THE ROLE OF GLASSY-WINGED SHARPSHOOTER SALIVARY ENZYMES IN INFECTION AND MOVEMENT OF *XYLELLA FASTIDIOSA*

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ABSTRACT

The purpose of this project is to determine whether a vector's enzymatic saliva aids the establishment of the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated into a plant; thus the bacteria would co-localize with the saliva. Previous work showed that watery saliva of glassy-winged sharpshooter (GWSS) degrades cell walls, and is injected into xylem during feeding. Saliva contains cell wall-degrading enzymes, notably β-1,4-glucanase (EGase), which can degrade pit membranes that impede cell-to-cell movement of bacteria. We plan to immunoprobe for salivary EGase, in grape stems fed-upon by GWSS that have acquired green fluorescent protein (GFP)-transformed *Xf*, to co-localize *Xf* with both sheath and watery saliva. This year, we: 1) improved protocols for histology of salivary sheaths using epifluroescence light microscopy, 2) dissected 500 pairs of salivary glands to extract EGase and raise antibodies, 3) showed histologically that the salivary sheath dissolves over time, resulting in cellular abnormalities typical of cell-wall loosening, and 4) showed that sheath and watery saliva are directly injected into and travel through xylem cells.

INTRODUCTION

Many researchers, including ourselves, are investigating how *Xf* moves from cell to cell**,** because it is a crucial mechanism for the earliest stages of infection. *Xf* can cause PD only if bacteria can 'break out' of the initial, imprisoning inoculation xylem vessel(s) to produce an increasing population that becomes systemic. Thus, initial infection success is dependent, in part, on *Xf* lateral movement through adjacent vessels. Recent evidence by Labavitch and colleagues studying *Xf* movement through stems supports the idea that pit membranes limit bacterial movement. Sufficient quantity of cell wall-degrading enzymes can digest parts of the primary cell wall network of the pit membrane, allowing bacteria to pass. The enzymes polygalacturonase (PG) and β-1,4-glucanase (EGase, often identified as cellulase in the literature) are produced by mature *Xf* populations, typical of the biofilms in xylem that are seen in later stages of infection, and may function in this way. It seems to us, however, that the few pioneer bacteria inoculated by a vector are unlikely to produce a sufficient titer of enzymes to digest through the pit membrane. Fewer than 200 cells are typically inoculated by sharpshooters (Hill and Purcell 1995).

Although it is routine to histologically image sheath saliva in fed-upon plants (e.g. Leopold et al, 2003, Backus et al 2005b), no researcher has ever directly visualized watery saliva in plants, for any hemipteran. Due to its fluid and dispersive nature, it is unstainable by conventional means. However, Backus and colleagues have defined electrical penetration graph (EPG) waveforms that represent salivation and ingestion (Joost et al. 2006). Correlating EPG with histology of probed plant tissues revealed the cell types into which saliva is injected (Backus et al. 2005). Watery saliva becomes mixed with, and spreads out from, the salivary sheath and enters the xylem. Labavitch and colleagues recently have found very high activity of EGase and other cell wall polymer-degrading enzymes in GWSS salivary gland fractions. The evidence that copious quantities of cell wall-degrading saliva are injected along with very few bacterial cells led Backus to hypothesize that the small number of pioneer bacteria initially inoculated are aided in their cell-to-cell movement (therefore their ultimate infection) by the enzymatic salivary secretions of their vector.

OBJECTIVES

1. Purify and characterize β-1,4-glucanase (EGase), a putatively cell wall-degrading salivary enzyme of GWSS, and develop antibodies for *in planta* localization of saliva.
2. Determine whether GWSS salivary proteins (injected into grape during EPG-controlled insect feeding) affect the distribution of recently inoculated *Xf*, as detected systemically by PCR and locally by immunocytochemistry.

RESULTS

Award notification for this new grant was received in June 2006. However, as usual, the official start of the project will be delayed until spring 2007, due to budgeting and paperwork circumstances beyond our control. Nevertheless, we made progress in these four months analyzing pertinent previous research, using Backus's in-house ARS funds.

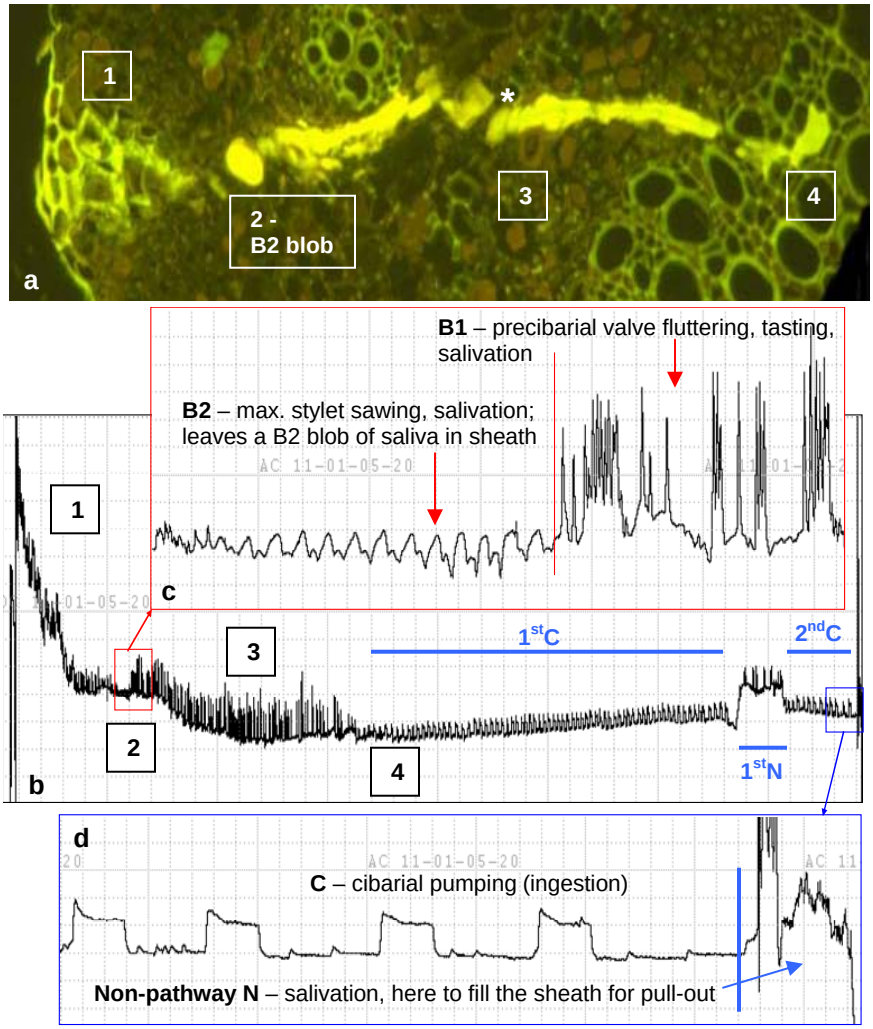

Figure 1. a. Montage of images (merged at *) from two adjoining sections of a long GWSS salivary sheath in healthy 'Cabernet sauvignon' grape petiole, excised 20 d after the probe. The sheath bypassed a small vascular bundle and penetrated straight to a secondary xylem cell in an interior vascular bundle. The cell is filled with sheath saliva because the probe was naturally terminated by the insect. Numbered labels identify spatial sheath correlations with temporal waveform correlations in part b. 200x. **b.** Entire EPG waveform trace recorded from this probe. **c, d.** Enlarged views of the red-boxed section of pathway waveform (**c**), and blue-boxed section of the last ingestion waveform (**d**), respectively, in part b, showing waveform details.

General Methodologies

Strong progress was made this year in developing new methods that will be applicable to this grant's research, as well as to other projects in the Backus lab. Among them are further fine-tuning of the histological methods used to visualize GWSS salivary sheaths in fed-upon grape tissues using confocal laser scanning microscopy (CLSM) and epiflurescence light microscopy (ELM), especially in relation to correlated EPG recordings of stylet penetration. We found that the salivary sheath autofluoresces very brightly at almost all wavelengths of light, and thus it can be imaged using both CLSM and ELM without time-consuming staining and counter-staining of tissues (Fig. 1a). The sheath is bright yellow under a GFP filter, and can be spectrally separated from the surrounding plant tissues (**Fig. 1a**) and GFP. We also further perfected the method for simultan-eous EPG recording, marking the probing site, histological preparation, then correlating waveforms (Figure 1b-d) with salivary sheaths. Landmarks in the sheath that correspond with known waveform types (e.g. the B2 blob; Figure 1 a-c) allow spatial and temporal correlations of behaviors (compare numbers on sheath and waveform pictures).

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

Study a: Enzyme purification

Salivary glands were dissected by Backus and her colleagues from nearly 500 GWSS, field-collected from ornamental shrubs in Bakersfield, CA, during the summer of 2006. Glands were frozen in extraction buffer at -20 C. Plans are to dissect another 300-500 pairs of glands before January 2007, when protein extraction, purification, and assaying of EGase will begin in the lab of Labavitch and his colleagues. Antibodies to purified EGase will be raised by a core facility at UC Davis, for later Objective 2 work.

Objective 2. Determine whether GWSS salivary proteins affect the presence/distribution of inoculated *Xf*.

Studies a and b: Immunocytochemistry of probes by clean vs. GFP-*Xf *inoculative GWSS

Our ultimate goal for this objective is to combine five challenging procedures into one experiment: 1) using ultra-clean GWSS, allow one group of insects to maximally acquire GFP-*Xf* and the other (control) group to remain clean, then 2) EPG-record a single, standardized probe as described in Backus (2006), then 3) excise, histologically prepare, and section the fed-upon grape tissue, then 4) probe the sectioned tissue with Cy5-fluorescently conjugated antibody to EGase (from Objective 1), and finally 5) use CLSM to simultaneously locate and image autofluorescent salivary sheaths and cell walls, GFP-*Xf* and Cy5-stained EGase/watery saliva. In addition, this study will include a time-course, in which fed-upon plants are held for varying time periods before excision and preparation for microscopy. In this way, we hope to visualize the location of both watery saliva (i.e. EGase) and sheath saliva in relation to presence, location and movement of *Xf* bacterial cells, during certain EPG waveforms. This year, we made substantial progress developing each of the individual protocols to be combined in the larger test, with the following findings.

The salivary sheath dissolves, resulting in cellular abnormalities typical of cell-wall loosening

In the plant inoculation experiment described more fully in Backus 2006, we developed the EPG and time course methods. We also used ELM to examine the appearances of the salivary sheaths after 0, 10, 20 and 40 days following an EPG-identified, standardized probe. We found that the outermost region of the salivary sheath (the "trunk" area, where the mandibular stylets are braced) shows signs of cell enlargement over time (Figure. 2a-d), similar to that caused by hemipterans that cause saliva-mediated, direct feeding damage via cell wall-degrading enzymes, such as *Empoasca fabae* and *Lygus hesperus* (Backus et al. 2005a, Shackel et al. 2005, respectively). This is strong, albeit indirect, evidence that such enzymes are active in GWSS saliva, ultimately leading to a cellular response typical of such agents.

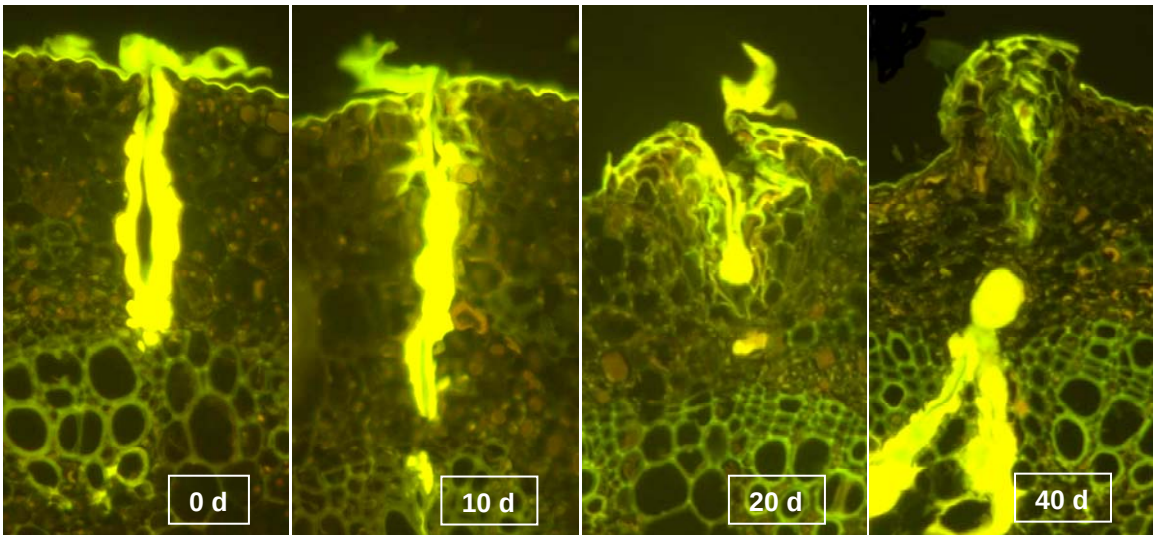

Figure 2. Naturally terminated, GWSS salivary sheaths in healthy 'Cabernet Sauvignon' grape. Although all sheaths terminated in xylem, not all chosen sections show that. **a.** Sheath excised at 0 d time period (actually, 2 – 3 min after end of probe). Note the distinctness of the sheath edges. **b.** Sheath excised 10 d after the probe. Note slight spread of saliva into adjoining cells, especially cell walls, and less distinct edges of the sheath, which is beginning to dissolve. **c.** Sheath excised 20 d after probe. Note strong dissolution and wide area of saliva into adjoining cells and cell walls, enlargement of some cells, and start of swelling of tissue. **d.** Sheath excised 40 d after probe. Note less sheath saliva (now absorbed) and strong cellular enlargement, swelling of adjacent tissue. 200x.

Sheath and enzymatic watery saliva are directly injected into xylem cells, and they can travel

ELM images of salivary sheaths from the plant inoculation-time course study (described in Backus 2006) usually showed sheath saliva within an ingestion xylem cell at the point of stylet entry, as in Figure 1. This is because the wired insects were allowed to naturally terminate their probes, and sharpshooters typically fill their sheaths as they back their stylets out of the plant (Backus et al. 2005). In addition, sheath saliva also was commonly seen in sections adjacent to the sheath's entry point. In the most extreme example to date, sheath saliva was found in each of 22 contiguous sections, i.e. up to 220 µm away from the salivary sheath (Figure 3). While a sheath branchet to a xylem cell was typically 20 – 40 µm wide, the stylet entry point was usually within one 10 µm-wide section. It is reasonable to hypothesize that less viscous, more dispersive watery saliva could travel even further within xylem vessels.

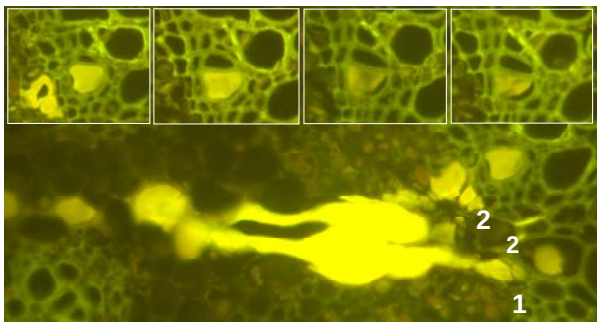

Figure 3. Naturally terminated, GWSS salivary sheath into healthy 'Cabernet Sauvignon' grape, excised 2-3 min after the probe. The sheath had four branches, two shown here, into secondary xylem cells filled with sheath saliva (1 and 2). Saliva in branch 2 moved 22 sections away from this entry point. Representative appearances of the sheath saliva in sections 3, 8, 18 and 19 (from left to right) are shown in insets. 200x.

We also have EPG evidence that salivation occurs in xylem ingestion cells. This year we definitively identified the sharpshooter waveforms representing watery salivation into a xylem cell (Figure 4, below). We performed an experiment to determine when smoke tree sharpshooter (STSS) ingestion waveforms are definitely from xylem. Preliminary data analysis is described in Backus 2006. But the following finding is pertinent to this project. Several correlated salivary sheaths had single branches leading directly to single xylem cells. In one example, the sheath (not shown) led to a large, lignified secondary xylem cell that appeared empty, i.e. it lacked occluding sheath saliva (because the insect's stylets were abruptly pulled out before it could fill the cell). This was the first xylem cell contacted; there was no evidence of branching into or extension from a different cell. Therefore, the two correlated ingestion

(C) waveform events (1st C and 2nd C) must have both occurred in the same cell. It follows then that the interruption waveform (N) between them (2nd N) must represent in part (non-stainable) watery salivation into that xylem cell (Figure 4).

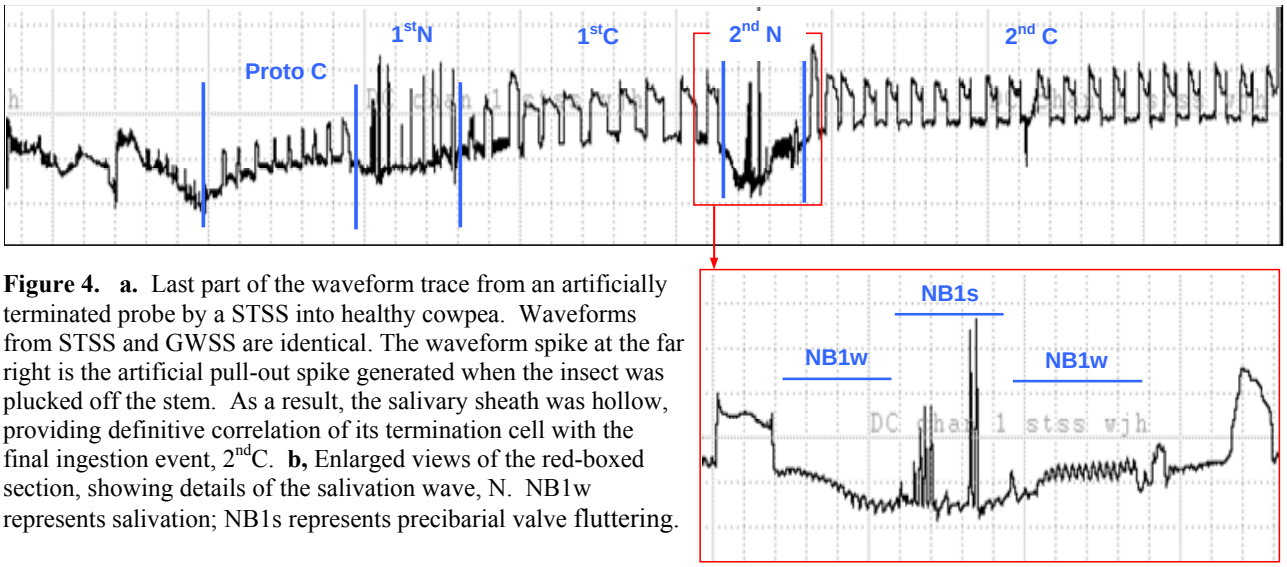

Figure 4. a. Last part of the waveform trace from an artificially terminated probe by a STSS into healthy cowpea. Waveforms from STSS and GWSS are identical. The waveform spike at the far right is the artificial pull-out spike generated when the insect was plucked off the stem. As a result, the salivary sheath was hollow, providing definitive correlation of its termination cell with the final ingestion event, 2nd C. **b,** Enlarged views of the red-boxed section, showing details of the salivation wave, N. NB1w represents salivation; NB1s represents precibarial valve fluttering.

CONCLUSIONS

The described findings support the following hypotheses, which will be further tested this year: 1) watery saliva is injected during the earliest stages of stylet penetration, as well as further along the pathway and into a xylem ingestion cell, 2) GFP-*Xf* exit the stylets during all parts of the probe, and become embedded in the salivary sheath, as well as injected directly into xylem cells, and 3) the bacteria move into areas first traversed by the watery saliva. Findings from this study will help solve the PD/GWSS problem by opening up all-new avenues for transgenic host plant resistance. Novel transgenes could be developed by engineering an inhibitor of the salivary components that aid inoculation.

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SIGNIFICANCE OF RIPARIAN PLANTS IN THE EPIDEMIOLOGY OF PIERCE'S DISEASE

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ABSTRACT
We examined the relationship between the occurrence of Pierce's disease (PD) in Napa Valley vineyards and both adjacent and distant vegetation types. Because the vector, *Graphocephala atropunctata* (blue-green sharpshooter, BGSS), is mobile and has a broad host range, disease risk is influenced by vector migration among vegetation types. Therefore, certain combinations of vegetation types surrounding vineyards are more likely to be associated with PD. To test this hypothesis, we surveyed for PD in a total of 41 vineyards located adjacent to either riparian woodland (vector habitat), urban land (vector habitat), other vineyards (vector habitat), or oak woodland (habitat status unknown). The proportions of the four vegetation types distant from the sites (within 0.5, 1, 1.5, and 2 km) were quantified with a geographical information system. Vineyards were surveyed for PD in 11/05. Pathogen presence was confirmed by ELISA. Multiple binary logistic regression showed that both adjacent and distant vegetation type significantly predicted PD presence. Vineyards were more likely to have PD if they were adjacent to riparian woodland and surrounded by more vineyards or urban land. These results suggest that vineyards and urban lands may be important in PD epidemiology. Given that uninfected vineyards adjacent to riparian woodland were also surrounded by large amounts of riparian and upland woodland, it is also possible that riparian woodland in more forested landscapes hosts lower vector densities or a lower proportion of infective vectors. Alternatively, more expansive woodland may be associated with lower PD risk because it decreases the spread of infective BGSSs.

INTRODUCTION
Riparian areas contribute to Pierce's disease (PD) in North Coast vineyards, as evidenced by a correlation between disease incidence and proximity of vines to riparian woodland (Purcell 1974). Purcell (1975) concluded that the bluegreen sharpshooter (BGSS) acquires *Xylella fastidiosa* (*Xf*) mainly from riparian hosts in spring, as the pathogen is not detectable in vines early in the growing season (Hopkins 1981). Our findings of few infected riparian hosts in spring suggest that feeding on such hosts is unlikely to result in *Xf* acquisition by the BGSS at this time (Baumgartner and Warren 2005). Therefore, either BGSSs acquire *Xf* from riparian hosts in summer or autumn, when *Xf* populations are sufficient, or they acquire *Xf* from other hosts.

The generalist feeding habit of the BGSS (Hewitt et al. 1949) makes it difficult to predict which hosts are important inoculum sources (competent reservoirs). *Xf* has a broad host range that includes all winegrape varieties and some riparian plants (Hewitt et al. 1949, Severin 1949, Freitag 1951), but its limited persistence and low titers in most species means that not all hosts are competent reservoirs (Purcell and Saunders 1999, Baumgartner and Warren 2005). *Xf* hosts have been identified mainly from greenhouse studies (e.g. Hill and Purcell 1995). In the field, such hosts are situated within plant communities (vegetation types), where their relative abundance can vary. In addition, the *Xf*-conducive environment in a greenhouse likely over estimates the host range. Therefore, field-based investigations are needed to identify vegetation types that contribute most to the spread of PD.

Our aim was to determine the relationship between PD and the spatial arrangement of vineyards among other vegetation types (landscape structure). Landscape structure is a key factor in the spread of vector-borne mammalian diseases, such as Lyme disease (Allan et al. 2003), bubonic plague (Collinge et al. 2005), and malaria (Guerra et al. 2006), and the invasive forest pathogen, *Phytophthora ramorum* (Meentemeyer et al. 2004). Our approach was to randomly select 41 Napa Valley vineyards adjacent to riparian woodland (vector habitat), urban land (vector habitat), other vineyards (vector habitat), or oak woodlands (habitat status unknown). The proportions of the four vegetation types distant from the sites (within 0.5, 1, 1.5, and 2 km) were quantified with a geographical information system (GIS; ArcGIS v9.1, ESRI, Inc., Redlands, CA). Each site (standardized to a 500-vine block) was sampled for PD in October 2005. *Xf* presence was confirmed by Dr. Barry Hill, using ELISA (Hill and Purcell 1995). Multiple binary logistic regression was used to identify combinations of adjacent and distant