thus, 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. Here, we report our construction of two series of integration vectors (the pAX1 series and pAX2 series). These vectors allow the insertion of a gene of interest and an antibiotic resistance cassette into specific nonessential regions of the *Xf* chromosome. Complementation using these vectors will provide researchers with a clearer interpretation of the precise role of specific genes in the PD infection cycle by eliminating issues concerning plasmid copy number, polar effects on operons, secondary mutations, and plasmid stability.

OBJECTIVES

Specific Objective 1: Develop a stable plasmid vector for *Xf*

1A. Evaluate the potential of various plasmid addiction systems for ability to convert plasmids known to replicate in *Xf* into stable vectors.

1B. 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.

Specific Objective 2: Evaluate the stability of the newly development plasmid vectors when propagate in *Xf en planta*.

RESULTS

To better understand how *Xf* survives in and interacts with its hosts, many research laboratories have been working to identify genes important for virulence and nutrient acquisition. However, rapid progress in this area is affected by the lack of genetic and molecular tools necessary to investigate the contribution of *Xf* genes to the infection process. One extremely important tool that is needed to advance these studies is molecular tools that will allow genes of interest to be stably maintained by *Xf* throughout the infectious cycle. The primary goal of this project was to develop this type of molecular tool. Our early studies focused on examining the impact of various stability elements on the maintenance of autonomously replicating plasmids in *Xf* in the absence of antibiotic selection. We found that addition of the plasmid addiction module *hok/sok* resulted in a slight increase in plasmid stably *in vitro*. However, plasmids containing *hok/sok* were eventually lost from *Xf* in the absence of selective pressure, eliminating the usefulness of these plasmids for studies *en planta*. During the period under review, we have focused on developing a chromosomal based complementation system, which relies on vectors that allow the integration of genes into nonessential regions of the *Xf* chromosome. A major advantage of integration vectors is that the introduced genes are stably maintained as part of the bacterial chromosome in the absence of antibiotic selection. Here we report our progress on the development of these vectors.

Development of integration vectors for complementation analysis

In many Gram-negative bacteria, complementation analysis is performed using plasmid vectors that are capable of autonomous replication in *E. coli*, but are unable to replicate in the host bacteria. These integration vectors normally contain a multiple cloning site for inserting the gene of interest and an antibiotic cassette that are flanked by DNA sequences from a nonessential region of the bacterial chromosome. The antibiotic resistance cassette is included on these vectors to facilitate the identification of strains containing the integrated DNA fragment. 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. 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.

The first set of integration vectors we developed is the pAX1 series. The pAX1 plasmids contain an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between two divergently transcribed genes PD0702 and PD0703. Based on the genomic sequence of *Xf-PD*, these genes are divergently transcribed and both genes are predicted to contain multiple frameshift mutations (Van Sluys *et al*. 2003). Although they target the same region of the *Xf* chromosome, each vector in the pAX1 series carries a different antibiotic resistance marker: pAX1Cm (chloramphenicol), pAX1Km (kanamycin), and pAX1Gm (gentamicin). These vectors are presented in Figure 1.

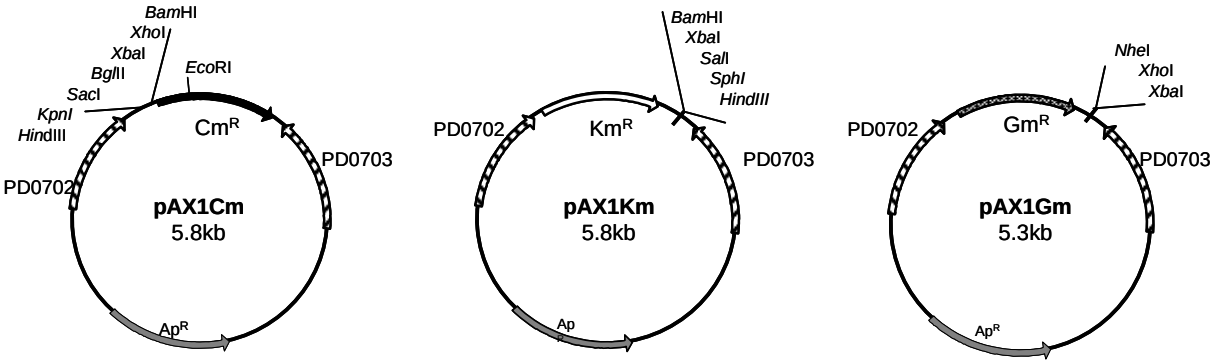

Figure 1. Restriction maps of pAX1 series of integration vectors

We have introduced all three pAX1 vectors into *Xf* and selected for antibiotic resistant transformants. Because these vectors are unable to replicate in *Xf*, the antibiotic resistant transformants must have arisen as the result of recombination between the integration vector and the *Xf* chromosome. We were able to confirm that the recombination event had occurred at the appropriate chromosomal location by PCR. We then examined the impact of the insertion in the PD702/703 intergenic region on the growth phenotypes of the resulting three strains *in vitro*. The properties of the three strains are presented in Table 1. 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. These strains 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.

Table 1. The integration strains exhibit a wild type phenotype.

Strain	Integration vector	Resistance marker	*In vitro*
Wildtype Temecula	---	---	Slow growth, biofilm
TAM22	pAX1Cm	Cm	Slow growth, biofilm
TAM91	pAX1Km	Km	Slow growth, biofilm
TAM105	pAX1Gm	Gm	Slow growth, biofilm

We also examined the usefulness of the pAX1 vectors for complementation analysis in *Xf*. For this analysis, we first generated a null mutation by gene disruption in the *cpeB* gene, which encodes catalase. The resulting catalase-defective *Xf* mutant (Δ*cpeB*) exhibits greater sensitivity to hydrogen peroxide than the wild-type Temecula strain. We then cloned a wild-type copy of the *Xf* catalase gene into the MCS of pAX1Cm and introduced the resulting construct into the Δ*cpeB* mutant. As shown in Figure 2, the complemented strain showed a similar level of resistance to hydrogen peroxide as a wild type strain establishing the usefulness of our insertion vectors for complementation analysis in *Xf*.

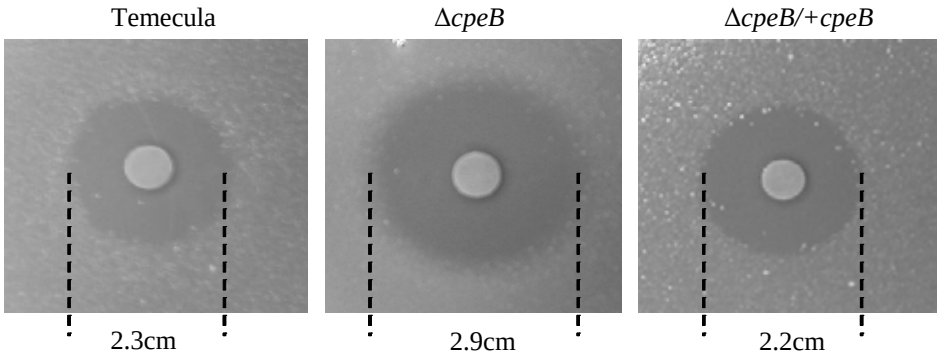

Figure 2. Complementation analysis of a catalase null mutation
P-values: Temecula vs. Δ*cpeB* ($P<0.01$); Temecula vs. Δ*cpeB*/+*cpeB* ($P>0.3$)
ΔcpeB/+*cpeB* vs. Δ*cpeB* ($P<0.04$)

To facilitate complementation analysis in strains containing double mutations, we are also developing a new series of integration vectors that target a different region in the *Xf* chromosome. These plasmids (the pAX2 series) contain an antibiotic resistant gene and a multiple cloning site (MCS) flanked by sequences homologous to the intergenic region between PD1160 and PD1161. Based on the genomic sequence of *Xf-PD*, both of these genes are predicted to contain frameshift mutations (Van Sluys *et al*. 2003). Like the pAX1 series, each pAX2 vector carries a different antibiotic resistance marker: pAX2Cm (chloramphenicol), pAX2Km (kanamycin), and pAX2Gm (gentamicin). Experiments are currently underway to examine the *in vitro* properties of strains carrying an insertion in the PD1160/1161 intergenic region and to test the usefulness of this set of vectors for complementation analysis in *Xf*.

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THE IRON STIMULON OF *XYLELLA FASTIDIOSA* INCLUDES GENES FOR TYPE-IV PILUS AND COLICIN V-LIKE BACTERIOCINS

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ABSTRACT

Xylella fastidiosa (Xf) is the etiologic agent of a wide range of plant diseases including citrus variegated chlorosis (CVC), a major threat to citrus industry in Brazil. The genomes of several strains of this phytopathogen were completely sequenced, enabling largescale functional studies. DNA microarrays representing 2608 (91.6%) coding sequences (CDS) of *Xf* CVC strain 9a5c were used to investigate transcript levels under growth in different iron availabilities. When treated with the iron chelator 2,2´-dipyridyl, 193 CDS were considered as up-regulated and 216 as down-regulated. Upon incubation with 100 µM of ferric pyrophosphate, 218 and 256 CDS were considered as up- and down-regulated, respectively. Differential expression for a subset of 44 CDS was further evaluated by RT-qPCR. Several CDS involved with regulatory functions, pathogenicity and cell structure, were modulated in both conditions assayed suggesting that major changes in cell architecture and metabolism occur when *Xf* cells are exposed to extreme variations in iron concentration. Interestingly, the modulated CDS include those related to colicin V-like bacteriocin synthesis and secretion and to pili/fimbriae functions. We also investigated the contribution of the ferric uptake regulator Fur to the iron stimulon of *Xf*. The promoter regions of strain 9a5c genome were screened for putative Fur boxes and candidates were analyzed by electrophoretic mobility shift assays. Taken together, our data support the hypothesis that Fur is not solely responsible for the modulation of the iron stimulon of *Xf* and present novel evidence for iron regulation of pathogenicity determinants.

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Section 4:

Pathogen and Disease Management

AN ANALYSIS OF C-DI-GMP SIGNALLING IN *XYLELLA FASTIDIOSA* VIRULENCE

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ABSTRACT

Pierce's disease (PD) poses a grave threat to many commercially important plants, including grapevine, and has placed the wine industries of Texas, California and other states at risk. Although *Xylella fastidiosa* (*Xf*) is recognized as the causal agent of the disease, the mechanism by which this xylem-limited, insect-transmitted bacterium induces sickness in plants remains almost completely unknown. Here, we present results from experiments that explore the role that cyclic diguanylate (c-di-GMP), a small regulatory molecule produced by many human and plant bacterial pathogens, plays in regulating *Xf* virulence. Specifically, we describe our efforts: (1) To generate *Xf* strains harboring deletions in genes encoding putative c-di-GMP signaling proteins; (2) To examine the effects of c-di-GMP on *Xf* gene expression; (3) To show that *Xf* biofilm formation *in vitro* is inhibited by c-di-GMP treatment. Taken together, these findings enhance our understanding of the molecular mechanisms mediating *Xf* virulence, and thereby, provide new insights into controlling PD.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative non-flagellated bacterium that causes a number of economically important plant diseases, including PD of grapevine, citrus variegated chlorosis, pear leaf scorch, and almond leaf scorch (Purcell and Hopkins 1996). Disease symptoms occur as a result of water stress and nutritional deficiencies caused by blockage of xylem vessels by bacterial biofilms. The bacteria are transmitted to plants by xylem-feeding insect vectors, such as glassy-winged and blue-green sharpshooters. To date, the molecular virulence mechanisms of *Xf*, as well as how it interacts with plant hosts, remain obscure. Our project focuses on exploring the role that the putative c-di-GMP signaling system in *Xf* genes plays in mediating these events.

Cyclic dinucleotide bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates cell-cell signaling, biofilm formation, motility, differentiation, and virulence (Tamayo et al 2007). High concentrations of c-di-GMP are associated with biofilm formation, exopolysaccharide production, attachment to surfaces, and attenuation of motility and virulence. c-di-GMP is produced from two molecules of GTP by diguanylate cyclase enzymes (DGCs). DGC activity resides in the GGDEF (Gly-Gly-Asp-Glu-Phe) domain of these proteins. c-di–GMP is degraded to GMP, via the linear pGpG by phosphodiesterases (PDEs). PDE activity has been show to reside proteins containing EAL (Glu-Ala-Leu) or HD-GYP (His-Asp, Gly-Tyr-Pro) domains. The interplay of DGC and PDE activities controls intracellular c-di-GMP concentration and hence c-di-GMP signaling. GGDEF, EAL, and HD-GYP domain containing proteins have been described in several bacterial species, and have generated significant interest as targets for the possible control of bacterial pathogenesis. In addition, a PilZ domain, which is thought to possess c-di-GMP binding activity, has recently been described (Amikam and Galperin 2006, Ryjenkov et al. 2006). Upon c-di-GMP binding, PilZ containing proteins are believed to regulate downstream events, including biofilm formation, motility and virulence (Cotter1 and Stibitz 2007). We are exploring the possibility that proteins participating in the c-di-GMP signaling system may play a role in regulating *Xf* virulence, and thereby, enable a strategy for Pierce's disease control.

OBJECTIVES

1. To examine whether c-di-GMP suppresses *Xf* biofilm formation in *vitro*, and disease progression *in planta*.
2. To examine whether putative *Xf* c-di-GMP biosynthesis and catabolic genes regulate bacterial biofilm formation in culture and disease *in planta*.

RESULTS

Cyclic di-GMP signaling proteins in *Xf*. Several GGDEF, EAL and HD-GYP domain containing proteins, which are implicated in c-di-GMP signaling, were identified in the genome of *Xf* Temecula strain. BLAST analysis revealed six proteins containing GGDEF domains, EAL domains and/or HD-GYP domains. Of the six proteins, two contained only a single GGDEF domain, one contained only a single EAL domain, two contained both a GGDEF and EAL domain, and one contained a single HD-GYP domain (Figure 1). The BLAST analysis also revealed that the proteins with GGDEF, EAL and HD-GYP domains contained additional signaling domains, including REC, conserved transmembrane regions (e.g., DUF21, 7TMR-DISMED2 and 7TMR-DISM_7TM), and a response regulator containing CheY-like receiver domain (COG3437), which senses and responds to environmental cues. Multiple proteins with PilZ domains, which are candidate receptors for c-

di-GMP, have been identified in several bacteria (Amikam and Galperin 2006, Ryjenkov et al 2006). BLAST analysis revealed two proteins with PilZ domains (PD1497 and PD0726) in the *Xf* Temecula genome.

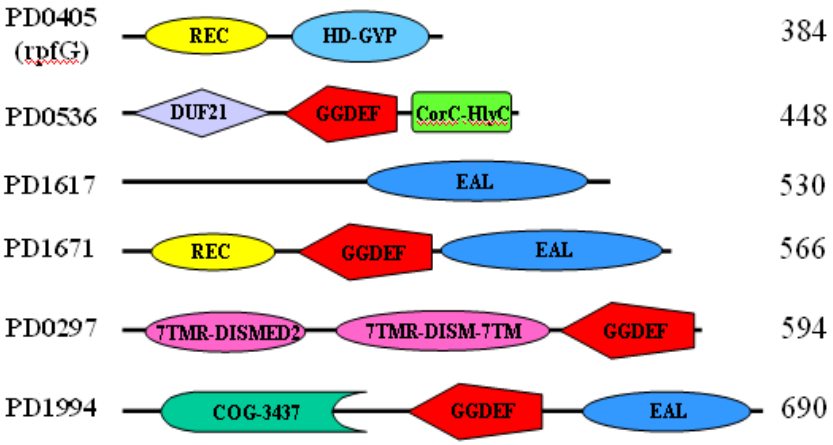

Figure 1. Architectures of GGDEF, EAL and HD-GYP domain proteins coded by the *Xylella fastidiosa* Temecula genome. The protein codes are shown to the left, and the number of amino acids in each protein number is shown to the right.

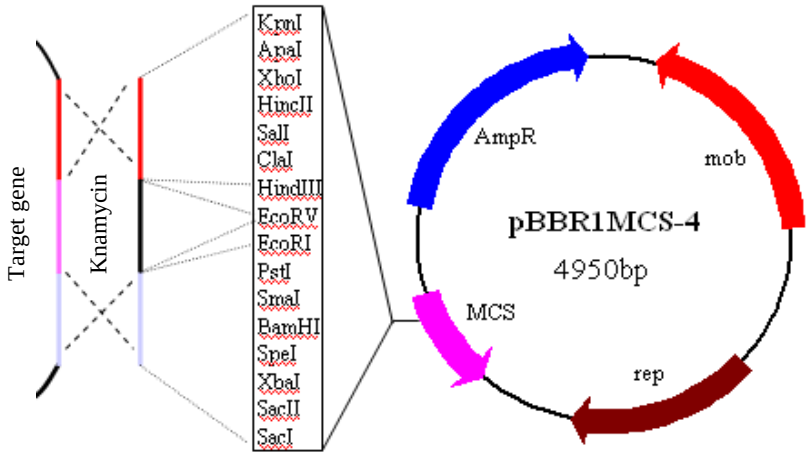

Figure 2. Construction of gene deletion vectors and schematic of double cross over strategy for the generation of gene deletions.

Generation of targeted gene deletion mutants of *Xf* Temecula. A replicative plasmid in *Xf*, pBBR1MCS-4, was used to construct several gene deletion vectors. The gene deletion vectors, p118LKR, p384LKR, p448LKR, p530LKR, p566LKR, p594LKR and p690LKR, have been constructed for deleting PD1497, PD0405, PD0536, PD1617, PD1671, PD0297 and PD1994 respectively. These vectors have been transformed into *Xf* (Figure 2). The transformation efficiency is about 30 transformants per microgram of plasmid DNA. The identification of knock-out mutants by PCR and Southern analysis is underway.

c-di-GMP suppression of biofilm formation in *vitro*. Bacterial biofilm formation is thought to play an important role in *Xf* pathogenesis and Pierce's Disease. To investigate the effect of c-di-GMP on biofilm formation, we treated bacterial cells with concentrations of c-di-GMP that have been shown to affect biofilm formation and virulence in other bacterial pathogens, including *Vibrio cholerae* and *Pseudomonas aeruginosa* (Tischler and Camilli 2005, Kulasakara et al 2006). We then quantified biofilm formation using the crystal violet method (Karaolis et al 2005). When cells were grown in PD3 medium for 10 days in the absence of c-di-GMP, significant amounts of biofilm formation was observed (our unpublished data). However, when cells were similarly grown in the presence of c-di-GMP, a significant decrease in biofilm formation was seen (our unpublished data). Our preliminary data therefore indicate that bacterial c-di-GMP synthesis and signaling may contribute to *Xf* biofilm formation.

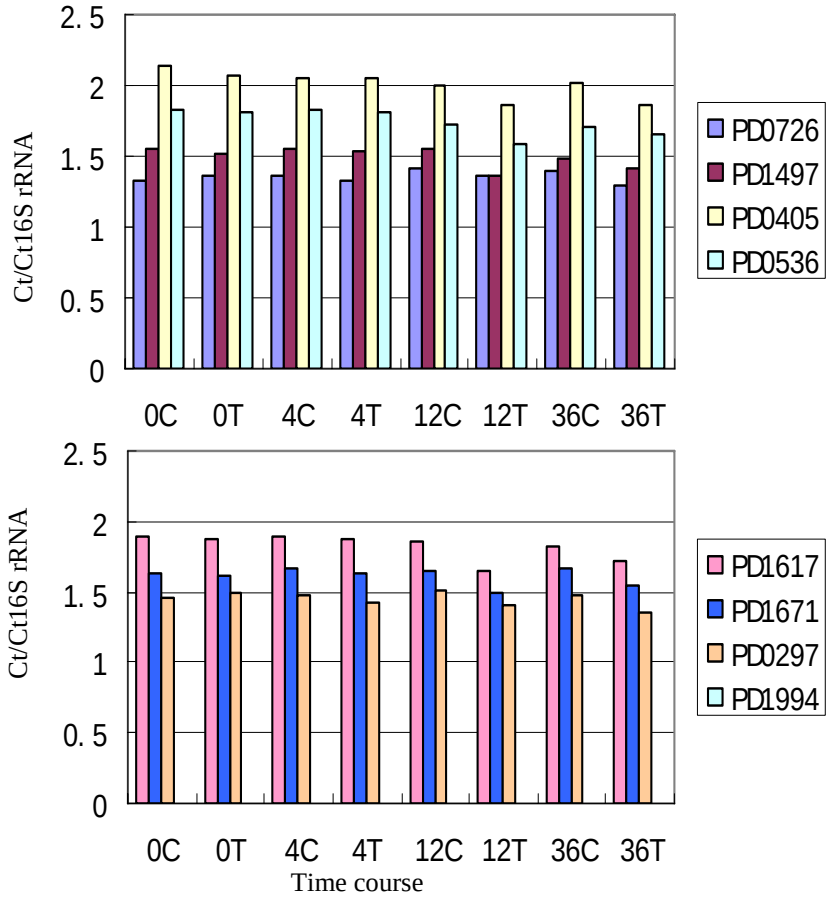

Figure 3. Expression analysis of the genes coding GGEDF, EAL, HD-GYP, PliZ domain proteins in response to c-di-GMP treatment.

Expression analysis of c-di-GMP pathway genes. To examine whether *Xf* gene expression changes in response to c-di-GMP treatment, we treated bacterial cells with c-di-GMP and then analyzed gene expression levels at different times post-treatment using real-time PCR methods. Eight genes (PD0726, PD1497, PD0405, PD0536, PD1617, PD1671, PD0297 and PD1994) that participate in the putative *Xf* c-di-GMP signaling pathway were analyzed, and revealed that the expression of these genes is not significantly altered by the tested concentrations of c-di-GMP (Figure 3).

CONCLUSIONS

We have initiated an effort to generate targeted deletions in genes contained in the putative *Xf* c-di-GMP pathway. In addition, we have shown that c-di-GMP addition to *Xf* liquid cultures inhibits bacterial biofilm formation. Finally, we have demonstrated that c-di-GMP treatment of *Xf* cultures does not alter the expression of genes contained in the c-di-GMP pathway. Taken together, these experiments provide a solid foundation for research into the role that the *Xf* c-di-GMP signaling system plays in mediating Pierce's disease.

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

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Reporting Period: The results reported here are from work conducted October 1, 2006 to October 8, 2007.

ABSTRACT
The aim of this project is to construct and express 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 (PD) of grapevine. As reported previously, we were able to select several *Xf*-cell-surface-binding peptides, and some of these showed marginal ability to interfere with *Xf* infection of grapevine. Although this result is encouraging, in this period we have aimed at discovering proteins, rather than peptides, that can bind to the *Xf* cell surface, in order to achieve greater affinity and more potent interference with *Xf* infectivity. Other observations revealed that an activity of *Xf* cells which results in the development of chlorosis in leaves of *Chenopodium quinoa* is due to the translation elongation factor EF-Tu, which in *Xf* is found in an unusual aggregated or conjugated form unlike the soluble form that EF-Tu takes in most other bacteria.

INTRODUCTION
It is likely that the development of grapevine cultivars resistant to *Xf* presents the best approach to long term, effective, economical and sustainable control of 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 is expected to provide protection against insect vector-delivered *Xf*. An effective anti-*Xf* protein may kill *Xf* cells or merely interfere with their ability to colonize or spread in the scion xylem. 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. We reported (Bruening et al. 2006) the selection of *Xf* cell-surface-binding peptides and showed that some selected peptides were able, marginally, to interfere with *Xf* infection when mixed with *Xf* cells prior to inoculation of grapevine. Stronger interaction, as likely can be provided by an *Xf* cell-surface-binding protein or protein-domain, should prove to be more potent in bioassays. We reported that amino acid sequences of motifs found in selected *Xf* cell-surface-binding peptides were consistent with interactions between *Xf* cell-surface proteins. Therefore, a strategy for identifying *Xf*-cell-surface-binding protein domains is to search a library of *Xf* cell-surface-protein domains. Given the power of panning procedures using bacteriophage M13 libraries and the small size of the *Xf* genome, there is no need to limit a library to protein domains from *Xf* cell-surface proteins; libraries from random fragments of total *Xf* DNA should be suitable. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. In the second subsection of Results we extend earlier research on the reaction of plants to invasion by *Xf* to study potential virulence factors derived from *Xf*. Virulence factors are potential targets for interfering with *Xf* infection.

OBJECTIVES
1. Discover or develop peptides and proteins with high affinity for 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 grapevine 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 against *Xf* cells in culture and in plants.
4. In collaboration with the Dandekar laboratory, prepare transgenic tobacco and grapevine expressing and xylem-targeting the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*

RESULTS
Selection of protein domains with high affinity for the *Xf* cell surface
Figure 1 describes the system we have developed for displaying domains of *Xf* proteins in the P3 protein of bacteriophage M13. In bacteriophage M13 panning procedures, a population (library) of M13 virions is created such that small sets of individual virions have the same displayed peptide, protein domain or protein, but there are many (10^4 to 10^9) such sets, each with a different display encoded in the bacteriophage M13 genome of the set. The entire population is exposed to the target,

in this case *Xf* cells. After extensive washing, M13 virions are released by exposure of the cells to pH 2.2 buffer. The released M13 virions are increased by infection of *E. coli* cells, and the panning cycle is repeated. To the extent that the original M13 population can be limited to members whose infectivity is dependent on inserts, the process of selection becomes more efficient and the chances of success are greatly enhanced. In the scheme outlined in Figure 1, two steps were designed to prepare M13 library members with the desired characteristics. A version of the gene for the P3 protein was prepared with a frameshift mutation. Insertion of a *Xf* DNA fragment capable of restoring the reading frame register is necessary to create a fusion protein with a full length P3 sequence. No insert or any insert that does not restore the reading frame register should result in a non-functional P3. The P3 protein is mobilized into complete bacteriophage M13 virions by inoculation of *E. coli* cells bearing the P3-encoding plasmid with the helper bacteriophage M3 described in Figure 1. Treatment of the resulting M13 virions with 27K protease will remove the N and M domains of the P3 protein derived from the helper bacteriophage, leaving the virions dependent on the P3 fusion protein derived from the plasmid. Tests show reductions in titer to 10^{-4} of the control value for 27KPro treatment of bacteriophage M13 with two cleavage sites as described in Figure 1.

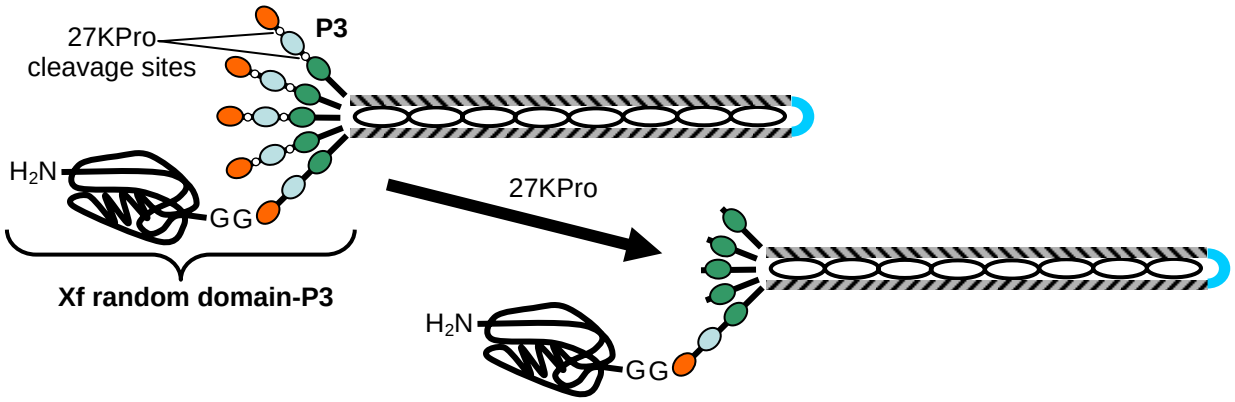

Figure 1. A solution to the problem of background infectivity during panning experiments with protein domains substituted into bacteriophage M13 protein P3. Random fragments of *Xf* DNA were inserted into a site in an out-of-reading-frame register (not infectious) version of bacteriophage M13 protein P3. About 1 in 18 such inserts can be expected to restore the reading frame with sequence of the sense polarity, and a fraction of these will contain *Xf* protein domain-encoding sequences possibly compatible with M13 infectivity. There are five P3 proteins per wildtype bacteriophage particle. To avoid steric and avidity effects from multiple copies of the *Xf* protein domain, ideally there would be only a single copy of that domain per M13 particle, and infectivity of the particle would be dependent on the presence of that P3 protein molecule. Conditions can be arranged so that most of the P3 molecules of a bacteriophage M13-infected cell are provided by a helper bacteriophage M13. The helper bacteriophage M13 was engineered to insert, into the P3 gene, sequences encoding two cleavage sites for the *Tobacco etch virus* 27K protease (27KPro), one site between the N domain (orange) and the M domain (blue), and one between the M domain and the C domain (green). Infectivity of M13 requires at least one P3 protein with intact N and M domains. A library of *E. coli* cells bearing a plasmid with *Xf* DNA fragment inserts is inoculated with the engineered helper bacteriophage M13. The progeny bacteriophage virions are treated with 27KPro to inactivate virtually all of the P3 molecules that do not bear an insert derived from *Xf* DNA.

Potential virulence factor(s) of *Xf*

Based on electrophoretic mobility, MopB, which likely is the major outer membrane protein of *Xf*, was tentatively identified as a chlorosis-inducing factor in pressure-infiltrated *Chenopodium quinoa* leaves. A procedure for partial purification of MopB was developed. When the MopB preparations were analyzed on a 10% or 11% polyacrylamide gel, rather than the more usual 12.5% gel, a faint band was observed trailing the main MopB band and was found to contain the *Xf* protein-synthesis-elongation-factor designated “temperature unstable” (EF-Tu). Material from this trailing band induced chlorosis in *C. quinoa*, and *Xf* MopB produced in transformed *E. coli* failed to induce chlorosis in *C. quinoa*. These observations suggest that the chlorosis-inducing factor in our MopB preparations may be *Xf* EF-Tu and not MopB. EF-Tu is one of a small number of highly conserved eubacterial proteins (“pathogen-associated molecular pattern,” PAMP) that have been discovered to induce defense responses in a variety of plants (Jones and Dangl 2006). Subnanomolar *E. coli* EF-Tu was found to induce alkalization in the medium of cultured *Arabidopsis thaliana* cells and to induce at 1 µM in pressure-infiltrated Arabidopsis leaves resistance to *Pseudomonas syringae* and the accumulation of defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu has been demonstrated to be amino-end-N-acetylated, which is unusual for a prokaryotic protein. (Kunze et al. 2004) also demonstrated that peptides corresponding to the first 18-26 amino acid residues of *E. coli* EF-Tu had the biological activities of the intact protein. Subnanomolar concentrations of EF-Tu amino end peptides from four plant pathogenic bacteria all induced alkalinization of Arabidopsis cell medium, whereas the corresponding peptides from *P. syringae* and *Xf* required a ~100X greater concentration. The recognition of EF-Tu was specific for brassicas among the plants tested (Kunze et al.

2004). The gene for the Arabidopsis EF-Tu receptor, ERF, when expressed in *N. benthamiana* leaves, conferred the ability to bind the EF-Tu amino-end epitope (Zipfel et al. 2006). Arabidopsis *erf* mutants showed enhanced disease susceptibility.

E. coli EF-Tu and *Xf* EF-Tu are 77% identical and 88% similar in amino acid sequence. The regions of identity between the *E. coli* and *Xf* EF-Tu sequences also showed >90% identity with >100 eubacterial sequences (Kunze et al. 2004). We found purified *E. coli* EF-Tu to be a strong inducer of chlorosis in pressure-infiltrated *C. quinoa* leaves (Figure 2, A-C). However, when we attempted to purify EF-Tu from *Xf*, we found that the bulk of the *Xf* EF-Tu and the bulk of the *E. coli* EF-Tu have different states of association. Procedures for purifying *E. coli* EF-Tu are not applicable to *Xf* EF-Tu. When *E. coli* cell suspension or crude extract of *E. coli* cells was heated in Laemmli SDS/mercaptoethanol disruption solution, as for SDS gel electrophoresis, antibody against *E. coli* EF-Tu detected a band with a mobility corresponding to the expected molecular weight of 43K (Figure 3, lanes 3', 4 and 5). Contrary to our expectation, we did not observe a band at 43K in immunoblots to which extract of *Xf* cells had been applied (Figure 3, lanes 1' and 2'). Rather, we found immunoreactive material in the gel well that adhered so poorly that streaks sometimes were observed emanating from the well. Weakly alkaline 10 M urea-SDS-DTT was more effective than Laemmli disruption solution in extracting the anti-EF-Tu immuno-positive material; prior treatment of the *Xf* cells with lysozyme did not increase the amount or mobility of immuno-positive material (data not shown).

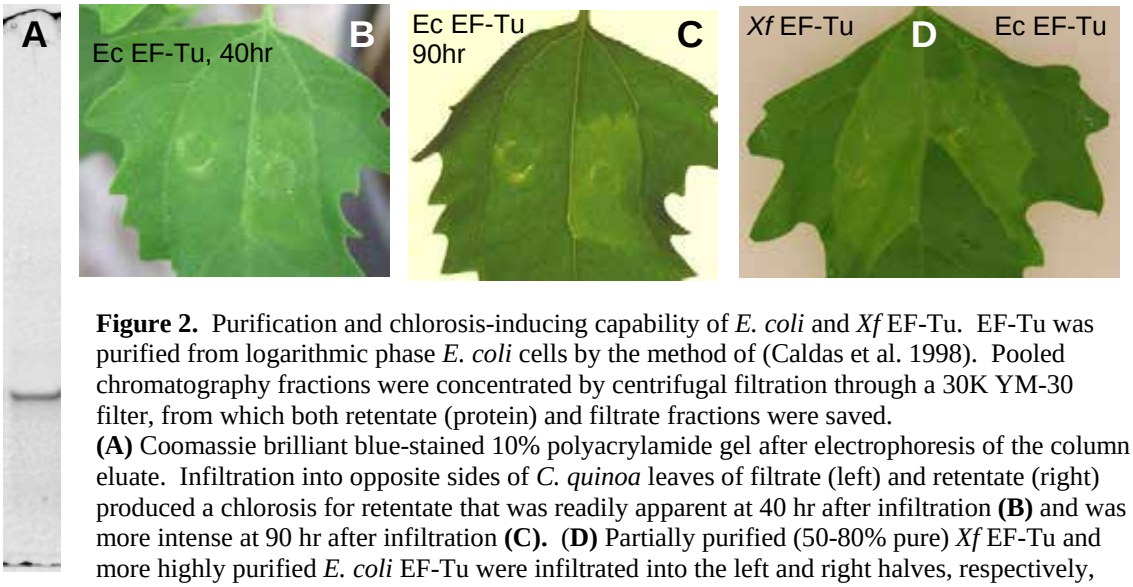

Figure 2. Purification and chlorosis-inducing capability of *E. coli* and *Xf* EF-Tu. EF-Tu was purified from logarithmic phase *E. coli* cells by the method of (Caldas et al. 1998). Pooled chromatography fractions were concentrated by centrifugal filtration through a 30K YM-30 filter, from which both retentate (protein) and filtrate fractions were saved.
(A) Coomassie brilliant blue-stained 10% polyacrylamide gel after electrophoresis of the column eluate. Infiltration into opposite sides of *C. quinoa* leaves of filtrate (left) and retentate (right) produced a chlorosis for retentate that was readily apparent at 40 hr after infiltration **(B)** and was more intense at 90 hr after infiltration **(C).** (**D)** Partially purified (50-80% pure) *Xf* EF-Tu and more highly purified *E. coli* EF-Tu were infiltrated into the left and right halves, respectively, inducing a similar chlorosis. The infiltrated protein corresponded to about 20 pmole of EF-Tu.

Centrifugation at 90,000 rpm for 90 min distributed the immuno-positive material between the supernatant and a clear, gelatinous precipitate (compare lanes 1' and 2', Figure 3). The fact that part of the immuno-positive material sedimented suggests that buoyant density is not impairing sedimentation. Therefore, *Xf* EF-Tu may be part of a very asymmetric structure of aggregated EF-Tu molecules or EF-Tu molecules conjugated to other molecules. These results suggest that a fundamental difference exists between the *Xf* and *E. coli* EF-Tu molecules. We postulate that *Xf* EF-Tu may occur predominantly as an aggregate that is exposed on the cell surface, where it can be detected by a plant equipped with the appropriate receptor(s). *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesion that is responsible for the binding of these bacteria to human cells, and, in the case of *M. pneumoniae*, antibody to EF-Tu was demonstrated to interfere with attachment to human cells (Dallo et al. 2002, Granato et al. 2004). *Xf* EF-Tu may have a similar function. As a model for EF-Tu on the surface of an *Xf* cell, we prepared a construction designed to place *Xf* EF-Tu on the surface of *E. coli* cells. The plasmid construction encodes an EF-Tu-P3 (Figure 1). In the absence of other bacteriophage M13 components, P3 expressed alone accumulates in outer membrane. In *E. coli* cells transformed to express P3 (calculated molecular weight 44.6K), a band of the appropriate mobility was observed after gel electrophoresis, immunoblotting and incubation with monoclonal antibody to P3, whereas no band was detected in the gel for the EF-Tu-P3 (calculated molecular weight 87.5K) fusion construction (data not shown). *Xf* EF-Tu-P3 fusion behaved like *Xf* EF-Tu from *Xf* cells (Figure 3, compare lane 5 with lane 4), whereas *E. coli* EF-Tu showed its expected mobility in the gel (Figure 3, lanes 3', 4 and 5). Partially purified *Xf* EF-Tu and nearly pure *E. coli* EF-Tu induced a similar chlorosis in *C. quinoa* (Figure 2, D).

CONCLUSIONS

Based on results with *Xf*-cell-binding peptides, selection of *Xf*-cell-binding proteins likely will provide reagents capable directly, or as conjugates with other protein motifs, of interfering with *Xf* replication. These proteins are to be expressed in grapevine rootstock for transport into the xylem for targeting of *Xf* cells. The bulk of the protein EF-Tu of *Xf* accumulates in

an aggregated or conjugated form that presumably is incompatible with EF-Tu function in protein synthesis. A few eubacteria enlist EF-Tu for cell-surface adhesion, and some plant species recognize the presence of bacteria by detecting EF-Tu. *C. quinoa* apparently recognizes EF-Tu from *Xf* and *E. coli*. Therefore, it is reasonable to suggest that the unusual form of EF-Tu in *Xf* may mediate pathogenesis or symptom induction in grapevine.

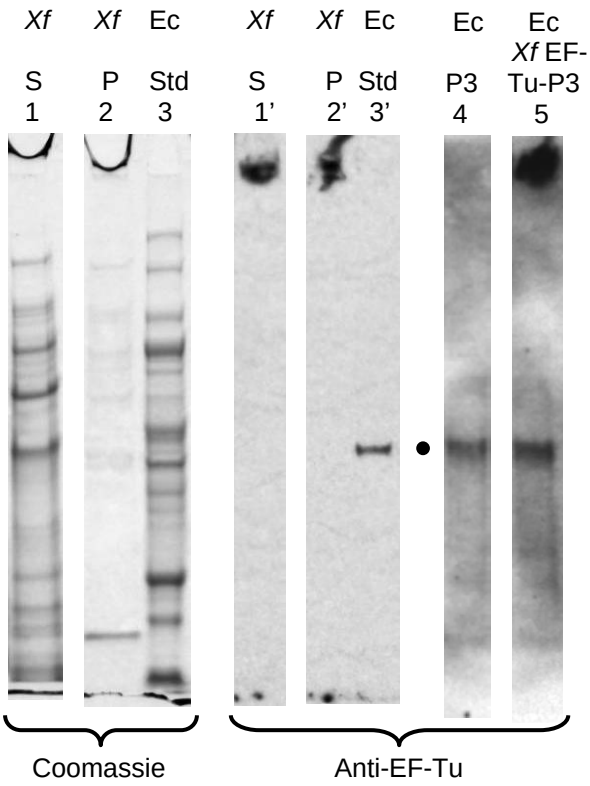

Figure 3. *Xf* EF-Tu and of *E. coli* EF-Tu show distinct properties. Lanes 1-3 are from a Coomassie brilliant blue-stained gel; immunoblots (lanes 1'-3', 4 and 5) were visualized using goat anti-EF-Tu (*E. coli*) IgG (gift from Dr. David Miller, Institute for Basic Research in Developmental Disabilities, Staten. Island, NY), rabbit anti-goat IgG coupled to horse radish peroxidase, and "supersignal" (Pierce) substrate. Mutant HxfB-*Xf* cells grown in liquid culture were extracted with 10 M urea, 0.1 M dithiothreitol (DTT), 6 mg/mL SDS, 96 mM Tris-HCl buffer, pH~8.7, at 90-95˚C for 4 min. Lanes 1 and 1' received an aliquot of the supernatant, and lanes 2 and 2' the precipitate, from a 90,000 rpm, 90 min centrifugation of the extract. Lanes 3 and 3' received colored protein standards mixed with a 1 hr, 100,000 x g supernatant of lysozyme-treated *E. coli* cells (Cull and McHenry 1990, Caldas et al. 1998). Samples for lanes 4 and 5 were *E. coli* cells collected by centrifugation. The *E. coli* strains for lanes 4 and 5, respectively, were transformed to express bacteriophage M13 protein P3 and a *Xf* EF-Tu-P3 fusion. All samples were heated in Laemmli SDS/mercapto-ethanol disruption buffer before electrophoresis through 10% (lanes 1-3; 1'-3', gels run simultaneously) or 12.5% (lanes 4-5, same gel) polyacrylamide gel. The dot between lanes 3' and 4 locates the expected position for EF-Tu. *Xf* EF-Tu from wildtype *Xf* cells grown on solid medium gave results similar to those for HxfB- cells from liquid culture (data not shown).

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

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Reporting Period: The results reported here are from work conducted October 1, 2006 to September 30, 2007.

ABSTRACT

Chardonnay is a *Vitis vinifera* scion variety that is susceptible to Pierce's disease (PD). We are evaluating the effect of rootstock variety on PD symptom expression in Chardonnay grown in an experimental vineyard at Weslaco, Texas with high natural PD pressure and abundant vectors, including glassy-winged sharpshooter. The rootstocks under evaluation are Dog Ridge, Florilush, Tampa, Lenoir, and Freedom. Natural *Xylella fastidiosa* (*Xf*) infection 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 *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 PD pressure (Loomis 1952, 1965, Magoon and Magness 1937). If grape rootstocks could contribute PD resistance or tolerance to their scions, this would be a major benefit to viticulture in PD prone areas. Elite wine, juice, and table grape varieties could be grown in areas where viticulture is currently restricted to PD resistant and tolerant varieties whose consumer appeal is low.

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

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

RESULTS AND CONCLUSIONS

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

Symptoms on leaves were assessed in August 2007 and vines given a numerical score from 0 to 5, with 0 representing no symptoms, 1 = minor symptoms up to 15% of leaves with marginal necrosis (MN), 2 = 15-30% of leaves with MN, 3 = 30-

50% of leaves with MN, 4 = 50-75% of leaves with MN, 5 = over 75% of leaves with MN or vine dead. All vines in the study (total vines = 47) showed MN symptoms. In general, symptom development was minor, with 46 of 47 vines in category MN = 1. Only one vine was assigned to category MN = 2, a vine grafted on the rootstock Tampa.

The rapid development of PD symptoms on the vines in this project following natural inoculation under vineyard conditions reflects the suitability of this location for PD management projects.

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

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Reporting Period: The results reported here are from work conducted July 2004 to July 2007.

ABSTRACT
Xylella fastidiosa (*Xf*) must survive multiple winters in an almond tree to reach sufficient populations for vector acquisition and economic impact in the orchard. The effects of cold temperatures on the establishment and persistence of *Xf* infections were measured in field and greenhouse-grown almond trees, with multiple *Xf* strains, and almond leaf scorch (ALS)-resistant and susceptible tree varieties. Potted trees infected with *Xf* were over wintered outside, or in cold rooms at 1.7ºC or 7ºC, for one, two, or four months. Cold exposure time negatively influenced potted tree recovery from *Xf* infection, while cold intensity did not: 21% of trees recovered after one month, 13% after two months, and 7% after four months. In the field, trees at UC Davis (UCD) and Intermountain Research and Extension Center in Tulelake (IRC) inoculated with either grape or almond-strain *Xf* had similar initial infection rates and bacterial populations, but ALS symptoms were much more severe at UCD, especially in 'Peerless' trees. At UCD, 10% of trees with almond-strain, and 78% of trees inoculated with grape-strain *Xf* were infected. Both strains initially infected trees at equal rates at IRC (64% almond, 40% grape). Winter conditions killed all *Xf* infections at IRC and all but one at UCD. These results partially support the hypothesis that almond-strain *Xf* is common in northern California almond orchards because almond strains initially infect trees at low rates, but survive the winter more frequently than grape strains to cause persistent ALS. Grape and almond strains initially infected field-grown trees similar to previously-reported infection rates. However, no almond infections and only one grape *Xf* infection over wintered in the field, even when the winter was mild. Previous studies found that almost all almond strain infections survived the winter, and 88 to 42% of grape-strain infections over wintered in field grown trees. Field inoculations at UCD, and potted tree inoculations were repeated in 2007, to get more data on initial infection establishment and over wintering rates.

OBJECTIVES
1. Compare establishment and over wintering of grape and almond strain *Xf* in susceptible and resistant almond cultivars.
2. Determine the effects of cold treatment on the over winter survival of *Xf* in almonds.

RESULTS
Controlled temperature study
Five months after inoculation with ALS 6 *Xf*, 72% of inoculated trees (89 of 125) developed almond leaf scorch, averaging 12.8 (SE =1.25) symptomatic leaves per infected tree, and a median population of 4.4 x 10^6 CFU/g. None of the buffer-inoculated trees were infected with *Xf*, and they averaged 0.3 scorched or yellowed leaves per tree. Fourteen of 89 infected trees died during or after cold treatment, compared to 1 of 27 buffer-inoculated trees ($\chi 2$ with Yates' correction = 3.17; $P > 0.05$; df = 1). Six of 29 trees were negative for *Xf* after one month cold treatment, compared to 4 of 30 after two months, and 2 of 30 after four months **(Figure 1A)**. There were not significant differences in the number of recovered trees regardless of temperature or time ($\chi 2$ with pairwise comparisons).

ALS symptoms were twice as severe after cold treatment as before. In 2005, buffer-inoculated trees averaged 0.26 scorched or yellowed leaves per tree (SE = 0.25), compared to 12.8 symptomatic leaves per *Xf*-infected tree (SE = 1.23). In 2006, there were an average of 1.29 (SE = 0.42) scorched and yellowed leaves per buffer inoculated tree and 30.24 (SE=2.57) symptomatic leaves per *Xf* infected tree. Symptoms were worse in trees following four months of cold treatment, compared to trees with one and two months **(Figure 1B)**.

The time of dormancy, but not the temperature, influenced symptom severity (2-way ANOVA, Standard Least Squares: Time $P = 0.0002$, df =2; Temperature $P = 0.593$, df =2; Temperature*Time $P = 0.39$, df = 4; root mean square error = 17.65). *Xf* populations in infected trees were similar across all treatments (2-way ANOVA, Time $P = 0.74$; Temperature $P = 0.19$; Time*Temperature Interaction $P = 0.39$) with a median population of 2.82 x 10^6 CFU/g. Trees in the 1.7ºC and 7ºC treatments were subjected to temperatures below the growth threshold of *Xf* for the entire period: 769 hours for one month, 1441 hours for two months, and 2905 hours for four months. Outside, trees were below 7ºC for 262 hours in the one month treatment, 557 hours in the two month treatment, and 673 hours for the four month treatments. Only trees kept outside were subjected to sub-freezing temperatures, for 10 hours, both in the two and four month treatments.

Field sites

In 2005, two to four months after inoculation, *Xf* was recovered from 49% of bacteria-inoculated trees (76 of 155), and from none of the buffer-inoculated trees. There was no difference in the proportion of infected trees at UCD or at IRC (36 of 76 at UCD; 40 of 79 at IRC; $\chi 2 = 0.17$; $P > 0.05$; df =1; Figure 4), nor in median *Xf* populations in infected trees (4.0×10^6 colony-forming units per gram of petiole tissue at UCD; 1.2×10^7 CFU/g at IRC; t-test with $\log_{10}$-transformed data; $P = 0.18$; df = 75). However, disease symptoms were more severe at UCD, especially in 'Peerless' trees, averaging 8.8 (SE = 2.1) symptomatic leaves per infected tree, compared to 2.3 leaves per infected 'Butte' tree (SE = 0.7; $P = 0.02$; two-sample t-test; df = 35). At IRC, symptoms were negligible, as both varieties averaged 0.3 scorched leaves per infected tree (SE = 0.1; $P = 0.76$; two-sample t-test; df = 39). Background yellowing and scorching in uninfected trees was 0.11 leaves per tree (SE = 0.08) at UCD and 0.21 (SE = 0.1) leaves per tree at IRC **(Figure 2A).**

Grape strain *Xf* infected trees more frequently than almond *Xf* at UCD but not at IRC **(Figure 2B)**. At UCD, 4 of 37 trees inoculated with Dixon or ALS-6 developed infections, compared to 32 of 39 trees with Fresno-ALS or Medeiros ($\chi 2$ with Yates' correction = 38.71; $P < 0.001$; df = 1). At IRC, both almond and grape strains of *Xf* infected trees with the same frequency (16 of 39 inoculated with almond; 24 of 40 inoculated with grape strain; $\chi 2 = 2.84$; $P > 0.05$; df = 1). Grape and almond strains reached similar titers in infected plants, median 3.48×10^6 CFU/g for almond strain *Xf*, and 5.71×10^6 CFU/g for grape strain, both sites combined. All *Xf* recovered from inoculated trees matched the type initially inoculated; there was no *Xf* movement between trees at either field site.

Over the winter, two trees died at UCD, and 62 trees or inoculated branches died at IRC. Surviving trees (previously infected in 2005) at IRC were evenly distributed among PD, ALS and buffer isolate treatments, with 6 buffer-inoculated, 3 ALS 6-inoculated, 4 Dixon-inoculated, 4 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 in extremely cold climates (Purcell 1980). No *Xf* was recovered from trees at IRC in 2006. At UCD, *Xf* was recovered from only one previously-infected tree, the Medieros isolate in a 'Peerless' tree. At both sites, there was negligible leaf scorch and chlorosis in uninfected trees. 'Butte' trees at IRC were beginning to senesce at the time of assessment. Trees at UCD were subjected to 1076 hours of temperatures below 7ºC between inoculation and rating in 2006 (1070 over winter), including 44 hours below 0ºC (an average of 2928 hours elapsed between inoculation and rating in 2005, and 12,223 hours in 2006). Trees at IRC received four times as much cold, 4659 hours over winter between inoculation in July 2005 and rating for disease in September 2006 (4600 over winter) of 10,343 total hours. Trees spent 1852 hours below 0ºC at IRC.

DISCUSSION

One hypothesis explaining the prevalence of almond-strain *Xf* in northern California almond orchards (Hendson 2001, Shapland 2006) is that almond strains initially infect trees at low rates, but survive the winter more frequently than grape strains to cause persistent disease (Almeida and Purcell 2003). Data from this study supported half this hypothesis, since almond strains initially infected 11 to15% of trees, similar to the 21 to 33% infection rate previously reported (Almeida and Purcell 2003). Grape *Xf* isolates infected 79% of inoculated trees at UCD in this study, and 64 and 75% in field trials in Davis and Parlier. In this study, no almond infections and only one grape strain *Xf* infections over wintered in field plots, although almond-strain *Xf* over wintered in potted plants. Almeida and Purcell (2003) found that almost all infections with almond strains survived the winter and 88-42% of grape-strain infections over wintered, with field grown trees in Parlier, CA, a similar climate to UCD.

Since only one infection survived the winter in the field plots, there is so far no data to support the hypothesis that *Xf* infections over winter more frequently in susceptible 'Peerless' compared to 'Butte' since all but one *Xf* infections died over winter. The one surviving infection was in susceptible 'Peerless'. Repeated inoculations at UCD in 2007 will provide more data to test this hypothesis. The effect of cold on *Xf* survival was previously investigated in grapes but not in almonds. Exposure to very cold over wintering temperatures can eliminate *Xf* infections but also results in significant plant mortality (Purcell 1980). Potted almond trees in the controlled study exposed to four-month dormancy had more symptomatic leaves than trees dormant for one or two months. This is in contrast to previous studies in grapes (Feil and Purcell 2001), where *Xf* populations decreased 320-fold in only 18 days at 5ºC (41ºF). A second replication of the cold-chamber experiment is ongoing to test the hypothesis that threshold temperatures to kill almond-strain *Xf* are lower than those needed to kill grape *Xf* in almond trees.

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FUNDING AGENCIES

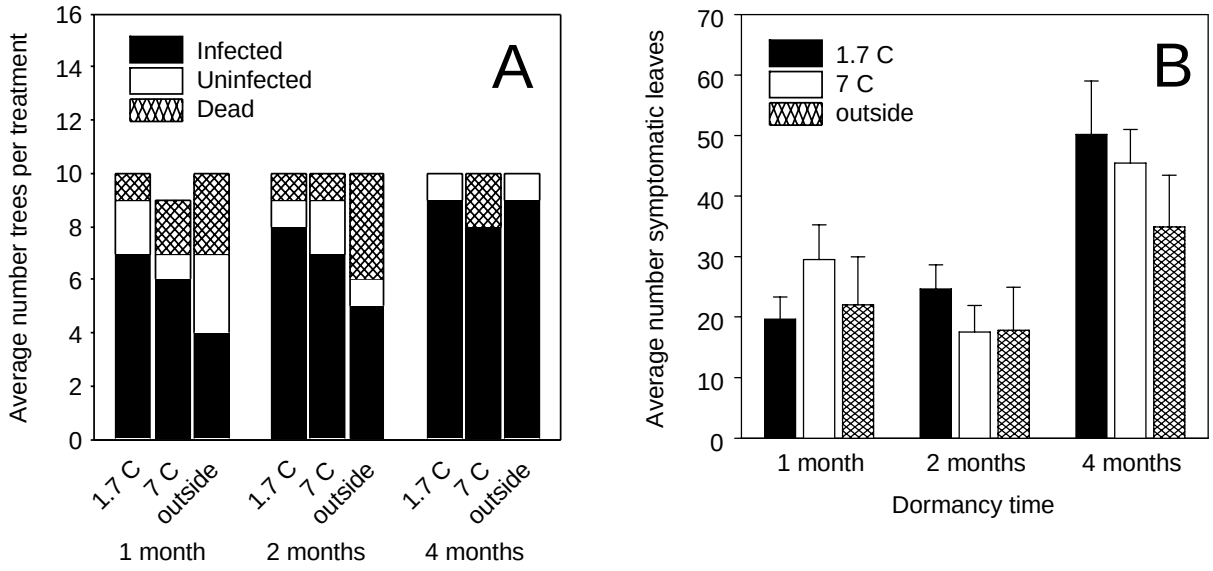

Figure 1. A) *Xf* infections and **B)** Almond Leaf Scorch symptoms in previously-infected potted almond trees after one, two or four months of dormancy at 1.7ºC, 7ºC, or outside in Parlier, CA.

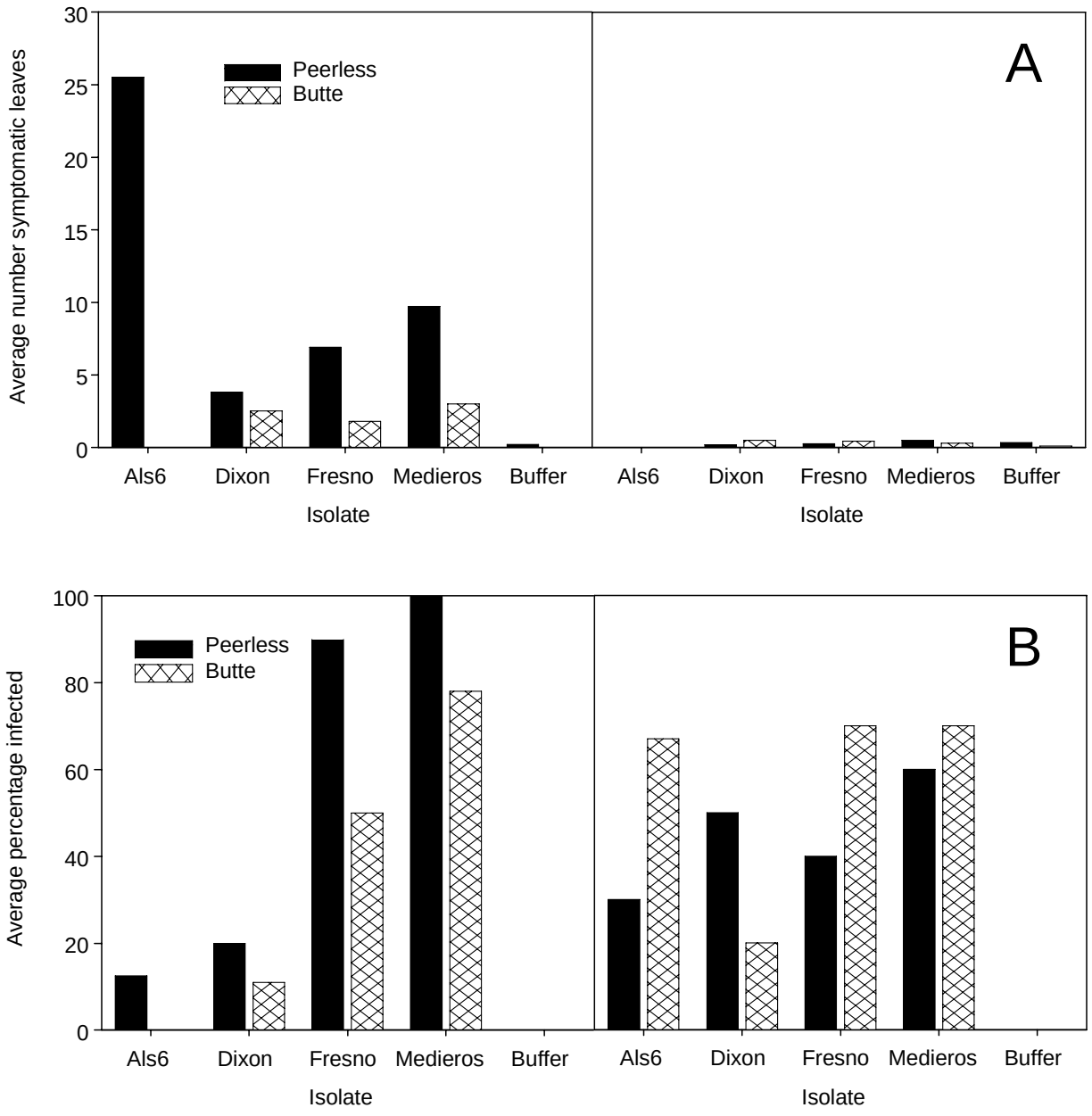

Figure 2. A) Almond leaf scorch symptoms and **B)** *Xf*-infections in 'Butte' and 'Peerless' almond trees at Davis (left) and Tulelake (right) field sites two to four months after inoculation.

EVALUATION OF SIGNAL SEQUENCES FOR THE DELIVERY OF TRANSGENE PRODUCTS INTO THE XYLEM OF GRAPEVINE

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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. This is a continuing project that began in late 2005. In our earlier research we collected xylem exudates 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 five of them were found in the TIGR *Vitis vinifera* gene index. Two of these sequences for xylem proteins Chi1b and one similar to NtPRp27 were fused to the mature pear polygalacturonase inhibiting protein (pPGIP)-encoding gene. We also made three other constructs incorporating signal peptides from the xylem sap protein XSP30, the rice amylase protein Ramy3D that we have described in earlier reports along with pPGIP lacking a signal peptide as control. We have successfully transformed *Vitis vinifera* 'Thompson Seedless' (TS) grape with the five vectors and callus and embryo cultures for all five vectors have been obtained. The expression of these chimeric genes is being evaluated in permanent transformations in order to evaluate their ability to target pPGIP to the xylem. We have successfully obtained Chi1b and Nt transformed TS plants that are PCR positive for the transgene. Using a zone inhibition assay, we have determined that seven out of 15 plants tested so far are positive for polygalacturonase inhibiting activity. Micropropagation of promising transformants and bench grafting of transgenic rootstock to wildtype scions are currently underway. 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 endoplasmic reticulum (1). Generally, signal peptides are interchangeable and secretion of non-secreted proteins becomes possible by the attachment of a signal peptide at the N-terminus of the mature protein that allows the entry into the vesicular transport system (2). Numerous reports of successful recombinant protein production using signal peptides in transgenic plants have been reported; however, changing the signal sequence of recombinant proteins can affect the degree of protein production. For example, the efficiency of secretion of heterologous proteins in transgenic tobacco was improved by replacing the heterologous endogenous signal peptide with a signal peptide from a tobacco protein (3).

In previous research, we have found that the product of the pPGIP encoding gene, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (4). pPGIP has a peptide sequence that directs its secretion to the apoplast and its presence in xylem vessels may represent protein secreted into the vessels through pit membranes that serve as transfer pathways from neighboring parenchyma cells. Then if pPGIP is secretion competent in grapes, it constitutes a good alternative to inactivate *Xf* genes products like polygalacturonase (PG), the enzyme required for *Xf* to successfully infect grapevines and as critical virulence factor for *Xf* pathogenesis in grapevines (5). In previous experiments we found GFP, when fused with the signal peptide XSP30, a xylem specific protein from cucumber (6) to either not be recognized or not be secretion competent. We intend to use the mature pPGIP as a secretion competent product fused to the signal peptides to be analyzed.

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 (7). cDNA sequences of five of them were found in the TIGR *Vitis vinifera* gene index. However, it was possible to predict the signal peptide in two contigs only (TC 39929 and TC 45857, annotated as Chi1b and similar to NtPRp27 respectively). 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) (8) 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).

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 (9). 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 (10).

The proposed work described in this proposal carefully corresponds to research priorities developed by the National Academies in their publication, "California Agriculture Research Priorities: Pierce's Disease" as outlined in Chapter 4, Recommendations 4.3, 4.4 and 4.5 and Chapter 3, Recommendation 3.3. Additionally, the objectives of this research project are relevant to the research recommendations from the CDFA PD/GWSS research scientific review final report from August 2007 as described on page 21 section F.1 by the CDFA Research Scientific Advisory Panel, specifically inhibition of *Xf* PG enzyme. 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.

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*.
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 embryo callus cultures of transformed grapevines.
   b. analysis of the expression and secretion of pPGIP in grapevines bearing roots and grafted to wild type grapevine scions.

RESULTS

The first three objectives have been accomplished and summarized in the introduction. These results pertain to our progress in accomplishing objective 4. The permanent transformation of *Vitis vinifera* 'Thompson Seedless' (TS) has been initiated

for all five vectors. The methods for *Agrobacterium*-mediated transformation have been reported earlier by us (10). We have obtained transformed callus cultures for all five vectors (Table 2) and analysis of the expression and secretion of pPGIP in embryo callus cultures of transformed grapevines is in progress. All of the callus lines will be tested for the presence of the transforming DNA gene segments using PCR, transcripts will be quantitated using TaqMan RT-PCR to identify high expressing callus lines. Five individual lines of callus cultures from each of the five vectors have been initiated into liquid cultures to evaluate the secretion of PGIP into the culture medium as would be expected of the apoplast targeted protein. PGIP secretion and activity will be evaluated using the zone inhibition assay with PG (Figure 2) (11). Select callii that have been transformed from each of the five constructs have been induced to undergo embryogenesis. These callus cultures that are embryogenic will be selected on kanamycin and these embryos will be used to obtain shoots for individual plants. All the methods that we will be using for the analysis of the callus or the plants have been described by us (4).

Table 2. Status of *Vitis vinifera* 'Thompson seedless' transformants

	Vector insert	Callus	Embryo	Plant	Positive PCR for NPT II
1	pDU05.1002	yes	yes	no	To be tested
2	pDU05.0401	yes	yes	no	To be tested
3	XSP	yes	yes	no	To be tested
4	pDU06.0201	yes	yes	yes	10/10
5	pDU05.1910	yes	yes	yes	17/22

We have already obtained plants containing vectors #4 and #5 (Table 2). Seventeen of the 22 plants transformed with vector #5 and 10 out of 10 plants transformed with vector 4 have tested positive via PCR for the transgene using nptII primers (Table 2). Plants will be regenerated and the same detailed testing that is used for the callus and embryo cultures will be done with the plants. We have tested 15 of the plants transformed with vector #4 and #5 for PGIP activity using the zone inhibition assay with PG and seven plants were showing PG inhibition activity. Micropropagation of the more promising plants is already underway. Initially the micropropagated plants will be evaluated; these will then be transferred to soil and transferred to the green house for growth. The vines will be allowed to grow in the green house for four to six months and the xylem fluid will be extracted with a pressure bomb. We also plan to do some grafting experiments where selected transformed lines will be bench grafted with wild type TS scion, we have done this type of experiment previously to evaluate the movement of the PGIP protein from the rootstock up into the xylem of the wild type scion (4). Since we found in this earlier research that pPGIP with its endogenous signal peptide is xylem competent we are using it as a positive control (4). We have successfully bench grafted transformed rootstock containing pPGIP with its endogenous signal peptide to wild type 'TS' scion (Figure 1) and micrografts using plants transformed with vectors four and five are in progress. An additional year of work may be required to accomplish this last evaluation that requires grafted plants.

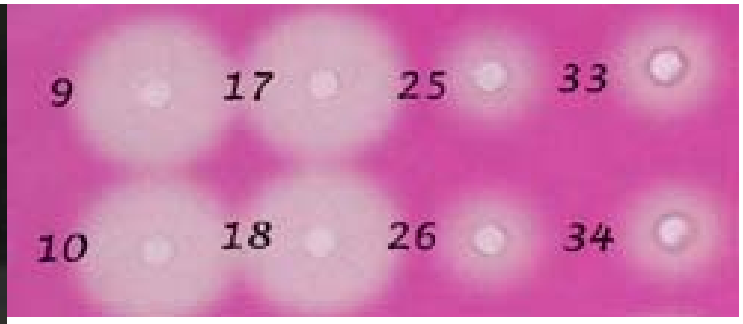

Figure 1 (left). Photograph of a TS root stock and scion successfully grafted together. The red arrow indicates the graft point. **Figure 2 (above).** Zone inhibition assay results. Wells 9, 10, 17 and 18 are showing no polygalacturonic inhibition activity and wells 25, 26, 33 and 34 are positive for polygalacturonic inhibition activity.

CONCLUSION

We have accomplished the first three objectives and we have already made significant progress toward achieving our fourth objective. We have successfully transformed TS with the five PGIP vectors and obtain both callus and embryo cultures. In the case of vectors #4 and #5 we have obtained plants as well. The analysis of the expression and secretion of pPGIP in

embryo callus cultures of transformed grapevines is underway. Results from our initial analysis look promising. Micropropagation and bench grafting of transformed TS has been initiated. 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.

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FUNDING AGENCIES

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GROUND VEGETATION SURVEYS TO DETERMINE RESERVOIR HOSTS OF *XYLELLA FASTIDIOSA* IN NORTH CAROLINA

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Reporting Period: The results reported here are from work conducted May 2007 to October 2007. The project is not complete; field surveys and laboratory analyses will be conducted in October and November 2007.

ABSTRACT

Ground vegetation surveys were conducted in three North Carolina vineyards to determine plant composition of the vineyard floor and to test for the presence or absence of *Xylella fastidiosa* (*Xf*) in these plants using ELISA and SYBR-Green based quantitative real-time (QRT) PCR methods. Ten out of 27 total plant species surveyed in the spring of 2007 tested positive for *Xf* using ELISA techniques. Analyses using QRT-PCR have not been completed. The fall survey has been completed in two of three vineyards. The results of this project are significant because previous control methods have focused on removing vegetation surrounding NC vineyards. This is the first research in NC to provide evidence that *Xf* is present on reservoir hosts within the vineyard. This work will establish the first official plant host list for *Xf* in North Carolina.

INTRODUCTION

Xf is found commonly among vegetation in the southeastern US, where the bacterium is native (Hopkins 1989). However, no official reservoir host list for *Xf* has been developed for North Carolina, where the wine industry has more than doubled from 2002 to 2007 (North Carolina Winegrowers Association 2005). It is common for NC vineyard managers to allow native vegetation to proliferate on the vineyard floor, or to plant grasses as groundcover. In the past, control recommendations regarding reservoir hosts have focused on removing vegetation surrounding the vineyards. The focus of this project is directed towards identifying potential reservoir hosts of *Xf* within the vineyard, as these plants may be more easily managed to reduce inoculum. It is important to identify plant species that consistently test positive for *Xf* so that control recommendations can include selecting groundcovers that are not among them.

OBJECTIVES

1. Determine the diversity and occurrence of plant species present on the vineyard floor in North Carolina vineyards.
2. Determine the presence or absence of *Xf* in plants collected from the vineyard floor using ELISA techniques and determine *Xf* concentrations found in positive samples using QRT-PCR techniques.

RESULTS

Objective 1

A transect was established near the center of each vineyard by laying a 50m measuring tape at approximately a 45° angle along the vineyard floor. Ten sites 1m in length were selected randomly along the 50 meter transect. At each of these 10 sites, a wooden $1m^2$ frame was placed on the ground and the percentage of each plant species within the frame was estimated and samples of each species were taken. Subsamples were put aside for identification and the samples were ground and tested for *Xf* with ELISA. Subsamples of the ground plant material are in cold storage for later analysis with QRT-PCR. In the spring, two surveys were conducted at each location, with the measuring tape arranged so that the two lines formed an "X" transect along the vineyard floor. For fall surveys, a third transect was added by sampling a straight line approximately 1m from the edge of one outer row of the vineyard. Fall surveys and analyses are still underway. The results reported here are for spring surveys only. Preliminary results from fall sampling, not shown, indicate that vineyard floor composition is significantly different in spring and fall. Vineyard floor composition for each of the three vineyards surveyed is shown below in Figure 1. Species that comprised less than 2% of the vineyard floor are grouped into the category "other."

Objective 2

Samples from each species found were ground and tested for *Xf* with ELISA. Plant species were sampled in proportion to the number of times which they were encountered. For example, for each $1m^2$ block, one sample was taken of each species, therefore many species were recurrent and hence were sampled repeatedly. Ten out of 27 species surveyed in spring tested positive for *Xf* with ELISA. Table 1 shows which species tested positive, the location(s) from which the plants were sampled, the number of positives out of the total number of samples tested, and the percentage of the vineyard floor that is comprised of that particular species.

CONCLUSIONS

The results of this project show that NC growers who allow weeds to develop in the vineyard floor may be supporting *Xf* inoculum within their vineyard. As a result, weed control may become part of a management plan for PD in NC. We have begun preliminary studies to identify a suitable groundcover that is not a reservoir host of *Xf*.

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Funding for this project was provided by the North Carolina Grape Council, the Golden Leaf Foundation, and the North Carolina Agricultural Research Service.

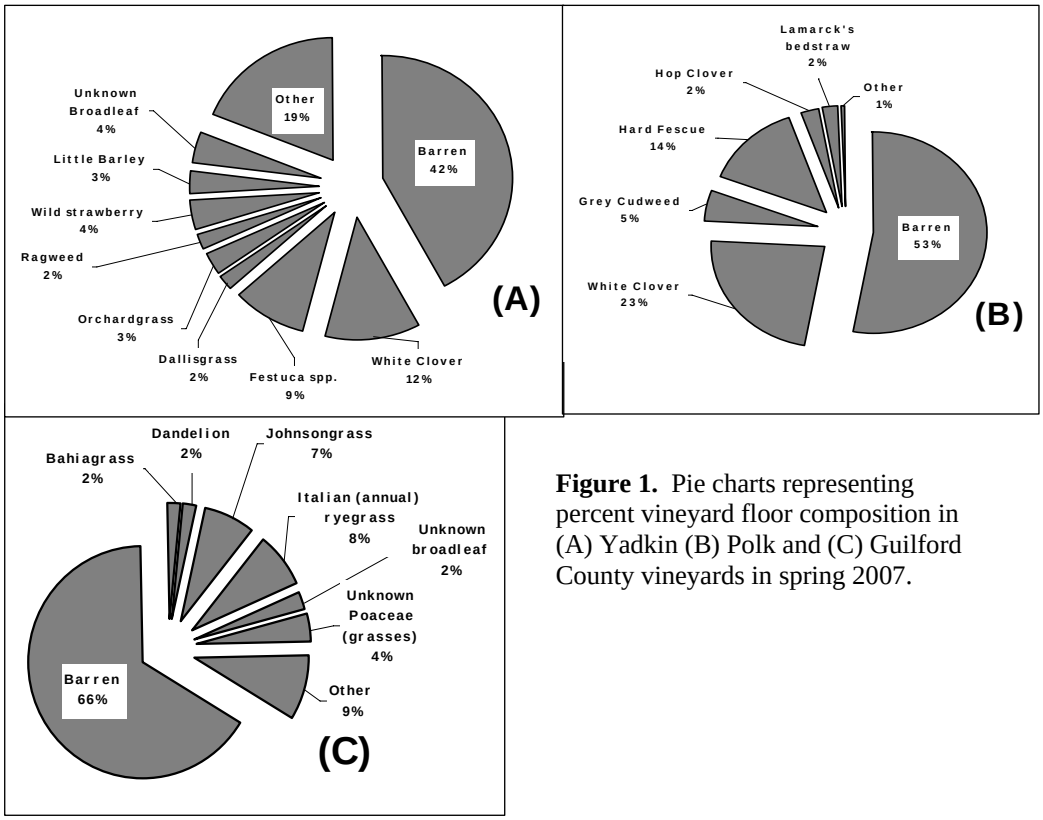

Figure 1. Pie charts representing percent vineyard floor composition in (A) Yadkin (B) Polk and (C) Guilford County vineyards in spring 2007.

Table 1. List of host plants tested for *Xf* from three NC vineyards in Guilford, Yadkin and Polk counties in spring 2007. Red font indicates plants that tested positive for *Xf* with ELISA.

Common Name	Scientific Name	Family	No. Positive/Total Sampled			Percent of Vineyard Floor		
			Guilford	Yadkin	Polk	Guilford	Yadkin	Polk
Bahiagrass	*Paspalum notatum*	Poaceae	0/4			2%		
Bermudagrass	*Cynodon dactylon*	Poaceae	1/1	1/1		1%	1%	
unidentifiable broadleaf	--			1/5			4%	
Dallisgrass	*Paspalum dilatatum*	Poaceae		0/3			2%	
Dandelion	*Taraxacum officinale*	Asteraceae	0/9			2%		
Fescue	*Festuca sp.*	Poaceae		1/7			9%	
Rabbitfoot clover	*Trifolium arvense*	Fabaceae		0/2			1%	
Poverty rush	*Juncus tenuis*	Juncaceae		0/1			1%	
Greenbriar	*Smilax rotundifolia*	Smilacaceae		0/3			1%	
Grey Cudweed	*Gamochaeta claviceps*	Asteraceae			5/11			5%
Hard Fescue	*Festuca spp.*	Poaceae			2/8			14%
Hop Clover	*Trifolium spp.*	Fabaceae			2/7			2.30%
Horse nettle	*Solanum carolinense*	Solanaceae		0/1			0%	
Italian (annual) ryegrass	*Lolium multiflorum*	Poaceae	0/9			8%		
Johnsongrass	*Sorghum halepense*	Poaceae	0/10	0/1		7%	1%	
Lamarck's Bedstraw	*Galium divaricatum*	Rubiaceae			4/4			2.30%
Little Barley	*Hordeum pusillum*	Poaceae	1/1	0/7		1%	3%	
Orchardgrass	*Dactylis glomerata*	Poaceae		0/6			3%	
Oxalis, yellow woodsorrel	*Oxalis stricta*	Oxalidaceae		0/1			0%	
Prairie fleabane	*Erigeron strigosus*	Asteraceae	0/1	0/1		0%	1%	
Prostrate spurge	*Chamaesyce maculata*	Euphorbiaceae		0/2			1%	
Ragweed	*Ambrosia artemisiifolia*	Asteraceae		0/2			2%	
Red Clover	*Trifolium pratense*	Fabaceae		0/2			1%	
Rough bluegrass	*Poa trivialis*	Poaceae	1/1			1%		
Smooth hawksbeard	*Crepis capillaris*	Asteraceae	0/2			1%		
White Clover	*Trifolium repens*	Fabaceae		1/14	5/18		12%	23%
Wild Garlic	*Allium vineale*	Alliaceae		0/4			1%	
Wild Strawberry, Indian Strawberry	*Duchesnea indica*	Rosaceae		0/3			4%	

ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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ABSTRACT
In previous work, marker exchange mutagenesis of the single *tolC* gene in Pierce's Disease (PD) strain Temecula (PD1964) was shown to result in a total loss of pathogenicity on grape. The tolC mutant strains were not recovered after inoculation into grape xylem, strongly indicating that drug efflux is critical to survival of this fastidious pathogen. The multidrug efflux role of TolC was investigated, and the *tolC* mutant strain M1 was found to be much more sensitive to antimicrobial compounds than the wild type Temecula strain. TolC in *Xyllela fastidiosa* (*Xf*) is the common outer membrane component of two drug efflux systems (AcrF/A and AcrC/D) and two "toxin" secretion systems (CvaA/B and HlyB/D). Knockout mutations of *acrD* and *acrF* resulted in reduced symptoms of pathogenicity, in keeping with a redundant role in drug efflux. Importantly, knockout mutations of *cvaA, cvaB* and *hlyB/D* also elicited reduced symptoms of pathogenicity, indicating a potentially offensive role for Type I secretion in conditioning *Xf* pathogenicity.

INTRODUCTION
In Gram-negative bacteria, multidrug resistance (MDR) efflux pumps are composed of three protein components, two of which are localized in the inner membrane, and one, TolC, that traverses both the periplasm and outer membrane (Koronakis et al. 2004). The process of MDR efflux is energy dependant and utilizes either ATP or the transmembrane electrochemical gradient. At least five characterized families of MDR efflux pumps exist in bacteria: the ATP-binding cassette (ABC) family (Davidson and Chen 2004), the major facilitator (MF) family (Pao et al. 1998), the small multidrug resistance (SMR) family (Paulsen et al 1997), the resistance-nodulation-cell division (RND) family (Tseng et al. 1999), and the multidrug and toxic compound extrusion (MATE) family (Brown et al. 1999). All utilize TolC as a common periplasm/outer membrane protein component.

In addition to (defensive) MDR efflux, TolC is also essential for type-I dependent secretion of a variety of degradative enzymes and offensive effectors, some of which are antibiotic and others involved in plant or animal pathogenicity. These include a variety of hydrolases (proteases, phosphatases, esterases, nucleases and glucanases) and protein toxins, including hemolysins and bacteriocins (Koronakis et al. 2004). Orthologs of *tolC* are highly conserved among diverse Gram-negative pathogenic bacteria, and strains typically carry multiple homologues per strain (Sharff et al. 2001), including all sequenced strains of *Xanthomonas, Pseudomonas* and *Ralstonia.*

Xylella fastidiosa is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell and Hopkins, 1996). Two of the most serious of these are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). Analyses of the CVC and PD published genomes showed that there was no type III secretion (*hrp*) system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins.

OBJECTIVES
There are two main purposes for Type I secretion (refer Figure 1) : multi-drug resistance or MDR efflux (in this case, defense against presumably anti-microbial chemicals in the xylem sap of grape), and toxin secretion (offensive, to promote pathogenicity). The outer membrane protein TolC has been shown to be essential for MDR efflux and pathogenicity in *Erwinia chrysanthemi* (Barabote et al., 2003) and more recently in *Xf* (Reddy et al., 2007). The purpose of this study was to further investigate the MDR efflux role and to determine a potential role for Type I secretion of (offensive) toxins by *Xf*.

1. Pathogenicity tests following disruption of four Temecula genes, in addition to *tolC*. The additional genes encode substrate-specific, periplasmic portions of at least four Type I Secretion Systems found in the Temecula genome:
   a. multi-drug resistance (MDR) efflux pumps, specifically, *mexC* (PD0202), *acrF* (PD0783) and *acrA* (PD0784), and
   b. toxin secretion, specifically, hemolysin (*hlyD*; PD1413) and colicin (*cvaA*; PD0496).
2. Assay the minimum inhibitory concentration of agents known to have an effect on bacteria that have been compromised in MDR efflux capability, including a) the detergent SDS, b) the hydrophobic chemical DOC, c) the antimicrobial agent from Rhubarb, Rhein, d) the isoflavonoid genistein, e) the alkaloid berberine and f) the grape phytoalexin resveratrol. If any of these assays are successful in inhibition of mutants, they may be very useful to help confirm complementation, since they would be more rapid assays than pathogenicity tests. However, they would not be a substitute for the

pathogenicity tests needed to confirm potential offensive and/or defensive roles of the Type I secretion systems in PD strain Temecula.

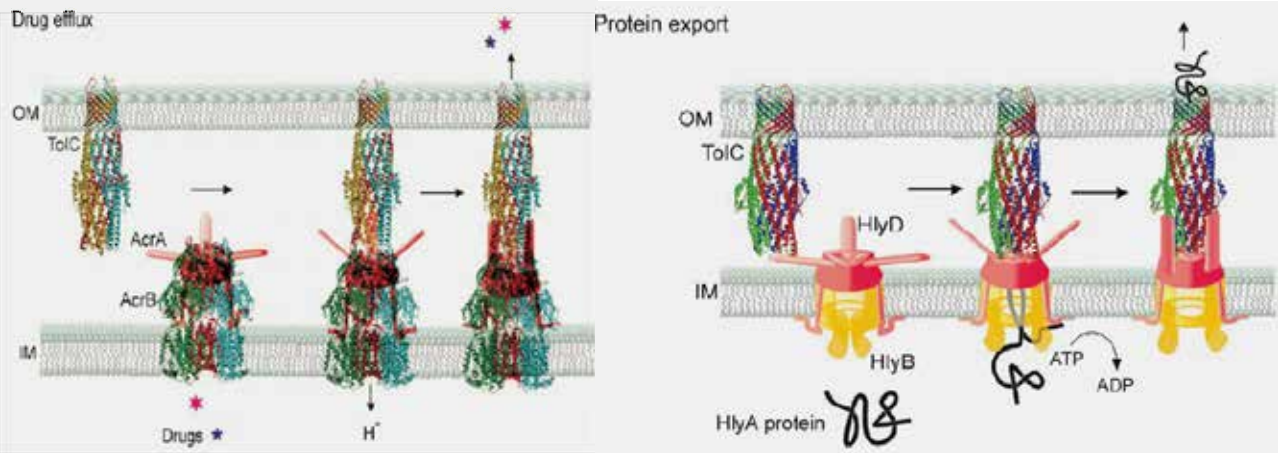

Figure 1. Type I machine for MDR ("Drug) efflux in *Xf* utilize *tolC* and *acrF/A* or *acrC/D* (left). Type I machine for protein export or secretion in *Xf* utilize *tolC* and *cvaA/B* or *hlyB/D* (right). Figures from Koronakis et. al. (2004).

RESULTS

1. **Pathogenicity tests following disruption of:**

a. MDR efflux pump genes *mexC* (PD0202), *acrF* (PD0783) and *acrA* (PD0784). The MDR efflux system genes *mexC* (PD0202) and *acrF* (PD0783) were successfully disrupted by marker exchange as described (Reddy et al., 2006) and used in assays described below. Despite many repeated attempts, we failed to disrupt *acrA* (PD0784); it is possible that *acrA* knockouts are lethal on the growth media used for selection. Since we have confirmed a critical role for MDR efflux in Reddy et al. (2007) and using *acrF*, we have dropped further attempts to disrupt *acrA*.

Plant inoculation assays using *mexC* and *acrF* were performed in collaboration with Dr. Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *Xf* Temecula strain and the mutant (*mexC*::*nptII*) strain in triplicates. The plants were maintained under green-house conditions and were evaluated for Pierce's disease symptoms at 60 and 90 days after inoculation. Not surprisingly, the *mexC::nptII* and *acrF::nptII* mutants on grapes had lost pathogenicity. All plants inoculated with the wild-type Temecula strain exhibited typical PD (not shown).

b. Protein export mutants *acrF* (PD0783), *acrD* (PD1404), *hlyBD;* PD1412-1413*), cvaA (*PD0496*) and cvaB* (PD0499). Mutations at these loci were generated using *nptII* as the marker and pGEM-T as the delivery vector. The mutants were verified Southern blot analysis and by using PCR analysis as described (Reddy et al., 2007; not shown).. Plant inoculation assays were performed as above. **All mutants listed were less pathogenic than the wild type, even after 90 days (refer Figure 2).**

2. Both *tolC*- and *acrF*- mutants were much more sensitive to antimicrobial chemicals berberine (an alkaloid DNA intercalating agent), genistein (an isoflavone phytoalexin precursor), rhein (an anthraquinone), and also to the surfactant Silwet L-77 than the wild type Temecula strain**,** confirming Reddy et al. (2007) that MDR efflux is required by *Xf* for pathogenicity (Table I).

3. Neither hlyBD, cvaB nor cvaA were sensitive to the antimicrobial chemical berberine, in keeping with their presumed role in Type I protein export, rather than drug efflux. The colicin Type I system secretion protein *cvaA* (PD0496) was successfully disrupted by marker exchange as described (Reddy et al., 2006) and used in assays as described above. However, in one experiment to date (to be repeated two additional times), no reduction of pathogenicity was observed in comparisons with wild type Temecula. These results provide a very preliminary indication that colicins may not be strongly involved in elicitation of PD symptoms.

Despite repeated efforts, we have failed to date to disrupt *hlyD* (PD1413), predicted to be involved in hemolysin secretion. We do not yet understand the reasons for this, but are working to obtain this mutation, which is important to test the hypothesis that Type I secretion of hemolysin results in some of the symptoms of PD.

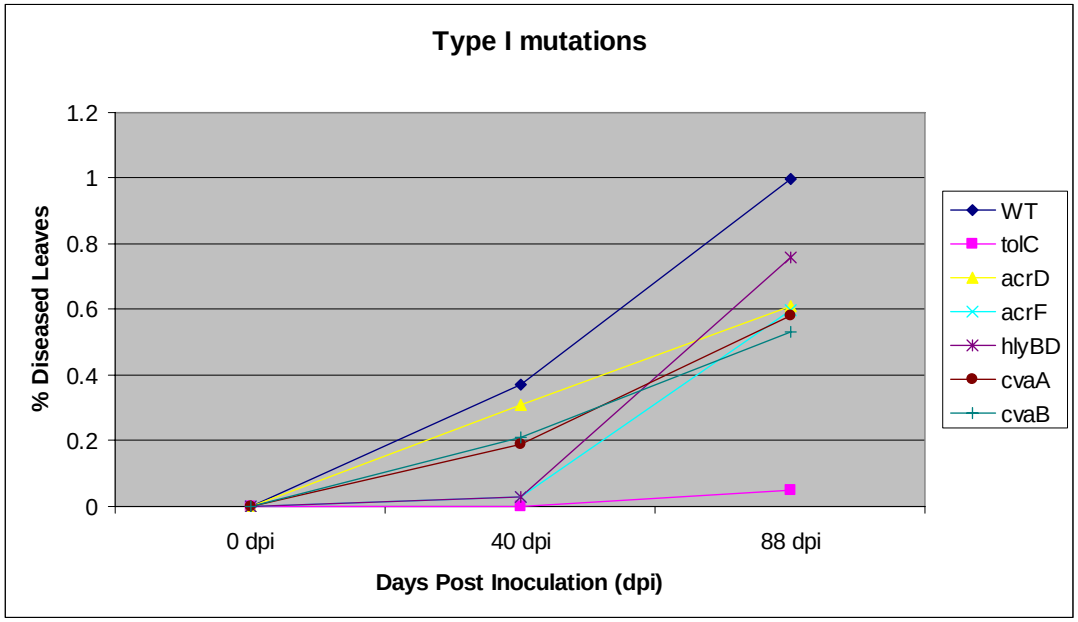

Figure 2. Grape var. Carignane inoculated with marker exchanged mutants of *acrF* (PD0783), *acrD* (PD1404), *hlyBD;* PD1412-1413*), cvaA (*PD0496*) and cvaB* (PD0499) and assessed for % diseased leaves at 40 and 88 days post inoculation. Complementation of tolC has been achieved, and complementation of the other mutations is in progress.

Table 1. Minimum inhibitory concentrations (MICs) of four phytochemicals and Silwet L-77 on Temecula, *tolC*– and *acrF*- Temecula mutants.

Chemical	MIC (µg/ml)			Fold difference
	Temecula	*tolC*⁻	*acrF*⁻	
Berberine	25	.02	.02	1000X
Genistein	5	0.5	NT	10X
Resveratrol	12.5	12.5	NT	1X
Rhein	50	.05	NT	1000X
Silwet L-77	>2000 ppm	20	20	>100X

CONCLUSIONS

This work demonstrates that not only is multidrug efflux critical to survival of *Xf* in grape, but also that Type I secretion is needed for full pathogenicity. Both multidrug efflux and Type I secretion depend upon a single tolC gene present in the *Xf* genome. Since TolC is exposed to the outer surfaces of bacteria, these combined results make TolC a vulnerable and specific target for both chemical and transgenic approaches to control Pierce's Disease.

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MANIPULATION OF ENDOPHYTIC BACTERIA FOR SYMBIOTIC CONTROL OF *XYLELLA FASTIDIOSA*, CAUSAL AGENT OF CITRUS VARIEGATED CHLOROSIS

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Reporting Period: The results reported here are from work conducted September 20, 2006 to September 20, 2007.

ABSTRACT

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

INTRODUCTION

Citrus variegated chlorosis (CVC) is a disease of sweet orange [*Citrus sinensis* (L.)] trees caused by *Xylella fastidiosa* subsp. *pauca* (*Xf*p) (Schaad et al. 2004). The disease continues to increase in severity, with 35% of the sweet orange trees in São Paulo, Brazil currently showing loss of yield. Endophytes colonize an ecological niche similar to that of phytopathogens, and this fact might favor them as candidates for biocontrol agents (Hallmann et al., 1997) because they have access to and could interact with phytopathogens (Azevedo et al., 2000). Many endophytic bacteria have been isolated from sweet orange (Araújo et al. 2002) but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *Xf*p in the xylem vessels of plants (Araújo et al., 2002; Lacava et al. 2004). The genus *Methylobacterium* is described as a main player in the interaction between the endophytic community and the pathogen *Xf*p (Araújo et al., 2002; Lacava et al. 2004). Cicadellinae leafhoppers, or sharpshooters, are considered xylem-fluid feeders (Young et al., 1968) and a clear association has been observed between their xylem-feeding habit and ability to transmit *Xf* (Costa et al., 2000; Almeida and Purcell, 2003). In Brazilian citrus groves, *Dilobopterus costalimai* Young, *Oncometopia facialis* (Signoret), and *Acrogonia citrina* Marucci & Cavichioli are the most common sharpshooters found, whereas *Bucephalogonia xanthophis* (Berg) is the most commonly trapped in citrus nurseries and young groves (Redak et al., 2004). A new genetic transformation tool, called paratransgenesis, has been used to prevent the transmission of pathogens by insect vectors to humans (Rio et al., 2004). Paratransgenesis means genetic alteration of symbiotic microbes that are carried by insects. The overall strategy of disease prevention is called symbiotic control and is a variation on the theme of symbiotic therapy (Ahmed, 2003). The key to symbiotic control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen. Bacteria of the genus *Methylobacterium* are known to occupy the same niche as *Xf*p inside citrus plants (Araújo et al 2002; Lacava et al. 2004), so during feeding, insects could acquire not only the pathogen but also endophytes from host plants.

OBJECTIVES

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

RESULTS

When the pCM88 was introduced into the strain *M. mesophilicum* SR1.6/6, up to 10^2 transformants per μg of plasmid DNA were obtained (now called SRGFP), indicating a high efficiency of transformation. The analysis of randomly selected SRGFP transformants revealed that pCM88 was stably maintained in medium without antibiotic, expressing both the

resistance to tetracycline and the *gfp* gene, after twenty generations in 120 h, 95%, decreasing the stability on 0,25 % per generations approximately (Fig 1). The original bacterial community of *B. xanthophis* was comprised of five groups: *Methylobacterium* sp., Actinomycetes, *Curtobacterium* sp., *Sphingomonas* sp. and *Bacillus* sp. (Fig 2). The *Methylobacterium* genus occurred naturally in *B. xanthophis*. The ecological niche occupied by the endophytic bacterium *M. mesophilicum* on *C. roseus* plants was determined by visualization with fluorescent microscope, of *in vitro* cultivated plants, 45 days after bacterium inoculation. A preferential colonization of plant xylem by this bacterium is clearly observed in fluorescence microscopy (Fig 3). The insects used in transmission experiments were monitored for the presence of the SRGFP strain 24 hours after acquisition. Bacteria isolation from insect heads revealed the average population density of *M. mesophilicum* of 4.6 10^3 CFU/insect $head^{-1}$, suggesting that the bacteria is capable of colonizing the foregut of the insect as it was not washed way by the sap flux. The ability of the sharpshooter *B. xanthophis* in transmitting *M. mesophilicum* was accessed by insect acquisition of endophytic strain SRGFP and further feeding in *C. roseus* plants cultivated in greenhouse. Forty-five days after the insect feeding on plants, leaves on which insects were trapped, were submitted to bacterial isolation. The population density of *M. mesophilicum* found in *C. roseus* leaves 45 days after insect transmission presented an average of 2.8 10^3 $CFU.g^{-1}$ of fresh tissue. Analyzing inoculated plants, from 45 plants used in insect traps, six presented the SRGFP strain colonizing inner tissues endophytically. It indicates that *B. xanthophis* is able to transmit the endophytic bacteria by the same way it transmits *Xf*, with an efficiency of transmission of 13.3%.

CONCLUSIONS

The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf*p makes it possible to study the potential interactions between these bacteria in the insect body and makes *M. mesophilicum* an interesting candidate for the symbiotic control of the CVC agent, e.g., through a paratransgenesis approach.

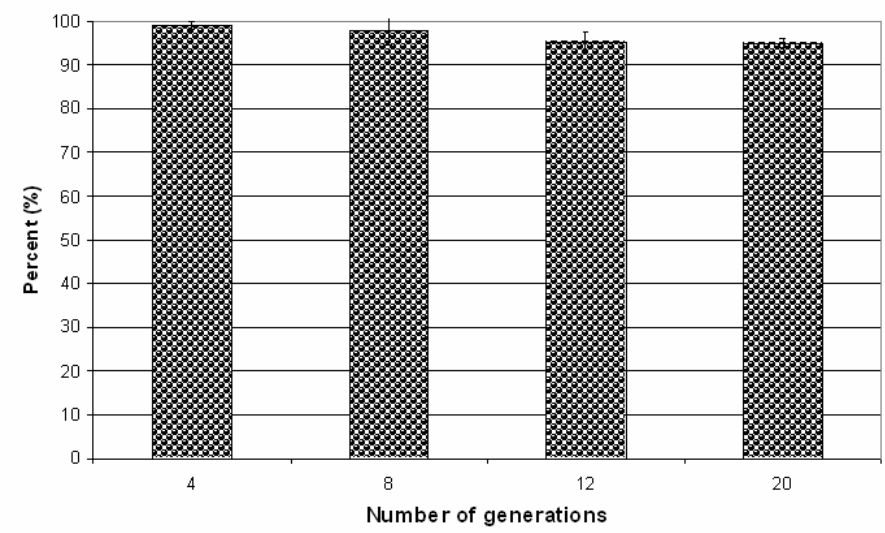

Figure 1. Plasmidial stability of pCM88 on *Methylobacterium mesophilicum*. The percent was obtained collecting random samples after 24, 48, 72 and 120 hours of culture cells of SRGFP strain growing without antibiotic tetracycline.

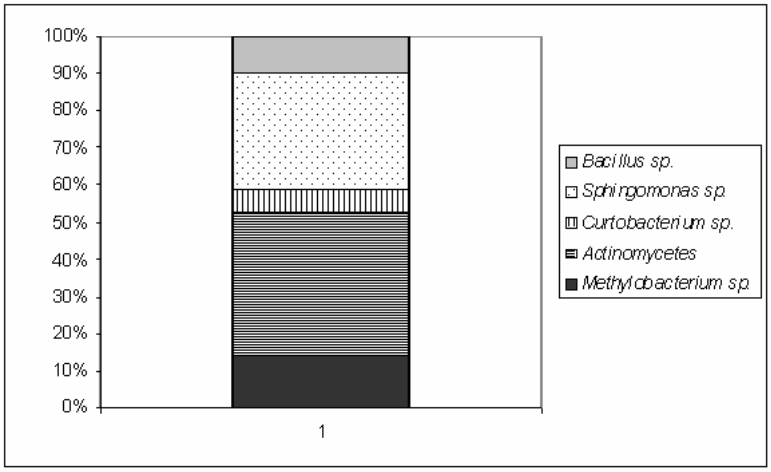

Figure 2. Most frequently group of bacteria isolated from *Bucephalogonia xanthophis*.

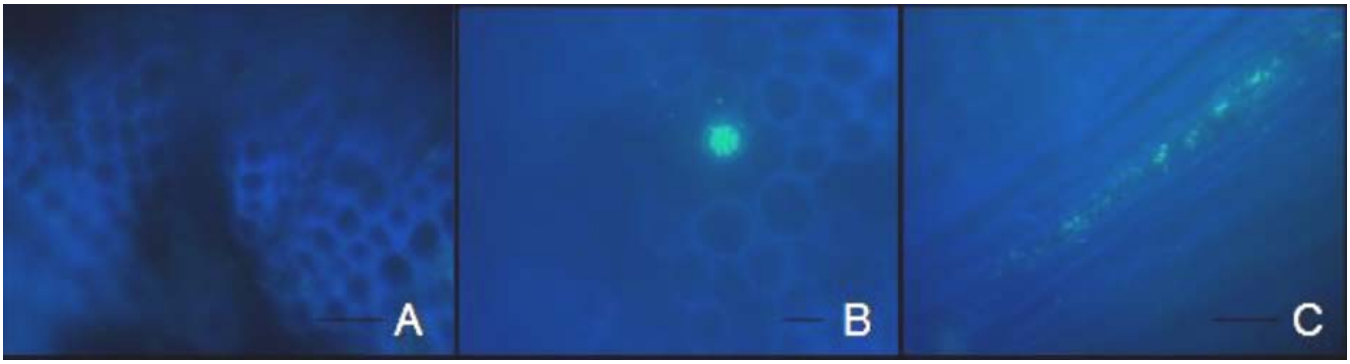

Figure 3. Fluorescent microscopy evidencing the ecological niche occupied by endophyte *Methylobacterium mesophilicum* in *Catharanthus roseus* plants 45 days after inoculation. A) Xylem vessels of a control plant, Scale bar = 10µm; B) Colonized xylem vessel, Scale bar = 5µm; C) Xylem vessels longitudinal cut, Scale bar = 10µm.

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ENVIRONMENTALLY CREATED IMMUNITY AGAINST PIERCE'S DISEASE

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ABSTRACT
Pierce's disease (PD) is caused by a xylem limited gram-negative *Xylella fastidiosa* (*Xf*) bacterium. Various species of sharpshooters, including the important glassy-winged sharpshooter (GWSS), transmit *Xf*. Currently, there is no cure for PD. Use of bacteriophage could be a good strategy for the management of PD. Here we are presenting the results of study use of apparent bacteriophage for the management of PD.

INTRODUCTION
Strains of *Xylella fastidiosa (Xf)*, a gram-negative bacterium, cause a number of important plant diseases including Pierce's disease (PD) in grapevine, citrus variegated chlorosis (CVC) in citrus, phoney peach disease, periwinkle wilt, and leaf scorch disease in plum, elm, maple, sycamore, and coffee (Hopkins 1989).

The principle vector for the transmission of *Xf* is the glassy-winged sharpshooter (GWSS) (*Homalodisca vitripennis*). The pathogen attaches to the cibarium and precibarium of sharpshooters by means of an extracellular matrix (ECM) and is transmitted from infected plants to healthy plants when the sharpshooters feed (Brlansky et al. 1983).

Phages have been reported to be used for the plant protection against different plant bacteria (Jones et al. 2007). Svircev et al. (2006) controlled fire blight of pear by utilizing a strain of *P. agglomerans* for delivering and sustaining a mixture of four phages, which were able to lyse strains of both *P. agglomerans* and *E. amylovora*, the causal agent of fire blight. Certain grapevines in PD areas appear to be resistant to the disease near other grapevines that show symptoms. We hypothesize that a bacteriophage survives in the soil and from there gets acquired by the plant where it kills the *Xf*.

OBJECTIVES
1. To isolate suspected bacteriophages for possible use in the management of PD.

RESULTS
Field observation has led us to suspect that an environmentally created immunity grapevines can impart resistance to PD. This resistance is not inherent to the plant but thought to form when certain combinations of environmental conditions are present in way that imparts symbiotic /apparent bacteriophage type immunity to the grapevine.

To test our theory we chipped up late season cane material and added it to water and soil from around the base of grapevines in vineyard showing apparent resistance to PD. This material was dried and then water was added. The resulting infusion was then filtered (using 0.2 μm filters) to remove all bacteria and fungi including their spores but allowing viral particles to pass through. We added the resulting liquid to PD3 media along with *Xf* bacteria and then allowed the mixture to incubate for 10 days. The finished product was clear indicating that our potion had inhibited bacterium growth. To test the potion further we autoclaved some and then repeated the test, this time *Xf* grew normally.

Currently we are continuing the study in the greenhouse. If *Xf* can be prevented from reverting downward in affected grapevine, the flush of pathogen the following spring will be prevented. By reducing the rate of downward movement of the PD bacterium we hope to provide a tool for reducing the number of chronic infections in grapevine which leads to vine death.

Since downward migration of the bacterium is directly correlated with chronic infections of grapevine. This is due to the fact that the bacterium must reach areas of the plant that are not trimmed away annually in order to find permanent residence. These areas are generally in the root ward direction from the point of infection.

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

Additional Note: All of the field tests were conducted under a permit from Environmental Protection Agency (TERA R-03-01). A report of tests was submitted to EPA and sponsors.

related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related (PR) proteins (Kuwabara, et al, 2002). Thus it may be possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap was extracted from healthy and *Xf*-inoculated, cold-stressed and control CS and PN vines using the pressure bomb, proteins were concentrated by cold-acetone precipitation, and protein profiles determined by 1-dimensional polyacrylamide gel electrophoresis (PAGE). We found that good PAGE profiles were obtained by concentrating 150ul of sap to 30ul of sample each sample. Thus we are able to have multiple PAGE profiles from any particular temperature/variety/disease that can be compared to healthy control vine sap. While most of the proteins were similar for the various temperatures, a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants and these proteins were end terminally sequenced by the UCD Molecular Structure Facility. The potential effect of these unique proteins on *Xf* viability will be assessed in Objective 3.

Table 2. pH of grapevine xylem sap collected from cold chamber treated vines.

pH	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	5.94	5.45	5.78	6.00
Cabernet Sauvignon	6.31	5.84	5.86	5.78

Table 3. Osmolarity of grapevine xylem sap collected from cold chamber treated vines.

Osmolarity	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	85.0	39.5	52.7	34.7
Cabernet Sauvignon	61.9	39.5	44.8	31.6

Objective 3

In this objective we assessed the effect of many of the physical, physiological and biochemical parameters we determined in Objectives 1 and 2 on *Xf* viability *in vitro*. In 2004-2005 we assessed the effect of various buffers on the viability of *Xf* cells *in vitro* using media such as PD3 and various buffers such as sodium phosphate and potassium phosphate. *Xf* cells were placed in potassium phosphate buffer with the pH of: 5.0, 5.4, 5.8, 6.2, 6.6 and 6.8 to assess the effect of pH on the survival of *Xf*. Viability was assessed by plating the exposed cells on PD3 medium. Results of these studies are presented in the 2004, 2005 and the 2006 PD Research Symposium Reports Interestingly, the osmolarity of PD3, a common media used for growing *Xf*, is approximately 113mmol/kg, whereas the osmolarity of dormant xylem sap averages between 25-60 mmol/kg. This suggests that *Xf* is able to survive at various osmolarities.

Objective 4

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as ABA, that induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998;). Some of these cold-shock proteins have been shown to inhibit the growth of certain fungal pathogens (Kuwabara, et. al 2002), but no work has been done on their effect on bacterial pathogens. These same cold-shock genes can be induced under non-freezing temperatures by the exogenous application of ABA (Kuwabara, et al. 2002).

We determined the concentration of ABA in cold-stressed and control vines from the four field sites and the cold room experiments. ABA concentration was determined using an immunological assay (Phytodetek ABA Test Kit, Agdia) that has a sensitivity approximately 0.0064-0.16 picomoles ABA/ml and only requires a small volume of sap. We found that ABA concentrations in the April xylem sap collections were the lowest in the coldest field locations. ABA levels were higher in the February sap collections than in the April collections for the field locations. ABA concentrations in the spring xylem sap collections were the lowest in the coldest cold chambers.

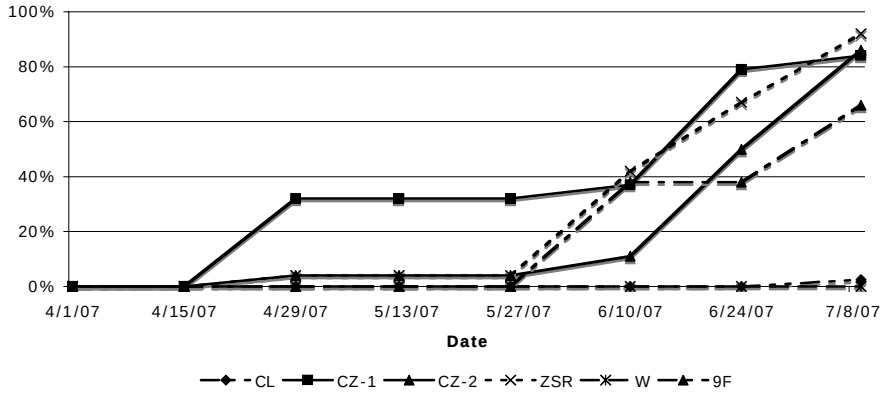
In 2005 CS and PN grapevines were grown in one-gallon pots in the screen house to determine the effect of applying exogenous ABA on the development and/or severity of PD. At the suggestion of Sue Abrams, an ABA expert at Plant Biotechnology Institute, National Research Council Canada, we contacted Valent Bioscience Corporation who has an active research and development program on the use of ABA on agricultural crops. We met Valent representatives at UCD and described our proposed research. They were interested in the project and agree to provide us with two types of ABA, one of which (VBC-30030) is a proprietary material. One set of treatments used regular ABA (VBC-30054) as a spray (1000ppm) and a soil drench (100ppm). The second material (VBC-30030) is an ABA analog that persists longer (1 week verses 1 day) and is more active than VCB 30054. VBC-30030 was used as a spray (100ppm) and soil drench (10ppm). There were 16 healthy and 16 *Xf* -infected Cabernet Sauvignon and 16 healthy and 16 *Xf*-inoculated Pinot Noir vines used in each of the treatments plus a set used as untreated controls.

For the 2006-2007 season, vines were prepared as described above and were subjected to slightly different applications. One of the treatments used VBC-30054 as a spray at 2000ppm at one week intervals for 3 weeks. The other treatments were the same as described for the 2005-2006 season. VBC-30054 was applied as a drench at 100ppm. VBC-30030 was applied as a

Table 1. Percentage of positive plants of survey results from Temecula vineyards.

	number of vines	1-Apr-07	15-Apr-07	29-Apr-07	12-May-07	26-May-07	10-Jun-07	24-Jun-07	9-Jul-07
CL	41	0%	0%	0%	0%	0%	0%	0%	2.50%
CZ-1	19	0%	0%	32%	32%	32%	37%	79%	84%
CZ-2	28	0%	0%	4%	4%	4%	11%	50%	86%
ZSR	24	0%	0%	4%	4%	4%	42%	67%	92%
W	3	0%	0%	0%	0%	0%	0%	0%	0%
9F	8	0%	0%	0%	0%	0%	38%	38%	66%

Figure 1.: A graph of percentage of positive plant of survey result from Temecula vineyards



DISCUSSION

The result of the survey was obvious that the Imidacloprid-untreated vineyards turned positive and Imidacloprid-treated vineyard was negative for *Xf*. Thus the treatment of Imidacloprid in late April was necessary to control the spread of PD in a vineyard. If vineyards were not treated in April, then *Xf* would be spread and cause substantial loss.

The organic vinyard owners are concerned about the use of non-organic insecticides. At this point there is no effective organic insecticide to control PD comparable to imidacloprid. We are searching for a nonrecombinant endophyte that can be approved for use to control *Xylella* in organic vineyards.

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FUNDING AGENCIES

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

BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPEVINE WITH BENIGN STRAINS OF *XYLELLA FASTIDIOSA* SUBSP. *PIERCEI*

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

ABSTRACT
This project involves the evaluation of the biological control of Pierce's disease (PD) with a strain of *Xylella fastidiosa* (*Xf*) that is benign to grapevine. The benign strain, EB92-1, will be evaluated in two vineyards in Southern California/Temecula that are hotspots for PD and in two vineyards in the Napa Valley. Different methods of utilization of the biocontrol strain are also being evaluated, including using cuttings from mature vines that are infected with the EB92-1 strain, injection of plants in the vineyard compared with those injected prior to transplanting, and injection of the rootstock, scion, or both.

INTRODUCTION
Pierce's disease (PD) of grapevine is a chronic problem for the California grape industry and has become more of a threat to the industry with the introduction of the glassy-winged sharpshooter (GWSS). The only feasible control for PD is resistance. Through 10 years of research on the biological control of PD of grapevine in Florida by cross protection with weakly virulent strains of *Xylella fastidiosa (Xf)*, we demonstrated that this also is a potential means of controlling this disease. The overall goal of this project is to develop a biological control system for PD of grapevine that would allow the production of *Vitis vinifera* (*V. vinifera*) in California and other areas where PD and GWSS are endemic.

OBJECTIVES
1. To evaluate strain EB92-1 of *Xf* subsp. *piercei* which has provided effective biocontrol of PD in previous greenhouse and vineyard tests in Florida for possible commercial application for the biological control of PD of grapevine in the vineyard in California.
2. To compare different methods of treatment with strain EB92-1 of *Xf* subsp. *piercei* for the biocontrol of PD in *V. vinifera* in the vineyard.

RESULTS
This project is being initiated. We are in the process of locating two test vineyards in the Temecula/Southern California area, where the PD is chronic and severe. We are also locating three test vineyards in the Napa Valley area. Plants for the test vineyards will be obtained this winter/early spring, injected with the biocontrol strain when new growth is two-three feet in length and transplanted into the vineyard in the spring of 2008.

Experiments to evaluate different methods of treatment with EB92-1 were established in the MREC vineyard in Apopka, Florida during the summer. Four treatments were applied to the cultivar Merlot/101-14 on May 29 and the plants were transplanted into the vineyard on June 21. The treatments were 1) injection of EB92-1 into the scion only, 2) injection of EB92-1 into the rootstock only, 3) injection of EB92-1 into both the rootstock and scion, and 4) nontreated. Five treatments were applied to the cultivar Chardonnay CL96/3309 on June 13 for the greenhouse treatments and on July 26 for the vineyard treatment. The plants were transplanted into the vineyard on July 3. The treatments were 1) injection of EB92-1 into the scion only in the greenhouse, 2) injection of EB92-1 into the rootstock only in the greenhouse, 3) injection of EB92-1 into both the rootstock and scion in the greenhouse, 4) nontreated, and 5) injection of EB92-1 into the scion only in the vineyard. In a third experiment, Chardonnay cuttings from the MREC vineyard were grafted onto Salt Creek rootstock rooted cutting from the vineyard. The grafted plants were transplanted into the vineyard on August 14. The treatments included 1) Chardonnay cuttings from mature vines that had been treated three years ago with EB92-1 on Salt Creek, 2) Chardonnay cuttings from mature nontreated vines on Salt Creek, and 3) Chardonnay cuttings from mature nontreated vines on Salt Creek, with the scion injected with EB92-1 in the vineyard on August 29.

CONCLUSIONS
The project was initiated in July. There are no conclusions to report.

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

EVALUATION OF GRAPEVINE ENDOPHYTIC BACTERIA FOR CONTROL OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2006 to September 2007.

ABSTRACT
In this reporting period we finished testing selected endophyte isolates for antagonism with our *in vitro* assay. Isolates of *Bacillus pumilus, B. subtilis, Pantoea sp.,* and *Pseudomonas sp.* were the most effective inhibitors. In this year's movement assays we identified one more isolate, *Kocuria* sp, that could move into grape petioles. We subsequently used this isolate as one treatment in the mechanical inoculation part of the 2007 biological control experiment. While Pseudomonas species were good inhibitors, none were capable of moving past the point of inoculation.

Results were obtained from two biological control experiments completed in December 2006. We found that our vines in the field, originally part of the 2003 biological control experiment did not retain endophyte protection. All vines developed severe Pierce's Disease (PD) symptoms when challenged with X*ylella fastidiosa* (*Xf*). In the 2006 biological control experiment, we tested the protective capabilities of 3 *Bacillus* sp. strains, 3 *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomona*s sp. After 15 weeks post challenge with *Xf*, all vines across all treatments developed PD symptoms and tested positive for *Xf* infection. Based on this experiment and previous research in our lab, results suggest that mechanical, rather than insect, inoculation may overwhelm endophyte protection.

Our biological control experiment established in 2007 evaluates five endophtytes for their protective abilities against *Xf* in the vine: *Kocuria* sp., *Bacillus subtilis, B. cereus, Curtobacterium flaccumfaciens*, and *Pantoea agglomerans*. Endophyte treatments were split into two groups, those challenged with *Xf* by mechanical inoculation and those challenged by infective blue green sharpshooters (BGSS). We mechanically inoculated with 10^4 *Xf* cell suspensions so as not to overwhelm endophyte treatments. Symptoms have been rated on a weekly basis to compare disease progression and petioles have been cultured to examine if any endophyte treatment is capable of reducing *Xf* concentrations in the vine. Initial results indicate that both mechanical and insect inoculations were efficient with no fewer than 11 out of 15 plants infected. Concentration of *Xf* in petioles is similar among endophyte treatments and controls.

INTRODUCTION
The environment inside grape vine xylem vessels is a distinct ecological niche that supports a sparse microbial community. *Xf*, the causative agent of PD, is one 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). Some of these bacterial endophytes are antagonistic against *Xf in vitro* and may be niche competitors within grapevine xylem. Our library includes endophytes, such as *Pantoea agglomerans* and several *Pseudomonas* sp., already tested as biological control agents in other crop systems (Stockwell et al., 2002, Barka et al., 2002). We have also isolated strains of *Curtobacterium flaccumfaciens*, which is potentially associated with the control of citrus-variegated chlorosis, caused by a genetically different strain of *Xf* (Lacava, et al, 2004). While several endophyte isolates were capable of inhibiting *Xf* growth in vitro, those able to systemically colonize the vine were rarer. Furthermore, our results suggest that *Xf* inoculated at high concentrations may out-compete endophytes and overwhelm any biological control they might provide. In this year's biological control experiment we sought to achieve lower concentrations of *Xf* in endophtyte treated vines by using infective BGSS to inoculate some groups of plants and use lower *Xf* concentrations for mechanically inoculated vines. We also hoped to achieve better endophyte colonization of vines by using multiple inoculations. 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.

a. prevention of infection.
b. suppression of Pierce's disease symptoms in greenhouse and field studies.
c. 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

Conclusion of Antagonism assays The optimized screening protocol developed in the last reporting period was more efficient and during this reporting period we concluded antagonism assays on our endophyte library. This library included bacteria isolated from vines used for the first biological control experiment in 2003, new isolates collected spring 2006 from escape vines in Napa, and isolates collected in 2000-2002. In total 124 new isolates were tested in this reporting period. Fifty eight of these showed some ability to clear or reduce *Xf* growth and 34 of these isolates were identified as *Bacillus subtilis* by 16S rDNA sequencing or morphology. Isolates of *Bacillus subtilis*, various other *Bacillus* species, *Pseudomonas* sp, and *Pantoea* sp. showed the largest zones of inhibition in the antagonism assays (Table 1).

Table 1. Summary of representative bacterial isolates screened in 2005-2007 showing some degree of *Xf* inhibition.

Endophyte Isolate #	Identification	Zone of clearing[a]	Endophyte Isolate #	Identification	Zone of clearing[a]
Average 38 isolates	*Bacillus subtilis*	2mm- complete inhibition	200	*Pseudomonas sp.*	complete
197	*Pseudomonas viridiflava*	20mm-complete	11	*Pantoea agglomerans*	rg 3-8mm
393	*Pseudomonas viridiflava*	rg over entire plate	37	*Pantoea agglomerans*	rg 1-2mm
403	*Pseudomonas syringae*	complete	4	*Pantoea sp.*	rg over entire plate
N37[c]	*Pseudomonas syringae*	complete	W157[b]	*Bacillus pumilus*	rg 6-10mm
205	*Pseudomonas sp.*	complete	843	*B. pumilus*	8mm rg
329	*Pseudomonas sp.*	complete	139	*Bacillus sp.*	rg 10-15mm
168	*Paenibacillus sp.*	4mm rg	473	*Stenotrophomonas sp.*	4mm rg
177	*Paenibacillus sp.*	5mm			

[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.
[c] "N" indicates an isolate collected from escape vines in Napa spring 2006
rg = reduced growth in these areas, ie. *Xf* colonies aren't cleared but are much smaller compared to controls

Assessment of endophytes' ability to colonize and move systemically in grape xylem Isolates used in the 2007 movement assays included, *Bacillus megaterium*, three isolates of *Curtobacterium sp.*, two isolates *B. Subtilis*, two isolates of *Pantoea agglomerans*, *Stenotrophomonas* sp., *Bacillus pumilus*, *Pseudomonas syringae*, *Kocuria* sp., *Bacillus cereus*, and *Paenibacillus* sp (Table 2). Two Chardonnay vines per isolate were inoculated at two places on the stem near the third or fourth internode, reinoculated three days later, and then allowed to grow in the greenhouse. After seven weeks, movement past the point of inoculation (POI), was measured by culturing stem sections onto solid media. Four of the isolates were rifampicin resistant mutants: one isolate of *B. subtilis*, one isolate of *P. agglomerans*, one isolate of *Curtobacterium flaccumfaciens* and *Bacillus cereus*. All isolates maintained high concentrations at the POI and 8 out of 14 isolates moved up to 10cm past the POI. Only W218, *Bacillus subtilis* and *B. cereus* were able to colonize up to 30cm, and only D753, *Kocuria* sp., was capable of colonizing the petiole. We hypothesize that 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. However, endophyte colonization may not be consistent throughout a plant and may be affected by health and developmental stage (Rosenblueth and Martínez-Romero, 2006). Our Chardonnay vines used for this experiment were three years old and inoculated at the end of the dormant period. All of these factors could have restricted endophyte growth.

Table 2. Summary of isolates in movement assays completed in 2007.

Isolate	Identification	POI cfu/ml	Petiole[a] cfu/ml	10cm cfu/ml	30cm cfu/ml
N6	*Curtobacterium sp.*	8.74×10^5	0	9.15×10^2	0
W94-rif	*Curtobacterium flaccumfaciens*	9.00×10^4	0	3.30×10^3	0
D753	*Kocuria*	1.41×10^5	6.99×10^3	6.15×10^3	0
W121-rif	*B. cereus*	3.70×10^4	0	3.00×10^2	2.00×10^2
W218	*B. subtilis*	2.54×10^5	0	1.34×10^5	4.00×10^3
D843	*B. pumilus*	5.03×10^5	0	1.40×10^4	0
W127	*Pantoea agglomerans*	3.20×10^4	0	1.38×10^4	0
D37	*Pantoea agglomerans*	1.60×10^6	0	8.00×10^1	0

[a] First leaf petiole up from the POI.

Assessing Biological Control of Endophytes Against *Xf*.

Results of the biological control experiment for 2006 Seven groups of 15 Thompson seedless grapevines each were inoculated with different endophyte treatments to evaluate these isolates as potential biological control agents against *Xf* (Table 3). These isolates were chosen based on prior movement assays and antagonistic ability *in vitro*. We tested the protective capabilities of three *Bacillus* sp. strains, three *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomonas* sp. Endophytes tested are summarized in Table 3. Seven weeks post endophyte treatment, vines were inoculated with a 10^8 cfu/ml suspension of Stagg's leap strain (STL) of *Xf*. Pierce's disease symptoms rated at 15 weeks and 18 weeks post inoculation showed that across all treatments, vines developed similar symptom severity as compared to the non-endophyte control.

Although endophyte treatments in this experiment were not effective control against Pierce's disease, it is possible that a 10^8 cfu/ml cell suspension may overwhelm any protective effect that the endophytes might provide. While it is not known exactly how many *Xf* cells an infective insect introduces into the plant, populations as high as 10^5 cells have been cultured from a single BGSS head (Hill and Purcell, 1994). A cell suspension of 10^8 cfu/ml, or 10^6 cells in a 20µl drop, is far more cells than an insect would transmit to plants. Secondly, it is possible that endophyte protection was limited because isolates tested in the biocontrol experiment had not fully colonized the plant. Thompson seedless plants used for this experiment were, on average, one-two meters tall, and seven weeks was not enough time for these isolates to completely move up the vine. Plant defense response to pinprick injury may also slow endophyte colonization.

Table 3. Summary of results for 2006 Biocontrol Experiment

Endophyte treatment	Identification (16S rDNA)	*Xf* Infected Vines	Average severity rating 15 weeks	Average severity rating 18 weeks
147	*Bacillus subtilis*	15/15	2.1	3.2
100	*Bacillus subtilis*	13/15	2.7	3.6
169	*Bacillus subtilis*	14/15	2.8	4.0
329	*Pseudomonas* sp.	15/15	2.0	3.4
197	*Pseudomonas viridiflava*	15/15	2.8	3.8
329/197	*Pseudomonas* sp./ *P. viridiflava*	15/15	2.4	3.7
Control	*No endophyte*	14/15	2.0	3.1

Continuing evaluation of biocontrol experiment initiated in 2003 During this reporting period we finished an experiment that continued evaluation of vines growing in the field that were originally part of a biological control experiment in 2003. In the 2003 experiment, Cabernet Sauvignon vines were inoculated with six endophyte treatments and challenged with infective BGSS. Vines with significantly lowered symptom severity were planted in the field. We wanted to determine if, after two years in the field, these vines were still protected against Pierce's disease. In spring of 2005, propagated bud wood cuttings from these vines were challenged via mechanical inoculation with Stagg's leap strain *Xf*. Symptom rating at 14 weeks showed that all vines inoculated with *Xf* had developed severe symptoms and these propagated cuttings showed no continued protection against Pierce's disease. Again, the concentrated *Xf* inoculum may have obscured possible endophyte effect. However, given the diversity of genera isolated from these same vines during fall 2005, we know the endophyte community has changed since these vines were originally used in the biocontrol experiment. Protection effected by the original endophyte treatment could have been diluted or inactivated.

Biological control experiment 2007 In this year's biological control experiment, we wanted to make sure that endophytes fully colonized test vines and that *Xf* was inoculated in a lower concentration such that potential endophyte protection would not be overwhelmed. We also wanted to compare potential endophyte protection between mechanically inoculated vines and vines inoculated with *Xf* infective insects. Five endophyte isolates were chosen for this year's biological control experiment: *Pantoea agglomerans* (D11), *Bacillus subtilis* (D147), *Curtobacterium flaccumfaciens* (W94), *Bacillus cereus* (W121), and *Kocuria* sp. (D753). Both isolates W121 and W94 were spontaneously generated rifampicin resistant mutants.

To achieve better colonization, endophytes were pin-prick inoculated into grapevines, first at the third internode and then at 15cm intervals up the stem. After seven weeks, one group of endophyte treated vines was mechanically inoculated with STL *Xf* and one group was inoculated with infective adult BGSS, *Graphocephala atropunctata* with the assistance of the Almeida lab at UC Berkeley. We tried to avoid overwhelming endophytes with high concentration of *Xf* in the mechanical inoculations by inoculating with 10^5cfu/ml instead of 10^8cfu/ml. Unfortunately culturing confirmed that we had inoculated with only a 10^4cfu/ml suspension which we felt was too low. Given these results we reinoculated vines three weeks later. Again the concentration was too low, 10^4 cfu/ml, and we inoculated a last time and achieved a cell concentration of 10^5 cfu/ml. In summary, vines were inoculated three times with STL *Xf* over a seven week period. Insect transmission was achieved with four infective BGSS per plant that were allowed to feed on endophyte treated or control vines for four days. First symptoms on all vines were rated eight weeks after the first inoculation.

Table 4 indicates endophyte treatments and preliminary results for the 2007 biological control experiment. Insect transmission was efficient although symptoms in these vines are developing more slowly that in mechanically inoculated vines. Unfortunately, mechanically inoculating three times, even with low concentrations, may have introduced too many *Xf* cells. Petioles cultured at 12 weeks post inoculation across all treatments contain similar concentrations of *Xf*. Symptom ratings will continue through November and *Xf* will be isolated and quantified from petioles a second time. Surviving vines will be cultured to determine, if possible, the concentration of endophytes in the vine.

Table 4. Endophytes tested and preliminary results for 2007 Biocontrol Experiment

Endophyte treatment	Identification	Vines infected BGSS inoculated	Vines infected mechanically inoculated	Average *Xf* concentration petiole BGSS inoculated	Average *Xf* concentration petiole mechanically inoculated	Average rating 12 weeks post BGSS inoculation	Average rating 12 weeks post first mech. inoculation
D147	*Bacillus subtilis*	11/15	15/15	$1.62x10^7$	$4.83x10^7$	1.1	2.2
D11	*Pantoea agglomerans*	13/15	15/15	$1.21x10^7$	$1.04x10^7$	1.2	2.4
W121-rif	*B. cereus*	14/15	14/15	$1.38x10^7$	$4.41x10^6$	1.4	2.1
W94-rif	*Curtobacterium flaccumfaciens*	14/15	15/15	$2.32x10^7$	$5.80x10^7$	1.3	2.4
D753	*Kocuria sp.*	---	14/15	---	$4.66x10^6$	---	2.1
Positive control	Water inoculated	13/15	13/13	$9.49x10^7$	$5.35x10^7$	1.5	2.8
Negative Control	Water inoculated endophyte, no *Xf*	---	8/8	---	0	---	0

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

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ABSTRACT
We are attempting to characterize the biochemical factors that mediate the cold curing phenomenon of grapevines infected with *Xylella fastidiosa (Xf)*. We are working towards completing project objectives that were initiated in July of 2004. To better understand the cold therapy phenomenon, we examined Pierce's Disease (PD) disease severity, curing rates and biochemical changes in control and *Xf* infected Pinot Noir and Cabernet Sauvignon grapevines grown in four locations in Northern California and four cold chamber temperatures. After the cold treatments, xylem sap was extracted using a pressure bomb and the sap analyzed for pH, osmolarity, abscissic acid (ABA), glucose, sucrose, fructose, calcium and magnesium ion concentrations. Differences between varieties and between temperature treatments were observed. In the field and cold chamber experiments, pH and osmolarity of xylem sap from cold treated vines was lower than what is found in PD3 culture medium used to grow *Xf*. PD severity was lowest and curing rates were highest for the coldest temperatures.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes (PD) in grapevines. PD is currently found in many regions of California and the southern United States. 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. For example, PD does not occur in New York, the Pacific Northwest or at high altitudes in South Carolina, Texas and California where the winter temperatures on average drop below 0 degrees Celsius (Hopkins & Purcell, 2002). 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' (CS), were cured by cold therapy treatments compared to susceptible varieties such as 'Pinot Noir' (PN). 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 Pierce's disease affected grapevines without unacceptable plant mortality.
2. Analyze chemical changes such as pH, osomolarity, total organic acids, proteins and other constituents that occur in 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 osomolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

Objective 1
All experimental work used PN (PD-susceptible) and CS (moderately resistant to PD) grapevines, the same varieties used by Purcell (1977, 1980) and Feil (2002) in their previous cold therapy studies.

Temperatures inside of the growth chambers were recorded using HOBO data loggers (Onset Computer Co. Borne, MA). In addition, temperature data is also being monitored by sensitive chart recorders on the cold chambers and by adjacent weather stations at all of our field plots. In the -5C cold chamber, custom thermocouplers were made and inserted into the xylem of 10 grapevines to determine if there were differences between the ambient chamber temperature and the internal xylem temperature. No significant differences in xylem temperature were detected.

To evaluate the cold curing phenomenon under different field temperatures we established the following experiment. Twenty-two vines of Cabernet Sauvignon and twenty-two vines of Pinot Noir for each of the four sites (176 vines total), were grown during the spring and summer in five gallon pots at UC Davis. One half of the vines were mechanically inoculated with *Xf* in June, the other half were mock inoculated with water for comparison and proper statistical analysis. In 2005-2006 (1st replicate) and 2006-2007 (2nd replicate), the vines were transported to the four field sites (in order of warmest site to coldest site), UC Davis (Yolo County), UC Hopland Research Station (Mendocino County), McLaughlin Reserve (Lake County) and Foresthill (Placer County) with the onset of cool fall temperatures in early November. In the 2005-2006 season, dormant canes were collected from the vines in February and April, xylem sap was extracted using a custom-made pressure bomb, and the expressed sap was subjected to the tests described in the objectives below. The vines were returned to UCD in the spring and planted in the field. The vines were then rated for PD symptoms and the presence of *Xf* in the fall. (The 2005-2006 results can be found in the 2006 Pierce's Disease Symposium Proceedings). Disease ratings were the lowest and percent curing was the highest in the coldest field locations (Table 1).

Grapevines, using the same varieties and inoculated as described for the field studies but grown in six" standard pots, were exposed to different temperature regimes in cold rooms located at the Department of Pomology, UC Davis during the winter months. Plants were subjected to one of four temperature regimes.

Regime 1: -5°C Regime 2: +0°C
Regime 3: +2.2°C Regime 4: +5°C

There were 40 plants per treatment regime, 10 *Xf*-inoculated plants and 10 control plants for both varieties (160 plants total). After three months of treatment, xylem sap was extracted from the plants, and the vines were planted in the Plant Pathology field at UC Davis. Late in the summer, the plants were evaluated for PD symptoms to determine the most effective temperature regime for curing PD without causing unacceptable plant mortality. Supporting the findings of the field study, disease ratings were the lowest and percent curing was the highest in the coldest cold room temperature regimes for both replicates (Table 1).

Table 1. PD-cured plants compared to the number of *Xf*-inoculated grapevines that survived until spring after winter chilling at the field locations or after spending 3 months in the cold chambers.

	Field Locations				Cold Room			
	Davis	Hopland	McLaughlin	Foresthill	+5C	+2.2C	0C	-5C
Pinot Noir	3/11	9/11	8/11	8/11	3/9	3/10	4/10	6/6
Cabernet Sauvignon	1/11	8/11	7/11	10/11	0/9	4/10	5/10	7/7

Objective 2

As described in the 2005 and 2006 PD Symposium Reports, we conducted similar analyses on canes collected from our field sites as well as the vines that were placed at various cold temperatures in growth chambers. Each potted vine was sampled in February and in April when the potted vines were returned and planted at UCD. The volumes of xylem sap that were expressed from individual canes from the potted 1-year old vines used in the field and growth chamber experiments were small, ranging from 1 to 200ul/cane. Our hypothesis is that changes in the pH and osomolarity of xylem sap in vines that undergo cold treatment may have significant effects on *Xf* viability. The results of this study show that the pH of xylem sap from both cold chamber and field cold treated vines is lower than culture media used to grow *Xf* (Table 2). Osmolarity of PD3 media is 113 mmol/kg in comparison to the osmolarity of xylem sap, 25-45 mmol/kg. For the cold chamber experiments the pH of CS xylem sap was significantly higher than PN sap overall (Table 3). Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in –5C cold chamber vines, whereas Ca+ levels were greater in the warmest treatments. Osmolarity was greatest in the coldest treatments and decreased with increasing temperature. Conversely, in PN grapevines, glucose and fructose levels were the lowest in the coldest treatments. Ca+ levels showed a similar trend with CS vines, with increased Ca+ levels in the warmer temperature treatments. Temperature appeared to have a less direct effect on osmolarity in Pinot Noir grapevines.

We also determined the relative water content of canes from cold-stressed and UC Davis vines by measuring the fresh weight of the canes, dehydrating them in an oven, and measuring the dry weight. Freezing temperatures are known to dehydrate plant tissue and this dehydration could affect the ability of *Xf* to overwinter in xylem tissue. No significant differences between fresh and dry weights were found.

Previous research on a number of plant species has shown that several plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998), and in some plants these low temperature-induced proteins are structurally

related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related (PR) proteins (Kuwabara, et al, 2002). Thus it may be possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap was extracted from healthy and *Xf*-inoculated, cold-stressed and control CS and PN vines using the pressure bomb, proteins were concentrated by cold-acetone precipitation, and protein profiles determined by 1-dimensional polyacrylamide gel electrophoresis (PAGE). We found that good PAGE profiles were obtained by concentrating 150ul of sap to 30ul of sample each sample. Thus we are able to have multiple PAGE profiles from any particular temperature/variety/disease that can be compared to healthy control vine sap. While most of the proteins were similar for the various temperatures, a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants and these proteins were end terminally sequenced by the UCD Molecular Structure Facility. The potential effect of these unique proteins on *Xf* viability will be assessed in Objective 3.

Table 2. pH of grapevine xylem sap collected from cold chamber treated vines.

pH	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	5.94	5.45	5.78	6.00
Cabernet Sauvignon	6.31	5.84	5.86	5.78

Table 3. Osmolarity of grapevine xylem sap collected from cold chamber treated vines.

Osmolarity	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	85.0	39.5	52.7	34.7
Cabernet Sauvignon	61.9	39.5	44.8	31.6

Objective 3

In this objective we assessed the effect of many of the physical, physiological and biochemical parameters we determined in Objectives 1 and 2 on *Xf* viability *in vitro*. In 2004-2005 we assessed the effect of various buffers on the viability of *Xf* cells *in vitro* using media such as PD3 and various buffers such as sodium phosphate and potassium phosphate. *Xf* cells were placed in potassium phosphate buffer with the pH of: 5.0, 5.4, 5.8, 6.2, 6.6 and 6.8 to assess the effect of pH on the survival of *Xf*. Viability was assessed by plating the exposed cells on PD3 medium. Results of these studies are presented in the 2004, 2005 and the 2006 PD Research Symposium Reports Interestingly, the osmolarity of PD3, a common media used for growing *Xf*, is approximately 113mmol/kg, whereas the osmolarity of dormant xylem sap averages between 25-60 mmol/kg. This suggests that *Xf* is able to survive at various osmolarities.

Objective 4

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as ABA, that induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998;). Some of these cold-shock proteins have been shown to inhibit the growth of certain fungal pathogens (Kuwabara, et. al 2002), but no work has been done on their effect on bacterial pathogens. These same cold-shock genes can be induced under non-freezing temperatures by the exogenous application of ABA (Kuwabara, et al. 2002).

We determined the concentration of ABA in cold-stressed and control vines from the four field sites and the cold room experiments. ABA concentration was determined using an immunological assay (Phytodetek ABA Test Kit, Agdia) that has a sensitivity approximately 0.0064-0.16 picomoles ABA/ml and only requires a small volume of sap. We found that ABA concentrations in the April xylem sap collections were the lowest in the coldest field locations. ABA levels were higher in the February sap collections than in the April collections for the field locations. ABA concentrations in the spring xylem sap collections were the lowest in the coldest cold chambers.

In 2005 CS and PN grapevines were grown in one-gallon pots in the screen house to determine the effect of applying exogenous ABA on the development and/or severity of PD. At the suggestion of Sue Abrams, an ABA expert at Plant Biotechnology Institute, National Research Council Canada, we contacted Valent Bioscience Corporation who has an active research and development program on the use of ABA on agricultural crops. We met Valent representatives at UCD and described our proposed research. They were interested in the project and agree to provide us with two types of ABA, one of which (VBC-30030) is a proprietary material. One set of treatments used regular ABA (VBC-30054) as a spray (1000ppm) and a soil drench (100ppm). The second material (VBC-30030) is an ABA analog that persists longer (1 week verses 1 day) and is more active than VCB 30054. VBC-30030 was used as a spray (100ppm) and soil drench (10ppm). There were 16 healthy and 16 *Xf* -infected Cabernet Sauvignon and 16 healthy and 16 *Xf*-inoculated Pinot Noir vines used in each of the treatments plus a set used as untreated controls.

For the 2006-2007 season, vines were prepared as described above and were subjected to slightly different applications. One of the treatments used VBC-30054 as a spray at 2000ppm at one week intervals for 3 weeks. The other treatments were the same as described for the 2005-2006 season. VBC-30054 was applied as a drench at 100ppm. VBC-30030 was applied as a

drench at 10ppm. A set of plants of unsprayed plants are being used as the untreated control to allow for meaningful comparisons.

Vines were inoculated with *Xf* in June using a standard pinprick inoculation method (Hill and Purcell, 2000) and the presence of *Xf* infection confirmed by IC-PCR later in the summer. ABA treatments were applied in November when the vines still had leaves but ambient temperatures were cooling off. The pH, osmolarity, and proteins profiles of xylem sap extracted from the treatments will be determined as described above in Objective 2. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced as previously described. PD symptoms will be rated in late summer and *Xf* infection, or lack thereof, will be confirmed by IC-PCR.

CONCLUSIONS

This study begins to document some of the biochemical/physiological changes that occur within control and *Xf*-inoculated grapevines that are exposed to various cold temperatures and attempts to better understand the cold curing phenomenon.

This study has documented that some of the temperatures examined in this study are able to induce cold curing of Pierce's disease-infected grapevines and cause significant changes in the chemistry of the xylem sap. Further studies could potentially utilize the associations between biochemical changes documented here and PD-curing to induce their expression under non-freezing temperatures and provide a novel approach for managing Pierce's disease.

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INHIBITION *OF XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

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ABSTRACT
Polygalacturonases (PG) (EC 3.2.1.15), catalyze the random hydrolysis of 1, 4-alpha-D-galactosiduronic linkages in pectate and other galacturonans. *Xylella fastidiosa* (*Xf*) possesses a single PG gene, *pglA* (PD1485) and *Xf* mutants deficient in the production of PG result in lost pathogenicity and a compromised ability to systemically infect grapevines. We have cloned the *pglA* gene into a number of protein expression vectors and a small amount of active recombinant PG has been recovered, unfortunately most of the protein expressed is found in inclusion bodies in an inactive form. The goal of this project is to use phage panning to identify peptides that can bind to and inhibit *Xf* PG. Once peptides are discovered that can inhibit PG activity in vitro these peptides will be expressed in grapevine root stock to determine if the peptides can provide protection to the plant from Pierce's disease.

INTRODUCTION
Polygalacturonases (PG) have been shown to be virulence factors of a number of plant pathogenic bacteria including *Ralstonia solanacearum, Xanthomonas campestris*, and *Erwinia carotova* (Huang and Allen 2000; Dow et al. 1989; Lei et al. 1985). *Xylella fastidiosa (Xf)* possesses a single PG gene *pglA* (PD1485), and mutation of this gene results in lost pathogenicity and reduced ability to systemically infect grapevines (Roper et al. 2007). In order for *Xf* to systemically infect a grapevine it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and *Xf* PG allows the bacterium to breakdown the pectin in these membranes. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress *Xf* PG activity thus limiting the ability of *Xf* to spread systemically through grapevines and cause Pierce's disease (PD).

To accomplish this we will use phage display of a random dodecapeptide library attached to the coat protein gp38 of M13 phage in a phage panning experiment using active recombinant *Xf* PG as the target. After three rounds of panning, phage that show a high binding affinity for *Xf* PG will be screened for their ability to inactivate PG activity in vitro in reducing sugar assays. Once a suitable inhibitory peptide is discovered it will be cloned into an *Agrobacterium* binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with *Xf* and compared to non transgenic plants in PD symptom progression. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock and see if they can also provide resistance to grafted scions.

OBJECTIVES
1. Isolate a sufficient amount of biologically active *Xf* polygalacturonase enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phage that possess high binding affinities to *Xf* PG from a M13 random peptide library.
3. Determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to *Xf* PG can inactivate PG activity in vitro.
4. Clone anti-*Xf* PG gp38 protein into an *Agrobacterium* binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson Seedless grapevine.
5. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.
6. Mechanically inoculate transgenic plants with *Xf* and compare PD development with inoculated, non-transgenic control plants.

RESULTS
Objective 1. Currently we have obtained a small amount of active recombinant *Xf* PG, however, most of the expressed protein is in the form of insoluble and inactive inclusion bodies. Fortunately it is possible to measure the activity of the small amount of active PG that we have obtained by using visible spectrum reducing sugar assays such as the dinitrosalicylic acid (Figure 1) (Wang et al. 1997, Sumner 1921) and 3-Methyl-2-benzothiazolinonehydrazone methods (Anthon and Barrett 2002, Honda et al. 1981). This is a significant improvement over the tedious High-performance liquid chromatography (HPLC) assays that were previously used to demonstrate *Xf* PG activity (Roper et al. 2007). These methods should also be appropriate for the PG-inhibition assays once we have determined a suitable candidate peptide, however, we have yet to

produce enough active PG to use as a target for panning experiments. We are currently exploring different expression systems and refolding conditions in order to obtain enough active protein for the panning experiments.

Objectives 1-6. It will be necessary to obtain a significant amount of active *Xf* PG in order to carry out the panning experiments before the rest of the objectives can be completed. Fortunately the panning procedure will benefit from the experiences of Prof. George Bruening and Project Scientist Paul Feldstein who have used the phage display system extensively in their own research.

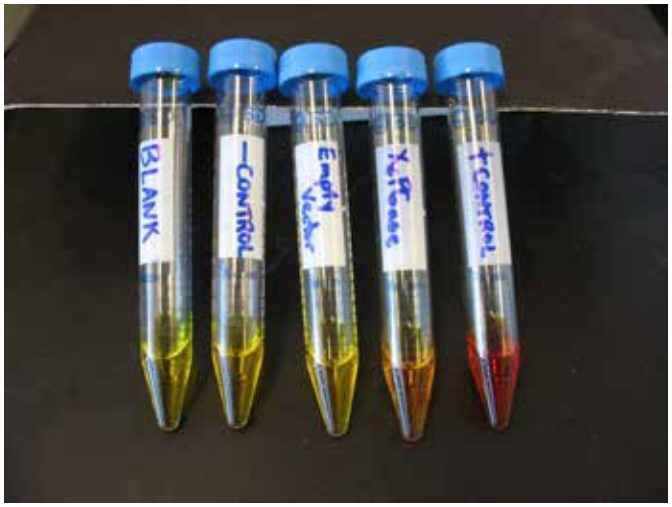

Figure 1. Dinitrosalicylic acid reducing sugar assay showing activity of *Xylella fastidiosa* Polygalacturonase (PG) versus positive control (commercial PG from *Aspergillus niger*) and empty vector negative controls.

CONCLUSIONS

We have made good progress thus far in finding suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different expression systems and continue to test different refolding protocols with the hopes of generating enough active PG to begin the phage panning experiments. Once a peptide is found that inhibits PG activity in vitro we can then transform grapevines with the peptide and determine if they provide plants with resistance to PD.

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

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Reporting Period: The results reported here are from work conducted October 2006 to September 2007.

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. We divided *Xf* HAs into smaller domains and generated antibodies against 3 possible adhesion domains (ADs) responsible for cell-cell and/or cell-surface binding. The abs were used in Western blot analysis and determined that HxfB was approximately 220kDa while HxfA was slightly larger. Western blot analysis showed that both HAs are secreted into the culture supernatant as well as being found in the bacterial outer membrane. Monomeric Fab fragments against AD1-3 and AD4 of HxfB reduced cell-cell aggregation when added to *Xf* cells growing in liquid culture.

Xf HxfB will be expressed in transgenic tobacco and grapevines where we hope the protein 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) hemagglutinins (HAs) are large secreted proteins 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* (1). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (1). The premise of this research is to determine whether over-expressing *Xf* HA adhesion domains in the xylem by transformation of grapevines, the HA will act as a "molecular glue" to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce's disease (PD).

Because of the large size of the HA genes (10kb), it is difficult to 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 should contain the cell-cell AD. Recombinant proteins derived from these fragments were expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera was 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 could later be transformed into plants.

OBJECTIVES
1. **a.** Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA) to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
   b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.
   c. Prepare an affinity column using HA domain antibodies and isolate native *Xf* HAs from culture cells. Establish the identity of affinity purified, putative HAs by N-terminal sequencing.
   d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.
   e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.
2. **a**. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.
   b. Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein.
   c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.
3. **a.** Transform tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.
   b. Mechanically inoculate HA-transgenic tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in transgenic tobacco with non-protected controls.

RESULTS

Objective 1a. Determination of native size and location of *Xf* HA in *Xf* cultured cells and grapevines by Western blot analysis using AD2 antibodies. Western Blot analysis with AD2 abs using a variety of protein samples showed a faint band of ~220kDa corresponding to HA. But the signal was very weak and there were non-specific interactions between other *Xf* cellular proteins and AD2 abs (2). Objective 1a was repeated using higher quality abs (see objective 2b).

Objective 1b. Determine if AD2-antibodies can facilitate clumping of *Xf*. Due to the low quality of AD2 abs, there was no difference in *Xf* cultures treated with AD2 abs versus cultures treated with preimmune serum (2). Objective 1b was repeated using higher quality abs (see objective 2c).

Objective 1c. prepare affinity column using HA domain antibodies to isolate native HAs from culture cells. Since HAs are secreted into the medium (see objective 2b), concentrated culture supernatant was used to isolate native HAs. Antibodies against AD1-3 and AD4 (see objective 2a) were bound to a Pierce protein A column and 1l of culture supernatant was concentrated and passed through the column according to the manufacturer's instructions. Neither denatured nor native supernatant protein samples interacted with the abs bound to the column and no HAs could be eluted from the column. Additional immunocapture strategies are being evaluated.

Objective 1d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells. In order to determine if HA fusion proteins can bind to *Xf* cells, ELISA plates have been coated with AD1-3 or AD4 fusion protein (see objective 2) and incubated with Wt, HxfA and HxfB mutant cells. Results of this one experiment, which needs to be repeated, suggests the denatured HA AD fusion proteins did not bind to and trap *Xf* cells. This suggests that the whole HA protein may be needed for binding to cells or that only cell bound HA has effective binding properties or that AD1-3 and AD4 did not contain the cell binding domain.

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

It has been shown for FHA, the filamentous hemagglutinin of *Bordetella pertussis* (3) 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 HxfB (1) and RGD (Arg-Gly-Asp) sites at position 2780 in HxfA and at positions 1805 and 3103 in HxfB were found (Figure1). The RGD site in FHA of *Bordetella pertussis* mediates binding of *B. pertussis* to lung epithelial cells (4).

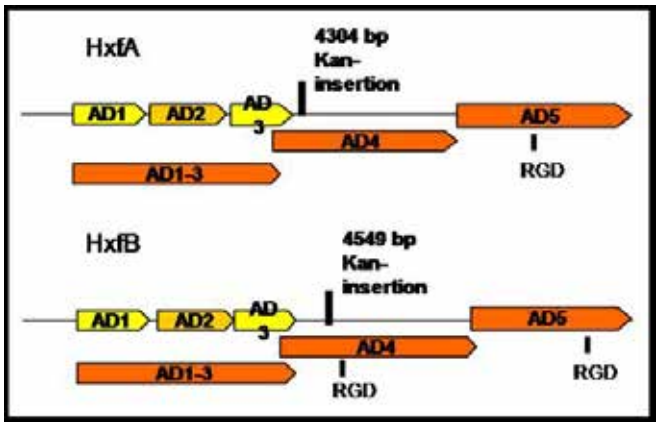

Figure 1. Identification of putative *Xf* HA adhesion domains (ADs) based on data base analysis. Antibodies against AD1-3 and AD4 of HxfB were generated and used in Western blot analysis.

Data base analyses led us to divide HxfA and HxfB into 3 domains named AD1-3, AD4 and AD5, each for HxfA and HxfB (Figure 1). All 6 ADs were cloned into the protein expression plasmid pet30b, the identity and integrity of the amplified fragments was verified by sequencing and the constructs were transformed into *E.-coli*. AD1-3 and AD4 of HxfB were expressed to high levels and the integrity of the affinity purified proteins was verified by sequencing.

Objective 2b. Prepare rabbit polyclonal antibodies against each HxfA/B domain fusion protein. Purified AD1-3 and AD4 antigen were each injected into two rabbits and sera obtained. In contrast to AD2 (objective 1), indirect ELISA showed that high titer abs were obtained in all injected rabbits.

Determine the native size and location of *Xf* HA in *Xf* cultured cells using AD1-3 and AD4 antibodies. Outer membrane proteins isolated from Wt, HxfA and HxfB mutants by sucrose gradients (protocol of Michele Igo) revealed that HxfA and HxfB are inserted into the outer membrane of *Xf*. Both HAs are ~220kDa with HxfA being slightly larger (Figure 2). In Wt samples a doublet of bands is detectable since the AD4 antibodies can detect both HxfA and HxfB. Supernatants of *Xf* cultures concentrated by PEG and extracted using phenol/methanol also revealed bands of 220kDa (Figure 2). HxfA is again slightly larger than HxfB. This shows that HAs are not only inserted into the outer membrane, but also are secreted into the culture medium.

To evaluate if HAs are also secreted into the xylem of infected grapevines, xylem sap was obtained by using a pressure bomb (5). The proteins were purified as described for supernatant proteins and subjected to Western blot analysis. No bands corresponding to *Xf* HAs could be detected in either Wt or HA mutant cells although other xylem proteins were detectable on a coomassie blue stained control gel.

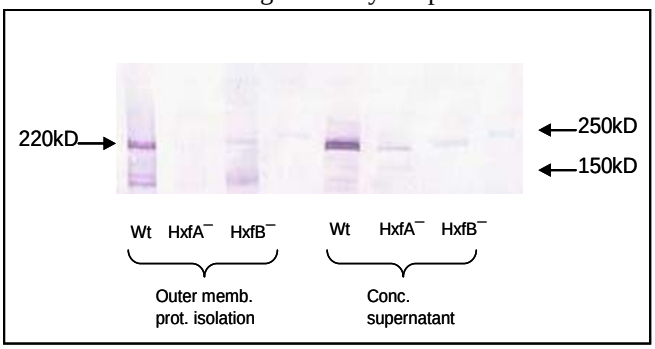

Figure 2. Western Blot analysis using AD4 abs shows that HAs are both inserted into the *Xf* outer membrane and secreted into liquid medium. HxfA is approximately 220kDa and slightly larger then HxfB.

Objective 2c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium. Incubation of *Xf* cells with AD1-3 and AD4 abs resulted in an increase of clumping due to cross linking of the cells. To identify the ADs on the HA protein that are responsible for cell-cell aggregation, monomeric Fab fragments were generated from AD1-3 and AD4 IgG. IgG was purified using protein A column chromatography and digested with papain. The Fab fragments were purified by protein A column chromatography and eluted from Tris/HCl gels. Monomeric Fab fragment, which contains the HA antigen recognition site was used in the clumping experiments. We would expect to see a decrease in clumping in the *Xf* cultures treated with Fab fragment if the abs were made against a possible HA cell-cell binding domain. We observed a decrease in clumping after inoculation of various amounts of both Fab fragments which suggests that the cell binding domains reside in AD1-3 and AD4 (Figure 3).

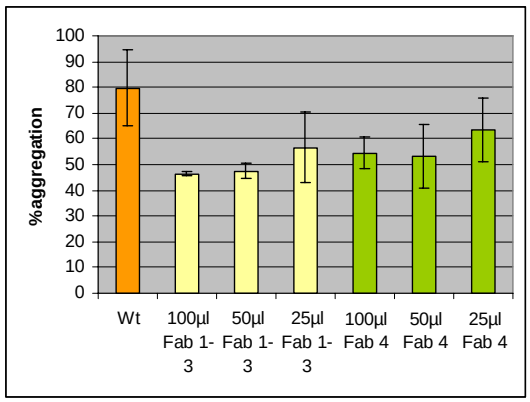

Figure 3. Blocking of cell-cell aggregation by adding of Fab fragments against AD1-3 and AD4 suggests that cell-cell binding domains are contained in AD1-3 and AD4.

Objectives 3 and 4 Our data suggests that large portions of HA protein will be needed for efficient clumping of *Xf* cells. Therefore we will try to transform plant hosts (tobacco and grapevines) with a DNA fragment containing both AD1-3 and AD4. Arrangements with the Plant Transformation facility on the UC Davis campus have been made and plasmids needed for transformation have been obtained from the Dandekar lab. These plasmids will be used to fuse the pear signal peptide pGIP to the HA fragment which will direct the HA fusion protein into the xylem using a method called splicing by overlap extension. This method was successfully used by the Dandekar lab to transform grapevines (6).

Additional work not included in original project objectives: HAs contain an N-terminal secretion domain for secretion mediated by bacterial typeV secretion (two-partner secretion pathway, TPS). In silico analysis identified a putative β-barrel forming secretion partner of HAs that is inserted in the outer membrane (PD1933) and we created knock-out mutants (insertion and deletion) of PD1933. These mutants were grown and supernatant proteins were isolated as described in objective 2b. The samples were used in Western blot analysis for detection of secreted HAs. No HAs were detected in the supernatant of mutant cells, whereas HA proteins were found in wt cells. This indicates that HAs in *Xf* are secreted via the TPS pathway and that PD1933 represents their secretion partner.

We are also in process to generate a HxfA/HxfB double mutant with a chloramphenicol cassette obtained from Ayumi Matsumoto (Michele Igo).

CONCLUSIONS

By generating high quality abs we could show that *Xf* HAs are processed upon or during secretion because the predicted size of the ~10kb HA gene product (365 kDa) was not be detected, instead proteins of approximately 220kDa were identified. We also showed that *Xf* HAs are inserted into the bacterial outer membrane as well as secreted into liquid medium. Since the isolation of native HAs using HA abs in affinity column chromatography was not initially successful, we will now use FPLC to isolate native HAs for additional experiments. Native HAs will be sequenced to identify the processing/cleavage site. The cell clumping experiments using Fab fragments suggest that cell-cell binding domain(s) reside in AD1-3 and AD4. Therefore a portion of the HA gene containing AD1-3 and AD4 will be expressed in 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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NATIVE SECRETION SYSTEMS FOR THE GRAPEVINE ENDOPHYTE *PANTOEA AGGLOMERANS* USEFUL FOR THE DELIVERY OF ANTI-*XYLELLA* EFFECTOR PROTEINS

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ABSTRACT
Symbiotic control is a strategy to deliver anti-pathogen effectors to plants or their insect vectors by modifying naturally occurring symbiotic bacteria. In order to deliver these effectors, secretion systems must be developed, especially for Gram – negative bacteria that contain an inner and outer cell membrane. We report here the development of secretion systems based on secreted proteins isolated from the grapevine endophyte, *Pantoea agglomerans* (*P. agglomerans*).

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

One way to deliver anti-*Xf* protein factors from symbiotic bacteria is by secretion. Secreted anti-*Xf* 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.

We report here the evaluation of three proteins secreted from the grapevine bacterial symbiont *P. agglomerans* for use as secretion partners of anti-*Xf* protein effectors.

OBJECTIVES
1. To create a system to secrete anti-*Xf* effector proteins from the grapevine symbiont, *P. agglomerans* based on one or more of its native secreted proteins.

RESULTS
While we have been successful in secreting a wide variety of proteins using the *Escherichia coli* (*E. coli*) hemolysin system, not all proteins secreted in this way have proved to be functional. We set out to develop a secretion system specifically for *P.* (=*Enterobacter*) *agglomerans*. To do this we first identified several major secreted proteins of *P. agglomerans*. We collected spent medium from log-phase cultures of *P. agglomerans* and subjected them to 2D electrophoresis. We were able to separate over 20 spots and picked each one for identification via MALDI-TOF using a facility at Harvard Medical School (Figure 1). We reasoned that since *P. agglomerans* was sufficiently similar to *E. coli* some of its proteins should be able to be identified in this way, which normally requires a sequenced genome. Of the 20 spots we picked, only three returned identities in which we were confident. Fortunately, these were among the most abundant secreted proteins. These were: *fliC* (flagellin, Figure 1); *flgL* (flagellar hook protein); and, *ssb* (single-stranded DNA-binding protein).

We designed degenerate PCR primers based on the peptide sequences obtained from MALDI-TOF and also designed another set of degenerate primers aimed at isolating the flanking sequences of the genes by an "arbitrary" PCR method. To date, we have assembled all of the sequence of *fliC* and have partial sequences for *flgL* and *ssbI*. The *fliC* gene is particularly interesting to us since it has been used as the basis of a secretion system in *E. coli* already and it is the single most abundant proteins secreted from *P. agglomerans*.

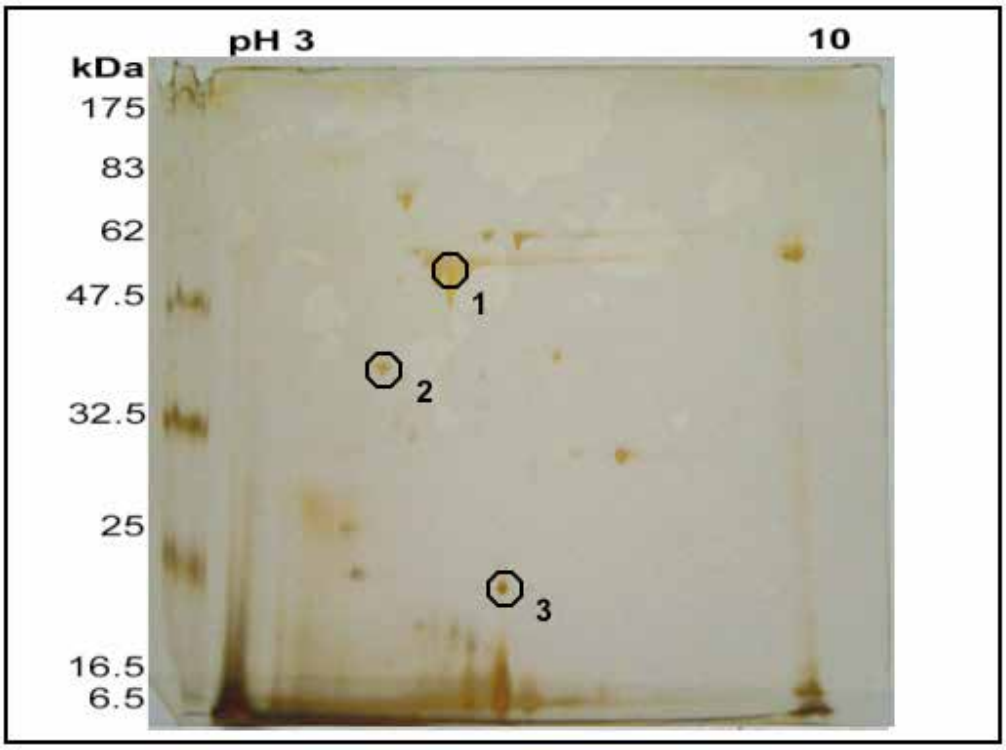

Figure 1. 2D-PAGE of *P. agglomerans* secreted protein profile in LB broth. A silver-stained gel of proteins isolated from late log phase cultures of *P. agglomerans*. The identity of circled spots was determined by MALDI-TOF analysis and peptide sequence comparison to *E. coli*. The identities of the proteins were as follows: 1. flagellin (*fliC*); 2. flagellar hook-associated protein (*flgl*); 2. single-stranded DNA binding protein (*ssb*).

We have begun to construct a secretion system for *P. agglomerans* based on the *fliC* gene. We designed several gene fusions between *fliC* and a single chain antibody (scFv) specific to bovine serum albumin (BSA). The fusion protein is relatively large and can be easily assayed for activity directly from spent media to determine if the protein is folding correctly. Similar experiments using the homologous gene in *E. coli* showed that only a portion of *fliC* is necessary to obtain secretion, perhaps as little as the 5'untranslated region (Majander et al., 2005). We designed constructs using the full-length *fliC* gene fused to the scFv and several C-terminal deletions of *fliC* down to as little as the 5'UTR. These will be tested for secretion by collecting the medium of log-phase cultures of *P. agglomerans* carrying the different fusion constructs. The smallest sequence of *fliC* that can successfully mediate secretion of the scFv will be used to test secretion of known anti-*Xf* effector proteins.

CONCLUSIONS

We were able to identify several abundant proteins secreted by the grapevine symbiont, *P. agglomerans*. These are under development for use in the secretion of anti- *Xf* effector proteins into grapevine xylem after colonization by the symbiont. The most promising of them is flagellin, which in E. coli can secrete proteins using only the 5' UTR of the *fliC* gene. Similar experiments will test this capacity of the *P. agglomerans fliC* gene.

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EXAMINATION OF GLASSY-WINGED SHARPSHOOTER ENTRY POINTS OF A HIGH-RISK VINEYARD IN THE TEMECULA VALLEY

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ABSTRACT
During the 1997-1990 Pierce's disease (PD) epidemic in Temecula, the Weaver vineyard had relatively modest losses in Chardonnay grapevines, while neighbors lost 100%. The Weaver vineyard is near citrus and therefore supposedly vulnerable. The owners thought an area in the vineyard opposite a driveway across from the vineyard that opened into a citrus orchard was a likely site of entry of glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* from the neighboring citrus orchard. Patterns of replacement grapevines were documented in the Weaver vineyard and tested statistically for any underlying patterns. The data gathered showed both parallel and perpendicular runs of replaced grapevines in concentrated PD replacement sectors of the vineyard. While there was an area of grapevine replacement opposite the driveway mentioned, there were other groupings of grapevine replacement on the opposite side of the Weaver vineyard. Thus the area in question opposite the driveway remains a curiosity and not the only area affected. What remains unknown is why the Weaver vineyard escaped the serious losses suffered by adjacent vineyards with the same variety of grapevine. The cause of this apparent resistance to PD is being studied further.

INTRODUCTION
The GWSS is a xylem feeder and transmits various strains of the bacterium *Xylella fastidiosa* to a number of economically important plants such as citrus, almond, and grapevine causing diseases [1-4]. In the late 1990's, a surge of GWSS swept through Southern California's Temecula Valley causing significant plant loses due to PD.

One vineyard, the Weaver vineyard, remained largely unaffected during this event, suffering only an 11% loss of vines, though it features classic high-risk properties. Characteristics of the Weaver vineyard that place it in a high-risk category include its proximity to two citrus groves [5] located directly across the street, and a susceptible grapevine variety, Chardonnay [6]. The effects of the citrus groves opposite the Weaver vineyard were of interest as the vine replacements in the vineyard showed signs of a possible GWSS entry point due to a driveway (Figure 1) that runs between the groves. Figure one was taken from the Weaver vineyard looking directly across the street into the neighboring citrus orchard. The Weaver vineyard anomaly of apparent resistance to PD could be due to such factors as endophyte concentration, plant morphology, genotype, or soil composition.

Figure 1. Driveway into neighboring citrus grove

METHOD
The pattern of rabbit guards indicating new replacement grapevines was recorded and analyzed with respected to proximity to each other and the vineyard's perimeter. There were 420 replaced vines, 38 of which were replaced due to rodent damage. Data of replaced vines were tested with the logistic regression equation. The rabbit guards were present in sectors in the vineyard.

RESULTS
An analysis of row and column effects was performed using logistic regression to determine the direction of entry in all significantly concentrated sectors. Sector A had only a row effect. Sector B had no significant column or row effect. Sector C is a combination of two sectors, which shared the same column data on the perimeter of the vineyard, and they had only a column effect.

Model I: The driveway entry point was located across from vineyard rows 47 and 48. Replacement grapevine data was tested using the logistic regression equation: $p = \text{logit}(\alpha + \beta_i \times Distance_i)$. Where α is the intercept, $ß_i$ is the correlation, and the distance *i* is the distance from the entry point to the replaced vine *i*. The *p-value* of the result was 0.0067 and the distance from the entry point to each replaced grapevine was found to be statistically significant (*p-value* less than 0.05).

Model II: The vineyard was evenly divided into 16 sectors. The concentration of replacement grapevines in each sector was tested against the average concentration using the logistic regression equation: $p = \text{logit}(\alpha + \beta_i \times Section_i)$. Where α is the intercept, $ß_i$ is the correlation and sector *i* is the concentration of replacements per sector. There were four sectors (sector *C* is a grouping of two separate but adjacent areas) with a significantly higher concentration (with *p-value* less than 0.05) of lost grapevines than average (figure 2.). All four sectors were near either a neighboring vineyard or a citrus grove. Sector A: *p-value* = 0.0026, concentration = 0.1857; Sector B: *p-value* = 0.0002, concentration = 0.2051; Sector C consists of two adjacent sectors: first *p-value* < 0.0001, concentration = 0.2308, second *p-value* = 0.0055, concentration = 0.1786.

Figure 2. The map of Weaver's vineyard. According to Model II, sector A-C are the highly significant areas of PD loss.

Model III: Data within significantly concentrated sectors (sector A-C) was tested for both horizontal (row) and vertical (column) effects and against the concentration within each row or column using the equation: $p = \text{logit}(\alpha + \beta_i \times Row_i + \beta_i \times Column_i)$ Sector A (rows 41-54 and columns 1-15) only showed a row effect in rows 45, 47, and 48 (R_i-R=1.3923, 1.9313, 1.1204 respectively, and *p-values* are less than 0.05). Sector B (rows 1-13 and columns 16-30) had no significant row or column effect (*p-values* are more than 0.05). Sector C (rows 14-26 and columns 46-61) showed a column effect only in columns 53, 54, 55, 57 and 58 (R_i-R=1.1357, 1.4427, 1.1357, 1.4427, 1.1357, and *p-values* are less than 0.05).

Model IV: This model was necessary since the replaced grapevine data included grapevines that were replaced due to rodent damage as well as vines replaced to PD-related loss. In this model, the probability of rodent damage was evenly subtracted from each grapevine (*P = C+(1-C)),* where *P* = the probability of PD-related loss, and *C* = the probability of rodent damage = 38/420 = 0.0905). Then the probability of PD-related loss for each grapevine was calculated using the modified logistic regression equation: $p = c + (1-c)\text{logit}(\alpha_i + \beta_i x_i)$. The color map of the probability was plotted (figure 3.).

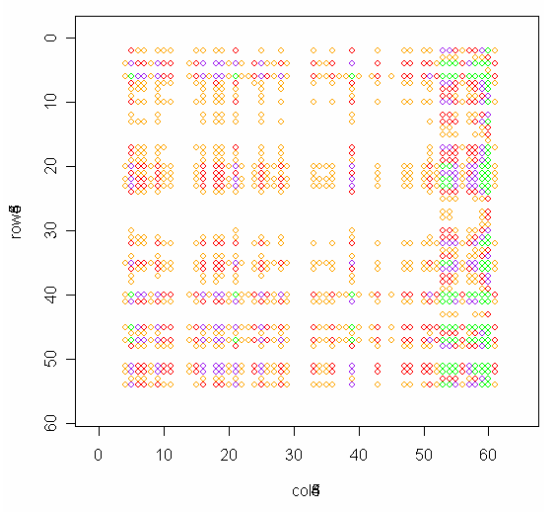

Figure 3. A color map of the probability of individual grapevine replacement due to PD according to Model VI. The range of probability is represented by: no color <0.2, red 0.2-0.25, purple 0.25-0.3, and green >0.3.

DISCUSSION

The concentrated sectors of the Weaver vineyard yield insight to GWSS behavior with respect to PD appearances. A theorized entrance point is supported by a perpendicular statistical pattern within a concentrated sector at the perimeter of the vineyard. Sector A of the Weaver vineyard shows this row effect, which is directly across from a road located between two citrus groves, a common GWSS harbor. GWSS tend to disperse intermittently while foraging [8], possibly to avoid predation or competition. The presence of a "corridor" absent of foliage may further aid the migration and dispersion of GWSS into a field. While the exact times of individual GWSS feeding and transmission are unavailable, regular and repeated contact with specific plants can be visualized, as these plants will be less likely to recover from PD symptoms and have to be replaced. Since the perimeter of a vineyard is necessarily more exposed to GWSS activity (the GWSS must cross the perimeter to enter the vineyard), one would expect to see more symptomatic vines where the GWSS enter.

Thirty-eight grapevines were removed due to rodent damage from possibly California ground squirrel, the jackrabbit, and/or the pocket gopher [9-11]. The vertical and horizontal logistic regression statistical analysis was performed to find a correlation between runs of data within concentrated areas. Only runs perpendicular to the perimeter could give rise to GWSS entrance points while parallel runs could be attributed to rodent damage. The runs of data in Sector C reflect the feeding behavior of rodents in that they are localized, parallel runs in close proximity to the rodent habitat. The habitat was identified by burrows, droppings, and activity.

Another possible cause of localized vine replacement could be due to GWSS nymph feeding behavior supporting vine-to-vine transmission locally. This behavior may explain the pattern observed in Sector B.

Future Works. It is important to recognize other environmental factors that may affect the introduction and spread of PD infection. Possible effects include parasitic wasps, endophytic bacteria antagonistic to the pathogen, plant morphology, and soil composition. Also, the soil may contain micronutrients capable of disrupting biofilm formation by the pathogen in the xylem of the grapevine. It is likely that the apparent resistance of Chardonnay grapevines in the Weaver vineyard to PD has several contributing causes. If some of these factors are discrete, such as endophytes protecting grapevines against PD, it may be possible to convert them into treatments to protect other vineyards. There is some precedent for this non-recombinant symbiotic control approach.

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FINDING PIERCE'S DISEASE INFECTED VINES IN LARGE VINEYARDS: A PROPOSED PIERCE'S DISEASE SAMPLING PROGRAM

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ABSTRACT
The goal of this project is to evaluate a sampling method for rapidly identifying vineyards to sample for Pierce's disease (PD) infection. Using aerial imagery to locate large "PD signature areas" we have searched 30,353 vineyard acres, identifying 76 vineyards for sampling. We have sampled 17 of these vineyards, finding PD in 8 of them. It is important to note that these infected sites had never been identified prior to our research. This is the first step toward sampling large areas for PD and it is the foundation for developing a sampling program for PD in California.

INTRODUCTION
Pierce's disease (PD), a disease of grapes caused by the bacteria, *Xylella fastidiosa* Wells et al., was described in California in the 1880s during an epidemic in Orange County (Pierce 1882). A second epidemic occurred in Tulare County in the 1930s (Hewitt et al. 1949), and until the mid-1990's, it was considered only a minor problem in vineyards close to riparian areas. In the early 1990s a new vector, the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (formerly *Homalodisca coagulata* Say), was introduced into the state (Sorenson and Gill 1996), and this species was associated with a devastating epidemic of PD in the Temecula Valley. Since 1994, at least 1,500 acres of vineyards have been lost to the disease in California; in the Temecula Valley alone, losses have been estimated at $13 million (Wine Institute 2002).

Locating infected vines also is the most important component behind vineyard-level management strategies implemented by individual growers. For example, vineyards with high PD incidence would logically require more aggressive vector management than vineyards with little or no PD in the field. This does not mean that growers should relax their vigilance in areas or vineyards with low PD incidence, but by knowing the distribution of PD in their field, growers could prioritize the areas needing the most immediate attention. Also, knowing the location of infected vines is necessary for growers to implement roguing strategies to reduce *Xf* inoculum in vineyards.

Presently, the only proposed strategy for finding PD is to census vineyards in search for symptomatic vines; infection is verified by ELISA. This type of census was evaluated in Kern County, and it provided a cost-effective strategy for identifying infected vines when incidence was low (Hashim and Hill 2003). However, it becomes prohibitively expensive to sample and test every symptomatic vine when the percentage of vines with PD-like symptoms exceeds 1% in the vineyard (Perring et al. 2005). It is especially difficult to use a symptom-based survey when other stress factors (e.g. drought and salt damage) that express PD-like symptoms exist in vineyards (Krell et al. 2006).

While many growers are fastidious and have been diligent in conducting field censuses and roguing infected vines, the majority of large vineyards in California have never been surveyed for PD. Even vineyards in close proximity to citrus which represent putative high risk areas have not been sampled. In this project, we will develop PD sampling strategies that are not biased by variable symptom expression, that are economical so they can be applied to large geographic areas, and that have sufficient detail so they can be used to locate diseased vines within vineyards.

OBJECTIVE

1) To evaluate the use of "PD-signatures areas," visualized with aerial photography, for prioritizing vineyards for more intensive PD sampling. While this project focuses on table grapes in Kern and Tulare County (due to the funding source), the protocol is adaptable to vineyards of all types throughout the state.

RESULTS

The goal of an area-wide-level PD sampling plan is to assess PD distribution over large geographic areas. Clearly it is impractical to visit every vineyard in California and search for PD infection, so a method is needed for prioritizing fields to sample. In previous studies, we found that 82% of the diseased vines in the Coachella Valley were adjacent to two to six consecutive missing, dead, or replanted grapevines in a row. We hypothesized that these holes were created by PD infection, and putatively termed them "PD signature areas," (Perring et al. 2005, Park et al. 2006). We learned that these signature areas can be seen in aerial photographs (Figure 1), and we evaluated the feasibility of using aerial images to identify fields with PD. In previous studies in the Coachella Valley, we identified 15 vineyards with putative signature areas and sampling revealed 7 of them were infected with PD. These vineyards represented new finds for our area-wide survey that had not been identified in the previous 4 years of surveying by vehicle. A subsequent study in September 2006 in Kern County identified nine vineyards that had not been sampled previously; all nine were selected because they had PD signature areas visible in aerial images. Tissue collected around the signature areas confirmed PD in seven of the fields.

Currently, we are in the process of sampling table grape vineyards in Kern and Tulare County (Figure 2). Using aerial imagery, we have searched 163 vineyard sites representing 12,584 acres in Kern County and 363 sites representing 17,769 acres in Tulare County. In Kern County, we identified 20 vineyards with large signature areas and to date, we have sampled 17 of these vineyards, collecting samples for ELISA analyses. Eight of these fields were positive. We are continuing to search the aerial imagery in Kern County for additional sampling sites. In Tulare County we have identified 56 sites for sampling, and we are in the process of contacting growers to gain access to their vineyards for sampling.

CONCLUSIONS

The CDFA Research Scientific Advisory Committee (2007) noted that adding Pierce's disease data to the current CDFA GIS database is an "important and highly desirable objective." With over 790,000 acres of grapes in the state, this is a sizeable goal. Our lab has been working for several years on sampling strategies for PD at the grapevine (Krell et al. 2006), vineyard (Park et al. 2006), and area-wide scales. Our results in the current study suggest that aerial imagery can provide a rapid and efficient method for surveying large land areas and prioritizing vineyards for PD sampling. This season, we have identified 76 vineyards for priority sampling out of 526 sites representing 30,353 acres. We have collected samples from 17 of the 76 sites and have verified PD in 8 of the vineyards that had not been identified previously. This area-wide sampling is the first step toward adding PD data to the CDFA database.

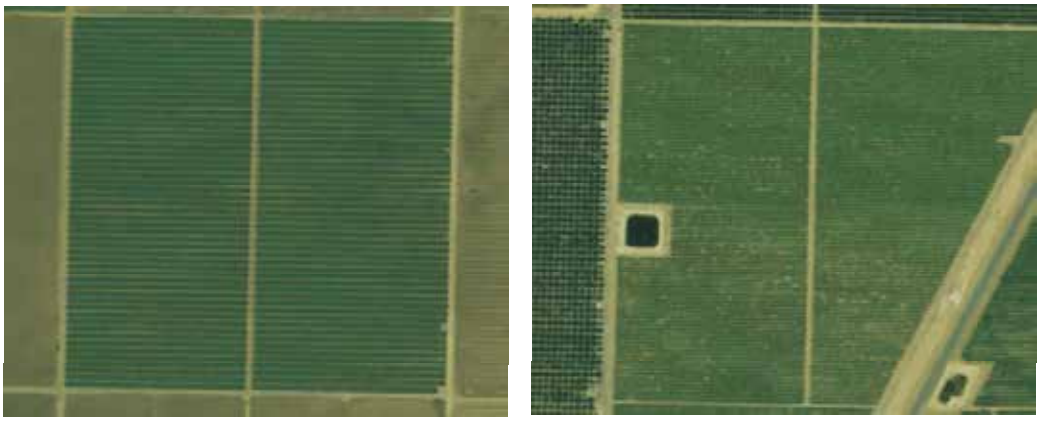

Figure 1. Aerial images of two vineyards in Kern County. The left side image was categorized as "healthy", meaning that it had a low priority for sampling while the right side image was categorized as having "PD signature areas", thus qualifying the vineyard for sampling.

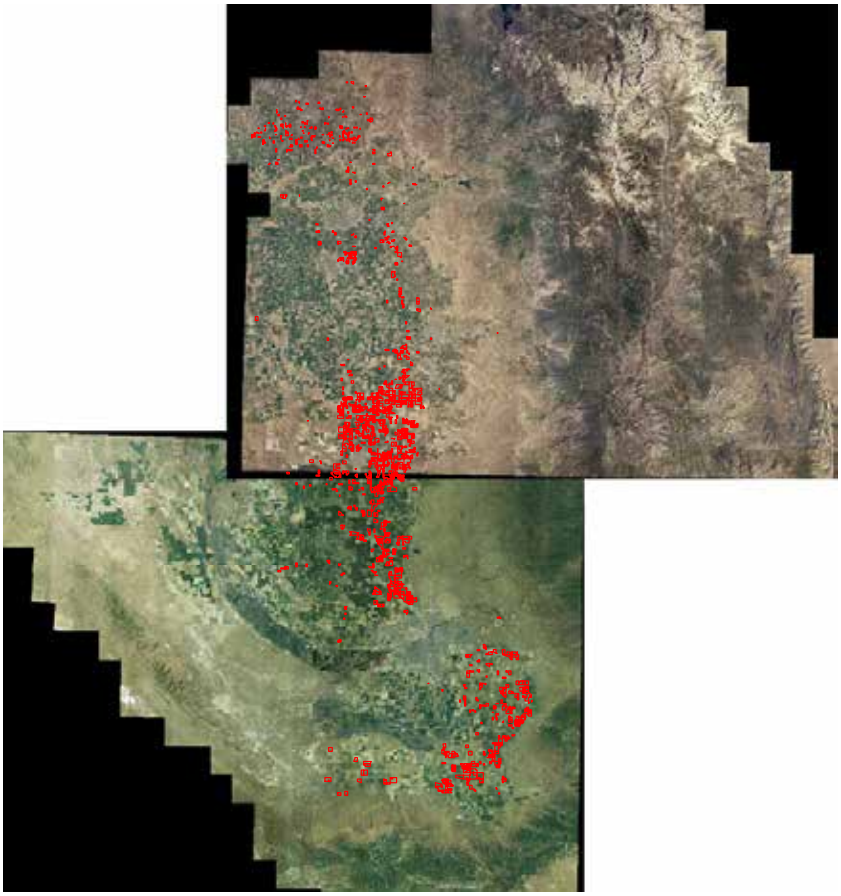

Figure 2. Aerial images of Tulare (top) and Kern (bottom) Counties showing the table grape vineyards (red areas).

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ASSAYS OF TEXAS VINEYARD SOILS FOR EFFECTS ON PIERCE'S DISEASE OF GRAPE

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ABSTRACT

Central Texas vineyards with clayey limestone-based soils have usually developed Pierce's disease (PD) caused by *Xylella fastidiosa* (*Xf*) sooner, and epidemics have been more severe compared to vineyards with sandy granite-based soils. Soils collected from two vineyards representing each soil group (granite: McCulloch and Gillespie Counties; limestone: Gillespie, Blanco Counties) and MetroMix 366 (SunGro Horticulture, Bellevue, WA) peat-based potting mix were planted with highly susceptible Chardonnay (bare-root 1-yr old plants). Blow-molded black plastic pots (28 cm dia. x 24 cm ht '2.9 gal.') were fitted with one fiberglass wick (knit rope, 1.9 cm diameter, fired 4 hr at 400 C) across the inside bottom of the pot with one end protruding 46 to 61 cm below the bench top to enhance drainage. Experimental design was an unbalanced split-plot with soils as main plots and inocula (five pots inoculated, three pots not inoculated) as sub plots. No-inoculum treatments (checks) were mock-inoculated with SCP buffer. Runs one, two, and three had 9, 2, and 10 replications, respectively. A screened structure (woven HDPE insect screen 20x20 mesh, 40% shade) excluded xylem-feeding insect vectors. White shade cloth (22%) was positioned over the screen for the duration of runs one and two, but was removed during run three after plants were established. Irrigation was with distilled or RO (after softening) water to minimize potential for minerals in water to alter soils. Minimal urea fertilizer (0.1 g urea per pot in RO water) was used periodically to maintain growth. Young cultures of *Xf* isolate GILBEC625-2 on PWG medium were suspended in SCP buffer until visibly turbid and standardized (0.200<OD<0.300, 600 nm). For run one, one 10-µl drop was placed on each of two adjacent internodes (20 µ/plant) and probed gently to xylem-depth with sterile 28- or 30-gauge syringes. Runs two and three were repeat-inoculated one or two days later on the opposite sides of the same internodes (40 µ/plant). Symptoms of PD were recorded (one to five index, one for no symptoms, five for dying/dead plants) and petiole samples were tested with ELISA (Agdia, Inc., Elkhart, IN). Data were analyzed with PC SAS PROC GLM. Run one was evaluated in November 2005 and June 2006. Run two was evaluated in June 2007. Run three was evaluated in August 2007. Soil effects were never significant for ELISA OD, incidence (proportion plants with ELISA OD>0.300), or PD symptoms intensity. Inoculum effects were always significant ($P<0.05$) for OD, incidence, and symptom intensity as expected because the comparison was some vs. none. Soil x inoculum interaction was never significant for OD, incidence, or symptom severity. We conclude that soil type has no direct effect on PD in Central TX. Our previous work showed that vineyard sites with granite- or limestone-based soils vary for certain important plant species. Some supplemental plant host species for *Xf* (*Helianthus annuus*, *Ambrosia trifida* var. *texana*, *Iva annua*) were mostly absent on droughty granite-based soils, but very frequent on higher-water-holding-capacity limestone-based soils. We propose an indirect soil effect on PD due to soil effects on plant communities. These three annual weeds sometimes occur in highway rights-of-way and farm staging yards in areas of granite-based soils, apparently because seeds were introduced on mowing and other equipment. These may represent significant corridors for vectors and the PD pathogen into usually low risk sites.

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Section 5:

Crop Biology and Disease Epidemiology

ROLE OF ALFALFA IN THE EPIDEMIOLOGY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

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ABSTRACT
Alfalfa (*Medicago sativa*) occurs widely throughout the Central Valley, often adjacent to grape and other plants susceptible to *Xylella fastidiosa* (*Xf*). Although a previous epidemic of Pierce's disease (PD) in the Central Valley was associated with migration of infective insects from alfalfa fields to vineyards, little is known about alfalfa-*Xf* interactions and their importance to disease epidemiology. In this project we are studying the suitability of alfalfa as a perennial host of PD and almond leaf scorch (ALS) strains of *Xf* and its role as a source of the pathogen for vector transmission. In a first study, we showed that several isolates of each strain can colonize alfalfa after mechanical inoculation, although the rate of infection and bacterial population over successive cuttings varied depending on the isolate. We are now testing transmission of an isolate of each strain to/from alfalfa to grape and almond by the green sharpshooter (*Draeculacephala minerva*), as well as studying the feeding sites of three sharpshooter vectors in relation to the distribution of *Xf* in the alfalfa plant. These studies should provide basic information on vector-alfalfa-*Xf* interactions, which may be considered in management strategies for pathosystems that include alfalfa as a host.

INTRODUCTION
In recent years, many aspects of *Xf* diseases have been studied, providing information that can be used to develop management practices. However, some aspects of the epidemiology of diseases like PD and ALS are poorly understood, such as the role of alfalfa (*Medicago sativa*) in the maintenance and spread of *Xf* in California. This bacterium colonizes alfalfa and it was associated with the alfalfa dwarf (AD) disease (Thomson et al. 1978). Alfalfa occurs throughout the Central Valley and it was directly associated with the largest PD epidemic that had occurred in this region (Hewitt et al. 1949) until the introduction of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*. Studies on the epidemics of the 1940s showed that PD was more frequent in areas adjacent to alfalfa fields, and that a disease gradient was observed, in which PD incidence was higher near alfalfa fields (Hewitt and Houston 1941). It was later found that the etiological agent of AD was transmitted by sharpshooter leafhoppers (Hewitt et al. 1946) and that spread to adjacent grapevines was associated to two sharpshooters commonly found on Bermuda grass and alfalfa fields, *Xyphon fulgida* (red-headed sharpshooter, RHSS) and *Draeculacephala minerva* (green sharpshooter, GSS) (Purcell and Frazier 1985).

In relation to PD, interest in AD was primarily focused on its importance as a reservoir of *Xf* to grape rather than studies aimed to understand the alfalfa-*Xf* association. Weimer described AD in a series of publications (Weimer 1932, 1936, 1937a), but at that time the disease was presumed to be caused by a virus (Weimer 1937b). Focus on AD was briefly renewed when Davis et al. were able to culture *Xf* from infected grape and almond plants (1978, 1980). Thomson et al. (1978) were also able to culture *Xf* from alfalfa, although Koch's postulates were not fulfilled for the disease. Since then, no further information was reported on AD. We do no know which *Xf* strains cause disease in alfalfa, or which strains can multiply in this plant. The only work done on this aspect of the disease showed that *Xf* isolated from alfalfa caused disease in almond (Thomson et al. 1978). Recently, it was shown that grape and almond *Xf* are genetically and biologically different (Almeida and Purcell 2003). Grape strains cause disease in grape and almonds, whereas almond strains do not cause disease in grape. This is of paramount importance in explaining the movement of *Xf* from vineyards to almond orchards and vice-versa. However, the role of alfalfa in this pathosystem remains unclear, as we do not know if these strains multiply in alfalfa to numbers high enough for insect acquisition and subsequent transmission to grape or almond. In this project we are evaluating the fate of grape and almond strains of *Xf* in alfalfa, as well as their acquisition and inoculation by vectors on this host plant, in order to determine the importance of alfalfa crop as a source for these strains.

OBJECTIVES
1. Assess multiplication, movement and survival of grape and almond strains of *Xf* in alfalfa.
2. Evaluate pathogenicity of *Xf* strains to alfalfa.
3. Determine vector transmission efficiency of *Xf* to/from alfalfa to grape and almond.

RESULTS
Objective 1. Colonization of alfalfa by grape and almond strains of *Xf*
In this study, we are assessing the multiplication and survival in alfalfa (cv. WL625HQ) of 12 isolates of *Xf* from grape, 10 isolates from almond and two from alfalfa (Table 1). Potted alfalfa seedlings were pin-inoculated in the stem (2" above soil

level) with a 10^8-10^9 CFU/mL suspension of each isolate; we kept the plants in a vector-proof greenhouse and cut at 1.5" above soil after each flowering stage. *Xf* infection and concentration at the base of the stem was determined by primary isolation on solid PWG medium (Hill & Purcell 1995) at 8, 14 and 21 wks after the inoculation (just before the 1st, 2nd and 3rd cuts, respectively). At each cut, plants were evaluated for height, number of internodes and harvested dry mass, in order to determine strain pathogenicity to alfalfa. One last evaluation of *Xf* infection and alfalfa growth will be carried out at 35 wks after inoculation (before the 5th cut).

The results obtained so far show that both grape and almond strains of *Xf* can multiply and survive in alfalfa for at least three cuts. The percentage of infected plants varied widely for grape (0-100%) and almond (11-100%) strains, and the mean bacterial concentrations ranged from 10^6 to 10^8 CFU/g of alfalfa tissue for both strains (Table 1). By the 3rd cut, however, most grape isolates showed mean concentrations around 10^8 CFU/g, whereas concentrations of almond isolates were generally 10 times lower. Reductions in plant height and dry mass in relation to healthy controls (mock-inoculated plants) were apparent at the 3rd cut for at least four grape isolates and one of alfalfa (Figure 1). Statistical analyses for testing the effects of different isolates on alfalfa growth parameters will be carried out after the 5th cut.

Objective 2. Distribution of *Xf* strains in alfalfa in relation to vector feeding sites

We are conducting another study to determine bacterial movement and distribution in relation to vector feeding sites in alfalfa. Initially, a choice experiment including three sharpshooter species [GSS, GWSS and the blue-green sharpshooter (BGSS), *Graphocephala atropunctata*] was carried out to examine vector preference for feeding on different parts of the alfalfa plant. This choice experiment showed that GSS feeds both on the basal and upper portions of the stems, while GWSS prefers to feed on the stem at the medium and upper part of the plant and BGSS feeds exclusively on the upper part of the plant (leaves and stems). Based on the vector seeding sites, we designed a second experiment to measure the movement and distribution of two almond and two grape isolates of *Xf* in alfalfa stems. Potted alfalfa seedlings were mechanically inoculated with cell suspensions of each isolate, as described above. At 6 (1st cut) and 12 (2nd cut) wks after inoculation, the bacterial concentrations at the base of the inoculated stem, in the tap root, and at the base and tip of an adjacent stem will be determined by culturing. As an additional measurement of systemic movement, we will confine healthy sharpshooter vectors at the base and tip of the adjacent stem for an acquisition access period (AAP) and then tested for transmission to indicator plants and assayed for infectivity by PCR. Final results are pending.

Objective 3. Transmission efficiency of *Xf* strains to/from alfalfa

A third study is being carried out to evaluate transmission efficiency by GSS of a grape and an almond isolate of *Xf* to/from alfalfa to grape and almond plants. Healthy adults of GSS were confined on source plants of these isolates for a 48-h AAP and then transferred to test plants of alfalfa, grape and/or almond for an inoculation access period of 48 h. For the grape isolate, alfalfa, almond and grape are being tested as source and test plants, whereas for the almond isolate (not pathogenic to grape), only alfalfa and almonds are being tested. These experiments were already set up, but the results are not available yet.

CONCLUSIONS

Grape and almond isolates multiply and persist in alfalfa to population levels $\geq 10^7$ CFU/g of tissue, which exceed the minimum threshold (10^4 CFU/g) required for *Xf* acquisition by sharpshooter vectors (Hill & Purcell 1997). *Xf* infection reduces height and dry mass of alfalfa. Pending experiments will determine the importance of alfalfa as a source of *Xf* for vector acquisition.

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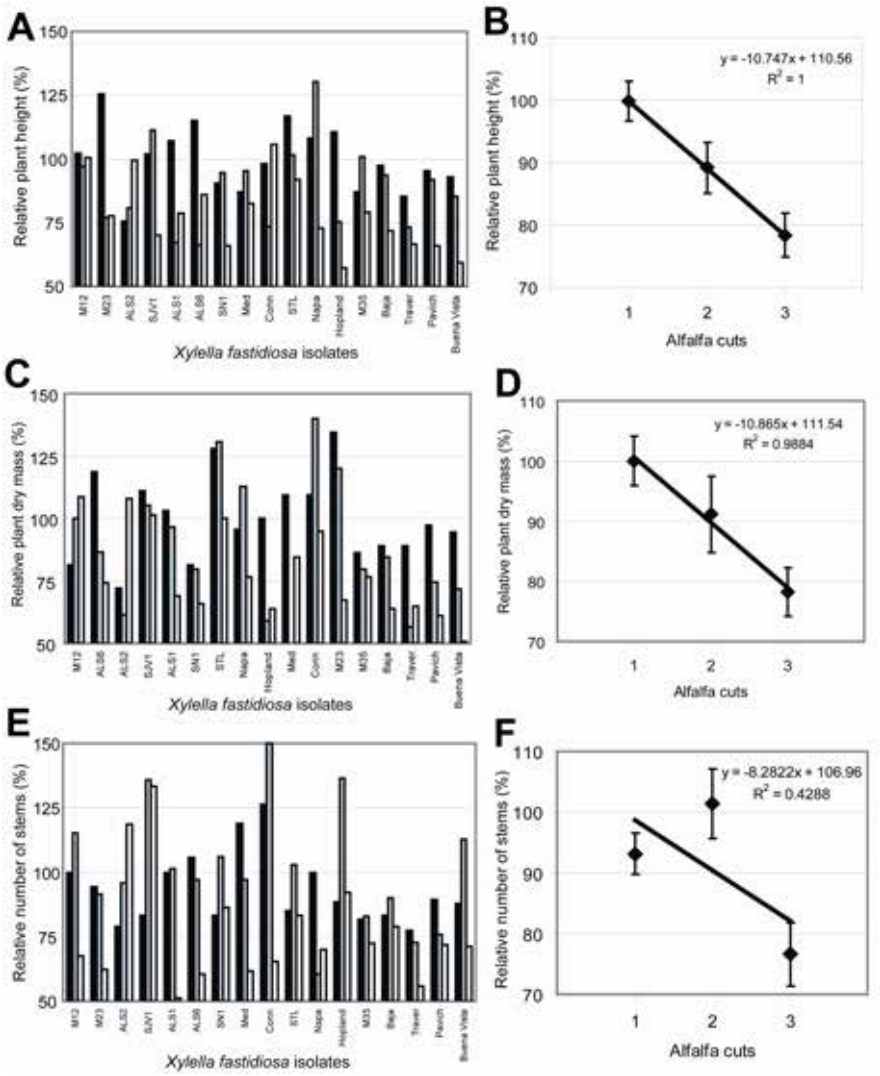

Figure 1. Growth parameters of infected alfalfa plants at successive cuttings following mechanical inoculation of *Xf* isolates. A) Plant height in relation to healthy control; B) Regression of number of cuts versus relative plant height; C) Dry mass in relation to healthy control; D) Regression of number of cuts versus relative dry mass; E) Number of stems in relation to healthy control; F) Regression of number of cuts versus relative number of stems. Black, gray and white columns represent the 1st, 2nd and 3rd cuts, respectively (graphs A, C and E). In graphs B, D and F, means (±SE) are based on pooled data of all isolates.

Table 1. Rate of infection and bacterial concentration of *Xf* isolates in plants of *Medicago sativa* at successive cuttings after mechanical inoculation.

Inoculation date Isolate (County)	Host of origin	Proportion of infected plants 1st cut (8 WAI[a])	 2nd cut (14 WAÍ)	 3rd cut (21 WAI)	Log CFU/g tissue (±SE) 1st cut (8 WAÍ)	 2nd cut (14 WAÍ)	 3rd cut (21 WAÍ)
03/15/2007							
Hopland (Mendocino)	Grape	6/12[b]	1/7	100.0 (9/9)	6.6±0.5	8.0	8.6±0.2
Napa silverado (Napa)	Grape	5/11	4/9	44.4 (4/9)	6.7±0.6	7.8±0.4	8.2±0.4
STL (Napa)	Grape	7/9	4/8	57.0 (4/7)	7.2±0.4	8.7±0.2	7.6±0.5
SN1 (?)	Alfalfa	7/9	6/8	87.5 (7/8)	8.1±0.5	8.6±0.2	7.9±0.3
M12 (?)	Almond	7/9	5/8	44.4 (4/9)	6.3±0.4	7.6±0.2	7.3±0.5
Mock	-						
03/30/2007							
M23 (?)	Grape	7/7	7/7	6/7	8.0±0.4	8.8±0.2	8.6±0.1
Medeiros (Fresno)	Grape	6/6	6/9	6/6	8.1±0.1	8.6±0.2	8.4±0.2
Conn (Napa)	Grape	9/9	4/8	4/8	7.3±0.4	6.5±0.5	7.4±0.5
ALS1 (San Joaquin)	Almond	8/8	8/8	7/7	7.9±0.2	8.2±0.2	8.7±0.2
ALS6 (San Joaquin)	Almond	7/7	6/8	5/7	7.1±0.3	7.9±0.4	7.4±0.2
Butte (Butte)	Almond	9/9	9/9	Ct[c]	8.3±0.2	7.6±0.3	-
ALS4 (San Joaquin)	Almond	7/7	4/7	1/8	7.7±0.2	6.8±0.2	7.7
ALS9 (San Joaquin)	Almond	6/9	5/8	Ct[c]	8.0±0.2	7.6±0.4	-
Mock	-						
04/10/2007							
Buena Vista (Kern)	Grape	9/9	9/9	9/9	8.4±0.2	8.4±0.1	8.7±0.1
Traver (Tulare)	Grape	8/9	9/9	7/7	8.7±0.0	8.6±0.1	8.8±0.2
Pavich (Kern)	Grape	8/9	9/9	9/9	8.3±0.1	8.6±0.2	8.2±0.1
Baja#5 (Mexico)	Grape	6/9	7/8	7/9	7.3±0.2	8.1±0.3	8.7±0.1
Temecula (Riverside)	Grape	1/9	0/8	2/7	5.2	-	8.4±0.5
UCLA (Los Angeles)	Grape	5/9	0/9	1/9	6.3±0.3	-	5.7
M35 (?)	Alfalfa	9/9	7/9	6/7	8.3±0.1	8.0±0.6	7.6±0.4
SJV1 (San Joaquin)	Almond	4/9	2/7	5/9	6.0±0.2	7.3±0.5	7.2±0.6
ALS2 (San Joaquin)	Almond	6/9	3/9	4/9	7.4±0.2	7.8±0.4	7.1±0.3
Glenn (Glenn)	Almond	6/9	5/8	1/9	7.9±0.2	8.4±0.2	5.3
Dixon (Solano)	Almond	3/9	3/9	1/9	7.5±0.2	8.3±0.5	8.0
Mock	-						

[a]WAI: weeks after inoculation.
[b]Number of infected plants over the total number inoculated.
[c]Data not obtained due to plate contamination during bacterial isolation.

SIGNIFICANCE OF RIPARIAN PLANTS IN THE EPIDEMIOLOGY OF PIERCE'S DISEASE

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ABSTRACT
Xylella fastidiosa (*Xf*) can infect all winegrape varieties and some riparian plants, but its limited persistence and low titers in many species means that a small subset of all hosts are likely important inoculum sources. We examined the relationship between the abundance of riparian host species in riparian areas and the incidence of Pierce's disease in Napa Valley vineyards. Our preliminary finding of only one species, *Vinca major* (periwinkle), being positively correlated with a high incidence of Pierce's disease suggests that eradication of all twelve species recommended for removal, according to the guidelines of riparian vegetation management, may be unnecessary.

INTRODUCTION
In California's North-coastal grape-growing region, the Pierce's disease pathogen, *Xf*, is vectored by a native insect, *Graphocephala atropunctata* (blue-green sharpshooter; BGSS; Purcell 1975). There are no effective controls for Pierce's disease on the North Coast. Uptake of a soil-applied insecticide, imidacloprid, is effective against the invasive vector *Homalodisca coagulata* (glassy-winged sharpshooter; GWSS) in the San Joaquin Valley and southern California (Castle et al. 2005), but is known to be poor in North Coast soils (Weber et al. 2005). The egg parasitoids released in southern California to control GWSS do not attack the BGSS (Boyd and Hoddle 2006). Furthermore, no commercially-viable, resistant winegrapes yet exist.

Riparian areas contribute to Pierce's disease in North Coast vineyards as evidenced by a correlation between disease incidence and proximity of vines to riparian areas (Purcell 1974). Eradication of riparian hosts, a technique known as riparian vegetation management (Anonymous 2000), offers the promise of decreasing the pathogen reservoir and vector densities outside of vineyards. However, there are some gaps in the knowledge that make it difficult to predict the efficacy of this approach for control of Pierce's disease.

Xf hosts have been identified primarily from greenhouse studies (Hill and Purcell 1995). Greenhouse studies are important for evaluating a pathogen's host range. However, the moderate greenhouse climate and the high pathogen concentrations used for inoculations likely over-estimate a pathogen's host range in the field. The generalist feeding habit of the BGSS (Hewitt et al. 1949) further complicates our ability to predict which hosts identified from greenhouse studies are significant inoculum sources (i.e., competent reservoirs). In the field, such hosts are situated within plant communities, where their relative abundance and, thus, importance in the spread of a pathogen, can vary. Therefore, field-based investigations are needed to identify vegetation types that contribute most to the spread of Pierce's disease.

Xf populations within infected hosts affect the probability of a vector first acquiring the pathogen while feeding on such hosts, then transmitting the pathogen to other hosts (Hill and Purcell 1997). While *Xf* populations tend to be higher in some hosts (e.g., *Vitis vinifera* cv. Chardonnay) than in others (e.g., *Artemisia douglasiana;* mugwort), they are known to fluctuate according to temperature (Feil and Purcell 2001) and plant hormones (Hopkins 1985). In order for a host to serve as a significant inoculum source, vector feeding must be synchronous with *Xf* populations at or above the acquisition threshold ($\geq 10^4$ colony forming units (CFU) per g petiole tissue; Hill and Purcell 1997). Such fluctuations make it difficult to determine that all hosts identified in the greenhouse are likely to host high pathogen populations during high rates of feeding activity. Given that *Xf* is not detectable in vines early in the growing season (Hopkins 1981), it is possible that overwintering BGSSs acquire the pathogen from feeding on infected riparian hosts in spring (Purcell 1975). In spring, we found that *Xf* was not detectable in two riparian hosts [California grapevine (*Vitis californica*), blue elderberry (*Sambucus mexicana*)] at two North Coast locations where infected plants of both species were placed the previous summer (Baumgartner and Warren 2005). *Xf* was detected in spring in three riparian hosts [California blackberry (*Rubus ursinus*), Himalayan blackberry (*Rubus discolor*), periwinkle (*Vinca major*)], but no plants of any of these species supported *Xf* populations $\geq 10^4$ CFU/g petiole. Our findings suggest that seasonal fluctuations in *Xf* populations limit the ability of species identified as hosts in the greenhouse to serve as hosts in the field.

We previously examined the relationship between Pierce's disease and the spatial arrangement of vineyards and other habitats (i.e., landscape structure). Our objective was not to disprove the association between riparian areas and Pierce's

disease, but to clarify the influence of other habitats (vineyards, residential neighborhoods, wineries, etc...) known to support both the pathogen and the vector. Little work has been done on the influence of landscape structure on the spread of plant diseases (e.g., the invasive forest pathogen, *Phytophthora ramorum*; Meentemeyer et al. 2004). Nonetheless, landscape structure is known to be 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). Lyme disease and Pierce's disease share some similarities. Both pathosystems are characterized by a generalist pathogen, a generalist vector, and numerous alternate hosts that occupy different habitat types. Natural habitats with more diverse host populations are likely to support lower densities of alternate hosts via a dilution effect, and this is thought to contribute to lower incidence of Lyme disease in urban areas surrounded by such habitats (Schmidt and Ostfeld 2001). Based on the results of binary logistic regression analyses, we found that Pierce's disease was more likely to occur in Napa Valley vineyards situated in a landscape surrounded by more vineyard and residential development, regardless of their proximity to riparian habitat (Greenleaf et al. In review). Given that vineyards and urban areas support high densities of BGSS and *Xf* hosts, specifically grapevines (Davis et al. 1978) and landscape plants (Hewitt et al. 1949; Severin 1949; Freitag 1951), it is possible that these habitats are more important in the spread of Pierce's disease than previously thought. Indeed, reports of pathogen titers in various hosts show that grapevines support among the highest (Hill and Purcell 1995).

In 2006 and 2007, we investigated the relationship between plant community composition in riparian areas and disease incidence in adjacent vineyards (Figure 1). Our study was the first to investigate which riparian plants are correlated with a high incidence of Pierce's disease in vineyards.

OBJECTIVES

1. Quantify the relative abundance of plant species in riparian areas adjacent to vineyards with a high incidence of Pierce's disease and vineyards with a low to zero incidence.
2. Quantify the incidence of Pierce's disease in the vineyards.
3. Examine the correlation between the relative abundance of riparian species and Pierce's disease in adjacent vineyards.

RESULTS

Based on preliminary analyses of ELISA results from 20 of 50 vineyards, we found that Pierce's disease is significantly more likely to occur in vineyards adjacent to riparian areas where periwinkle is more abundant ($p < 0.05$; Figure 2). In riparian areas adjacent to vineyards with Pierce's disease, periwinkle was 21 times more abundant, on average, than in riparian areas that were adjacent to disease-free vineyards. In contrast, neither California blackberry, California grape, blue elderberry, Himalayan blackberry, California mugwort, mulefat, nor stinging nettle was associated with a high incidence of Pierce's disease in nearby vineyards ($p > 0.1$). Real-time PCR analyses are in progress for symptomatic grapevines and riparian hosts at all 50 sites.

CONCLUSIONS

In applying knowledge of the *Xf* and BGSS host ranges to development of management strategies for control of Pierce's disease in the field, we find that there are some important gaps to fill. Over the past several years, our efforts have been aimed at testing hypotheses that fill such gaps, in order to help growers evaluate their riparian vegetation management efforts. Our preliminary results from this previous year show that periwinkle is significantly correlated with a high incidence of Pierce's disease, and that other riparian hosts known to harbor the pathogen are not. It is possible, therefore, species may be more important in the spread of Pierce's disease than previously thought.

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Figure 1. Study design for sampling the plant community composition in riparian areas. We studied 19 randomly selected vineyards in Napa County; each was adjacent to riparian vegetation. In Fall 2005, at each of these sites we examined grapevines for Pierce's disease symptoms and confirmed the presence of *Xf* by ELISA, with the help of Dr. Barry Hill. In July 2006, we surveyed the riparian vegetation adjacent to each vineyard. At each site, we established four transects. Along each transect, we measured the percent cover per species within each of 20 quadrats (0.5 x 0.5 m; Figure 1). A geographic information system (GIS) was used to measure the amount of residential and winery land within 1.5 km of each site. In 20 quadrants positioned along each of four transects, as drawn in the riparian area pictured at left, we measured percent cover of all riparian species present. The rectangular box in the vineyard section was surveyed for Pierce's disease.

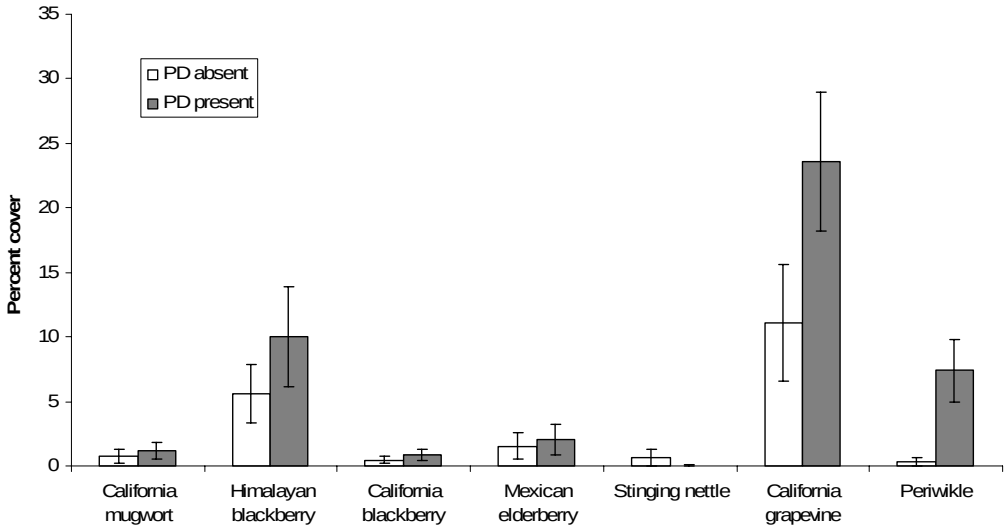

Figure 2. Abundance of riparian plants adjacent to vineyards with and without Pierce's disease. Means and standard errors are shown. Periwinkle was significantly more abundant adjacent to vineyards with Pierce's disease compared to vineyards without Pierce's disease ($p < 0.05$); differences were not significant for other plants ($p > 0.1$).

ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

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ABSTRACT
Navigating the intellectual property (IP) rights of commonly used research tools is essential to prevent downstream legal or regulatory obstacles for deployment of new technologies. This is particularly true in the area of agricultural biotechnology and specifically as related to plant transformation technologies. In light of the complex patent landscape and recognizing the robust intellectual property portfolio among universities and public research institutions, this project seeks to leverage the public portfolio of technologies to support the development of a patent pool of technologies available for plant transformation. This research project will develop and test a grape-specific transformation system for the generation of 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 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 project 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
Transformation vector system
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 eliminate 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. PIPRA's legal and scientific staff performed a review of the intellectual property landscape surrounding recombinase-based plant transformation systems and this scientific and legal information was subsequently used to design a Par A recombinase- based marker removal transformation vector suitable for asexually propagated crops, such as grapes. Although recombinase-mediated gene excision systems have been filed for patent protection (Moller et al. 2004), preliminary evaluation and licensing discussion indicate that the specific technologies incorporated in this vector are available for non-exclusive licensing. Thus far, PIPRA designed and initiated synthesis of the recombinase transformation vector (Figure 1).

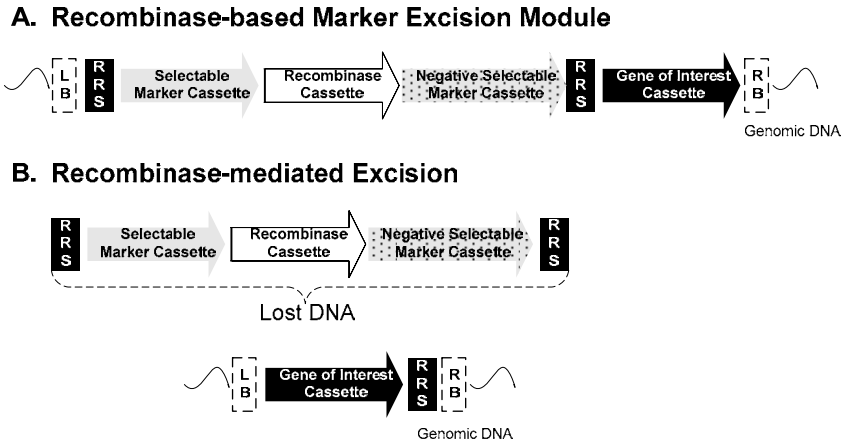

Figure 1. Diagram of the Recombinase-Excision Strategy. Panel A: Recombinase-based transformation construct contains a gene of interest cassette and two selectable marker cassettes flanked by recombinase recognition sequences (RRS). Panel B: Inducible expression of the recombinase excises the selectable marker cassette. Transgenic lines with successful recombination events can be isolated by treatment with a negative selection agent and expression of the gene of interest, i.e. marker gene, GFP.

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. PIPRA has now tested two recently described plant-derived markers (Dirk et al. 2001, 2002, Miki and McHugh 2004, 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. The *Arabidopsis* ABC transporter, *Atwbc19*, provides kanamycin resistance levels comparable to the bacterial-*NptII* gene when overexpressed. 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 licensing discussions to include these technologies in the transformation vector system. In addition, we initiated the experiments to test the plant-based, *DEF2* and *Atwbc19*, and more routinely used hygromycin and glufosinate selectable markers. For these experiments, young embryogenic grape (Thompson Seedless) callus were plated on callus induction medium containing increasing levels of inhibitors (Figure 2). Callus was plated into 24 well plates containing increasing concentrations of kanamycin, hygromycin, and glufosinate. To test the efficacy of the *DEF2* inhibitor in grapes, increasing concentrations of actinonin (0, 15, 30, 60, and 120 mg/L) were added to the media. Callus was subcultured every two weeks onto fresh inhibitor containing media. Plates were evaluated for callus development at eight weeks. Thus far, we are observing some reduction in callus growth with increasing levels of all the inhibitors, especially hygromycin (Figure 2). The preliminary results indicate that

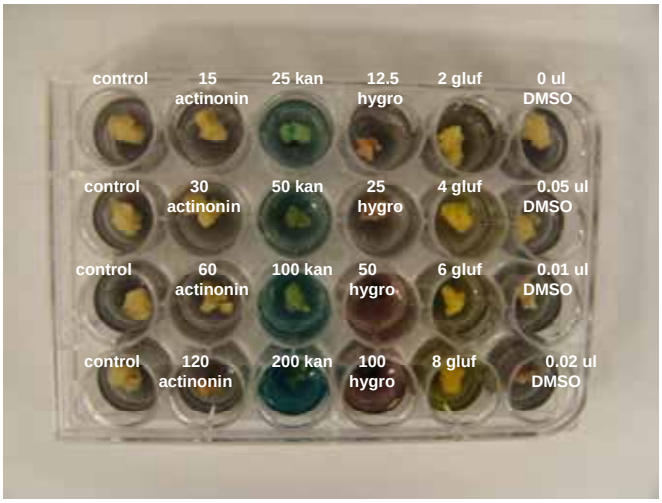

Figure 2. Grape callus growth on various selective agents (actinonin, kanamycin, hygromycin, and glufosinate) (mg/L). Control samples contain no inhibitor (control) or a solvent used for the actinonin inhibitor, DMSO.

higher concentrations of actinonin are required to achieve better selection in grapes. While the growth reduction with actinonin is encouraging, we also observed some reduction in growth with increasing DMSO concentrations, a chemical utilized in the actinonin buffer.

Promoters

PIPRA identified constitutive promoters (Purdue's MAS, University of California's UC FMV34S) suitable for grape transformation. In addition, we identified three 3'UTRs that are in the public domain and that could be used for the proposed vectors; 3'UTRs from pea rubisco E9, nos, CaMV35S.

***Agrobacterium* alternatives**

Of a limited number of high efficiency plant transformation methods, the method of choice for essentially all researchers is *Agrobacterium tumefaciens*-mediated transformation. 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 has been to identify alternate strategies to the use of both *Agrobacterium* and T-DNA borders as components of the gene transfer vehicle.

We have primarily explored the possibility of utilizing early 1980's technology based on the use of *Rhizobium trifolli* to transform plants. *Rhizobium trifolii*, *Rhizobium*, *Sinorhizobium meliloti*, and *Mesorhizobium loti* species have all been demonstrated to introduce new genetic material into plants. 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). We have assessed the legal landscape surrounding the use of these non-agro strains for plant transformation and used the advice of a patent attorney to evaluate the use of *Rhizobium* strains as a legal alternative to *Agrobacterium* strains for plant modification application. In brief, the legal information suggests that the *Rhizobium trifolli* strain, originally disclosed and patented in the early 1980's, are in the public domain because the patents have expired. However, in spite of the likely viability of this gene delivery strategy, attempts to access the original *Rhizobium* strains deposited by Dr. Hooykaas and colleagues at the Centraal Bureau voor Schimmel cultures (CBS) have been unsuccessful to this point.

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 two to three 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, accesses an IP portfolio that represents ~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 PD 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

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ABSTRACT
The Public Intellectual Property Resource for Agriculture (PIPRA) and the California Department of Food and Agriculture Pierce's Disease and Glassy-winged 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 developed 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 provide scientists an integrated view of the technical and legal aspects involved in their projects.

INTRODUCTION
PIPRA is a not-for-profit research organization hosted by the University of California, Davis. PIPRA currently represents 47 public sector organizations from thirteen different countries and its mission is to enable access to agricultural 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 (PD/GWSS Board) began collaboration in 2005 to address IP issues surrounding PD research and development. California's wine industry is a $51.8 billion industry in terms of total economic impact on the State of California, and a $125.3 billion industry in terms of total economic impact on the national economy [1]. Therefore, the threat PD poses to California's 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 strategy. Consortia of universities and other public research entities, however, typically do not have well 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. 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.

Details of objectives set out regarding maintenance of the PD/GWSS-PIPRA IP and Literature databases, as well as an IP and technical audit of a PD related technology will be included. Additionally, ongoing and future objectives will be discussed in detail.

OBJECTIVES

1. Maintenance of the PD/GWSS-PIPRA IP and Literature databases.
2. Development of an annual impact statement of sponsorship based on IP and publication indicators.
3. Conduct an IP and technical audit of a commercially promising PD related technology.

RESULTS

Objective 1: Maintenance of the PD/GWSS-PIPRA IP and Literature databases

The PD/GWSS-PIPRA Database currently contains over 6,000 IP records and over 2,500 scientific publications. This electronic library of IP and scientific publications has been updated on a quarterly basis to include the most recent IP disclosures, science publications, and licensing information available to PIPRA.

New additions made to the database include the conference proceedings from the 2006 Pierce's Disease Research Symposium. This update was unique in that PIPRA employed a different method to extract information from the published symposium proceedings. This new method allowed PIPRA to capture additional information which was not displayed in the 2005 version of the database, such as the focus area in which the proceeding appeared in, funding acknowledgements, and reference citations. This sort of information is valuable to both the CDFA PD/GWSS board and PIPRA because it allows for determining metrics which can then be used in the broad context of an impact statement. This improved method of data extraction was also used on all previous symposium proceedings, thus replacing all of PIPRA's old symposium records with new and more thorough records.

Maintenance and data updates of the PD/GWSS-PIPRA Database will continue on a quarterly basis. Furthermore, PIPRA anticipates making alterations to the current database interface so that it can clearly display the newly captured information.

Objective 2: Development of an annual impact statement of sponsorship based on IP and publication indicators

In 2003 a National Academy of the Sciences panel, reviewed the research conducted by the PD/GWSS research community. A report of this review was published in 2004 and made some suggestions as to the direction PD research should take. Since the publication of this report, no internal or external assessment was conducted to follow up on the progress of PD research. PIPRA suggested conducting one such assessment in the current service year by interpreting research outputs as indicators of PD research impact and progress. Typically, impact statements aim to answer two questions, what was/is the issue and what has been done to address it. While in the process of developing this impact assessment, PIPRA became aware of an independent external assessment (Research Scientific Advisory Panel [RSAP]), headed by Dr. Nancy Irelan, to evaluate progress of CDFA PD/GWSS Board sponsored research activities. Because the PIPRA impact statement and RSAP assessment have converging goals, collaboration between both groups was setup to design and execute a more thorough assessment of all agencies funding PD/GWSS research activities.

Assignment of tasks was divided into two parts. Nancy Irelan was responsible for recruiting the members of RSAP as well as recruiting the cooperation of program administrators and individual researchers to participate in this assessment. PIPRA was responsible for 1) collecting funding and scientific information on every PD/GWSS project sponsored by CDFA, USDA-APHIS, USDA-ARS, and the UC PD program, and 2) presenting this material in a concise two page summary format for RSAP members to review. Information necessary for this assessment were collected from multiple sources. Fundamental information, which is inclusive of project names, associated researchers, funding amounts and years, were provided to PIPRA by the administrators of each funding agency. This information was then used for querying bibliographic information on scientific publications and patents which seemed directly related to funded research. PIPRA searched for scientific publications and patents across multiple, large-scale databases available online, namely: ISI Web-of-Science, PubMed, PD/GWSS-PIPRA Database, and Delphion Patent Database.

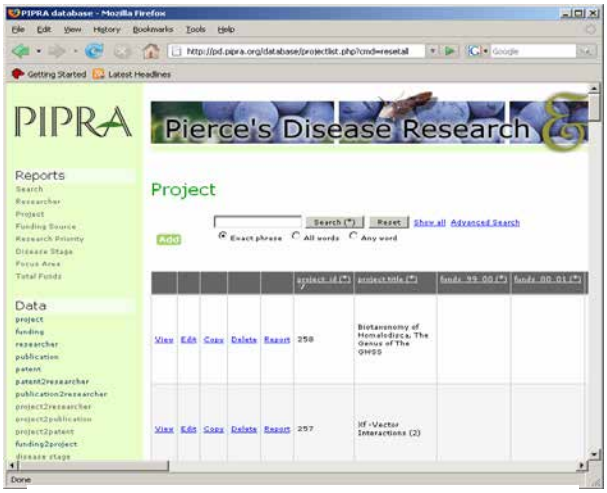

Figure 1. PD/GWSS-PIPRA Database.

A principal function of this database is to allow funding agencies to track the progress of research as a result of their funding. Another function is to allow researchers to update their peers on progress of research conducted, as well as to research duplication. Within this database, researchers may view all of the past and current PD projects, including the project objectives, publications, patents and related research projects. Researchers can also directly review and modify entries in the database related to their projects and other biographical information. This increases the efficiency and accuracy of updating the database.

Objective 3: Conduct an IP and technical audit of a commercially promising PD related technology
PIPRA conducted an IP audit pertaining to a promising PD technology, funded by the CDFA PD/GWSS Board. This audit consisted of a thorough IP landscape and prior art analysis along with an analysis of all contractual agreements affecting research and development of the target technology.

After a solution to PD has been developed, securing IP rights in relation to the invention will be vital. This means that all IP rights in the technology and the components, including the manufacturing process and method of application of the invention will need to be identified, in order to fully protect the technology. This should reduce the hurdles in the process of commercialization of the invention. The CDFA PD/GWSS Board along with RSAP recommended that PIPRA conduct analysis on a promising technology developed by Dr. Steven Lindow and colleagues.

Dr. Lindow and colleagues developed a novel genetic construct which can be used to disrupt extracellular bacterial signaling, preventing *Xylella fastidiosa* from effectively proliferating within its host. A patent application for the technology had been filed on behalf of Dr. Lindow by UC Berkeley. At the time this report was being written, the patent application was still unpublished by the United States Patent and Trademark Office, thus no information regarding its prosecution status was available. Analysis of the IP surrounding Dr. Lindow's technology revealed an extensive list of biological components which may require licensing in order to commercialize the technology. Some of these biological components may be replaceable with functionally equivalent components that carry fewer IP restrictions. PIPRA can work with Dr. Lindow collaboratively to design a plant transformation vector that is technically and legally suitable for research and development of this agricultural biotechnology.

Attention should also be given to regulatory challenges Dr. Lindow's technology may face prior to commercialization. A consumer-friendly approach should be taken when introducing a "genetically modified grape" so to increase consumer acceptance. Dr. Lindow's technology may be better accepted by grape growers and consumers if the technology is contained to the rootstock rather than spread onto the whole plant. This will allow the grapes resulting from plants with rootstock containment will be able to circumvent a "GM" label. It was recommended that the IP landscape around Dr. Lindow's technology be revisited prior to any commercialization so that infringement risks may be minimized.

CONCLUSIONS
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 commercialization issues in mind. PIPRA also recognizes that these resources are only a part of a successful IP management strategy. Over the course of the next year, a number of objectives have been set out. These include maintenance of the PD IP and Impact database, streamlining PD research progress reporting, and an overall assessment of current IP policies and practices within the research community.

As previously noted, maintenance and updating of the PD IP and Impact databases will take place on a quarterly basis. At present, PIPRA is working to further improve the IP database through the use of a 'tier' system for the patent records included in this database. The database contains over 6000 patent records, all of varying relevancy to PD. At present, there is no way of sorting by relevancy to Pierce's disease. To make the database more useful, it has been proposed that a tiered system will be developed to address the issue of relevancy among the various patent records. In this way, any user of the database will be able to conduct an IP search and then sort the records by relevancy to PD. This work is ongoing at present.

Other ongoing and future work includes the work conducted on the objective of streamlining PD research reporting for research assessment. Currently, researchers must send in a report by certain deadlines. These reports must then be manually entered into the database. It has been proposed that an electronic research reporting system be developed to streamline the process of research reporting. To this end, a prototype demonstration of an electronic research reporting system has been made available online at: http://pd.pipra.org/database/cdfa-demo.html. This form allows for online submission of reports. When the form is filled out, it submits the information by email to PIPRA. This online form is composed of a number of fields, in which researchers are able to fill in the required information, as set out by the CDFA's guidelines. Additionally, there is a field included that allows researchers to upload PDF versions of their reports, which will then be submitted to PIPRA. There are many benefits to this type of system. First, it is simple. Second, the most important fields are filled in electronically, but the researcher submits a PDF and therefore has control of the formatting of the document. Finally, there is almost no maintenance required and the form can be easily hosted on any website, including CDFA's.

Another option would be to create a Microsoft Word document template. This template would include fields that could then be filled in individually by researchers. This option may prevent the loss of formatting and would allow data -- such as author names, abstracts, etc -- to be extracted automatically by software created by PIPRA. The final option would be to create a PDF document template. This has the same benefits as the MS Word template, but does not, in general, support including images and complicated markup. A decision will have to be reached regarding the form this electronic research

reporting system will take, and whether or not it is needed by the research community. Again, this work will be ongoing through the year.

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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FUNDING AGENCIES

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

FUNCTIONAL TESTING AND CHARACTERIZATION OF PIERCE'S DISEASE-INDUCED PROMOTERS FROM GRAPE

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Reporting Period: The results reported here are from work conducted October 1, 2006 to September 30, 2007.

ABSTRACT

Several projects working toward understanding the genetic basis for susceptibility or resistance at the molecular level in grape to Pierce's disease (PD) 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 inability to regulate expression of these genes in time and space relative to the presence of the pathogen. We report here the initial characterization of transgenic grape plants with unique DNA sequences from grape (promoters) that specifically regulate the expression of a marker protein (GFP) in grape tissues that are inoculated with *Xf*, but not with a related bacterium, *Xanthomonas campestris* (*Xc*).

INTRODUCTION

Among the potential solutions to 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 *Xf*. This means candidate genes with the potential to suppress or block PD, when driven by *Xf* 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. In contrast, constitutive promoters are expressed in all cells all the time. *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 would reduce the possibility of unintended side affects in non-target tissues (Figure 1). The results displayed herein confirm that we have detected, cloned, expressed and validated the fact that two of the promoters tested to date are activated by the presence of *Xf* in the xylem of both detached branch and whole plant assays of plants expressing promoter-GFP fusions. Mock inoculation and inoculation with the related *Xc* under the same conditions do not activate the promoters.

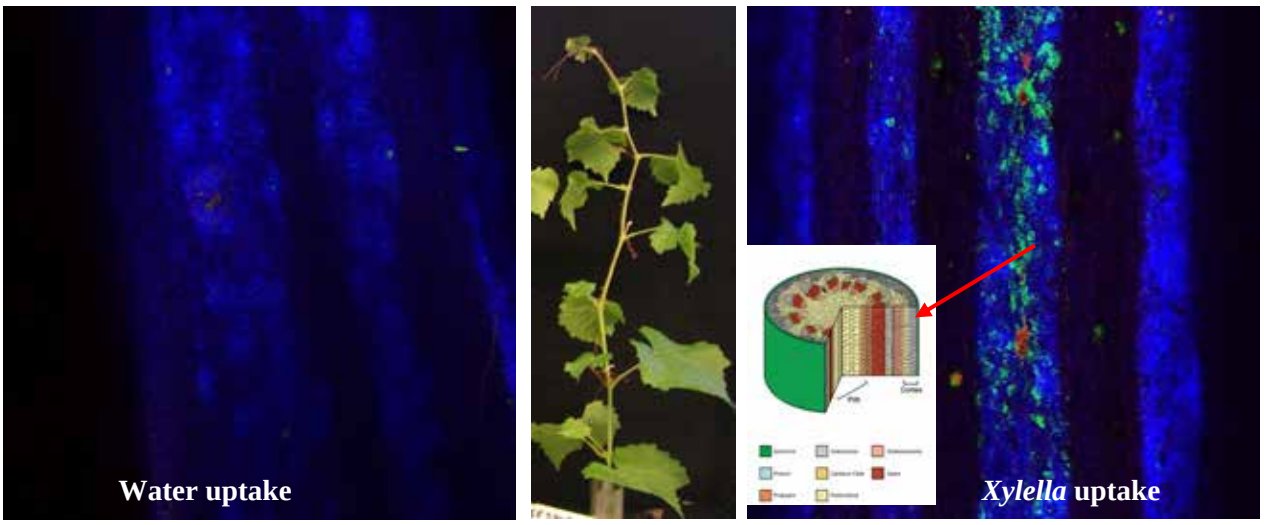

Figure 1. Expression of promoter G7061-11 fused to GFP detected after seven days in petioles of leaves attached to a detached branch into which the Temecula strain of *Xf* (10 ml of 10^7 cfu/ml) was taken up through the cut end of the branch. Water control and uptake of *Xc* shows no promoter activation under the same conditions.

OBJECTIVES

1. Characterization of the transgenic plants expressing promoter constructs, identified previously in this project, designated as G8946, G9353 and G7061.
   a. Determine the extent to which the transgenic promoter-GFP fusions reproduce the patterns of expression for the endogenous promoters.
   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.
   c. Assess the specificity of the promoters for activation by *Xf* compared with *Xc*.
2. Develop a new generation of expression cassettes that control the specificity of gene expression and the subcellular destination of candidate proteins.

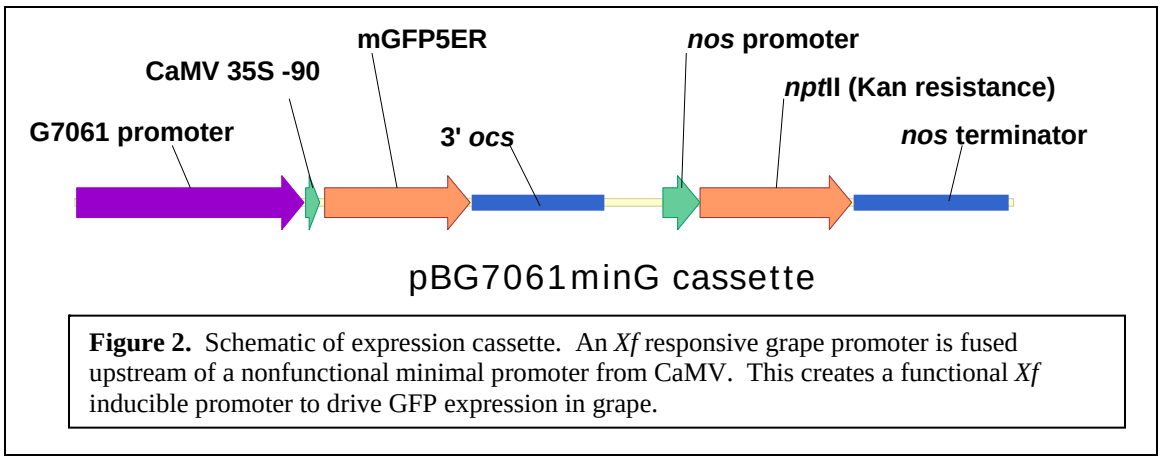

Figure 2. Schematic of expression cassette. An *Xf* responsive grape promoter is fused upstream of a nonfunctional minimal promoter from CaMV. This creates a functional *Xf* inducible promoter to drive GFP expression in grape.

RESULTS

With prior funding from this program, an Affymetrix GeneChip was used to characterize the expression of ~15,000 *Vitis vinifera* genes in response to *Xf* infection and drought stress. Promoters for three of the *Xylella*-responsive vascular tissue localized genes, referred to as G8946, G9353 and G7061, were sequenced from bacterial artificial chromosome (BAC) clones. Regions 5' to the coding sequence were isolated by PCR and used to produce promoter-GFP-reporter fusions (Figure 2) for transformation into the *Xf* susceptible Thompson Seedless grape background. We have received transformants (Table 1) from the UC Plant Transformation Facility within the past year from constructs of the initial promoters fused to GFP

Table 1. Transgenics received.

VARIETY: Thompson Seedless	# Lines or Independent Transformants
7061minGFP	22
8946minGFP	18
9353minGFP	9
Callus derived control	7
total	53

Each of the individual plants was verified by PCR of genomic DNA to contain the appropriate construct. The transgenic plants were then grown in the greenhouse and cuttings made to produce a small population of each transgenic plant (ramets). While the ramets of the transgenics were being made over a several months period preliminary assays were conducted using, the cut-branch and detached leaf assays described in our 2006 PD-GWSS symposium report. Briefly, these assays enabled the bacteria to enter and spread though the grape vascular system of a cut branch or petiole in days to with a few weeks accompanied by PD symptoms. These short term pathogenicity tests were used to assess the relative level of expression of the transformed promoter-reporter gene fusions in the *Xf*- induced tissue (Figures 3 and 4). For the cut leaf assay, young, fully expanded, leaves are 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 per ml water. The detached branch uptake method is similar to the leaf method using individual branches with attached leaves are cut from the plant. Analysis of gene expression is by confocal microscopy showing evidence of the GFP-fused transgenes being expressed in the presence of *Xf* but not in the water control (Figure 1). RT-PCR of the endogenous genes in Thompson seedless indicated that this promoter was expressed

in the presence of *Xf*. This is confirmation that both the endogenous gene and the promoter gene fusion in transgenic plants are expressed in the presence of *Xf*. Comparable tests with *Xc* failed to show activation of the promoter and appeared the same as the water control.

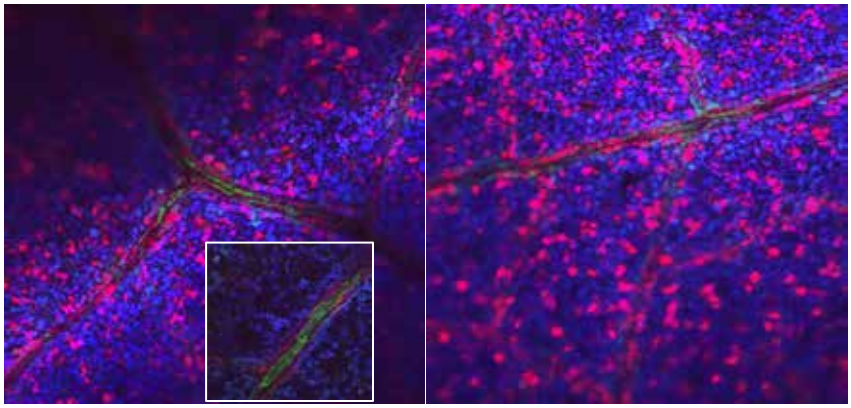

Figure 3. Confocal microscopic analysis at 4x of the activation of the G7061 *Xf* responsive promoter in the laminar tissue following uptake of the bacterium via the petiole in cut leaf assay. The promoter construct is fused to GFP for visualization of activation. The images above were captured two weeks after exposure to the bacteria. Green = GFP; Blue = chlorophyll autofluorescence; Red= polyphenolics released in dying cells. Insert shows an expanded view (25x) of a vascular element with intense GFP fluorescence indicating promoter activity.

The results to date indicate that in both assays methods, the promoter activation is *Xylella* responsive and appears near, but not directly in contact with the bacteria as indicated by preliminary analysis of the bacteria by RT-PCR. The PCR based method for detection and quantifying the bacterial presence in relation to promoter activation enabled both the bacteria and the promoter-GFP fusion transgene to be assayed in the same tissue. In comparison of the leaf and branch uptake methods, the results at this point suggest that the detached leaf assay is somewhat faster than the branch uptake with similar results but is more sensitive to activation of the promoter in the water control tissues than the branch uptake method. In both cases, it appears that, at least for the promoter assays done to date, that this method of placing the bacteria in the vascular system and detecting a response to the presence of the bacteria at the level of plant gene expression is valid. Interestingly, the two promoters illustrated in Figure 2 are both active in the presence of *Xf* but are expressed in different cells types within the cross-section of the petiole. These cell-specific locations are consistent with the previous *in situ* detection of RNA of these genes from the Cook lab (1), the original analysis that provided the basis for selection of the promoters used herein.

As indicated earlier, the leaf scorching indicative of PD occurs within several weeks in the detached tissue assays. Confocal imaging of GFP tagged *Xf* in these same tissues detected only very small amounts of bacteria in the stems and none in the leaf lamina where the genes were detected as being expressed. This indicates that the activation of these promoters is highly sensitive to the presence of bacteria but is active at a distance from where the bacteria were easily detected. However, we also observed that these promoters were frequently activated in water controls of detached leaves making interpretation of expression patterns difficult with this system. Therefore, we are now focusing on whole plant assays (Figure 5).

The whole plant assays are conducted on full-sized (~1m tall) greenhouse grown plants. Inoculations are done by piercing the petiole of mature leaves with a 25G needle attached to a syringe of $2x10^7$ Temecula strain of *Xf* per ml water. While still through the petiole, a small drop of *Xf* solution is expelled from the needle and the needle is gently withdrawn from the petiole leaving a 3-5ul drop of bacteria to be sucked into the petiole by negative vascular pressure. This method results in sufficient amounts of bacteria into the xylem of the pierced petiole to be detected by confocal microscopy to visualize the activated promoter (data not shown).

CONCLUSIONS

Transgenic technology offers the possibility of modifying specific traits (e.g., PD susceptibility) based on the introduction of novel genes. In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression provides an extremely powerful tool to study and characterize host response to *Xf* in intact tissue. 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. In the absence of site or response-specific promoters, transgenic strategies for control of PD can use only so-called constitutive promoters. The expression cassettes we are developing will allow precise regulation of gene expression, in particular tissues (e.g., vascular tissue) and/or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection), and direct secretion of transgenic proteins or small RNAs to the apoplastic compartment where the pathogen resides.

Results summarized in this report indicate that within the first set of three potential *Xf*-inducible promoters; at least two (G7061 and G9353) have been confirmed to be induced in detached tissue in the presence of the bacterium within one to two weeks after exposure to the bacterium. The detached tissue assays appear to have utility in assaying for bacterial-induced transgene expression. Whole transgenic plants expressing the first three promoter-GFP fusions have been inoculated and the

results show that one of the promoters (G9353) is induced within four days of inoculation. Further characterization of the biotic regulation of their expression, including proximity to *Xf*, is ongoing. A new set of potential promoters have been identified and are being assembled into a Gateway binary-based system for higher through put expression and analysis.

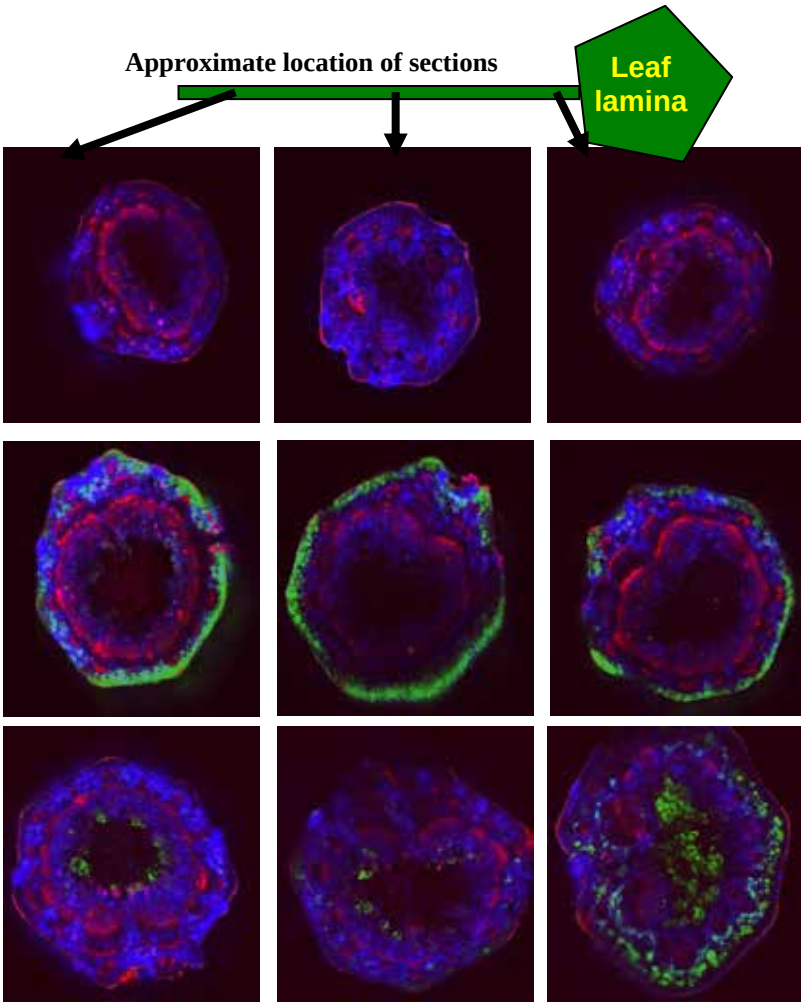

Figure 4. Confocal microscopic analysis of the activation of two putative *Xf* responsive promoters following uptake of the bacterium via the cut leaf assay. The promoter constructs are fused to GFP for visualization of activation. The petiole cross section images were captured two weeks after exposure to the bacteria. The green color is GFP indicating that the promoter was activated, while the blue color indicates chlorophyll autofluorescence and the red color is polyphenolics

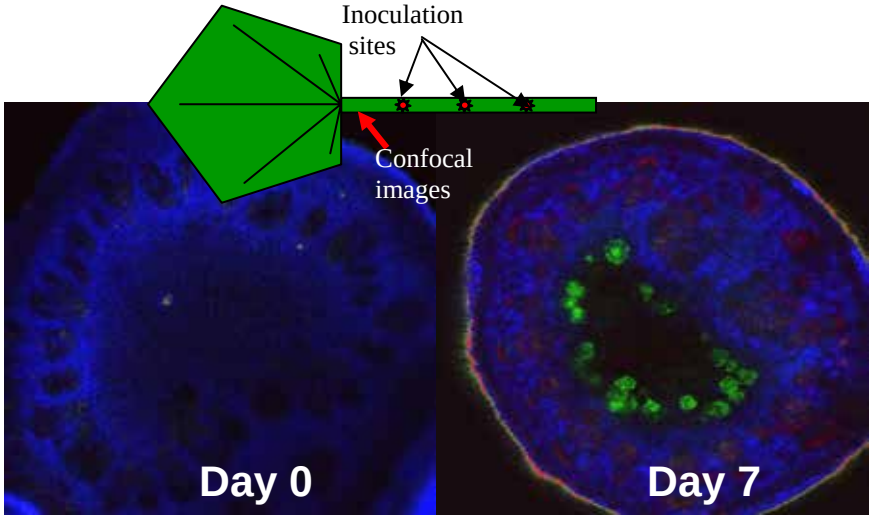

Figure 5. *In planta* activation of promoter G9353. Leaves of the transgenic plant 9353#2 were inoculated with *Xf* by petiole piercing while still attached to the plant. No detectable activation of the promoter is seen just prior to inoculation (Day 0). While after 7 days cells near the xylem can be seen expressing GFP. Water control and *Xc* were negative.

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FUNDING AGENCIES

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

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Reporting Period: The results reported here are from work conducted October 1, 2006 to September 30, 2007

ABSTRACT
The goal of this research is to identify, clone, and express plant genes that can functionally suppress the symptoms of Pierce's disease (PD) in grape. It is well established that several relatives of grape, including *Vitis californica*, and other host plants can harbor otherwise lethal titers of *Xylella fastidiosa (Xf)* without exhibiting PD symptoms. Hence, *Xf* does not have to kill in order to colonize the vascular system and, death-based symptoms are not necessary for *Xf* to exist as benign endophyte. We now have identified several grape genes from a programmed cell death (PCD) suppressive functional cDNA library screen that block PCD when over-expressed in the presence of known pathogen-derived chemical inducers of PCD. These genes were then put into grapes transgenically being driven by the constitutive 35S promoter. First cycle experiments indicate that three of these genes have homology to genes from other sources whereby their presence is associated with situations in which PCD is suppressed. For example, VVPR1A, is expressed in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. The images presented here indicate that the first three of these putative anti-apoptotic genes tested suppress PCD induced by *Xf* and the commensurate PD disease symptoms. In addition, the data confirm that the bacteria remains present in the asymptomatic transgenic plants at a titre level equivalent to that supported by *V. californica* and at levels that were lethal to untransformed or GFP-transformed grape plants used as controls.

INTRODUCTION
We confirmed before beginning this project that the symptoms of PD are due to the induction of apoptosis or PCD. In animals PCD is regulated by many genes but the functional regulators in plants are unknown. We then designed a conditional life-death screen to attempt to identify plant genes that could modulate the activity of the signaling interactions between the bacteria and the grape plant that lead to PCD. The questions posed were: a) are there genes in the plant that respond to *Xf* signals by triggering PCD in certain grape genotypes, b) can this response be blocked genetically, and 3) if so, does this then allow the bacteria to return to the endophytic state, leaving the plant otherwise unaltered but free of disease symptoms or 4) does suppressing PD symptoms negatively affect the ability of the bacteria to colonize the vascular system?

The basic premise is that strategies for disease suppression and characterizing the bacterial-plant interaction were high priority areas in the Pierce's Disease/GWSS Research Program and as noted in the NAS report. Pierce's Disease is defined as plants expressing several symptoms resulting from cell death (leaf scorch) or changes in tissue differentiation (green islands) with the potential to kill plants over time once the infection is established. However, it also is established that several relatives of grape, including *Vitis californica*, and other host plants can harbor otherwise lethal titers of *Xf* without exhibiting PD symptoms.

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 *Xf* in the xylem.

OBJECTIVES
1. Produce grape transgenic plants over-expressing candidate anti-apoptotic plant genes obtained through conditional life-death cDNA library screens.
2. Measure the effect of blocking PD symptoms with anti-apoptotic transgenes on bacterial population and movement *in planta*.
3. Determine grape gene expression changes in transgenic compared with non-transgenic plants infected with *Xf*.

RESULTS
Genes identified as potential anti-PCD genes from the conditional life-death screen.
Previous funding on this project lead to the development of a functional cDNA screen to identify plant genes, which when over-expressed as transgenes, suppress cell death triggered by chemical inducers of PCD. The genes in Table 1 have been described in earlier reports to this symposium. These genes were then transferred to the UC Davis plant transformation facility to insert these genes in cv Freedom and Thompson Seedless, chosen for their high relative susceptibility and ease of transformation. Within the past year we have obtained 42 Freedom and 195 Thompson seedless independent transgenics with a variety of these anti-PCD genes being expressed from the 35S, a strong constitutive promoter.

Grape transgenic plants over-expressing candidate anti-apoptotic plant genes (Tables 2 and 3). All plants were tested in the laboratory for the presence of the test gene and expression of the gene by Northern analysis before moving to the greenhouse. Individual plants were then cloned into ramets of each line and tested again for expression of the transgene before being inoculated with *Xf*.

Cv.Freedom grape plants expressing anti-apoptotic genes were inoculated after creating ramets of these transgenic lines. The transformed plants were individually inoculated March thru June of 2007. The inoculation method was by needle puncture of the stem to allow uptake of 10-20 ul of *Xf* at $2x10^8$ cfu/ml of the Temecula strain. The plants were monitored for symptoms and bacterial movement by PCR. They were scored for disease severity in September 2007 using a 5 point scale (1=dead and 5= asymptomatic) (Figure1 and Table 4) and photographed.

The effect of anti-apoptotic transgenes on *Xf* bacterial populations and movement *in planta* was measured by RealTime quantitative PCR of the stem of primary branches (Table 4). 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. Based on initial experiments to ascertain which tissue to sample for *Xf* quantitation, we have sampled the stem of primary branches of individual plants. This also allows repeated sampling of an individual plant over the course of the experiment. We find that *Xf* bacterial concentrations are similar for all of the asymptomatic plants including *V. californica*

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

Table 2. VARIETY: Freedom

Genotype	# of Independent Transformants	# of Plants
FR - CBP14	16	293
FR - CB456	7	112
FR – CBGFP control	10	140
FR - CB390	9	126
FR-Walker control	1	122
FR-FPS control	1	39
total	44	832

Table 3. VARIETY: Thompson Seedless now available with putative anti-PCD genes under control of the 35S promoter.

Genotype	# Lines or Independent Transformants	# of Plants
TS – CBPR1A	24	30
TS CBP14LD	27	27
TS - CB376	28	29
TS - CB456	27	31
TS - I35	14	15
TS - SGFP-RIN	10	12
TS – CB390	22	24
TS - CBWG23	23	25
TS - CBWG71	20	23
TS – Control	7	72
TS - GFP	1	1
total	203	289

Table 4

Genotype	# of Inoculated independent transformants	# of Plants evaluated to date	Category 3-5 plants similar to images in Figure 1	Mean bacterial load per gm of stem in asymptomatic category 5 branch
FR - CBP14	5	32	58%	10^6
FR - CB456	2	15	71%	10^6
FR - CB390	3	24	76%	10^6
FR – CBGFP (control)	4	29	0%	not applicable
V. californica	asymptomatic host	3	100%	10^4

CONCLUSIONS

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program and the NAS report. The 2007 RFP for research on Pierce's Disease lists as one focused area of research the effect of blocking disease symptoms, defined primary as cell death, initially of foliar tissue with leaf scorch and matchsticks, culminating under heavy disease pressure in death of the entire plant. The overall goal of our research is to determine the molecular basis for the symptomatic cell death and attempt to genetically block the Xylella- triggered cell death as a means of blocking the deleterious effects of the disease. We have identified novel genes from cDNA libraries of either grape or heterologous plants that, when over expressed in grape, prevented symptom development due to the presence of *Xf* in the xylem. We have previously reported 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 PCD. The anti-PCD genes isolated through earlier research in our laboratory are the focus of the current studies that now demonstrate that blocking PCD initiated by *Xf* can block disease rending the grape plant an asymptomatic endophytic host. The current experiments provide initial information that the effect of the genes appears to be on symptom expression and not a direct effect on the bacteria. Therefore, the symptom suppressive genes do not act as antibiotics and do not affect the natural endophytic ecology of the bacteria in the xylem. In essence, an endophyte gone bad has been returned to the state of a benign endophyte.

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FUNDING AGENCIES

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

Figure 1. Anti-PCD genes expressed in transgenic grape (cv. Freedom) plants suppress symptom appearance in PD susceptible plants without affecting the presence of *Xf* in the asymptomatic branches. The transgenic control plants and non-transgenic plants were uniformly killed under the same conditions with the same level of initial inoculum.

THE PIT MEMBRANE BARRIER TO *XYLELLA FASTIDIOSA* MOVEMENT IN GRAPEVINES: BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS

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Reporting Period: The results reported here are from work conducted from July 1, 2006 to September 30, 2007.

ABSTRACT
The overall goal of the work in this project is to characterize the role of the pit membranes of grapevine xylem vessels in limiting the systemic movement of *Xylella fastidiosa*. Work carried out in the project in the last year has made use of monoclonal antibodies that recognize specific cell wall polysaccharides (pectins with varying degrees of methyl esterification and xyloglucan [XyG]) to identify polysaccharides in grapevine pit membranes. The demonstration that these polysaccharides are present is consistent with earlier observations indicating that polygalacturonase (PG) and endo-ß-1,4-glucanase (EGase) are used by the pathogen to digest pit membranes as its population expands and spreads systemically because these enzymes would be expected to digest pectins and XyG. Another study in this project year was carried out to see if we could increase the information content of the MRI analyses we have been doing on Pierce's Disease- (PD) infected grapevines. Our earlier work in a now concluded CDFA-supported project had shown that MRI could non-destructively identify xylem vessels that are cavitated (i.e., air-filled) and non-functional. However, our studies and those of other PD researchers have shown that more permanent obstructions (tyloses and pectin-rich gels) also occur in the vessels of PD-infected vines. Work in this project year has shown that adjustments in the instrument protocols used to obtain MRI images of grapevine stems may allow discrimination between these different potential vessel obstructions

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 three PD Symposia indicate that PG and EGase enzymes, likely produced by *Xf* resident in xylem water-conducting cells (also Roper et al. 2007) 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 been introduced, into leaves where disease symptoms then become evident. Work in 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. To 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. To examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.
3. To 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 reported on our work on this Objective in last year's symposium proceedings (Labavitch, 2006). The one EGase-inhibiting protein reported thus far is from tomato. It specifically inhibits EGase enzymes that digest XyG. It was provided to us by a colleague, Dr. Will York, at the Complex Carbohydrate Research Center at the University of Georgia. However, while the *Xf* EGase cloned and expressed by Dr. Caroline Roper does digest XyG polysaccharides, presumably targeting XyGs in pit membranes (see Objective 2, below), it is not inhibited by the tomato EGase-inhibiting protein. Further work on this aspect of the work must await the identification of new EGase-inhibiting proteins from other plants.

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

Before joining our research program, postdoctoral researcher Qiang Sun had collaborated with us to show that the introduction of PG and EGase to the xylem of healthy grapevine stems resulted in the digestion of pit membranes resulting in perforations that would likely permit unhindered passage of *Xf* cells. Dr. Sun's work in the past year made use of antibodies that bind specifically to epitopes in pectin and XyG polysaccharides to provide the first demonstration that pit membranes contain polysaccharides typically found in the primary cell walls of dicots. While this has been a long-standing presumption, Dr. Sun's use of immuno-localization techniques has confirmed it. Because the *Xf* PG and EGase are likely to digest these polymers, Sun's work explains why these enzymes had digested grapevine pit membranes.

These studies were carried out using monoclonal antibodies that recognize (1) homogalacturonan (HG) with a low level of methyl-esterification (antibody JIM 5), (2) HG with a high level of methyl-esterification (antibody JIM 7), (3) HGs that are cross-linked via Ca^{2+}-cross bridges (antibody 2F4) and (4) fucosylated xyloglucan (antibody CCRC-M1). After reaction with the primary antibody (i.e., the antibody that recognizes specific wall polysaccharide structures), the bound primary antibody is revealed by using a secondary antibody labeled with green fluorescent FTIC that can be observed using a confocal or fluorescence microscope (Figures 1, 2 & 3). In some of the images we have also used these antibodies to ask what the cell walls surrounding tyloses are composed of (Figures 1 & 2, right images). This is an important question related to vascular system obstructions in PD-infected grapevines. Tyloses form early in inoculated vines and numerous tyloses have been reported to accumulate on grape vessels, often completely obstructing them. Tyloses develop from parenchyma cells that are adjacent to vessels and the primary walls of these parenchyma cells share the vessel-parenchyma pit membranes illustrated in some of the images above (Figure 1) and below (Figures 2 & 3).

The MRI analysis of grape stems developed by Dr. Alonso Pérez-Donoso proved to be very useful for detecting cavitated vessels but could not distinguish between water- and pectin-filled vessels. Drs. Greve and Sun first prepared glass capillary tubes filled with either water or water solutions of pectins with a high degree of methyl esterification (71-72%; this is **Pectin #1**)) or a low degree of esterification (33-40%; this is **Pectin #2**). An additional variable in the experiment (Figure 4) was the concentration of each pectin that was tested. Capillaries with 1%, 2% and 4% solutions of pectins #1 and #2 were prepared. Figure 4 shows the array of the capillaries that was placed in the core of the NMR instrument. The pattern of the array identified in Figure 4A, with water in the larger, central capillary is the same in panels B & C.

The images in Figure 4A used the NMR settings routinely used by Alonso in his work with grape stems (Pérez-Donoso et al., 2007). For panel B, the NMR operational parameters were adjusted. An additional adjustment was made in panel C (see figure legend). In panels B and C, note that as TI (the time of inversion delay) is increased the image of the water-filled capillary fades and the images of the pectin-filled capillaries begin to be discriminated from one another, based on pectin concentration and type. The images in panel C demonstrate that the signal is stronger for the more highly Me-esterified pectin #1.

The encouraging results demonstrated by the images in Figure 4 led to an additional experiment in which the grapevine xylem perfusion system used by Perez-Donoso and Greve was used to fill the xylem systems of different 'Chardonnay' grape vines with air, water, a 4% water solution of the low Me-esterified pectin (pectin #2 in Figure 4), and then a 4% gel of that pectin (made by adding $CaCl_2$, once the 4% pectin solution had been perfused into the xylem). The distinctions revealed in the capillary experiments were then shown to work with grape stems (Figure 5).

Objective 3. To repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

Work on these objectives was started in Spring-Summer, 2007 but was not completed. Dr. Sun was made an offer he could not refuse. At the start of August, 2007 he and his family moved to Steven's Point, WI where Qiang began work as an Asst. Professor. The full season series of measurements will be made in Spring-Summer, 2008.

CONCLUSIONS

1. The cell wall substrates for the *Xf* PG and EGase have been demonstrated to be structural components of grapevine pit membranes.
2. Preliminary tests have shown that modifications of the way grapevine stem MRIs are obtained should be able to distinguish between air-embolised (i.e., cavitated) vessels and vessels that are filled with water or pectin.

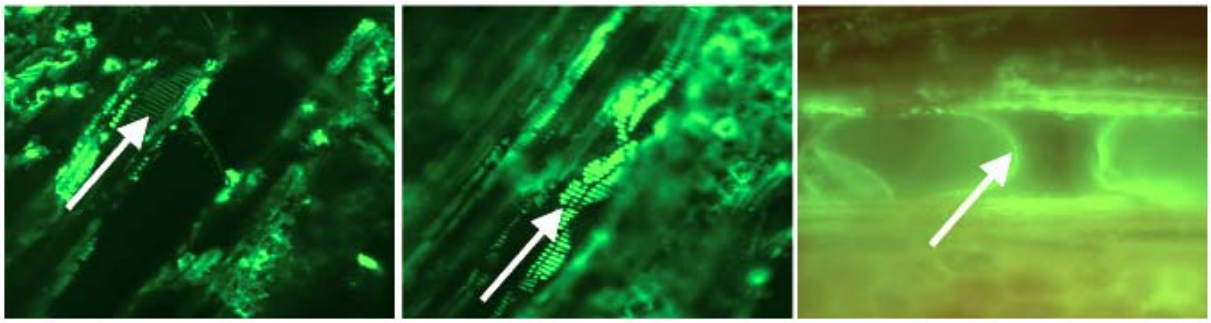

Figure 1. The use of JIM 5 reveals the presence of weakly Me-esterified HGs in the walls of grapevine intervessel pit membranes (left image), in vessel-parenchyma pit membranes (middle image) and in developing tyloses (right image). The arrows in the left and middle images point to the rows of pit membranes that are aligned in the vertical orientation along the length of a vessel. In the right image, the arrow points to the wall of one tylose in a vessel that contains at least two tyloses. The left and center images were made using the confocal microscope. The right image is from the fluorescence microscope.

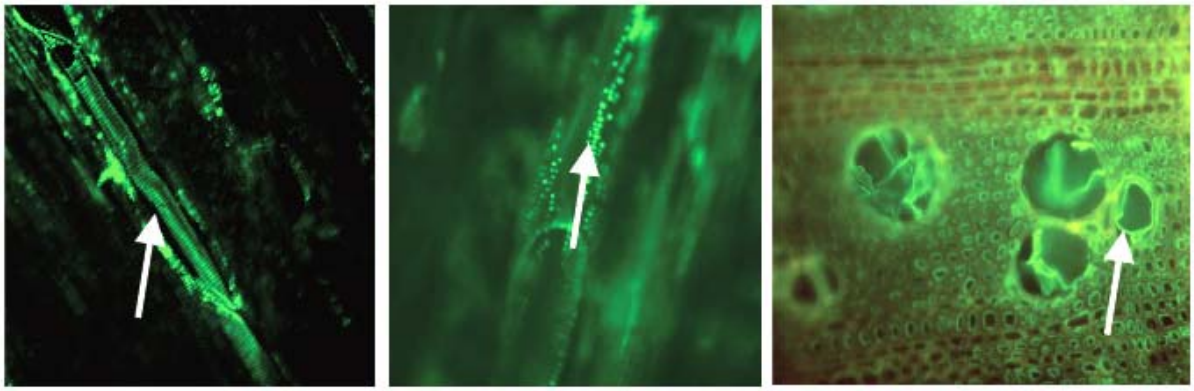

Figure 2. The use of CCRC-M1 reveals the presence of xyloglucan in the walls of grapevine intervessel pit parenchyma pit membranes (middle image) and in developing tyloses (right image). The arrows in the left and middle images point to the rows of pit membranes that are aligned in the vertical orientation along the length of a vessel. In the right image, the arrow points to the wall of one tylose in a vessel that contains at least two tyloses. The left and center images were made using the confocal microscope. The right image is from the fluorescence microscope.

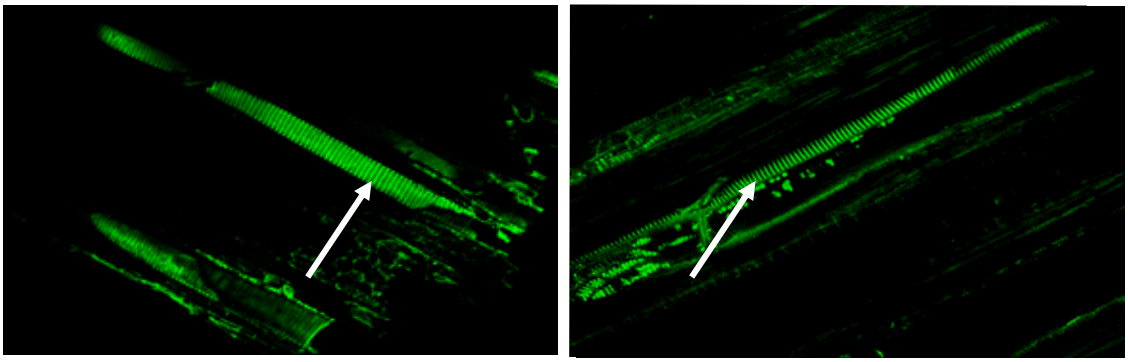

Figure 3. These images of intervessel pit membranes make clear the ladder-like arrangement of pits and pit membranes in the vessel walls. The left image shows the fluorescent fucosylated xyloglucan revealed by the CCRC-M1 antibody. The right image shows the fluorescent Ca-cross linked HG network revealed by the 2F4 antibody. The images were made using the confocal microscope.

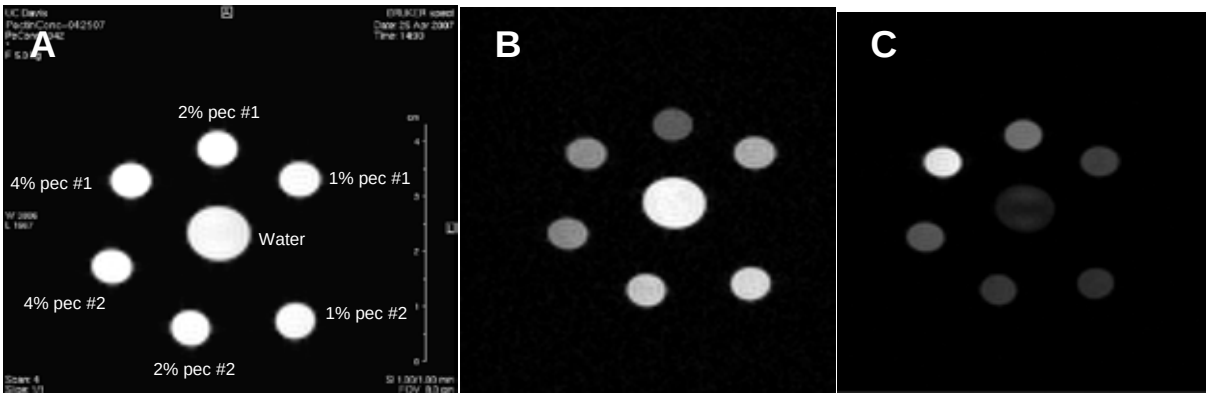

Figure 4. In panel A, the NMR TR (time of repetition) was set at 1906 msec and TE (time of echo) at 15 msec. In panel B the TR and TE were 3005 msec and 10 msec, respectively and TI (time of inversion delay) was set at 800 msec. In panel C, the TR and TE were as in panel B and the TI was set at 1000 msec.

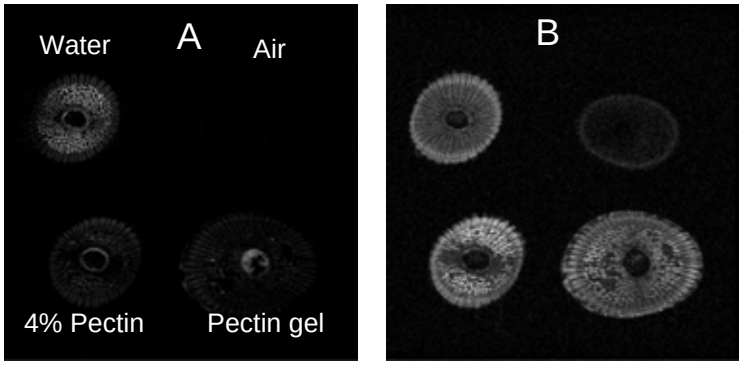

Figure 5. Stems segments (ca. 5 cm in length) were perfused with the indicated solutions. The images in panel A were obtained with the NMR settings shown for Figure 4B. The images in panel B were obtained with the settings used for Figure 4C.

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MICROARRAY ANALYSIS OF GLOBAL GENE EXPRESSION OF *VITIS VINIFERA* IN RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION

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Reporting Period: The results reported here are for work conducted from March 2007 to October 2007.

ABSTRACT
In previous years we have analyzed gene expression profiles of Pierce's Disease (PD) resistant and susceptible genotypes of *Vitis arizonica* hybrids in response to infection by *Xylella. fastidiosa* (*Xf*), the bacterium causing PD. Here we report gene expression of the PD susceptible European grapevine, *V. vinifera* in response to *Xf* infection. RNA was extracted from healthy and infected leaf tissues at 4, 8 and 10 weeks post inoculation. Isolated RNA was converted into cDNA and used for microarray-based global gene expression analysis. Data analysis results indicated that there were a total of 2,385 differentially expressed transcripts from early (4 week), middle (8 week) and late (10 week) stages of disease development. Of these 2,385 transcripts, 1,050 transcripts were up-regulated (2 to 100 fold) and 1,335 transcripts were down-regulated (0.5 to 0.02 fold) across the three stages. Comparative analysis of the differentially regulated transcripts has identified common and distinctive features of the host response from *V. arizonica* hybrids and *V. vinifera* to *Xf* infection that are important for understanding of PD resistant and susceptible mechanisms. An online relational database is now publicly available that has *Vitis* transcriptome data along with other relevant information and bioinformatics tools.

INTRODUCTION
The impact of PD on the California grape industry has been significant since the introduction and establishment of a more effective vector, *Homalodisca coagulata*, the glassy-winged sharpshooter (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. Our recent studies further confirmed that PD response differs between resistance and susceptible genotypes at molecular and physiological levels (Lin et al., 2007; Fritschi et al, 2007). 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 genome-wide 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 an understanding of such gene function and interactions. The goal of this study was to identify and characterize the molecular events in the grape/*Xf* interaction using genome wide transcriptome profiling between resistant and susceptible genotypes and among the different tissue types.

OBJECTIVES
1. Perform a microarray gene expression analysis.
2. Develop a grape transcriptional relational database.

RESULTS AND DISCUSSIONS
Objective 1 - Microarray gene expression analysis.
Experimental set-up: Total RNA from leaf tissues of *V. vinifera* from 4, 8 and 12 weeks post-infection with *Xf* was hybridized to nine slides in a two-color experiment using the monochromatic dyes Cy5 and Cy3. For each time point, there were three slides (biological replicates) including a dye flip.

Data analysis: For each gene 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 first normalized with RMA algorithm (Robust multichip average) and data was further subjected to quantile normalization. SAM software was used to identify statistically significant genes expression changes using a cut-off value of two-fold differential expression and a q-value of 0.5. Clustering of the significantly differentially expressed genes was carried out using TMEV software.

Overview of transcriptional responses: A total of 2,385 transcripts and 1,955 individual genes from early (4 week), middle (8 week) and late (10 week) stage of infection were differentially regulated. Of these 2,385 transcripts, 1,050 transcripts are up-regulated (2 to 100 fold) and 1,335 transcripts are down-regulated (0.5 to 0.02 fold) across the three stages (Table 1). Below we briefly describe the expression pattern of the differentially expressed genes.

Table 1. Summary of the gene expression profile of *V. vinifera* infected with *Xf*. Expression profile data for the spotted genes was generated using the RMA algorithm (Robust multichip average) and was further subjected to quantile normalization. SAM software was used to identify statistically significant genes expression changes using a cut-off value of 2-fold differential expression and a q-value of 0.5.

	Microarray Results		
Expression Pattern	**4 weeks**	**8 weeks**	**12 weeks**
Upregulated	86 (2 to 6 fold)	100 (2 to 100 fold)	864 (2 to 60 fold)
Downregulated	300 (0.12 to 0.49-fold)	162 (0.05 to 0.49-fold)	873 (0.02 to 0.49-fold)

1. Early disease development response
Out of the 1,955 transcripts that were differentially regulated, 158 were exclusively up or down-regulated four weeks after infection. Of these, 33 genes were up-regulated (2 to 5.4-fold) and 125 genes were down-regulated (-2 to -5.9-fold). Some of the up-regulated genes included ABC transporter, adenosine 5' phosphosulfate reductase, polygalacturonase and the down-regulated ones included flavonol synthase, calcium binding protein and heat shock protein 18.

2. Mid-disease development response
There were 57 transcripts that were exclusively up or down-regulated eight weeks after infection. Of these, 17 genes were up-regulated (2 to 2.8-fold) and 40 genes were down-regulated (-2 to -9-fold). Some of the up-regulated genes included chlorophyll A-B binding protein, Glycoside hydrolase family 1 genes. The down-regulated ones included pectinesterase and Serine carboxypeptidase family protein coding genes.

3. Late disease development response
Similarly, after 12 weeks of infection, 1,345 transcripts were exclusively up or down-regulated 12 weeks after infection. Of these, 713 genes were up-regulated (2 to 47-fold) and 632 genes were down-regulated (-2 to -37.5-fold). Some of the up-regulated genes included CBF-like transcription factor, pathogenesis-related protein 5-1, Tyrosine protein kinase, chitinase genes. The down-regulated ones included subtilase, heat shock protein 22, suphate transporter and phosphate transporter, desiccation-related protein and salicylic acid-binding protein 2.

4. Over lapping transcriptional response
In addition, there were three genes that were differentially regulated in early and mid stages of disease development, 167 genes that were differentially regulated in mid and late stages, and 190 genes that were differentially regulated in early and late stages of disease development. 35 genes were found to be differentially regulated at all the three stages.

5. Comparison of *V. vinifera* vs. *V. arizonica* transcriptional responses
Comparison of the PD susceptible *V. vinifera* transcriptional responses to *Xf* infection with the responses observed from *V. arizonica* hybrids that are resistant (9621-67) and susceptible (9621-94) genotypes suggests common as well as distinct responses. Transcripts such as WRKY transcription factor 30, CBF like transcription factor, NDR-1 like protein, phi-1 (an AvrPto-Pto, or AP responsive gene) are commonly upregulated in *V. vinifera* and the resistant (9621-67) genotypes (Figure 1A). Similarly, genes such as polygalacturponase inhibitor like protein, amino acid carrier, sulfate transporter-2, integral membrane protein Nrampl, cholone phosphate cytidylyltransferase and CXE carboxylesterase are commonly downregulated between the *V. vinifera* and the susceptible (9621-94) genotypes. On the other hand, there are genes that are differentially regulated in *V. vinifera* species alone. This includes homologues of metallothionein like proteins, aspartic proteinase 2, starch phosphorylase, putative purine permease, and few hypothetical proteins that are down-regulated only in the *V. vinifera* species (Figure 1B). Similarly, transcripts of genes such as Glycoside hydrolase family-1, dirigent protein oxidase, nucleotide sugar epimerase, ubiquitin protein ligase, NAF protein kinase are only up-regulated in the *V. vinifera* species.

Objective 2 - Develop of a grape transcriptional relational database
VitisExpDB is an online MySQL-PHP driven relational database that houses annotated expressed sequence tags (ESTs) and gene expression data for *V. vinifera* and non-*vinifera* grape varieties. Currently, the database has over 300,000 EST sequences derived from 8 species/hybrids, their annotation details and gene ontology based structured vocabulary. The database has information on probe sequence and annotation features of the 60-mer gene expression chip consisting of ~20,000 non-redundant set of ESTs. There is data on 14 processed global microarray expression profile sets. Data from 12 of these Expression profile sets have been mapped onto metabolic pathways. A web interface with multiple search indices and hyperlinked result features has been developed. Several online bioinformatics tools have been added. In addition, users

can submit their ESTs to the database. VitisExpDB database is available at http://cropdisease.ars.usda.gov/vitis_at/main-page.htm

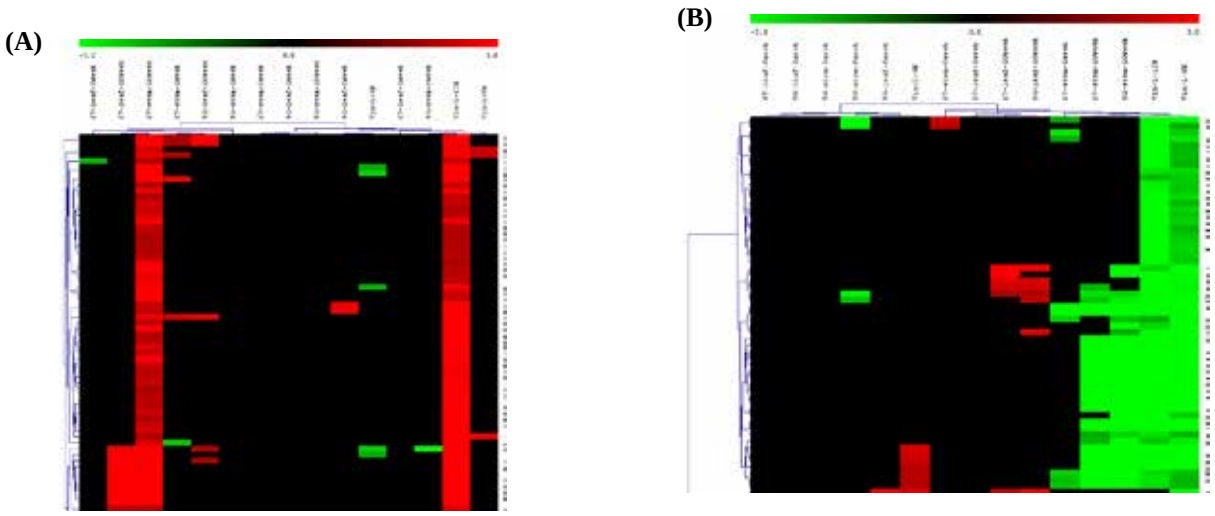

Figure 1. Expression profiling of the differentially regulated transcripts. Red indicates transcriptional activation and green represents repression. Transcripts that are not significantly regulated are shown in black. **(A)** Transcripts that are up-regulated in *V. vinifera* and the resistant genotype (9621-67) of the *V. arizonica* hybrids, **(B)**. Transcripts that are downregulated only in the *V. vinifera* species

Database architecture and Web interface

The server uses Red Hat Enterprise Linux 4 RPM (x86). The relational database was developed using MySQL 4.0 as the back end. The website is powered by an Apache server. A number of useful query interfaces for data mining, analysis and visualization have been developed. This includes simple and advance search forms that facilitate either single query or multiple query search options for both EST and microarrays components. The EST component of the database can be searched using GB number, GI number, Gene Ontology ID, enzyme number or putative function as a key word. Other additional parameters can be included to build a stringent query. A separate web page is provided for listing the homologous gene sets in the major *Vitis* varieties using nWayComp. To query the microarray data, a simple form can be used where the user can enter a GB number, an array ID(s) or a putative function. Alternately, an advance search form is designed where the user can build stringent Boolean searches such as a cut-off expression value or select a particular stage of the experiment, tissue or genotype, or based on Arabidopsis gene ID, for data retrieval. Under the microarray warehouse, a separate HTML page has been designed that has hyperlinked icons to various metabolic pathways. There are 25 different pathways for each of the studied 12 microarray experiments.

Online data analysis tools

Several online tools have been developed that will either interact with the database such as BLAST, CLUSTALW, Tandem Repeats Finder (TRF), and Cluster, or work independently such as CAP3, and other BioPHP modules. Annotated *Vitis* databases such as EST and microarray probe sequence sets have been added to the BLAST database that will help the grape scientific community for quick and efficient identification and annotation of their ESTs. The developed VitisExpDB also includes other online software tools such as bioPHP modules and CAP3 software that will help the user in carrying out DNA, protein and microarray data manipulation. An EST sequence submission form has been developed where users can submit their sequence directly to the database.

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 species, tissues and between resistant and susceptible genotypes, and early and late stages. While a broad spectrum and presumably non specific plant response was observed in *V. vinifera* species as well as in the susceptible *V. arizonica* genotype, a majority of this did not overlap with the resistance genotype response.

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ENDOPHYTIC BACTERIAL POPULATIONS IN GRAPEVINES RESISTANT TO PIERCE'S DISEASE OF GRAPEVINE

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Reporting Period: The results reported here are from work conducted May 2006 to September 2007

ABSTRACT
A specific strain of the bacterium *Xylella fastidiosa* causes Pierce's Disease of grapevine (PD). This disease has caused significant disruption to the wine industry centered in the Temecula, California region; at the height of the most recent PD epidemic in the late 1990s, 25% of the grapevines in this area were lost before emergency quarantine and control measures could be instituted. Under these circumstances, the 2006 discovery of a population of apparently PD-resistant grapevines in the area was of particular interest. The vines were all located in a single vineyard, which had total PD-related losses of approximately 10%, while a neighboring vineyard suffered a nearly 100% loss of the same variety. In addition, a similar phenomenon was observed in a grapevine population located in the Agricultural Operations grounds at the University of California, Riverside. While the cause of this apparent resistance is unknown, one possible explanation for this resistance is that it is being conferred by certain endophytic bacteria present in resistant vines but not in susceptible vines. In order to test this hypothesis, cane samples from both the apparently susceptible populations and the apparently resistant populations were surface sterilized and plated onto standard microbiological media. Any observed bacterial growth was diluted into standard liquid media and then streaked out in order to obtain pure cultures, which were identified using 16S sequencing.

INTRODUCTION
Specific strains of the bacterium *Xylella fastidiosa* (*Xf*) cause disease in almonds, grapevines, and a variety of other economically important plants (1,2,3). *Xf* is spread by the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, formerly known as *H. coagulata* (4,5).

In grapevines, one strain of this bacterium is the cause of Pierce's Disease (PD). Since the preferred host of GWSS is citrus, vineyards close to a citrus grove are at increased risk for the development of PD (6). In addition, Chardonnay vines are known to be more susceptible to PD than other varieties (7). The Weaver vineyard is planted with Chardonnay vines and is immediately across from two citrus groves, meaning that it is at high risk of developing PD. However, while adjacent Chardonnay vineyards suffered catastrophic crop failure, the Weaver vineyard had a PD-related loss of far less, approximately 10%. This observation was of special interest since many of the plants in this vineyard were old enough to have survived the initial PD epidemic that occurred after the GWSS was accidentally introduced into California. The Agricultural Operations vineyard at the University of California, Riverside contains both symptomatic and asymptomatic Chardonnay vines in close proximity. These vines are younger than the ones at the Weaver vineyard.

One possible explanation for this phenomenon is that it is being conferred by bacterial endophytes that live inside the apparently resistant plants but not in the more susceptible plants. The endophytic bacterium, *Curtobacterium flaccumfaciens* has already been shown to confer resistance to *Xf* in sweet orange plants (8).

To test this hypothesis, cane samples from asymptomatic and symptomatic grapevines at both locations were surface-sterilized and then plated on microbiological media. The genus of any resulting bacterial growth was then identified using 16S gene sequencing. The 16S gene has been widely used to classify unknown organisms (9). Because this gene evolves very slowly, it is most useful for classifying organisms at the genus level, but not at the species or subspecies level (10). Even so, it is widespread practice to include a species name when identifying bacteria based on this sequence. These designations can be considered putative in nature.

OBJECTIVES
The primary goal of this research was and continues to be to test the initial hypothesis through isolating bacterial endophytes from asymptomatic and symptomatic grapevines at both locations and using 16S sequencing to identify them.

RESULTS
Table 1 lists currently identified endophytic bacteria isolated from symptomatic and asymptomatic vines at the Weaver vineyard and the UCR Agricultural Operations vineyard (AgOps). Two pieces of data from the BLAST results are also included with the identifications; one is the bit score and the other is the E-value. Typically, the lower the E-value, the higher the probability that the similarities in the two sequences are due to a close genetic relationship and not to random chance. All species designations included are considered putative because 16S analysis does not permit resolution below the genus level.

Table 1

Code	Genus	Putative Species	Bit Score	E-value	Symptomatic	Source
45V16 2D C1	*Bacillus*	*niacini*	863	0	N	Weaver
45V16 2E C1	*Bacillus*	*thuringiensis*	2771	0	N	Weaver
46V16 2D C1	*Bacillus*	*cereus*	500	7.00E-138	N	Weaver
46V19 1C C7	*Bacillus*	*massiliensis*	2454	0	N	Weaver
46V19 2D C1	*Bacillus*	*cereus*	2736	0	N	Weaver
46V19 2F C1	*Bacillus*	M4	2605	0	N	Weaver
47V1 1B C1	*Bacillus*	LMG 20241	1844	0	N	Weaver
47V1 1C C2	*Bacillus*	*cereus*	2389	0	N	Weaver
47V1 1F C1	*Bacillus*	*thuringiensis*	2365	0	N	Weaver
47V1 2C C3	*Bacillus*	9B_1	2692	0	N	Weaver
47V3 1B C1	*Bacillus*	MB-9	2351	0	N	Weaver
47V3 1C C2	*Bacillus*	*drentensis*	1015	0	N	Weaver
47V3 1E C12	*Bacillus*	LMG 20241	401	2.00E-108	N	Weaver
47V3 2D C13	*Bacillus*	*gibsonii*	1009	0	N	Weaver
47V3 O C3	*Planococcus*	*maitrii*	2609	0	N	Weaver
47V8 1A C9	*Bacillus*	*cereus*	2561	0	Y	Weaver
47V8 R6	*Bacillus*	*thuringiensis*	2627	0	Y	Weaver
48V10 1B C1	*Bacillus*	*pumilus*	2591	0	N	Weaver
48V10 1B C2	*Bacillus*	EP23	2407	0	N	Weaver
48V15 1C C3	*Bacillus*	*cereus*	1084	0	N	Weaver
48V15 1D C2	*Bacillus*	*licheniformis*	2605	0	N	Weaver
48V15 2A C7	*Bacillus*	ge15	910	0	N	Weaver
48V19 2F C2	*Bacillus*	*thuringiensis*	2379	0	N	Weaver
49V9 1A C2	*Bacillus*	*subtilis*	979	0	N	Weaver
49V9 1C C2	*Bacillus*	*niacini*	2533	0	N	Weaver
49V9 1C C3	*Bacillus*	*niacini*	2670	0	N	Weaver
49V9 1D C3	*Bacterium*	8-gu2-10	880	0	N	Weaver
49V9 1F C1	*Bacillus*	*cereus*	1203	0	N	Weaver
49V9 1F C2	*Bacillus*	*cereus*	2660	0	N	Weaver
49V9 1F C7	*Bacillus*	*cereus*	1154	0	N	Weaver
49V9 2B C1	*Bacillus*	*cereus*	2577	0	N	Weaver
49V9 2C C1	*Bacillus*	GB02	650	0	N	Weaver
A-4 1A C1	*Erwinia*	*tasmaniensis*	722	0	N	AgOps
A-4 1E C2	*Bacillus*	*pumilus*			N	AgOps
A-4 2A C1	*Bacillus*	*endophyticus*	1352	0	N	AgOps
A-4 2A C12	*Bacillus*	*licheniformis*	2448	0	N	AgOps
B-3 1B C1	*Bacillus*	*thuringiensis*	2674	0	Y	AgOps
B-3 1D C2	*Bacillus*	*megaterium*	2750	0	Y	AgOps
B-3 2C C6	*Bacillus*	*megaterium*	2710	0	Y	AgOps
B-3 2C C7	*Paenibacillus*	*illinoisensis*	1070	0	Y	AgOps
C-1 1D C12	*Staphylococcus*	*epidermidis*	2545	0	N	AgOps
C-1 1E C1	*Bacillus*	*acidicola*	1029	0	N	AgOps
C-1 2A C6	*Bacillus*	*pumilus*	989	0	N	AgOps
C-1 2B C12	*Bacillus*	LMG 20241	946	0	N	AgOps
C-1 2B C13	*Bacillus*	*subtilis*	2441	0	N	AgOps
C-1 2D C12	*Bacillus*	*pumilus*	2753	0	N	AgOps
D-7 2D C12	*Bacillus*	*pumilus*	2640	0	N	AgOps
D-7 2F C16	*Erwinia*	*psidii*	543	6.00E-151	N	AgOps
E-1 2F C16	*Erwinia*	*tasmaniensis*	2127	0	Y	AgOps
F-15 1D C12	*Bacillus*	M31	2246	0	Y	AgOps
F-15 1D C7	*Pantoea*	MMB047	852	0	Y	AgOps
G-6 1A C2	*Bacillus*	*megaterium*	2020	0	N	AgOps

Table 1 (continued)

Code	Genus	Putative Species	Bit Score	E-value	Symptomatic	Source
G-6 1B C16	*Bacillus*	LMG 20241	385	3.00E-103	N	AgOps
G-6 1E C13	*Bacillus*	*subtilis*	1076	0	N	AgOps
G-6 1F C12	*Bacillus*	*licheniformis*	2561	0	N	AgOps
G-6 2D C2	*Bacillus*	M31	2246	0	N	AgOps
G-6 2E C8	*Bacillus*	*pumilus*	722	0	N	AgOps
G-6 2F C6	*Bacillus*	*oleronius*	2629	0	N	AgOps
G-6 2F C16	*Staphylococcus*	*caprae*	562	9.00E-137	N	AgOps
H-11 1D C2	*Bacillus*	PC1	2658	0	N	AgOps
H-11 1E C2	*Bacillus*	*benzoevorans*	2520	0	N	AgOps
H-11 2C C7	*Bacillus*	*benzoevorans*	1061	0	N	AgOps
I-6 1C C12	*Bacillus*	*herbersteinensis*	1110	0	Y	AgOps
J-9 1A C2	*Bacillus*	*pumilus*	2789	0	Y	AgOps
J-9 1A C7	*Bacillus*	*pumilus*	997	0	Y	AgOps
J-9 1C C1	*Brevibacillus*	*laterosporus*	2678	0	Y	AgOps
J-9 2A C16	*Erwinia*	*tasmaniensis*	2452	0	Y	AgOps

CONCLUSIONS

Members of the genus *Bacillus* were most frequently isolated from both symptomatic and asymptomatic plants at the Weaver Vineyard and the Agricultural Operations Vineyard. Bacteria tentatively identified as *Bacillus thuringiensis* were isolated slightly more frequently from asymptomatic vines than from symptomatic vines at both locations. Other bacteria much less commonly isolated from these populations included members of *Staphylococcus*, *Pantoea*, *Brevibacillus*, and *Planococcus*.

There also appeared to be several location-specific effects. For example, bacteria that were tentatively identified as *Bacillus cereus* were isolated solely from asymptomatic vines found at the Weaver Vineyard but were not recovered from any vines in the Agricultural Operations Vineyard. In addition, bacteria tentatively identified as *Bacillus pumilius* were much more frequently isolated from asymptomatic and symptomatic vines at the Agricultural Operations Vineyard than from either symptomatic or asymptomatic vines at the Weaver Vineyard. Finally, members of the genus *Erwinia* were also commonly isolated from symptomatic and asymptomatic vines growing at the Agricultural Operations Vineyard. However, members of this genus have not yet been isolated from any vines in the Weaver Vineyard. The reasons for these differences remain unclear.

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XYLELLA FASTIDIOSA TRANSMISSION BY GLASSY-WINGED SHARPSHOOTERS AND SMOKETREE SHARPSHOOTERS FROM ALTERNATE HOSTS TO GRAPEVINES

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ABSTRACT
The goal of this project is to evaluate the importance of many crops, cover crops and common weeds that are found in close proximity to vineyards as sources of *Xylella fastidiosa* (*Xf*) from which glassy-winged (GWSS) and smoketree (STSS) sharpshooters can acquire and transmit Pierce's Disease (PD) into grapevines. Acquisition from non-grapevine hosts and subsequent transmission to grape is of fundamental importance to primary spread of *Xf* in California vineyards. Identifying the plants that are contributing to primary spread enables growers to target these plants around their vineyards as a mechanism to reduce spread.

INTRODUCTION
Over 140 plants are known to host PD strains of *Xylella fastidiosa* (*Xf*) (Freitag 1951, Raju et al. 1980, 1983, http://www.cnr.berkeley.edu/xylella/temp/hosts.htm). Many of these plants are found in close proximity to vineyards, and some are even used as cover crops in vineyards. While considerable research has identified *Xf* hosts, little work has been done to determine if sharpshooters can acquire the bacteria from these hosts and transmit it to grapevines. If this does not occur, then the alternate host is of little consequence in PD epidemiology. Conversely, plants that contribute inoculum for sharpshooter acquisition and transmission to grape should be removed if growers wish to reduce primary spread into their vineyards.

To successfully implement a program to remove pathogen sources, we first must identify those sources. The introduction into California of GWSS, an insect with a broad host range, theoretically increases the probability of disease spread from the these alternate host plants to grape. For this to occur, GWSS must feed on the infected plant in such a way to acquire *Xf* from the plant, and successfully transmit the acquired pathogen to grapevines. While studies have shown mechanical and insect transmission to wide variety of alternate hosts (Freitag 1951, Purcell and Saunders 1999) they have demonstrated transmission from only a handful of alternate hosts to grapevines (Hill and Purcell 1995, 1997). We are unaware of research published on transmission of *Xf*, PD strain, from alternate hosts into grapevines using GWSS or STSS, a native California sharpshooter also found in grape growing regions, as the vector.

OBJECTIVES
Using GWSS and STSS vectors:
1. Evaluate the acquisition and transmission of *Xf* to grapevines from agricultural crop plants known to be PD hosts that are grown in the vicinity of vineyards.
2. Evaluate the acquisition and transmission of *Xf* to grapevines from weed plants known to be PD hosts that are grown in the vicinity of vineyards.
3. Evaluate the acquisition and transmission of *Xf* to grapevines from vineyard cover crop plants.

RESULTS
Plants for our studies were selected using the UC IPM lists of common weed and cover crop species found in and around California grape vineyards (Statewide IPM Program 2007) to determine the plants that would likely be found near vineyards. This list of plants then was cross-referenced with previously published inoculation studies and field surveys of *Xf* and we selected plants that developed systemic infections in 50% or more of the plants inoculated by Wistrom and Purcell 2005, and plants identified as *Xf* hosts in almond orchards (Shapland et al. 2006) and in vineyard areas of southern California (Costa et al. 2004). Additional plants were chosen because they are commonly found in or around vineyards, but they have not been previously inoculated with *Xf* or used in transmission studies for GWSS and STSS. Our plant list includes:

Table 1: Selected Plants

Common Name	Scientific Name	Plant Type
Alfalfa	*Medicago sativa*	Agriculture Crop
Bell Pepper	*Capsicum annuum*	Agriculture Crop
Cotton, Upland	*Gossypium hirsutum*	Agriculture Crop
Lima Bean, Fordhook 242	*Phaseolus lunatus*	Agriculture Crop
Tomato, Rutgers	*Solanum lycopersicum*	Agriculture Crop
Annual Bluegrass	*Poa annua*	Weed
Annual Bursage	*Ambrosia acanthicarpa*	Weed
Black Nightshade	*Solanum nigrum*	Weed
Cheeseweed	*Malva parviflora*	Weed
Chickweed	*Stellaria media*	Weed
Cocklebur	*Xanthium strumarium*	Weed
Common Groundsel	*Senecio vulgaris*	Weed
Common Morning Glory	*Ipomoea purpurea*	Weed
Common Sunflower	*Helianthus annuus*	Weed
Field Bindweed	*Convolvulus arvensis*	Weed
Filaree	*Erodium sp.*	Weed
Goosefoot	*Chenopodium sp.*	Weed
Horseweed	*Conyza canadensis*	Weed
London Rocket	*Sisymbrium irio*	Weed
Poison Hemlock	*Conium maculatum*	Weed
Prickly Lettuce	*Lactuca serriola*	Weed
Shepherd's Purse	*Capsella bursa-pastoris*	Weed
Spanish Broom	*Spartium junceum*	Weed
Speedwell	*Veronica sp.*	Weed
Stinging Nettle	*Urtica sp.*	Weed
Tree Tobacco	*Nicotiana sp.*	Weed
Alyssum	*Alyssum sp.*	Cover Crop
Annual Ryegrass	*Festuca sp.*	Cover Crop
Annual Fescue, Zorro	*Lolium multiflorum*	Cover Crop
Black Mustard	*Brassica nigra*	Cover Crop
Birdsfoot Trefoil	*Lotus spp.*	Cover Crop
Buckwheat	*Fagopyrum sp.*	Cover Crop
Burr Medic	*Medicago polymorpha*	Cover Crop
Cilantro	*Coriandrum sativum*	Cover Crop
Clover, New Zealand White	*Trifolium repens*	Cover Crop
Clover, Hykon Rose	*Trifolium hirtum*	Cover Crop
Cowpea, California Blackeye	*Vigna unguiculata*	Cover Crop
Fava Bean, Windsor	*Vicia faba*	Cover Crop
Field Pea, Miranda	*Pisum sativum*	Cover Crop
Oat, California Red	*Avena sativa*	Cover Crop
Sweetclover	*Medicago sp.*	Cover Crop
Vetch, Cahaba White	*Vicia sativa*	Cover Crop

Seeds have been obtained for all of the agricultural and cover crops and we have secured seeds for 11 of the 21 weed species listed in Table 1. We have completed inoculations of 7 plant species (Table 2). From the agricultural crop group, we have worked with bell pepper and tomato. Bell pepper appears to be a non-host for PD. Five of 20 PD-inoculated bell pepper plants tested positive with ELISA at 2-weeks, but they all tested negative at 4-weeks, suggesting a transient infection. Attempts to isolate *Xf* from bell pepper by culturing have been negative. A third culture attempt for all inoculated bell pepper plants in currently underway and final results are pending. Of the 20 tomato plants inoculated with PD, 1 died before the first assay, and 10 have tested positive with ELISA. PD has been successfully isolated from these plants and confirmed with PCR. Insect transmissions with both sharpshooter species have been done for bell pepper and data are being collected on this study. Transmissions from tomato to grapevine will be conducted in late October.

From the weed category, we have studied goosefoot and it appears to be a non-host for PD. Only 1of the 20 PD-inoculated plants tested positive and all cultures have been negative. A third isolation attempt is underway for all 20 goosefoot test plants and final results are pending. Experiments on four cover crop species (buckwheat, cowpea, fava bean, and field pea) are underway. All four tested positive for *Xf* with ELISA and bacteria was successfully cultured from these plants (Table 2). Two of the 20 buckwheat plants died before the two-week assay. Most of the field pea plants were dying by two-weeks post-

inoculation. Three were dead at four-weeks post-inoculation and at five-weeks post-inoculation there were only six plants alive, of which one was confirmed positive for PD, but it was in poor condition and could not be used for insect transmission. Field pea inoculations will be repeated in the spring 2008. Insect transmissions have been done for buckwheat and cowpea and we are in the process of evaluating those studies. Insect transmission from fava bean to grape is planned for late October 2007.

Table 2: First Test Group Needle Inoculation Results.

Plant Type	Plant Name	# Inoculated	# Positive 2-Week ELISA	# Positive 4-Week ELISA	Cultured
Agric. Crop	Bell Pepper	20	5	0	Negative
Agric. Crop	Tomato	19	5	6	Positive
Cover Crop	Buckwheat	18	5	12	Positive
Cover Crop	Cowpea	20	4	7	Positive
Cover Crop	Fava Bean	20	11	17	Positive
Cover Crop	Field Pea	20	4	9	Positive
Weed	Goosefoot	20	0	1	Negative
Control	Grapevine	20	9	16	Positive

CONCLUSIONS

This project addresses the 2006 Scientific Summit category of "*Understanding transmission of the disease,*" and relates directly to the acquisition and transmission of *Xf* by GWSS. It also has relevance to several of the recommendations developed by the National Academy of Science, National Research Council (2004). First and foremost, the definition of the Category 1 research option is that it "holds a reasonable promise of generating successful tools for management of PD/GWSS, either in the short term or the long term." By determining the plants that truly contribute to primary spread by sharpshooters, we can give growers another strategy (i.e. removing those plants) in an effort to reduce bacterial inoculum around their vineyards. This proposal also meets the general criteria defined in the NRC report in recommendation 2.2, of "contributing to PD/GWSS management and its sustainability," and it applies specifically to recommendation 3.9 of examining plants "for effective transmission rates from host to grape."

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BREEDING PIERCE'S DISEASE RESISTANT TABLE AND RAISIN GRAPES AND THE DEVELOPMENT OF MARKERS FOR ADDITIONAL SOURCES OF RESISTANCE

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ABSTRACT
Fifteen BC3 and two BC2 crosses between the *V. arizonica* source of Pierce's disease (PD) resistance and seedless table and raisin grape selections were made, seedless ovules were cultured and produced 3,396 berries, 4,459 ovules and 1,840 embryos. Two additional seedless crosses from Southeast United States (SEUS) and *V. tiliifolia* were also made. Two seeded BC1 crosses based on a SEUS PD resistance source were made, resulting in 349 seed. Ten 2006 BC2 families *(V. arizonica* source of resistance) consisting of 765 individuals were screened at the seedling stage in the greenhouse with SSR markers for resistance. A total of 262 were resistant and planted in the field. Resistance and susceptibility segregated in a 1:1 ratio in all but one family. Greenhouse screening of the BC2 raisin family 04-5554 confirmed that 17 of 18 individuals with resistant markers were resistant. Four of the 13 resistant fruiting plants had small aborted seed and they will be useful as parents for continued backcrossing. Progress in developing fruit quality improved as rapidly in resistant progeny as in susceptible progeny. An additional 33 plants and 241 embryos have been produced to increase the C33-30 x BD5-117 family size for maker development. This year 424 SSR primers were screened against the parents and resistant and susceptible bulks. From these 30 were confirmed to be polymorphic.

INTRODUCTION
Pierce's disease (PD) has existed in California since the late 1800s when it caused an epidemic in Anaheim. A number of vectors for PD already exist in California, and they account for the spread and occurrence of the disease. The introduction of the glassy-winged sharpshooter to California in the 1990's significantly increased the spread and damage caused by PD. Other vectors exist outside California and are always a threat. All of California's commercially grown table and raisin grape cultivars are susceptible to PD. An effective way to combat PD and its vectors is to develop PD resistant 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 is inferior to *V. vinifera* table and raisin grape cultivars grown in California. Greenhouse screening techniques have been improved to expedite the selection of resistant individuals (Krivanek et al. 2005, Krivanek and Walker 2005). Molecular markers have also been identified that make selection of PD resistant individuals from *V. arizonica* in these families even quicker (Krivanek et al. 2006). The USDA, ARS grape breeding program at Parlier, CA has developed elite table and raisin grape cultivars and germplasm with high fruit quality. Embryo rescue procedures for culturing seedless grapes are being used to help introgress the seedless trait with PD resistance quickly (Emershad et al. 1989). This collaborative research gives the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry.

OBJECTIVES
1. Develop PD resistant table and raisin grape germplasm/cultivars with fruit quality equivalent to standards of present day cultivars.
2. Develop molecular markers for *Xf*/PD resistance in a family (SEUS) other than those from *V. arizonica.*

RESULTS
Objective 1 This year the majority of seedless embryo culture crosses concentrated on using the *V. arizonica* source of resistance. Fifteen BC3 and 2 BC2 crosses were made and produced 3,396 berries, 4,459 ovules and 1,840 embryos (41% embryos/ovules) (Table 1). The percent embryos recovered is twice the amount normally recovered from seedless embryo rescue. One BC1 cross from SEUS source of resistance and one cross to increase the population from a unique source of resistance from *V. tiliifolia* were also made (Table 1). Two seeded BC1 crosses from SEUS source of resistance were made (Table 2). Fruit has been harvested and 349 seeds extracted for germination in January.

Ten BC2 families (89-0908 *V.* arizonica source of resistance) produced in 2006 and growing in the greenhouse as small plants were tested for molecular markers associated with the PdR1 locus on chromosome 14 (Table 3). A total of 765 individuals were tested with SSR markers and 680 showed markers on both sides of the PdR1 region as expected. Eighty-two percent had either resistant or susceptible bands making selection for resistance effective. A total of 262 individuals (39% of those showing markers) were resistant and planted to the field from the greenhouse. The susceptible individuals were discarded. All families segregated in a 1:1 ratio except for family 06-5552 which had a higher percent of resistant individuals. Greenhouse testing of selected individuals from the BC2 *V. arizonica* raisin family 04-5554 was completed.

Twenty-nine individuals were tested in the greenhouse. Four of the susceptible individuals based on molecular markers were tested and had > $6x10^6$ cfu/ml bacteria and >5 PD symptoms, indicating that they were susceptible. Seventeen of the 18 individuals identified as resistant with markers fell in the resistant class based on the greenhouse test (<250,000 cfu/ml bacteria and <2.8 PD symptom rating). This gives good confidence in the use of molecular markers for the *V. arizonica* (89-0908) source of resistance. Plants from this family fruited in the field for the first time this year. Thirteen were resistant and consisted of seven seeded, two with large aborted seeds, and four with small aborted seeds. This was comparable to the fourteen susceptible which consisted of 10 seeded, one with large aborted seeds, and three with small aborted seeds. The mean berry weight for the resistant individuals was 2.11 g compared to 1.96 g for the susceptible individuals. This shows that progress can be made as fast in developing resistant types with high fruit quality as in susceptible types, and that seedless resistant genotypes can be produced. This family is 87% *V. vinifera* and most of the seedlings are neutral in flavor without off flavors. Seedlings from 2005 crosses have also started to fruit. In the raisin family 05-5551 (seeded x seedless), all resistant seedlings produced seedless fruit, with 13 having large aborted seeds and 10 with small aborted seed traces. The susceptible individuals have not yet been analyzed for their seed content. The 23 resistant individuals were composed of 12 with white fruit and 11 with colored fruit which fits the expected ratio. The average fruit size between the resistant and susceptible individuals was comparable at 1.45 g and 1.48 g respectively. This also indicates that progress is being made equally in the resistant compared to the susceptible types based on berry size. Additional families are still being evaluated for their fruit quality. All PD selections from the California State University, Fresno have been propagated and moved to the USDA/ARS research center at Parlier, California.

Table 1. 2007 table and raisin grape PD resistant seedless crosses and the number of ovules and embryos produced.

Female	Male	Type		No. Emas-culations	No. berries Opened	No. Ovules	No. Embryos
89-0908 *V. rupestris* x *V.arizonica*							
Scarlet Royal	04-5012-2	Table	BC3	2,687	219	297	192
C45-25	04-5012-3	Table	BC3	2,997	107	117	34
A38-7	05-5551-39	Table	BC3	1,530	169	299	99
C49-96	05-5551-62	Table	BC3	942	0	0	0
C49-96	04-5012-3	Table	BC3	1,018	2	0	0
B48-17	05-5551-26	Table	BC3	1,421	94	19	3
A49-82	05-5551-110	Raisin	BC3	4 bags[a]	252	367	78
04-5554-08	A63-85	Raisin	BC3	4 bags	415	657	283
A57-27	05-5551-116	Raisin	BC3	3,365	74	2	0
A50-85	05-5551-109	Raisin	BC3	3,496	300	335	63
A61-97	04-5554-01	Raisin	BC3	2,097	61	63	14
A51-21	04-5554-01	Raisin	BC3	2,404	82	94	15
B82-43	05-5551-30	Raisin	BC3	1,827	251	344	59
B82-43	05-5551-62	Raisin	BC3	2,029	275	350	147
04-5554-08	A50-33	Raisin	BC3	4 bags	492	590	221
A81-138	Summer Muscat	Raisin	BC2	7 bags	265	450	309
A81-138	Selma Pete	Raisin	BC2	8 bags	338	475	323
Total				**25,831**	**3,396**	**4,459**	**1,840**
V. tiliifolia source of resistance							
C33-30	IAC572	Genetic family		5 bags	641	850	261
SEUS source of resistance							
C33-30	BD5-117	Genetic family		10 bags	588	724	241
01-5026-20	C56-11	Table BC1		7 bags	190	450	87

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

Table 2. 2007 table and raisin grape PD resistant seeded x seedless crosses and the number of seeds produced.

Female	Male	Type		No. Emasculations	No. Seeds
SEUS BD5-117 source of resistance					
01-5026-15	C56-11	Table	BC1	1099	25
01-5026-21	B49-128	Table	BC1	6 bags[a]	324

[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 made in 2006.

Family	Type Cross	No. Resistant[a]	No. Susceptible	No. Recombinants[b]	No data[c]	Total
06-5501	Table	40	56	21	7	124
06-5502	Table	24	22	21	9	76
06-5503	Table	30	42	18	14	104
06-5504	Table	27	33	9	8	77
06-5551	Raisin	37	37	4	5	83
06-5552	Raisin	37	20	29	9	95
06-5553	Raisin	50	59	15	13	137
06-5554	Raisin	1	1	0	0	2
06-5555	Raisin	0	0	0	2	2
06-5556	Raisin	16	24	7	18	65
Total		**262**	**294**	**124**	**85**	**765**

[a]Resistant = marker on both sides of *PdR1* region.
[b]Susceptible = no markers.
[c]No data = genotypes that amplified with one marker, off types and that failed with both 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 was produced. Additional plants are being made to increase the family size. In 2006, 33 plants were produced. In 2007, 724 ovules were cultured and produced 241 embryos that have been sub-cultured on fresh medium for growth into plants. Fruit from the 154 individuals has been collected and is being evaluated for berry size, seed/trace weight and fruit characteristics. This year 424 SSR primers were screened against the parents, a resistant bulk of five individuals and a susceptible bulk of five individuals. Of these SSR primers, 30 showed polymorphism amongst the resistant and susceptible parents and bulks. These polymorphic SSR primers have been retested against the parents and individuals from the bulks and continue to show polymorphism. The next step is to test all 154 individuals and parents with these 30 polymorphic SSR markers.

CONCLUSIONS

Families for the development of PD resistant seedless table and raisin grape cultivars continue to be produced and the first BC3 crosses in the *V. arizonica* source of resistance were made this year. This generation is 93% *V. vinifera*. The use of molecular markers has simplified and sped up the identification of PD resistant individuals from *V. arizonica.* Resistance based on molecular markers continues to segregate in a 1:1 ratio in *V. arizonica* BC2 families. Seedless table and raisin grape selections with PD resistance and improved fruit quality have been made. Thirty Polymorphic SSR primers have been identified in the BD5-117 family in the search for molecular markers from sources of resistance other than *V. arizonica.*

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MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES: THE XYLEM PATHWAYS AND MOVEMENT OF *XYLELLA FASTIDIOSA*. COMPARISON OF THE XYLEM STRUCTURE OF SUSCEPTIBLE/TOLERANT GRAPEVINES AND ALTERNATE PLANT HOSTS

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ABSTRACT
In grape, there are very few xylem differences between susceptible and tolerant varieties, except for smaller vessel diameters and more parenchyma rays in the tolerant variety examined. These findings in the tolerant grape suggest a restriction to bacterial movement imposed at the level of the vessels. The systemic and non-systemic alternate hosts studied here showed relatively similar xylem characteristics with large variations. Overall, the comparison of the basic xylem characteristics (vessel length, diameter, stem-petiole-leaf connectivity) is not conclusive and more studies are needed. First, more species needs to be studied as *Xylella fastidiosa* (*Xf*) is found in numerous species. Second, *Xf* movement and patterns of colonization needs to be determined in more details (time, distance, location). Third, differences in the vessel network, vessel overlapping, spatial organization of the pit fields, structure (thickness, porosity) of the pit membrane, need to be considered to fully understand the role of the xylem network in bacterial movement.

INTRODUCTION
The capacity for *Xf* to move in the xylem differs greatly among species (Freitag, 1951), ranging from moving efficiently throughout the plant to instances where the bacterium moves only a few centimeter from the infection point (Hill and Purcell, 1995; Purcell and Saunders, 1999; Costa et al., 2004; Wistrom and Purcell, 2005; Baumgartner et al., 2005). Our lab has observed the presence of long xylem conduits from stem to leaves in chardonnay and cowart (Thorne et al., 2006; Chatelet et al, 2006) and we reported last year that these conduits seemed to be shorter in alternate hosts in which bacterial movement is limited. Other aspects of the xylem system, for example, a higher number of tracheids, shorter and narrower vessels, the spatial organization of the vessels, and the number and location of paratracheal parenchyma cells could be part of a passive strategy to limit bacterial movement. Another strategy in non-systemic species, where the bacteria do not move, 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 objective of this study was to study the comparative anatomy of different species of plants supporting a range of *Xf* population sizes and movement characteristics described by previous studies (Purcell and Saunders, 1999; Costa et al., 2004; Wistrom and Purcell, 2005; Baumgartner et al., 2005). 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.

A range of species chosen from previous studies (Purcell and Saunders, 1999; Costa et al., 2004; Wistrom and Purcell, 2005; Baumgartner et al., 2005) was examined:
1. grapevines with a low infection rate, medium bacterial population and showing very little to no bacterial movement (non-systemic): *Vitis vinifera* cv. Cabernet sauvignon and *Vitis vinifera* cv. Sylvaner.
2. grapevines with a high infection rate, high bacterial population and showing systemic movement: *Vitis vinifera* cv. Chardonnay and *Vitis vinifera* cv. Pinot noir,
3. other plant 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),
4. plant 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).

RESULTS
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., 2006). However, there was no difference between the resistant and susceptible grapevine varieties (Table 1). Also, despite large variations, the alternate species categorized as supporting the systemic spread of *Xf* seemed to have longer open conduits compared to the non-systemic plants (Table 1). 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.

Table 1: Xylem vessel lengths measured as the total distance (mm) from the base of the petiole to the leaf margin divided by the distance moved by low pressured air and paint and expressed as a percentage for primary laminar veins, in grapevine varieties and in plants allowing or not the movement of *Xf*.

	Node 3	Node 7	Node 12	Node 16	Node20
Grapevine					
Sylvaner	71.9 (2.9)[a]	68.6 (2.1)[a]	71.1 (2.4)[a]	71.5 (2.6)[a]	69.5 (1.9)[a]
Cabernet Sauvignon	71.7 (2.8)[a]	69.9 (2.3)[a]	73.6 (3.1)[a]	70.6 (2.6)[a]	69.4 (2.9)[a]
Pinot Noir	71.8 (2.9)[a]	64.3 (3.8)[a]	68.7 (4.0)[a]	71.2 (2.9)[a]	69.9 (2.9)[a]
Chardonnay	52.9 (4.2)[b]	47.0 (2.9)[b]	54.9 (4.3)[b]	61.2 (2.0)[b]	62.5 (2.4)[b]
Systemic					
Morning glory		67.1 (2.0)[a,b]	73.1 (2.1)[a,b]	77.7 (1.6)[b]	86.6 (1.6)[a]
Periwinkle	30.6 (1.6)[c]	34.9 (2.2)[d,e]			
Orange	69.5 (1.6)[a]	68.9 (2.4)[a,b]			
Almond	52.7 (2.6)[b]	53.4 (3.5)[c]	53.9 (1.8)[c]	54.5 (2.9)[c,d]	54.8 (2.7)[b]
Sunflower		59.0 (3.4)[b,c]	55.9 (3.6)[c]	58.8 (5.9)[c]	58.9 (2.9)[b]
Tobacco			42.3 (4.2)[d]	50.0 (4.5)[d]	56.1 (2.7)[b]
Non-systemic					
Laurel	45.4 (2.9)[b]	39.6 (3.2)[d]			
White alder	30.2 (2.4)[c]	27.9 (2.1)[e]	29.7 (2.7)[e]		
Datura	70.9 (2.7)[a]	76.6 (2.6)[a]	70.3 (1.9)[b]	84.1 (2.5)[f]	
Eucalyptus	34.1 (4.2)c	32.2 (8.6)[d,e]	35.3 (2.3)[d,e]	34.8 (1.9)[a,b]	
Mugwort		19.9 (1.1)[f]	20.0 (2.2)[f]	20.8 (1.1)[e]	21.1 (1.4)[c]
Quinoa	66.1 (4.3)[a]	72.6 (4.5)[a]	79.9 (1.8)[a]	86.8 (1.4)[a]	80.8 (2.4)[a]

Leaf vessel length distribution (Figure 1, left) - The species with limited *Xf* movement have slightly shorter vessels. With the exception of morning glory (systemic) and quinoa (non-systemic), at least 40% of the vessels are less than 3cm. The longest vessels [24-27cm] were found in systemic plants: morning glory, sunflower and tobacco. The vessel lengths in grapevine leaves are within the range of those from alternate hosts.

Stem vessel length distribution (Figure 1, right) - The vessel length distributions in stems of systemic or non-systemic hosts are similar. In all alternate hosts except orange, 30 to 80% of the vessels were less than 3cm. In contrast in grapevine, the majority of the vessels (55%) were less than 6cm. Also, the longest vessel measured in alternate hosts was about 30cm (eucalyptus), while in grapevines it was about 100cm.

Vessel diameter distribution at the base of the stem (Figure 2, left)- The diameter distributions of the vessels at the base of the stem of the alternate hosts are similar, ranging mostly from 10 to 35µm, except for the vessels from sunflower (systemic) and datura (non-systemic) whose diameter ranged from 40 to 65µm. In contrast, the grapevines have most of their vessels ranging from 150 to 400µm at the exception of the resistant Sylvaner whose vessels are slightly smaller and mostly ranged from 80 to 250µm.

Vessel diameter distribution at the base of the petiole (Figure 2, right) - Petioles of the four grapevine varieties showed similar vessel diameter distributions with about 70% of the vessels ranging from 10 to 45µm. Alternate hosts (systemic and non-systemic) showed a lower vessel diameter distribution, in the range 0-25µm, with the exception of sunflower (systemic) and datura (non-systemic) whose diameter ranged from 30 to 55µm (Sunflower) and 15 to 45µm (datura).

Anatomical differences from stem cross-sections - Among grapevines, the only difference resides in the number of rays. The resistant grapevine (Sylvaner) has significantly more rays separating the vessels (Table 2) than the other grapevines in which bacterial movement is more efficient. Regarding the other plant species hosting *Xf*, no discernable differences between systemic and non-systemic were observed.

Table 2: Anatomical comparisons of stems of similar age

	Nb vessel at cane / stem base (SE)	Vessel density (SE)	% vessel ≤ 3cm (SE)	Longest vessel (SE)	Nb of rays / stem base (SE)
Grapevine					
Sylvaner	513 (38)^{a}	12 (2)^{a}	17 (5)^{a}	69 (9)^{a}	40 (2)^{a}
Cabernet Sauvignon	487 (27)^{a}	14 (1)^{a}	24 (2)^{a}	60 (3)^{b}	34 (1)^{b}
Pinot Noir	474 (27)^{a}	13 (2)^{a}	20 (3)^{a}	64 (9)^{a}	34 (2)^{b}
Chardonnay	433 (19)^{a}	10 (1)^{a}	26 (2)^{a}	72 (9)^{a}	35 (1)^{b}
Systemic					
Morning glory	298 (26)^{g}	15 (2)^{f,g}	66 (5)^{b,c}	13 (2)^{e,f}	84 (3)^{f}
Periwinkle	584 (5)^{b,c}	58 (5)^{c}	84 (2)^{a}	17 (1)^{c,d,e}	82 (2)^{f}
Orange	446 (4)^{d,e}	75 (5)^{b}	21 (1)^{g}	12 (1)^{f}	137 (1)^{d}
Almond	731 (11)^{a}	28 (2)^{e}	70 (2)^{a,b}	18 (1)^{b,c,d}	146 (2)^{c}
Sunflower	314 (22)^{f,g}	8 (2)^{g}	42 (7)^{e,f}	21 (2)^{b}	19 (1)^{i}
Tobacco	474 (23)^{d,e}	6 (1)^{g}	64 (7)^{b,c}	15 (2)^{d,e,f}	116 (1)^{e}
Non-systemic					
Laurel	434 (19)^{d,e}	14 (1)^{f,g}	37 (4)^{f}	20 (1)^{b,c}	56 (2)^{g}
White alder	657 (18)^{a,b}	87 (7)^{a}	71 (3)^{a,b}	6 (1)^{g}	170 (3)^{b}
Datura	485 (14)^{d,e}	25 (1)^{e,f}	45 (2)^{d,e,f}	27 (1)^{a}	32 (2)^{h}
Eucalyptus	507 (5)^{c,d}	65 (3)^{b,c}	53 (2)^{c,d,e}	28 (1)^{a}	198 (3)^{a}
Mugwort	489 (52)^{d,e}	40 (4)^{d}	58 (5)^{b,c,d}	12 (1)^{f}	18 (1)^{i}
Quinoa	391 (20)^{e,f}	20 (3)^{e,f,g}	35 (3)^{f}	18 (1)^{b,c,d}	30 (2)^{h}

CONCLUSIONS

From this study, it appears that the comparison of the basic xylem characteristics (vessel length, diameter, stem-petiole-leaf connectivity) in relation to the limitation of bacterial movement is not conclusive. In grape, the only differences consisted of smaller vessel diameters and higher number of rays in the tolerant grapevine variety, suggesting a restriction of the bacterial movement imposed at the level of the vessel elements. T he alternate hosts showed similar xylem features with a lot of variability. Overall, the comparison of the basic xylem characteristics (vessel length, diameter, stem-petiole-leaf connectivity) is not conclusive and needs to be extended. First, more species needs to be studied as *Xf* is found in numerous species. Second, *Xf* movement and patterns of colonization needs to be determined in more details (time, distance, location). Third, differences in the vessel network, vessel overlapping, spatial organization of the pit fields, structure (thickness, porosity) of the pit membrane, need to be considered to fully understand the role of the xylem network in bacterial movement.

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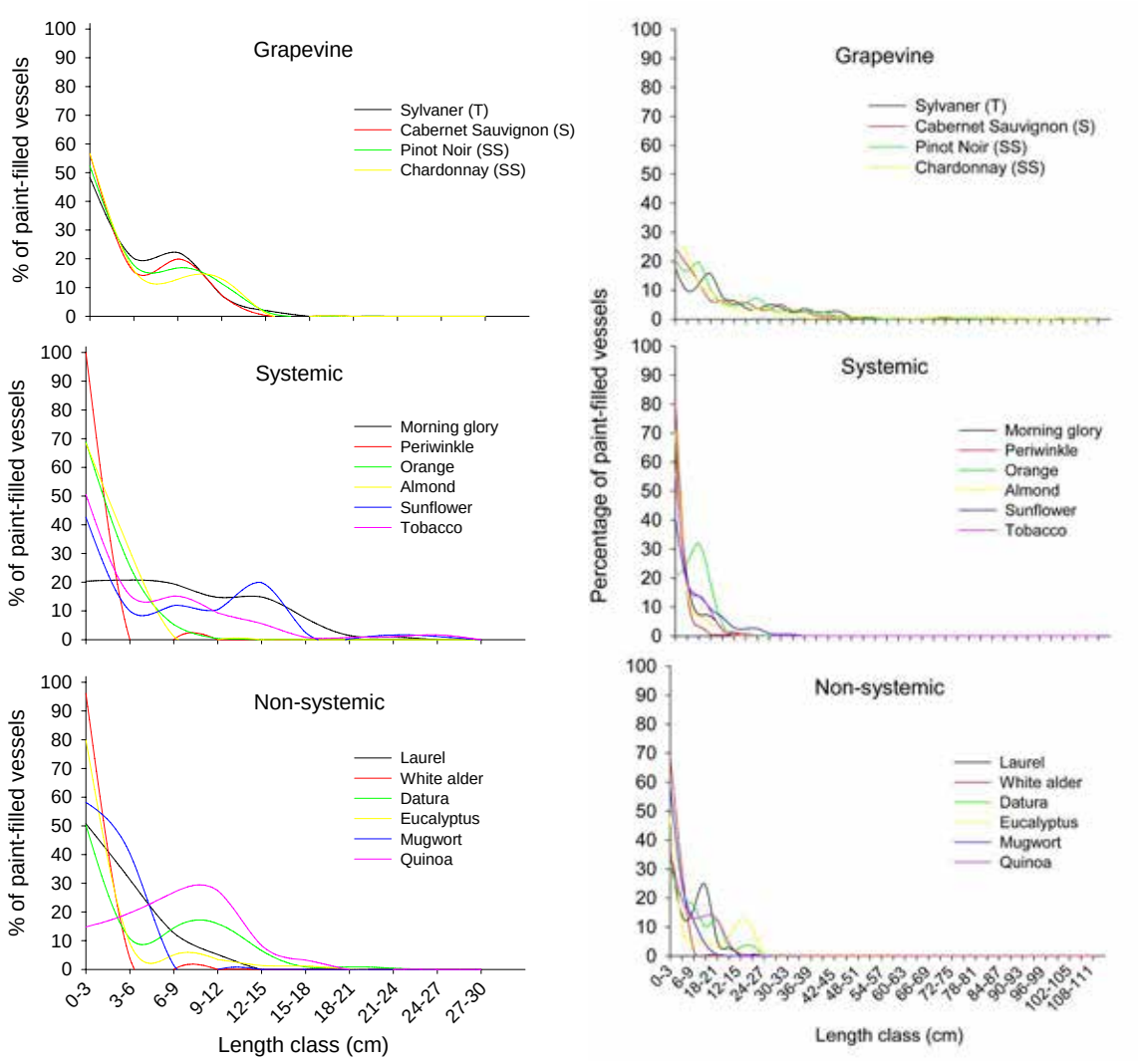

Figure 1: Vessel length distribution in the mature leave (left) and in the stem (right) of grapevines and alternate hosts to *Xf*. For each length class, the number of painted-vessels was calculated as a percentage of the total number of painted vessels at the petiole or stem base.

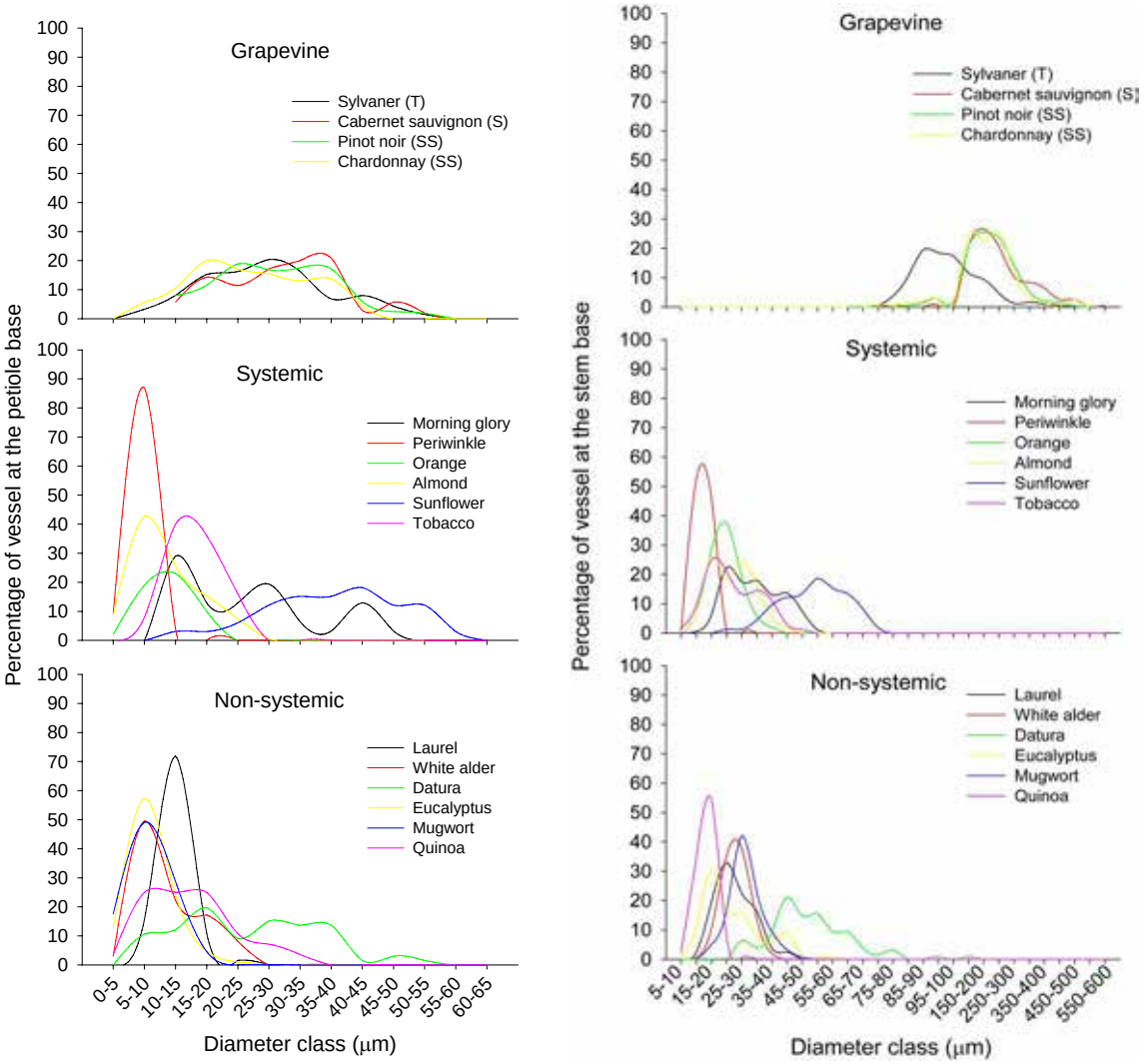

Figure 2: Vessel diameter distribution at the base of the petiole of mature leaves (left) and at the base of stems (right) from grapevines and alternate hosts to *Xf*.

ASSESSING THE POTENTIAL OF FORAGE ALFALFA CROPS TO SERVE AS PIERCE'S DISEASE PRIMARY INOCULUM SOURCES IN THE SAN JOAQUIN VALLEY

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ABSTRACT

The potential for forage alfalfa to serve as a primary inoculum source of Pierce's disease (*Xylella fastidiosa; Xf*) in the San Joaquin Valley of California was evaluated. Laboratory inoculation of fourteen cultivars of alfalfa indicated that all alfalfa cultivars tested were equally suitable hosts for *Xf*. Incidence of *Xf* in forage alfalfa averaged across all field sites located in the San Joaquin Valley of California was low (0.1%), although incidence in one field on one date was 9%. Green sharpshooter (*Draeculacephala minerva*), a known vector of *Xf*, was prevalent at field sites and preferred field edges. The three cornered alfalfa hopper (*Spissistilus festinus*), whose vector competency is unknown, was more abundant in field samples than green sharpshooter. Greenhouse transmission tests with the three cornered alfalfa hopper have not documented competent transmission. The results indicate that alfalfa has the potential to be an important inoculum source due to its suitability as a host for *Xf* and the presence of vectors, but that measured incidence of *Xf* in alfalfa is typically low.

INTRODUCTION

Pierce's disease (PD) of grape and almond leaf scorch disease threaten grape and almond production in California's San Joaquin Valley. Little is known about where potential insect vectors acquire the pathogen, when they move into orchards/vineyards, and when they spread the pathogen. Alfalfa is widely planted in the Southern San Joaquin Valley and alfalfa fields often border vineyards and almond orchards. Alfalfa is a host of *Xylella fastidiosa (Xf)* and often harbors high numbers of known vectors (Freitag and Frazier 1954). Due to the large acreage planted to this crop in the San Joaquin Valley, it's potential to serve as a host of *Xf*, and it's propensity to harbor vectors, we initiated studies to quantify the potential of alfalfa forage crops to serve as a primary source of *Xf* inoculum.

OBJECTIVES

1. Estimate *Xf* incidence in forage alfalfa planted adjacent to grape and/or almond.
2. Characterize the seasonal abundance and dispersal of green sharpshooters present within and emigrating from alfalfa.
3. Determine the relative susceptibility of selected alfalfa cultivars to infection by *Xf*.
4. Determine the vector competence of a potentially new insect vector, the three-cornered alfalfa hopper, *Spissistilus festinus*, (Hemiptera: Membracidae).

RESULTS

Objective 1. Estimate *Xf* incidence in forage alfalfa planted adjacent to grape and/or almond. We sampled alfalfa fields in Fresno, Tulare, and Kern County seasonally (Winter, Spring, Summer, Fall) to estimate the incidence of *Xf* starting in the summer of 2005 to the present. To date, 5,533 samples have been screened for the presence of *Xf* using conventional PCR (Minsavage et al. 1994). Of those samples, six have been confirmed positive. Two positives came from a collection in Fresno County during the summer of 2005. The other four positives came from another collection in Fresno County during the summer of 2007.

Objective 2. Characterize the seasonal abundance and dispersal of green sharpshooters present within and emigrating from alfalfa. The abundance and spatial distribution of the green sharpshooter was monitored in alfalfa fields in Fresno, Kern, and Tulare Counties throughout 2006 and 2007. Four transects of yellow sticky traps were placed in each

field. Traps were counted and replaced biweekly. Peaks in trap catches occurred in May/June and in July/August (Figure 1). Trap catches were generally higher on field edges than in the middle (Figure 1).

Objective 3. Determine the relative susceptibility of selected alfalfa cultivars to infection by *Xf*. Fourteen alfalfa cultivars were screened to determine their relative susceptibility to infection by four different *Xf* strains (Temecula, Dixon, M12, and M23). Six plants of each cultivar were needle inoculated with each strain of *Xf*. Plants were screened for infection using conventional PCR 12 weeks after inoculation. The pathogen was detected in at least three out of 24 plants for each cultivar and the percentage of plants infected averaged across the four *Xf* strains varied from 13 to 48% (Figure 2). After screening, only infected plants and control plants were kept. Plants were screened again in early fall and infections were confirmed in 35% of plants previously determined to be infected.

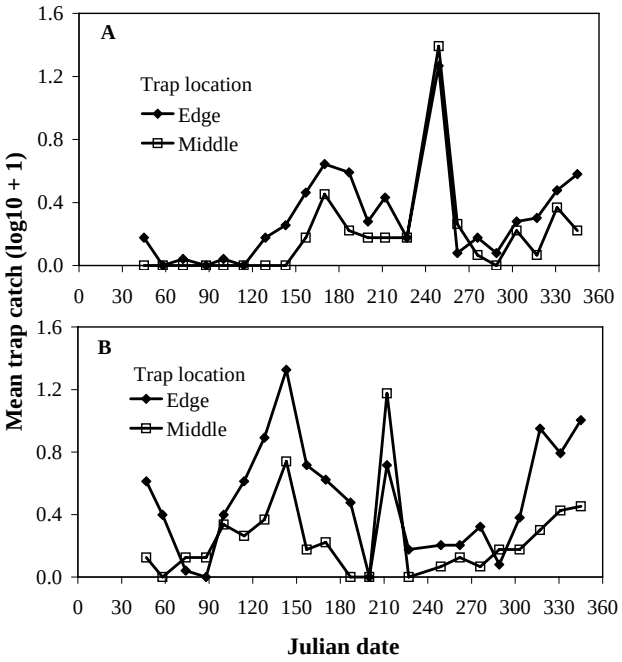

Fig. 1. Trap catches of green sharpshooter in alfalfa at sites located in (A) Tulare and (B) Fresno Counties. Traps were located in the middle of fields or on edges.

Objective 4. Determine the vector competence of a potentially new insect vector, the three-cornered alfalfa hopper, *Spissistilus festinus*.

Transmission assays were conducted in August of 2006. Three needle inoculated alfalfa plants were used as acquisition hosts. Approximately, 150 *S. festinus* adults were collected from the field and placed in a cage with *Xf* source plants for a four day acquisition access period (AAP). After the four day AAP, insects were caged in groups of five on uninfected alfalfa for a 4 day inoculation access period (IAP). After the four day IAP, all insects were frozen and plants held. This experimental protocol was repeated three times and a total of 430 *S. festinus* were tested on 86 test plants (86 plants x five insects per plant = 430). Plants were screened using conventional PCR 3 months and 5 months after the IAP for each experimental replicate. PCR screening failed to detect the presence of *Xf* in test plants and attempted cultures of *Xf* from a subset of plants also detected no infections. A subset of *S. festinus* heads was screened via PCR for the presence of *Xf* with no positives. Results of this experiment do not suggest that *S. festinus* is a competent vector of *Xf*. This experiment is currently being repeated.

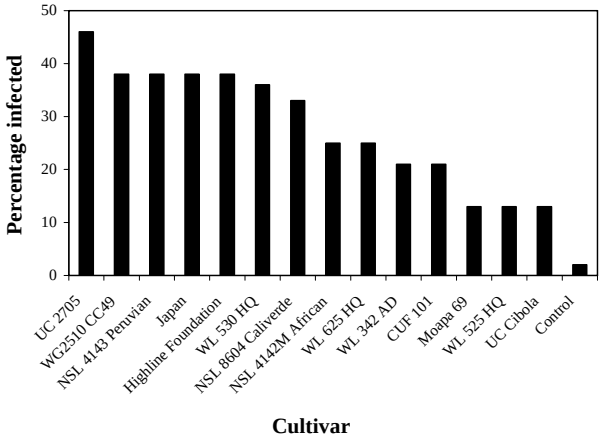

Fig. 2. Percentage of each alfalfa cultivar succesfully needle incoculated with *Xf* across all *Xf* strains tested.

CONCLUSIONS

All alfalfa cultivars tested were suitable hosts for *Xf* (Figure 2) and green sharpshooters were abundant in alfalfa fields (Figure 1). Incidence of *Xf* in field collections averaged over all sites and dates was low (6 out of 5,533 of tillers tested = 0.1%). However, four of six positive samples came from a single collection of 45 tillers collected from a field in Fresno County during the summer of 2007, giving an incidence of 9% in that field on that date. This suggests that incidence of *Xf* in alfalfa may typically be low, but that there may be localized 'hot spots' in some locations during some years. This observation combined with high vector activity in alfalfa suggests that continued investigation into the role of alfalfa in the epidemiology of xylellae diseases is warranted.

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FUNDING AGENCIES

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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

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ABSTRACT
In this report we present the results of refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 and 9621 population (both with resistance from *V. arizonica* b43-17). The *PdR1* locus is 0.2 and 0.4 cM from flanking markers in 9621 population and 0.9 and 5.0 cM in the 04190 population. BAC library screening is more effective and allows more precise selection of positive BAC clones when flanking distances are within one cM. BAC library development from the b43-17 genotype is complete. We developed two libraries each with one restriction enzyme (*Hind* III and *Mbo* I). The *Hind* III library consists of 34,504 clones with an average insert size of 140 Kb. The *Mbo* I library consists of 23, 000 clones with an average insert size of 130 Kb. We initiated the screening of the *Hind* III BAC library with two markers that flanked *PdR1* and identified 10 positive BAC clones with marker VVCh14-10. A total of 15 positive clones were identified with VVCh14-56. Three of the positive clones were flanked by both markers, implying that they contain the entire region harboring *PdR1*. All positive clones from *Hind* III library were large and range from 120-196 Kb. We are in the process of determining the size of positive clones identified with the VVCh14-56 marker. Ten positive clones are already in the process of BAC end sequencing and results will be presented in 2008. We also developed 48 new markers from the newly released Pinot noir genome sequence and 16 of these markers were polymorphic for both the 9621 and 04190 populations. These markers can be used for MAS screening and as well as mapping. We completed the characterization of the UC Davis Mexican *Vitis* collection, the source of the exceptional resistance to *Xf* that was collected by Dr. Olmo in 1961. We are using these unique selections in our genetic and molecular breeding to produce PD resistant table and wine grape cultivars.

INTRODUCTION
Previous reports described the mapping of resistance to *Xylella fastidiosa* (*Xf*) in four (9621, 0023, 04190, and 04373) populations. The preliminary AFLP-based 9621 genetic map and refined 9621 map with SSR, EST-SSR and ESTP markers have been published (Doucleff et al. 2004; Riaz et al. 2006). A manuscript detailing the genetic mapping of the PD resistance locus, *PdR1*, in three populations (04190, 04373 and 9621 populations all of which derive resistance from the single dominant gene resistance source *V. arizonica* b43-17) and an explanation of segregation distortion mechanisms is in preparation. A manuscript detailing the mapping of QTLs for PD resistance in 0023 population (which derives its multigenic resistance from *V. arizonica* b42-26) is also in preparation. A complete report on the origin of the "89 group" seedlings, two of which, D8909-15 and F8909-17, are the parents of the 9621 population is in press (Riaz et al. 2007). During the last three months, we have refined the position of *PdR1* in the 9621 and 04190 populations by increasing the number of recombinants and adding more markers. We completed mapping of the 04373 and 04191 populations and completed greenhouse screening of 64 genotypes in the 04373 population. We also completed the development of a BAC library from the homozygous resistant b43-17 and started BAC library screening with tightly flanking markers.

This report details the results of three genetic maps (9621, 04190, and 04373), the screening of 48 new markers developed from the newly released Pinot Noir genome sequence, and most importantly the development, characterization and screening of the b43-17 BAC library. A high quality BAC library with good coverage is essential for the isolation of the BAC clones that harbor the *PdR1* resistance gene(s). BAC end sequencing of these clones will allow us to develop a physical map in conjunction with the genetic map, develop more markers around the *PdR1* region, and lead to genetic engineering of susceptible *V. vinifera* grapes with *PdR1* gene candidates to evaluate their function.

OBJECTIVES
1. Develop genetic linkage maps for chromosome 14 around the *Xf* resistance locus, *PdR1*, in three populations 04190 (*V. vinifera* F2-7 x F8909-08), 04191 (*V. vinifera* F2-7 x F8909-17), both segregating for *Xf* resistance, and 04373 (*V. vinifera* F2-35 x *V. arizonica* b43-17).
2. Summarize inheritance of PD resistance from other genetic sources.
3. Develop a BAC library for the homozygous resistant genotype b43-17, parent of F8909-08, F8909-17 and the 04373 population.
4. Utilize map-based positional cloning to identify the resistance gene(s) candidates prior to testing in a transformation system.

RESULTS

Objective 1. As mentioned in the previous report, the resistant genotypes F8909-17 and F8909-08 inherited different sister chromatids from the homozygous resistant parent b43-17. It was noted that F8909-08 has a 50 cM region in which marker segregation is distorted and the same markers are distorted in b43-17 indicating that this is a region of segregation distortion. However, these markers on the F8909-17 map were not distorted in this region. Two mapping software programs were used; Join Map and TMAP, and marker order did not change. In the 9621 population, the flanking markers are 0.4 cM (VVCh14-56) and 0.2 cM (UDV095) away from *PdR1*. In the 04190 population, the flanking marker distance is 0.9 (UDV095, VVCh14-10) and 5.0 cM (UDV025). In previous reports the flanking marker distance was 2.0 and 6.0 cM away. Marker VVCh14-56 was not polymorphic for this population. We are in process of screening seven more markers developed from the Pinot Noir genome sequence in an effort to find additional polymorphic markers for the 04190 and 04373 populations. The 9621 map was developed from 425 genotypes (26 genotypes were not included due to a lack of screening data, these genotypes are being re-screened). A total of 361 genotypes were used for 04190 population and screening data was available for all of them. The majority of the markers were homozygous for the parental genotype b43-17. A total of 282 progeny from the 04373 population were used to create the 04373 map. This map of chromosome 14 spans 86 cM with a gap of 44 cM between two groups of markers (Figure 1). Sixty-four plants of 04373 population were selected for greenhouse PD screening. All these genotypes were resistant proving that b43-17 is homozygous resistant.

The 04191 population (*V. vinifera* F2-35 x F8909-17) provides genotypes with 50% *vinifera* background for breeding wine and table grapes as well as more recombinant plants for genetic mapping. It also allows the possible confounding impact of resistance coming from D8909-15 to be examined. Currently, there are 212 genotypes in this population. We are in the process of adding only those markers that are tightly linked to *PdR1*. We categorized resistant, recombinant and susceptible genotypes based on marker information, with recombinant genotypes being selected based on flanking markers. The plants were propagated and inoculated with *Xf*. All marker work is complete and mapping analysis will be carried out as soon as greenhouse screen results are available.

To develop new markers that map only to chromosome 14 and most importantly in the region associated with *PdR1*, we utilized the new released draft of the 11X coverage Pinot noir grape genome sequence, available on NCBI. This sequence information is a great resource and its use is not only confined to determining a DNA sequence. It will also allow predictions of gene positions and comparisons of gene functions. We searched the Pinot noir genome sequence database with the cloned sequences of 16 SSR markers tightly linked to *PdR1* and identified 16 contigs that provide coverage of 55.0 Kb (more detail in report June 2007). We developed 48 new primers and tested them on a small set of parental and progeny DNA from the three populations above. A total of 41 markers amplified cleanly and 16 of them were polymorphic in the 04190 and 9621 populations (data not shown). We added VVCh14-10 to entire set of 361 genotypes from 04190 and it co-segregated with UDV095 (Figure 1). We are in process of adding polymorphic markers to the entire 04373 mapping population, which did not have polymorphic markers in the *PdR1* region. These markers are also candidates for use in MAS screening projects.

Objective 2. 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 in previous reports. It is easier to manipulate single locus resistance traits in breeding and when attempting to use map based positional cloning of genes. Resistance from b43-17 is inherited as a single gene while resistance from b42-26 and its offspring D8909-15 is quantitatively inherited and appears to involve multiple genes that might be present on multiple chromosomes. Screening of a wide range of *V. arizonica* genotypes revealed other resistant selections, of which b40-14 is another promising homozygous resistant genotype. We screened 45 genotypes from an F1 cross of *V. rupestris* x b40-14 and all were resistant except three genotypes with intermediate results. In Spring 2007, we made crosses with these resistant F1 genotypes to other susceptible and resistant genotypes to verify the single dominant gene mode of inheritance. Seeds are harvested and progress will be presented in interim report 2008. Our plan is to select 188 genotypes from F2 population to develop a genetic map to position this potentially different PD resistance locus.

Objective 3. Genetic analyses determined that b43-17's *Xf* resistance segregates as a major single locus and that the full sibling progeny, F8909-08 and F8909-17, inherited different sister chromatids for chromosome 14. *PdR1* has been mapped in the F8909-17 genome, and it is possible that the PD resistance gene from F8909-08 is a different allele of the same gene or that it may be a different gene. Based on the genetic map information, the exact number of genes involved could not be determined and a physical map of the *PdR1* region is essential. We developed two BAC libraries (each with different restriction enzymes) from the homozygous resistant b43-17. Young leaves were used to isolate high molecular weight DNA. Two restriction enzymes, *Hind* III and *Mbo* I were used to digest the DNA. The development of two libraries was done to reduce the bias in the distribution of restriction sites in the grapevine genome. The use of two restriction enzymes on one library might generate DNA fragments too large or small to clone, and thus not a complete representation of the genome.

Hind III *and Mbo* I *libraries:* The *Hind* III BAC library consisted of a total of 34,560 clones that are stored in ninety 384 wells plates. The average insert size is about 140 Kb. The BAC cloning vector is pCC1 (Epicenter) and Invitrogen DH10B T-R competent cells were used. This library provides almost 12X genome coverage of grape genome (W=NI/G, where W is the

genome coverage, N is the total number of BAC clones, I is the mean length of DNA insertion and G is the genome size of grape). With only 3X coverage, the chance of finding a particular genomic sequence in a library is approximately 95%. The *Mbo* I library consisted of 23,040 clones with average insert size of 130 Kb. The same cloning vector and competent cells were used. This library provides about an 8X coverage of grape genome. With both libraries we have average of 10X genome coverage. Both libraries were also gridded onto 22.5 × 22.5 cm2 Hybond N+ filters. Clones were double-spotted using a 4x4 array. The grid pattern allowed all clones to fit on two filters for each library.

Library screening: The *Hind* III BAC library was screened with hybridization using ECL Direct Nucleic Acid Labelling and Detection System (Amersham, GE Healthcare, Buckinghamshire, UK). Standard protocol for screening was used as provided with the kit. Screening was carried out twice with two markers (VVCh14-10 and VVCh14-56), which are tightly linked to *PdR1* (Figure 1). VVCh14-10 was developed from Pinot noir genome sequence. We blasted the UDV095 clone sequence and obtained a 50,000bp contig that was used to develop more markers. VVCh14-10 segregated and amplified cleanly in the 04373 and 04190 populations. There were a total of 10 positive BAC clones. BAC DNA was isolated and verified by PCR-screening (Figure 2). To determine the size of the positive BAC clones, bacteria containing the positive BAC clones were cultured overnight and clones were isolated. BAC DNA was digested with restriction enzyme *Not* I (New England Biolabs) and analyzed with Chef gel DRIII system (BioRad, Calif.) run for 15.5 hours at 14C with 6 v/cm and pulse 5-35 s (Figure 3). Eight of the positive clones have more than 140Kb inserts. Two clones H23-P13 and H64-M16 had two additional bands. *Not* I is a GC rich eight-base cutter and there are two *Not* I sites on the vector, and releases about 8.1 Kb fragments with additional cuts at other *Not* I sites in the insert. Other bands represent additional *Not* I sites that are inside the grape genomic DNA insert. H23-P13 has an insert that is 145 Kb + 20 Kb + 25 Kb = 190 Kb. Similarly, H64M16 is probably ~155 Kb (100 + 25 +30).

The *Hind* III library was also screened with the other flanking marker VVCh14-56 using the same procedures. A total of 15 positive clones were selected based on an X-ray image. We are in process of PCR screening to verify and size the positive clones. Interestingly, three of the positive clones that were selected based on the VVCh14-10 screening were also positive for the VVCh14-56 marker. These three clones are H23-P13, H34-B5 and H64-M16 and they are 190Kb, 160Kb and 155Kb in size, respectively. VVCh14-10 and VVCh14-56 flank the PdR1 locus and the identified clones should contain the complete *PdR1* region. BAC end sequencing of these clones is in process and results will be presented in 2008.

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 *PdR1*; and finally 5) work towards map-based positional cloning of genes. We focused on chromosome 14 in a variety of genetic backgrounds to verify the single gene nature of *PdR1* expression, and are using quantitative trait loci (QTL) analysis in the 0023 population to study PD resistance from b42-26. These genetic linkage maps will enable us to characterize and clone different variants of PD resistance genes, 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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FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Because the 9621 map is also being used to place *Xiphinema index* resistance, support for that effort from the California Grape Rootstock Improvement Commission should be acknowledged, as well as funding from the Louis P. Martini Endowed Chair in Viticulture.

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
04190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17

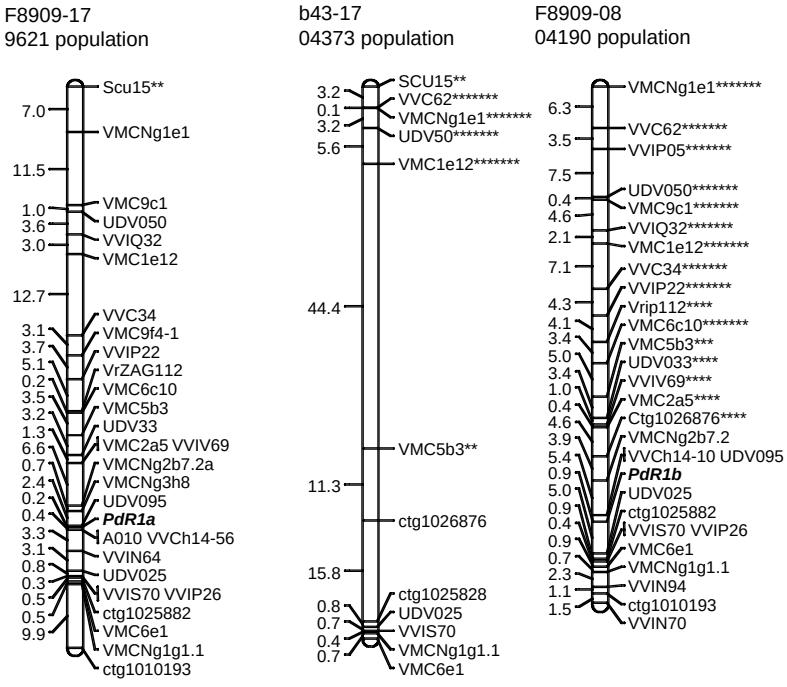

Figure 1. Genetic maps of F8909-17, b43-17 and F8909-08 genotypes

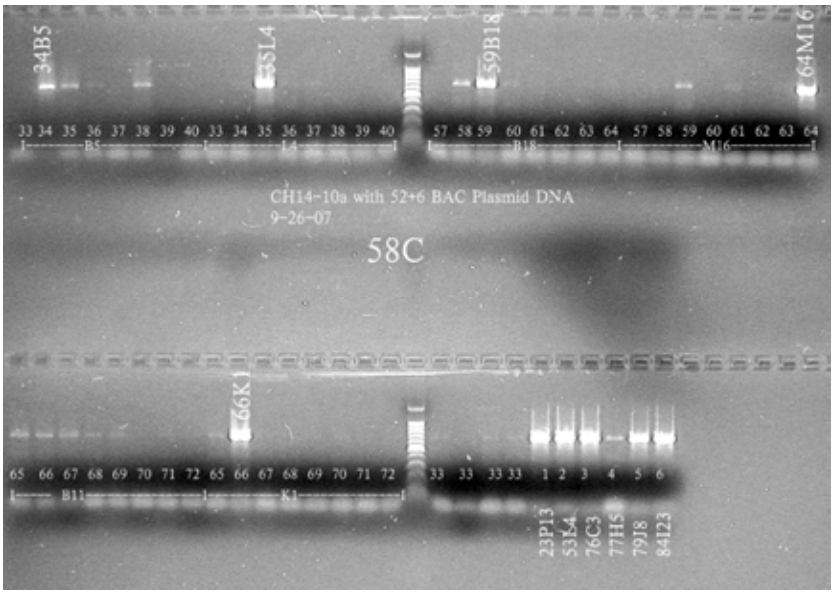

Figure 2. PCR screening of positive BAC clones with VVCh14-10 marker.

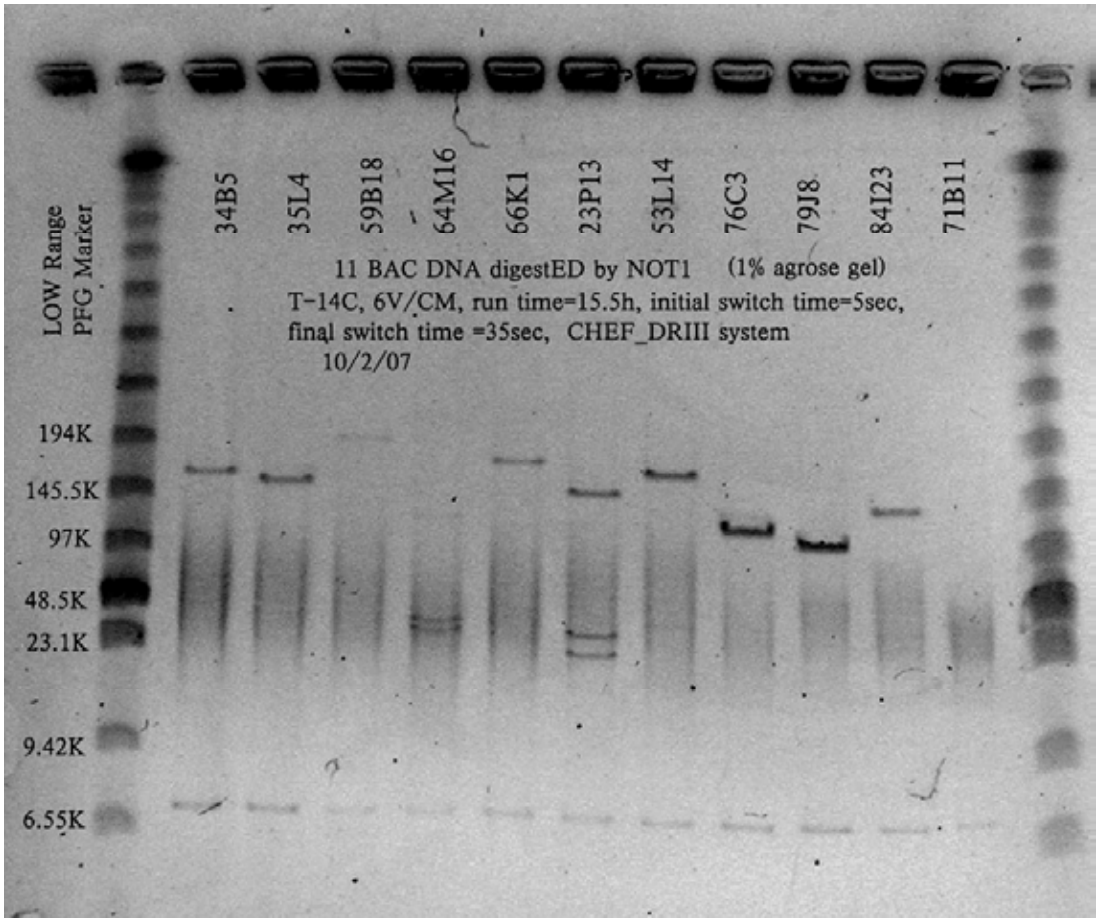

Figure 3. Size determination of Positive BAC clones.

BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT
Progress continues on Pierce's disease (PD) resistant winegrapes and has been greatly accelerated by the incorporation of marker-assisted selection (MAS) for the Pierce's disease resistance gene, *PdR1* (see companion report). The use of MAS and our acceleration of the seed to seed breeding cycle to three years have allowed very rapid progress towards PD resistant winegrapes. Populations from the 2006 crosses were screened with MAS for both PD and powdery mildew (*Run1*) where appropriate and only those with the markers were planted in the field. The 2007 crosses were made to: 1) produce populations with *PdR1* and 93.75% *vinifera*; 2) broaden the base of *vinifera* wine grape types with *PdR1* at the 87.5% *vinifera* level; 3) broaden the base of *vinifera* wine grapes with *PdR1* from the 8909-17 allele; 4) produce *vinifera* wine grape types with resistance from two other non-*PdR1 V. arizonica* resistance sources (b42-26 and b40-14); and 5) to produce additional rootstock lines with *PdR1* and *XiR1* (the *Xiphinema index* resistance gene). *Vinifera* wine grape types with *PdR1* and 87.5% *vinifera* in were planted in a Beringer, Napa county trial. Finally, small scale wine lots were made from four of eight selected 87.5% *vinifera PdR1* containing wine grape types. Fruit evaluation and must analysis were performed on numerous other promising progeny at this level.

INTRODUCTION
The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xf* resistance (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005), and in possession of unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, to allow the introduction of extremely high levels of *Xylella* fastidiosa(*Xf*) resistance into commercial grapes. They have produced seed that is 93.75% *V. vinifera*, from winegrape cultivars, with resistance from our b43-17 *V. arizonica*/*candicans* resistance source. There are two sources of *PdR1*, 8909-08 and 8909-17, both siblings of b43-17. These selections have been introgressed into a wide range of winegrape backgrounds over multiple generations, and resistance from southeastern United States (SEUS) species is being advanced in other lines. However, the resistance in these later lines is complex and markers have not been developed to expedite breeding.

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 The breeding cycle for the development of PD resistant grapes has been reduced to three years (seed to seed) using marker-assisted selection (MAS) with the b43-17 resistance sources and their progeny. The 2006 crosses, number of seedlings produced and the number of seedlings that went to the field are presented in the 2006 Proceedings and in March 2007 Report. Our goal at this point is to introgress our PD and *PdR1* resistance sources into a large number of *V. vinifera* winegrapes backgrounds. Until we get to the backcross 4 (BC4) (96.8% *V. vinifera*), there is not much point to growing very large numbers of progeny from any given cross. With the now standard three-year seed-to-seed cycle we will plant BC4 progeny in 2010.

Table 1 presents the crosses made and seed produced in 2007. The goals of this year's crosses were to: 1) Use the *PdR1* allele from the 8909-08 to make 93.75% *vinifera* level progeny; 2) Broaden the *vinifera* winegrape lines in the 8909-08 resistance source at the 87.5% *vinifera* level; 3) Combine *PdR1* with the powdery mildew resistance gene *Run1* at the 87.5% *vinifera* level; 4) Use 8909-17 and 8909-08 based resistance with diverse *vinifera* winegrapes to produce resistant progeny at the 75% *vinifera* level; 5) Use the 8909-15 resistance source with a broad range of *vinifera* winegrapes; and 6) Produce rootstocks with *PdR1* and broad-based nematode resistance.

Five groups of plants were greenhouse screened for *Xf* resistance in 2007. Group A tests (160 genotypes) were done to verify the expression of *PdR1* from b43-17 in the 04190 (*V. vinifera* F2-7 x 8909-08) population, and confirm *PdR1* in parents used in 2006. The Group B tests (76 genotypes) examined progeny of Midsouth and BD5-117 crossed to advanced *vinifera* wine

types. Both of these parents continue to produce resistant progeny, but very few and in ratios that suggest a complex inheritance. The progeny of Haines City (n=9) were all resistant by ELISA in the greenhouse screen, but do not contain *PdR1*. Eleven genotypes from Olmo's e-series BC2 that carry *Run1* (the powdery mildew resistance marker) were also tested, none were resistant and they seemed more sensitive to PD than typical *vinifera*. Group C tested the use of b43-17 as a rootstock and interstock to examine *Xf* transmission and expression through a graft union. b43-17 did not induce PD resistance in Chardonnay, but preliminary results found that it and A8909-05 (*V. rupestris* x *M. rotundifolia*) prevented PD expression in Chardonnay scions when they were used as interstocks. Group D tests (120 genotypes) focused on mapping population progeny to verify recombinants and establish resistance ratios. Recombinants from 04190 were tested and aided fine-scale *PdR1* mapping efforts; three recombinants from the 04191 (*vinifera* F2-7 x 8909-17) were also tested. The 05347 population (*vinifera* F2-35 x *V. arizonica* b42-26) was also tested (n=60) to establish the R/S ratios derived from b42-26, which is quantitatively inherited and provides an alternative and strong source of PD resistance. Progeny from these tests will be used for mapping studies and as parents with a non-*PdR1* resistance. Group E tests (150 genotypes) included additional 04190 progeny and remnants from the 9621 (8909-15 x 8909-17) that had not been tested. This group also tested advanced *PdR1* carrying parents, which were used in the 2007 crosses based on marker data alone, to confirm their resistance.

Objective 2 Although resistance from other backgrounds is complex and quantitative, which results in few resistant progeny from crosses to *vinifera* cultivars, we continue to advance a number of lines. In order to better understand the limits of other PD resistance sources the following resistance sources are being studied:

***V. arizonica* b42-26 –** *Xf* resistance in the 0023 (D8909-15 (*V. rupestris* x b42-26) x *vinifera* B90-116) population is strong, but is quantitatively inherited. Quantitative trait locus (QTL) analysis has identified a major QTL that accounts for about 20% of the variability (preliminary results). Previous efforts with the 0023 were focused on table grape breeding, and found that the 0023 population (F1, 1/4 b42-26) had about 30% resistant progeny. This population has a large number of weak genotypes, few females with viable seeds, and generally lacks fertility. The progeny of a cross of a resistant 0023 genotype crossed back to *vinifera* (BC1) were tested and only 7% were resistant. We are now testing 05347 (*vinifera* F2-35 x b42-26) to examine the b42-26 resistance source in a less complex background (without the confounding effect of *V. rupestris*). Crosses using elite *V. vinifera* wine type pollen were made to a number of females in this population in Spring 2007 (Table 1f).

***V. arizonica* b40-14 –** In 2006 we crossed F2-35 x b40-14, produced 1,385 seeds and established 198 seedlings for testing. We are planning on using this population, or one generated from its progeny for mapping efforts to better characterize this very strong, and morphologically and genetically different source of PD resistance (Table 1f).

Field and Wine Evaluations The A81 series (BC1, 75% *vinifera*) F8909-08 allele type of *PdR1* is in its second year of field testing at the Beringer Yountville test site; ELISA and visual symptom results have been consistent with greenhouse assays. Selections from the 045554 (BC2, 88% *vinifera*) have been made onto Dog Ridge (currently the only certified PD resistant rootstock) and were planted at Yountville on May 14, 2007. These genotypes have been marker tested and their PD resistance status confirmed by greenhouse testing. Twelve genotypes were resistant, four were recombinants (one resistant and three susceptible in the greenhouse test). They will be inoculated in Spring 2008.

Three of eight advanced red wine selections (U0501-12, U0502-01 and -10) containing *PdR1* that are 87.5% *vinifera* from crosses with Syrah and Chardonnay were replicated for small-scale fermentation this Fall. About four liters of wine from each were produced along with similar amounts of Cabernet Sauvignon and Pinot noir as *V. vinifera* controls and Midsouth and Lenoir as standard PD resistant controls. These selections were evaluated for their productivity, flowering and ripening dates, and berry and cluster weights. Vine, fruit and juice analyses are presented in Tables 2a, 2b and 2c, and images of the leaves and fruit are in Figure 1.

Numerous other genotypes from crosses involving elite *vinifera* wine cultivars were examined for fruit evaluation and must analysis. ETS Laboratories (www.etslabs.com) of St. Helena kindly donated their fruit analysis and phenolics panel, which uses a wine-like extraction to model a larger fermentation. Surprisingly, none of the U05 series analyzed contained significant levels of diglucoside anthocyanins, which are negative quality markers for hybrid wines with American grape species and which would create problems with exporting wines to the EU. Cuttings of the best of these will be taken in the winter so that we can get small-scale wine lots made for evaluation in 2009. Plants will be grown in both Davis and Napa when possible. A new MS student is examining the reasons for the lack of diglucoside anthocyanins in these selections to determine whether the *arizonica*-resistance sources possess these anthocyanins. A second MS student is in the second year of examining what wine quality parameters are useful at micro (2L), mini (20L) and macro (2,000L) scales. Determining how to best select for wine quality at the micro scale will be critical as we begin to evaluate large populations of PD resistant and 96% vinifera selections.

Powdery Mildew - Any new PD resistant variety should also be resistant to powdery mildew. We have been exploring powdery mildew resistance in a number of backgrounds including Olmo's VR (*vinifera* x *rotundifolia*) hybrids, which form the base of international efforts at characterizing *Run1*, the *rotundifolia*-based locus responsible for resistance to powdery mildew. Current season field evaluations of the 2006 crosses show the markers correlating perfectly with field resistance on leaf and cane. Berry and cluster observations will begin in Fall 2008. The goal with these individuals is to cross our advanced PD resistant selections with selections from these powdery mildew resistant progeny. We also made crosses to examine powdery mildew in two other backgrounds, Villard blanc and Tamiami. Powdery mildew resistance markers are being developed for these resistance sources by labs in Germany and the US.

CONCLUSIONS

This project continues to breed PD resistant winegrapes with the primary focus on the *PdR1*resistance source so that progress can be expedited with MAS. Populations with *Xf* resistance from other sources are being maintained and expanded, but progress is slower with these sources. We continue to supply plant material, conduct greenhouse screens and develop new mapping populations for our companion project on fine-scale mapping of PD resistance leading to the characterization of the *PdR1* resistance. Fall 2007 will see the first testing of wine from advanced selections with 87.5% *vinifera* from winegrapes.

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Figure 1. Pictures of the 87.5% *vinifera* PD resistant wine grape selections used for small-scale winemaking at UCD in 2007.

Table 1. 2007 crosses and numbers of seed produced.

Resistant Type	*Vinifera* Parent of Resistant Type	*Vinifera* Types used in 2007 crosses	# Seeds Produced
1a. Monterrey *V. arizonica/candicans* resistance source (F8909-08) to produce progeny with 93.75% *V. vinifera* parentage.			
U0501	Syrah	F2-7, F2-35	478
U0502	Chardonnay	F2-7, F2-35	2,769
U0503	Sauvignon blanc	Chardonnay, Palomino, Semillon	126
U0505	Cabernet Sauvignon	Chardonnay, F2-7, LCC, Merlot, Palomino, Petite Syrah	3,229
1b. Monterrey *V. arizonica/candicans* resistance source (F8909-08) to produce progeny with 87.5% *V. vinifera* parentage			
05310	Alicante Bouschet	Burger, Carignane, LCC	1,666
05312	Cabernet Franc	Zinfandel	194
05317	Tempranillo	Burger, LCC	371
05319	Zinfandel	Cabernet Franc, LCC	144
A81-17	A38-7	Carignane, Grenache noir, LCC	705
1c. Monterrey *V. arizonica/candicans* resistance source (F8909-08) and *Run1* powdery mildew resistance.			
U0501, U0504	Syrah	e-series, e78 and e88 allele patterns	499
U0502	Chardonnay	e-series, e78 and e88 allele patterns	837
U0505	Cabernet Sauvignon	e-series, e78 and e88 allele patterns	642
A81-17	A38-7	e-series, e78 allele pattern	603
1d. Monterrey *V. arizonica/candicans* resistance source (F8909-08) and *Vitis* PM resistance source.			
U0505, A81-17	Cabernet Sauv, A38-7	Villard blanc	348
1e. Monterrey *V. arizonica/candicans* resistance source (F8909-17 allele) to produce progeny with 75% *V. vinifera*.			
04373-02	F2-35 (Cab x Carignane)	Alicante Bouschet, Aligote, Carignane, Chardonnay, Zinfandel	597
04373-08	F2-35 (Cab x Carignane)	Aligote, Cabernet Franc, Carignane	938
04373-64	F2-35 (Cab x Carignane)	Grenache noir	293
1f. Other resistance sources. R89 is 50% *vinifera*, 25% resistance source. 05347 is 75% *vinifera* and 25% resistance source			
R89 (b40-14)	NR	Airen	238
05347 (b42-26)	F2-35	Aligote, Chardonnay, Grenache noir, Zinfandel	1,877
1g. Rootstock crosses to combine PD and nematode resistance.			
9621-257	9365-85		653
9365-43	9621-161		112

Table 2a. Phenotypic observations of reference varieties and select progeny with the *PdR1* resistance source.

Genotype	Parentage	Percent *vinifera*	2007 Bloom Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Ripening Season	Prod 1=v low 9=vhigh
Cab. Sauv.	Cab. Franc x S. blanc	100%	5/20/07	B	1.0	168	mid-late	6
Pinot noir	Historic	100%	5/7/07	B	1.1	259	Early	6
U0501-12	A81-138 x Syrah	87.5%	5/7/07	B	1.0	90	mid-late	4
U0502-01	A81-138 x Chardonnay	87.5%	5/1/07	B	1.6	128	mid-late	4
U0502-10	A81-138 x Chardonnay	87.5%	5/1/07	B	1.4	160	very early	7
Lenoir	*V. aestivalis* hybrid	<50%	5/12/07	B	0.8	201	Late	7
Midsouth	DGxGalibert 255-5	<50%	5/5/07	B	2.2	211	mid-late	6

Table 2b. Analytical evaluation of reference varieties and advanced selections with the *PdR1* resistance source. Diglucoside anthocyanins were detected in Midsouth and Lenoir. All analysis courtesy of ETS Laboratories, St. Helena, CA.

Genotype	L-malic acid (g/L)	°Brix	potassium (mg/L)	pH	TA (g/100mL)	YAN (mg/L (as N)	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
Cab. Sauvignon	2.19	24.9	2460	3.65	0.62	227	59	250	404
Pinot noir	2.43	26.5	2190	3.83	0.49	279	321	842	568
U0501-12	4.20	29.4	2900	3.87	0.68	420	88	802	979
U0502-01	2.90	25.9	2530	3.77	0.61	301	91	564	380
U0502-10	4.92	23.7	2220	3.48	0.85	301	87	588	845
Lenoir	4.32	26.9	2920	3.67	0.75	164	195	341	1801
Midsouth	4.60	18.2	2220	3.49	0.81	278	32	230	971

Table 2c. Sensory evaluation of reference varieties and advanced selections with the *PdR1* resistance source.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin (1=low, 4= high)	Seed Color (1=gr, 4= br)	Seed Flavor	Seed Tannin (1=high, 4= low)
Cab. Sauv.	pink-brown	light-med	fruity-CS	fruit jam	2	3	nutty-full	4
Pinot noir	pink-brown	medium	hay, honey	mildly fruity	1	4	spicy	4
U0501-12	red	med-dark	fruity	fruit jam	2	4	neutral	2
U0502-01	pink-brown	medium	fruity-PN	sweet fruit	1	3	spicy	1
U0502-10	pk-red-orng	med-dark	slight vegetal	mildly fruity	1	4	nutty,spicy	1
Lenoir	red	dark	mildly fruity	fruity	1	4	nutty	1
Midsouth	red-orange	med-dark	veg-fruity	neutral	1	4	neutral	4

IN VITRO STUDY OF THE EFFECTS OF GRAPE XYLEM SAPS AND CELL-WALL CONSTITUENTS ON GROWTH AND VIRULENCE-RELATED GENE EXPRESSION OF *XYLELLA FASTIDIOSA*

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Pierce's disease (PD) of grapevines is caused by the xylem-inhabiting bacterium *Xylella fastidiosa (Xf).* The widely cultivated *Vitis vinifera*-based grape cultivars are susceptible to PD. However, grape species *V. aestivalis*, *V. arizonica, V. shuttleworthii, V. simpsonii*, *V. smalliana,* and *Muscadinia rotundifolia*, are highly resistant to PD. In this study, we investigated the effect of PD resistant and susceptible grape xylem sap and several cell-wall constituents on bacterial growth, biofilm formation, and virulence-related gene expression *in vitro*. Xylem sap from susceptible grape species provided better support for bacterial growth and biofilm formation compared to xylem sap from resistant grape species. Bioassay of *Xf*, co-cultured in PW medium with various purified cell-wall constituents, demonstrated that cellulose, xylan, laminarin, and glucan significantly promoted bacterial growth whereas lichenan strongly suppressed growth. A study of pathogenicity and virulence-related gene expression using RT-PCR revealed that glucanase, protease, and a number of virulence genes were expressed differentially in response to treatment with xylem sap from resistant and susceptible grape species. This preliminary study suggests that differences in xylem cell wall properties and chemical composition of xylem sap between PD resistant and susceptible grape species may effect development of PD.

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