WHERE, WHEN AND HOW DO INGESTION AND OTHER FEEDING BEHAVIORS OF THE GLASSY-WINGED SHARPSHOOTER ALLOW INOCULATION OF *XYLELLA FASTIDIOSA*?

Project Leader:
Elaine Backus
USDA, ARS, PWA
Crop Diseases, Pests & Genetics
Parlier, CA 93648

Personnel:
P. Houston Joost
Dept. of Entomology
University of California
Riverside, CA 92521

Holly Shugart
USDA, ARS, PWA
Crop Diseases, Pests & Genetics
Parlier, CA 93648

Cooperators:
William H. Bennett
Private Consultant
Otterville, MO 65348

Thomas Miller
Department of Entomology
University of California
Riverside, CA 92521

Gregory Walker
Department of Entomology
University of California
Riverside, CA 92521

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

Jianchi Chen
USDA, ARS, PWA
Crop Diseases, Pests & Genetics
Parlier, CA 93648

Reporting Period: The results reported here are from work conducted October 1, 2004 to September 30, 2005.

ABSTRACT
Many nagging questions about the mechanisms of transmission of *Xylella fastidiosa* (*Xf*) by the glassy-winged sharpshooter (GWSS) exist, hindering development of predictive epidemiological models for ultimate GWSS impact on California crops, as well as rapid development of resistant crops. This new grant seeks to complete our answers to these questions. Only five months of funding from this grant was available to Backus this year, due to bureaucratic hurdles. Nonetheless, we were able to accomplish significant work with support from Backus's in-house ARS funds. Efforts focused on developing research infrastructure such as simultaneous availability of plants, insects and bacteria, and protocols for the experiments. We now receive monthly shipments of adult GWSS, housed in a CDFA-approved quarantine facility at CSU Fresno, fed on greenhouse-reared cowpea, sorghum and grape. We also maintain green fluorescent protein (GFP)-transformed *Xf* in colony and are mechanically inoculating it into grape (cv. 'Cabernet Sauvignon') on a regular basis. GWSS are caged on these acquisition plants for 6-8 days, and are assayed for presence and location of bacteria using protocols we developed for confocal laser scanning microscopy (CLSM). All protocols for electrical penetration graph (EPG)-monitoring of identified probes, including artificial termination of probes, marking of probe locations, and recovery of tissues and processing for CLSM, have been perfected. In addition, a protocol that was very difficult to develop has been nearly perfected, wherein a wired GWSS that has acquired GFP-*Xf* is monitored while probing transparent artificial diet. Views of its stylets and salivary sheath are simultaneously video-captured and synchronized with EPG waveforms. Preliminary results show that GFP-*Xf* is present and visually resolvable following injection by the stylets into artificial diet, but synchronization between video and EPG recording of behavior was not perfect in these earliest tests. The experiment to determine the time course of inoculation and movement of GFP-*Xf* from the site of injection is about to begin. However, completion of most of the other experiments will await start of a new post-doc in January 2006, to replace the previous post-doc after his departure from the project in May 2005.

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. Once we complete our definition of EPG waveforms in the present project, EPG will provide a powerful tool for development of crop resistance. Information gained also can be assembled into a predictive model for risk assessment, with implications for all levels of the *Xylella*-sharpshooter-grape pathosystem, including ecological, epidemiological and management. The overall goal of PI Backus's research is to identify the stylet penetration behaviors of the glassy-winged sharpshooter that contribute to *Xf* transmission, infection success and disease development, and to use that information for epidemiological risk assessment and to help develop new methods of host plant resistance. Two stylet penetration behaviors crucial for *Xf* inoculation are uptake of plant fluids into the gut (ingestion) and expulsion of bacteria-laden fluids (extravasation).

OBJECTIVES
1. Characterize ingestion behavior, especially to: (a) identify in which cell types various durations of ingestion (C) are occurring, and (b) how to recognize that by EPG alone.
2. Characterize extravasation behavior, especially to: (a) correlate the B1 waveform with fluid flow in and out of the stylets, and (b) determine in which plant cells this behavior occurs.

3. Characterize behavior-*Xf* interactions that permit inoculation, especially to: (a) identify the behaviors (ingestion, extravasation or both) during which bacteria are expelled, and (b) whether bacterial expulsion is into xylem, or any plant cell type penetrated, or both.

RESULTS

This new grant was funded in July 2004. The start of this project was delayed significantly due to circumstances beyond our control. Nevertheless, we still managed to make significant progress on some of the objectives.

General Methodologies

This year, we solved all previous problems with availability of experimental plants, insects and bacteria. Our quarantine insect facility at CSU Fresno was put into operation in October 2004, and has been receiving monthly shipments of cowpea/sorghum-reared adult GWSS from D. Morgan (CDFA Riverside) since December 2004. Cowpea and sorghum, as well as rooted cuttings of grape, cv. 'Cabernet Sauvignon,' are reared in disease-free, pesticide-free exclusion cages in a quarantine greenhouse at ARS Parlier. GFP-expressing *Xf* STL were kindly provided by S. Lindow (UC Berkeley), and are being maintained in culture by JC Chen (ARS Parlier). Protocols have been developed for mechanically inoculating grape with GFP *Xf*, establishing and maintaining PD infections, and sufficient acquisition by GWSS to allow inoculation of GFP *Xf* to be studied. The intensive infrastructure effort to make all of these experimental subjects available (simultaneously) has required more than half the effort of Backus's ARS-funded, full-time technician, H. Shugart, for the last year.

Objective 1 – Correlation of Ingestion with EPG Waveforms

Study a: Cell types in which ingestion occurs

Joost, Shugart and Backus developed most of the protocols needed for this experiment, including timing and collection of excretory droplets from EPG-recorded sharpshooters and histology of probed grape tissues. We have perfected the art of artificially terminating probes in mid-waveform (Backus et al. 2005); appropriate repetition of this simple but time-consuming protocol will be performed by the new post-doc. Shugart will perform the histology of salivary sheaths in probed plant tissue.

Study b: Recognizing ingestion from waveforms alone

Backus and W. H. Bennett completed testing and design of the final prototype AC-DC EPG monitor (ms. in prep.). The level of detail about waveform fine-structure is unprecedented, and will allow minor sub-types (possibly correlated with ingestion tissues) to be characterized. Also, Backus organized and taught an international workshop on principles and applications of EPG to 20 scientists from the US, Europe, S. America and Asia, in August 2005. Research performed during the workshop stimulated groundbreaking plans for future projects and collaboration with other researchers in Asia and S. America. Among other developments, the collaborative findings identified how to distinguish active vs. passive ingestion from waveforms alone, and showcased new technologies in computerized pattern recognition for extremely rapid waveform measurement and analysis. These developments will make possible near-future development of the Stylet Penetration Index and very rapid analysis by novice EPG users.

Objective 2 – Correlation of Extravasation with EPG Waveforms

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

The first test was completed by Joost. Movement of markers in a probed artificial diet solution shows indirectly that fluid flows both in and out of the stylets during B1. However, so far only sheath saliva flow is directly viewable (Joost et al. 2006). For the second test, preliminary attempts at electromyography of the precibarial valve and cibarial dilator muscles (by Joost with help from Miller) have been partially successful. They suggest that B1spikelets (B1s) represent precibarial valve muscle contractions, while C plateaus represent cibarial dilator muscle contractions. If so, this complements findings from our first UC PD grant (Backus et al. 2006) showing that the B1 waveform is ubiquitous during stylet penetration, interspersed within and among all other waveforms, and is performed in virtually all cell types. Thus, B1 may include movement of the precibarial valve (at least in part), which controls uptake of fluid into the precibarium for tasting, as well as expulsion (extravasation) of fluid after tasting is completed. However, protocols must be further fine-tuned before electromyography can be repeated. Work to complete the remainder of this objective will also be performed by the new post-doc.

Study b: Determine in which plant cells B1 occurs

Once the extravasation waveform is definitively identified in Exp. 2a, we will use artificially terminated, EPG-monitored probes coupled with histology to pinpoint the cell types in which it occurs during stylet penetration of grape. Like experiment 1a above, EPG will be done by the post-doc, the tissues processed by Shugart, and the data compiled and analyzed by the post-doc. Again, all protocols are developed.

Objective 3 – Characterize behavior-*Xf* interactions that permit inoculation

Study a: Identify the behaviors (ingestion, extravasation or both) during which bacteria are expelled

In the interest of time, we spent several months this year developing the protocols of this final and most difficult experiment. Joost, with help from Shugart and Backus, developed a technique for visualizing (via high-resolution epifluorescence microscopy) individual cells or clumps of GFP-*Xf* cells injected into transparent, artificial diet by an adult, wired GWSS held

in a specially-built apparatus. Activities of the stylets and bacteria in solution are simultaneously EPG-recorded and images captured via a microscope-mounted digital video camera and MediaCruise software.

Over 40 probes were simultaneously EPG-recorded and video-captured by Joost prior to his departure. Of those, two tantalizing probes expelled GFP-*Xf* into the diet, although visualization of each was not optimal. In the first case, an insect was rapidly removed from the acquisition plant in mid-probe, immediately wired up, and then placed directly into the diet/microscope apparatus. Within 2 sec of stylet insertion, both clumps and individual, rod-shaped cells of glowing, green bacteria were clearly seen as they were injected into the diet, presumably from a column of liquid that had been held in the food canal of the stylets. The expelled bacteria quickly dispersed in the liquid diet. Unfortunately, in that very early test, the bacteria were viewed but not video-captured, and the EPG waveform was not recording properly. In the second case, an insect was removed from the plant, allowed to feed for a few hours on diet to clear its gut, then placed on a new diet in the test apparatus. It probed so rapidly that EPG waveforms were recorded, but the stylet tips were off-screen at the instant of the bacterial expulsion. About a minute later, when the stylet tips were brought on-screen (and by then, the insect was performing B2, sawing out of the hardened salivary sheath), the entire length of the sheath was found to be glowing green, in some places very brightly (Figure 1A, B). The sheath is normally transparent, does not fluoresce at the same excitation wavelengths as GFP, and is dark against the dark confocal background. This insect had not yet begun ingesting. Evidently, bacterial expulsion occurred during pathway activities, when B1 is a prevalent waveform. This matches our findings from last year's inoculation studies (Backus et al. 2006).

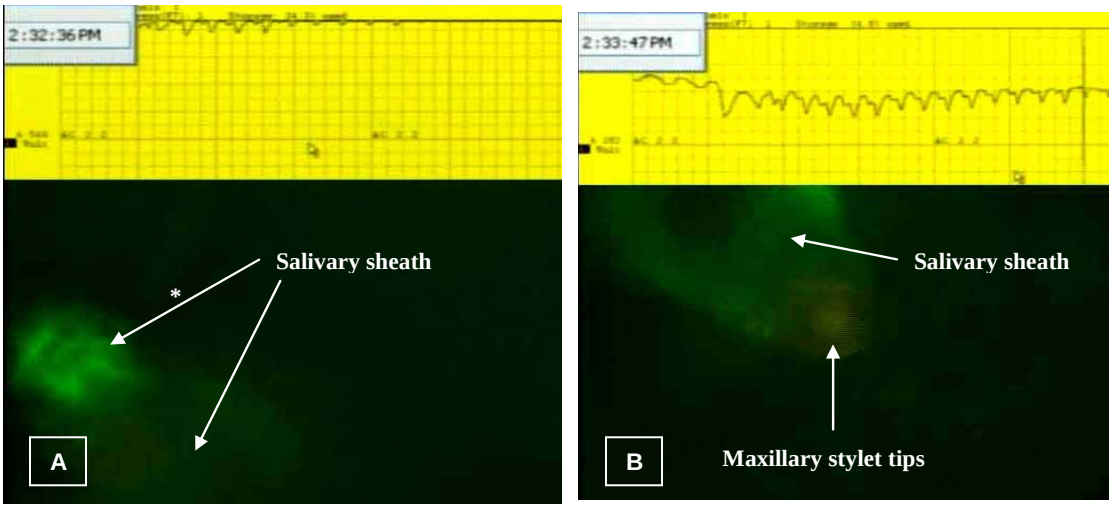

Figure 1A. GFP-*Xf*-containing salivary sheath in the lower left corner of the screen, with a basal portion that is glowing bright green (arrow with *), and more apical portion that is glowing less brightly (unstarred arrow). Sheath is slightly out of focus. The stylets are still in the sheath, but their tip is out of view. B2 waveform is being performed by the stylets (elsewhere), but the waveform has nearly peaked out. **B.** 1 min 11 sec later, the view has been adjusted and refocussed to reveal the tip of the stylets, and the offset has been adjusted to bring the waveform, once again in B2, back into view. The stylet tips are orangish-brown, just appearing out of the sheath at its tip. The apical area of the sheath is glowing slightly green, presumably with dispersed, perhaps fragmented, GFP-*Xf*.

These tantalizing preliminary results suggest that: 1) free-floating bacteria can be held in a water column in the stylets, then expelled immediately upon initiation of a probe, and 2) non-free-floating bacterial cells, presumably within biofilm adhering to the cuticular surface of the precibarium, can come loose during pathway (i.e. pre-ingestive) behaviors and be injected into the feeding substrate. These expelled bacteria can become lodged in or adhere to the salivary sheaths. These exciting results must now be replicated with improved focus and waveform synchronization.

We believe the low rate of success of inoculation (2/40 probes) in Joost's preliminary work was due to a low rate of acquisition by his experimental insects, because his acquisition plants were very unhealthy. Therefore, we performed tests to improve GFP-*Xf* acquisition success. GFP-expressing *Xf* were inoculated into two grape plants, and onset and severity of symptoms were observed. Once clear symptoms had developed but plants were still relatively healthy (about 3 weeks after inoculation), adult GWSS were caged on individual plants and allowed acquisition access periods (AAPs) of 3, 6, 9 and 12 days. Twenty heads were prepared, dissected, and examined via confocal laser scanning microscopy (CLSM) by Shugart, using protocols she has spent the last several months developing. We found that every insect had acquired *Xf* into the area of the precibarium and cibarium by 3 days, but that the *Xf* colony size and distribution increased during subsequent days on the plant, especially between days 6 and 9. On the other hand, GWSS mortality was significant (50% by day 9, 100% by day 12). Therefore, we decided to use 6-8 days' AAP as a standard for future inoculation tests.

The rest of the video-EPG correlation recordings will be performed by the new post-doc within the next year. All insect heads will be dissected and examined via CLSM by Shugart, to verify whether insects were or were not inoculative.

Study b: Determine into which plant cells bacteria are expelled

Again in the interest of time, we have developed all protocols for histologically tracing bacterial spread from an EPG-identified probe terminated after likely bacterial expulsion has occurred. This experiment is planned to be performed by Shugart in fall 2005. The major objective of this experiment is to determine the grape cell types into which bacteria are first inoculated, during a single probe. Therefore, we seek to maximize the likelihood of inoculation. Results from our previous study (Backus et al. 2006) suggest that 1 hour of ingestion (C) waveform following pathway results in a very high likelihood of *Xf* inoculation. Therefore, we have modified this objective's experimental design slightly, to use a 1-hour ingestion period as the decision point to artificially terminate an EPG-identified probe. Cowpea-reared GWSS will be given 7 days AAP on GFP-*Xf* infected grape. Selected insects will be wired each day. Shugart then will perform the experiment as outlined in the proposal, except that plant holding durations will be increased to 0, 10, 20 and 40 days.

CONCLUSIONS

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

- Answering questions about transmission mechanisms and vector efficiency that are crucial for epidemiological modeling for risk assessment, such as:
  - the mechanism of *Xf* inoculation and using EPG to observe it real-time as it occurs,
  - one determinant of inoculation efficiency, i.e. the role(s) of inoculation behavior vs. bacterial presence and/or detachment in the foregut,
  - when, for how long, and under what circumstances, GWSS ingests from xylem vs. other cell types
  - probability that bacteria will be inoculated into xylem (or other cell types) when inoculative vectors probe those cells.
- Answering questions about bacterial movement and establishment in the plant following natural inoculation by vectors, such as:
  - where in the plant the *Xf* bacterial cells are first inoculated, and how far they move from that point
  - the probability that one inoculation event by a vector will lead to spread of the bacterium and, ultimately, chronic infection.
- In a future grant, developing a Stylet Penetration Index for testing among host and non-host species or cultivars, diets, etc. for performance of transmission behaviors, ultimately leading to improved host plant resistance.

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FUNDING AGENCIES

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