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

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

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

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

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

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

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

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

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

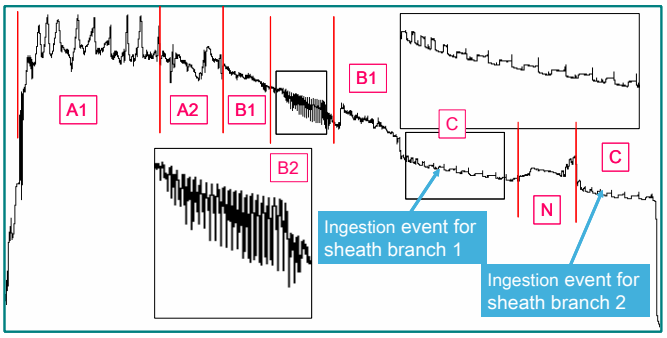
## INTRODUCTION
Almost nothing was known, until this work, about the stylet penetration (probing) behaviors of the glassy-winged sharpshooter (GWSS), and how they interact with populations of *Xylella fastidiosa* (*Xf*) to facilitate transmission to grapevine. This project is combining the three most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

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

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

***Objective 1 - Waveform Correlations:***

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



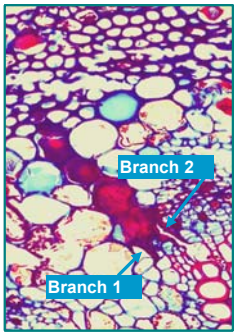




**Figure 1.** Waveform trace from an interrupted grape probe, with its correlated salivary sheath. The insect's stylets were pulled out after < 1 minute of the second C (ingestion) event. When stylets are removed so abruptly, the salivary sheath is left open. Thus, this event corresponds to branch no.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

***Objective 2 - Inoculation Behavior***

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

Types of probes:

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

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

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

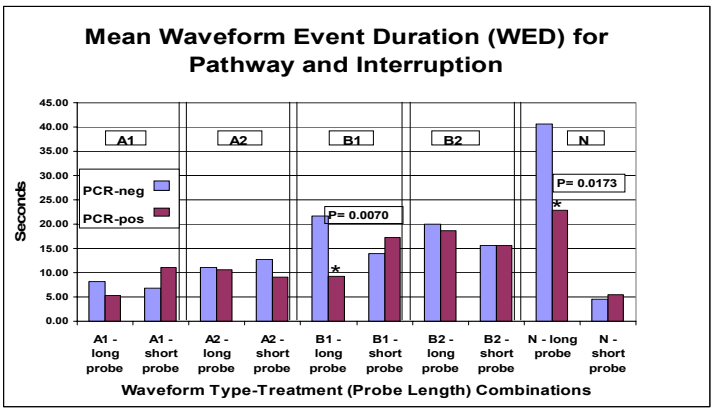
*Xf* detection methods: Probed plants were held in the greenhouse, then the 2-mm length of petiole immediately around the probe site was excised and tested via: 1) PCR (by Civerolo), after 6 week holding time, or 2) bacterial culturing (by Purcell), after 6 week holding time, or 3) symptoms, after 3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

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

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

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

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



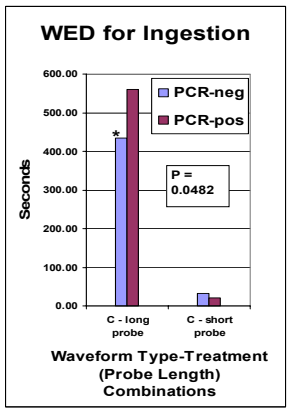
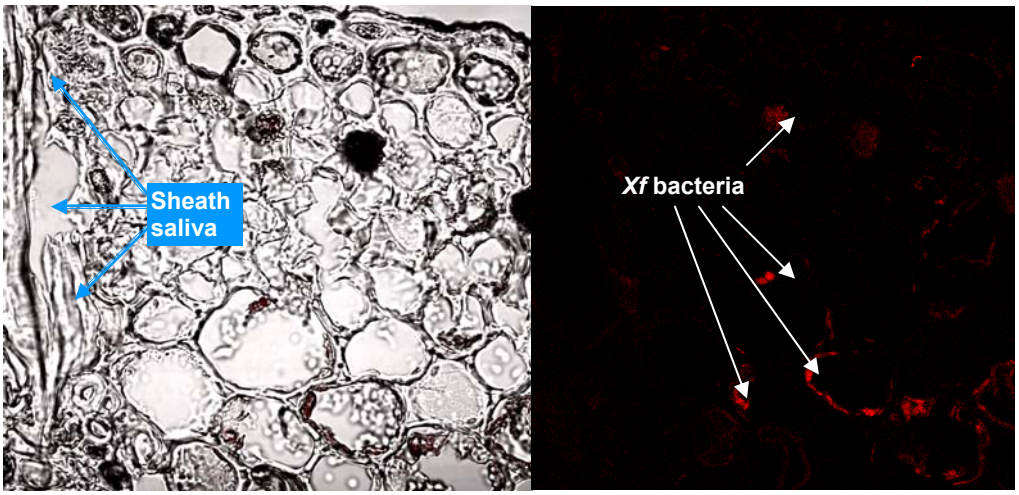



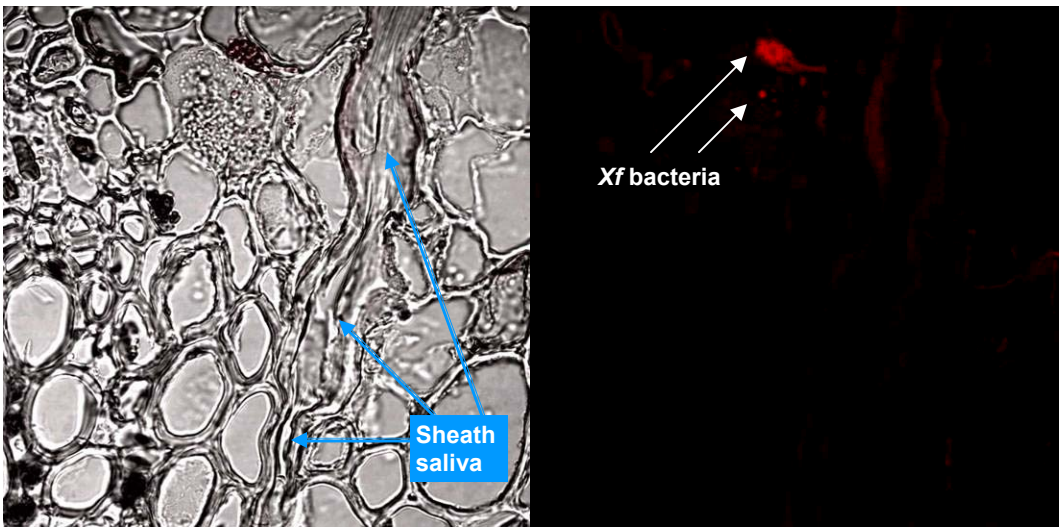

**Figure. 2.** Mean durations for each uninterrupted occurrence (= waveform event) of a waveform type, compared (ANOVA and LSD) between short and long probe treatments.

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



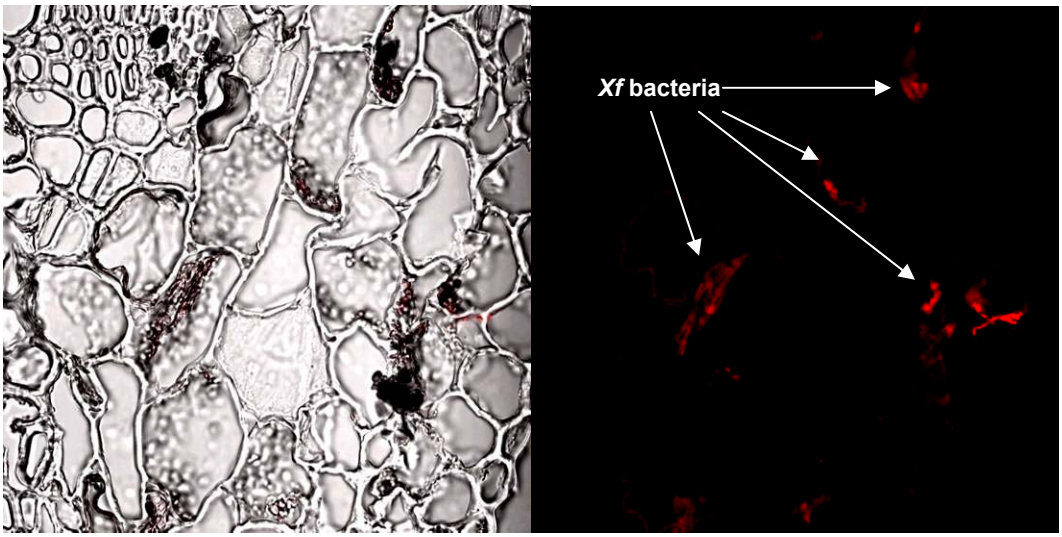

Numerous aggregations of what appear to be bacteria are visible in the transmitted light view (left). But, only a few aggregations clearly fluoresce, indicating binding with the *Xf* antibody. Size of aggregation and degree of fluorescence is low at 5 days.

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




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

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




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

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

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

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

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

***Interpretation of Overall Results***

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

**CONCLUSIONS**

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

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

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

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

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

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## FUNDING AGENCIES


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