SUPPORT FOR THE SALIVATION-EGESTION HYPOTHESIS FOR *XYLELLA FASTIDIOSA* INOCULATION: BACTERIAL CELLS CAN PENETRATE VECTOR SALIVA IN XYLEM

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ABSTRACT
Research is underway to develop varieties of grape that are resistant to Pierce's disease (PD) caused by *Xylella fastidiosa* (*Xf*). PD has become economically important since introduction of an exotic vector, glassy-winged sharpshooter (GWSS). However, achieving resistance to vector acquisition and inoculation is hampered by lack of knowledge of the *Xf* inoculation mechanism, despite nearly 70 years of research on this topic. Recent work suggests that bacterial cells spread from the cibarium (the retention reservoir) into the precibarium, from which inoculation occurs. Backus's recently published salivation-egestion hypothesis for inoculation proposes that saliva is brought up into the precibarium, where it is mechanically swept across taste organs by the precibarial valve. This mechanical action, combined with the enzymatic action of the saliva, dislodges bacteria in the precibarium, which are then forcefully egested (expelled) out of the stylets in a mixture of plant fluid and saliva that enters a xylem cell. The present preliminary study tested whether *Xf* bacteria can penetrate existing, hardened GWSS salivary sheaths. GWSS were allowed to feed for 24 hours on a grape stem, depositing salivary sheaths. After the insects were removed, the stem was needle-inoculated with *Xf* in the feeding area, then immunoprobed for *Xf* and examined by confocal laser scanning microscopy. Results showed *Xf*-lined needle punctures into xylem cells. *Xf* bacteria were drawn into xylem vessels and traveled varying distances from the punctures. Bacteria encountered and penetrated into undamaged, existing sheath saliva in xylem cells. The apparent ability of bacteria to penetrate into hardened sheaths suggests that *Xf* should be able to migrate out of newly-secreted, soft saliva, thereby providing indirect support for the egestion-salivation hypothesis.

LAYPERSON SUMMARY
Although the inoculation mechanism of *Xylella fastidiosa* (*Xf*) has been sought by scientists for nearly 70 years, it is still not known exactly how *Xf* is injected into a healthy plant by feeding sharpshooter vectors. This lack of knowledge hampers development of a novel means of host plant resistance to Pierce's disease, i.e. selection of plant traits that reduce the likelihood of inoculation by the glassy-winged sharpshooter (GWSS). Similar resistance to vector inoculation has been successfully developed in several crops with insect-vectored plant pathogens. Backus's recently published salivation-egestion hypothesis for inoculation proposes that *Xf* bacterial cells are forcefully egested (expelled) out of the vector's mouth parts in a mixture of plant fluid and saliva secreted into xylem cells. To determine whether bacteria could move into or out of saliva, GWSS were allowed to feed for 24 hours on a grape stem, depositing saliva in xylem cells. After the insects were removed, the stem was needle-inoculated with *Xf* near the feeding sites, then antibody-stained for *Xf* and examined by confocal microscopy. Results showed that *Xf* entered xylem cells and penetrated into sheath saliva already present in xylem. Bacterial penetration into such hardened saliva suggests that *Xf* should be able to penetrate out of newly-secreted, soft saliva. This finding provides indirect support for the egestion-salivation hypothesis.

INTRODUCTION
Despite nearly 70 years of research, it is still not known exactly how *Xylella fastidiosa (Xf)* is inoculated by feeding sharpshooter vectors. This lack of knowledge hampers development of a novel means of host plant resistance to Pierce's disease (PD), i.e. selection of plant traits that reduce the likelihood of inoculation by the glassy-winged sharpshooter (GWSS). Recent research (Backus and Morgan *ms. submitted*) demonstrates that bacterial cells spread/grow from part of the foregut (the cibarium, the retention reservoir) into another part (the precibarium) from which inoculation occurs. The egestion-salivation hypothesis for inoculation (Backus et al. 2009) proposes that a mixture of plant fluid plus newly secreted saliva is brought up into the precibarium, where it is mechanically swept across taste organs (Backus and McLean 1983, 1985) by the precibarial valve. This mechanical action, combined with the putative enzymatic action of β-1,4-glucanase in the saliva (Backus and Labavitch 2007), dislodges bacteria in the precibarium, which are then forcefully egested (expelled) out the stylets in saliva that is injected into a xylem cell just before fluid sucking (ingestion) commences. Research is underway to definitively test the egestion-salivation hypothesis. The present study was designed to finalize protocols for spectral separation of *Xf* and GWSS saliva, by studying the interaction between GWSS saliva and *Xf in planta*.

OBJECTIVES

1. Microscopically determine whether *Xf* cells can penetrate into existing hardened salivary sheaths in xylem.

RESULTS AND DISCUSSION

Eight GWSS were restricted to a marked, 5 cm-long area of grape (*V. vinifera* cv. 'Chardonnay') stem for 24 hrs. Approximately one hour after removal of cage and insects, a total of 200 µl of *Xf* culture (strain 'Temecula') were needle-inoculated within the marked, insect-probed area of stem. Within 30 – 60 minutes after inoculation, 15 ca. 3-mm blocks of stem tissue were excised. Blocks were microwave-fixed in paraformaldehyde, embedded in paraffin, sectioned at 10 µm, and mounted on glass slides. Wax sections were examined using epifluoresent light and a GFP filter cube using a Leica DM5000 compound light microscope (Deerfield, IL), which caused needle punctures, hardened salivary sheaths, and xylem cells to fluoresce against a dark background. Accumulations inside xylem cells that resembled bacterial biofilm also were slightly visible. Ten salivary sheaths from all over the stem were identified as lying close to needle punctures, so that *Xf* likely would be nearby. Eight of the ten sheaths were undamaged and intact; two (described below) had been punctured by the needle and were slightly damaged. Slides with sections containing these sheaths were immunoprobed for *Xf* using rabbit primary antibody (Agdia, Elkhart, IN) and goat secondary antibody with an Alexa Fluor 647 flourochrome (Invitrogen, Carlsbad, CA). BSA and secondary antibody controls were run with the test samples to determine levels of non-specific binding. Tissues were examined and imaged using a Leica SP2 AOBS confocal laser scanning microscope, and later edited in Adobe PhotoShop v. CS2 (Mountain View, CA) and Microsoft PowerPoint (Edmonds, WA).

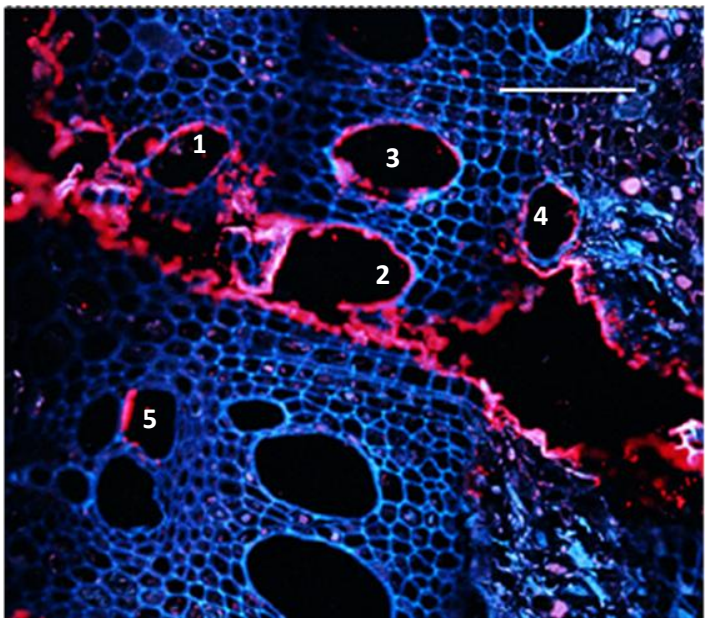

Figure 1. Needle puncture into grape stem. Red is immunostained Xf, pink is non-specific binding to certain vesicles in parenchyma cells, blue is autofluorescence of cell walls. Needle puncture directly into xylem cells (1 and 2) caused extensive cell wall damage, and introduced bacteria into xylem. Nearby, undamaged xylem cells (3, 4 and 5) were inoculated with *Xf* by other needle punctures, above or below this section. Scale bar 25 µm.

Needle punctures into grape stem caused extensive cellular damage, leaving large gaps that were lined with red-stained *Xf* deposits (**Figure 1**). Xylem cells in the direct path of needle-punctures had moderate to severe rupture of cell walls (**Figure 1,** 1 and 2). This process introduced bacteria into the xylem transpiration stream. Either an air embolism caused by cavitation or the intact transpiration stream would rapidly pull bacteria away from the site of puncture. Thus, intact xylem cells not continuous with broken xylem cells within one section often contained bacteria. Inoculation in this manner is demonstrated in cells 3, 4, and 5 (**Figure 1**), which are separate from the needle-punctured gap, yet lined with bacteria. These cells were likely inoculated by other needle punctures many sections above or below the imaged section.

GWSS salivary sheaths resembled those seen in other studies (**Figure 2a**). A salivary flange on the surface of the stem was confluent with the salivary sheath inside the stem. The hollow lumen of the

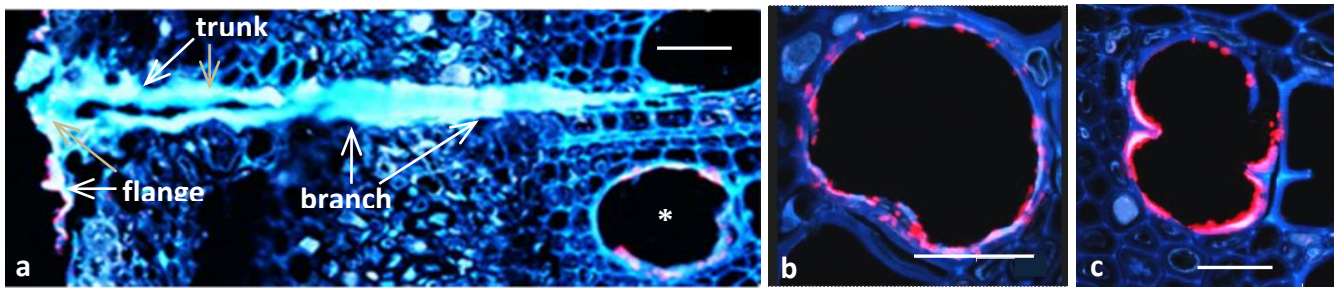

Figure 2. a. GWSS salivary sheath whose tip entered a xylem cell that did not contain bacteria (upper right, with scale bar). Sheath saliva is bright bluish-white due to stronger autofluorescence than cell walls. All other colors same as Figure 1. Intact, inoculated xylem cell (*, also imaged in part b) near the sheath. **b.** Close-up of same inoculated xylem cell (*) from part a, but in an adjoining section. **c.** Close-up of two intact, inoculated, immature metaxylem cells near the sheath. Scale bars 25 µm.

sheath trunk was visible as the sheath narrowed, indicating the furthest extension of the mandibular stylets. One or more branches extended from the trunk into the stem interior (**Figure 2a**) (Backus et al. 2005). Pictured is one of the four salivary sheaths that were immunoprobed but did not penetrate xylem cells into which *Xf* bacteria were later inoculated. However, inoculated xylem cells were nearby (**Figure 2a, b, c**).

Close-ups of intact, inoculated xylem cells showed that *Xf* bacteria not only lined the walls of the cells, but also penetrated the cell walls to varying depths (**Figure 2b**), sometimes the full width of the wall (**Figure 2c**). Such bacterial penetration strongly suggests that *Xf* can loosen the microfibrils of cellulose and other polysaccharides that form the secondary cell walls of xylem cells. It is known that the *Xf* genome codes for several cell wall-loosening enzymes such as β-1,4 endoglucanases, β-endoxylanases and β-xylosidases (three types of cellulases) and polygalacturonase (a pectinase) (Roper et al. 2007). It is also known that bacterial movement between xylem cells, establishing a systemic infection, occurs primarily through so-called pit membranes (actually primary cell wall containing pectin and cellulose, inside pits in the secondary cell wall) (Thorne et al. 2006). The pectin polymer lattice determines the pore size in pit membranes, explaining why the polygalacturonase gene has been found to be critical for establishment of systemic infection (Kirkpatrick et al. 2006, Roper et al. 2007). Nonetheless, our images suggest that (given enough time) individual or small numbers of bacteria also might be able to penetrate directly through the secondary cell walls of xylem, perhaps via action their secreted cellulases.

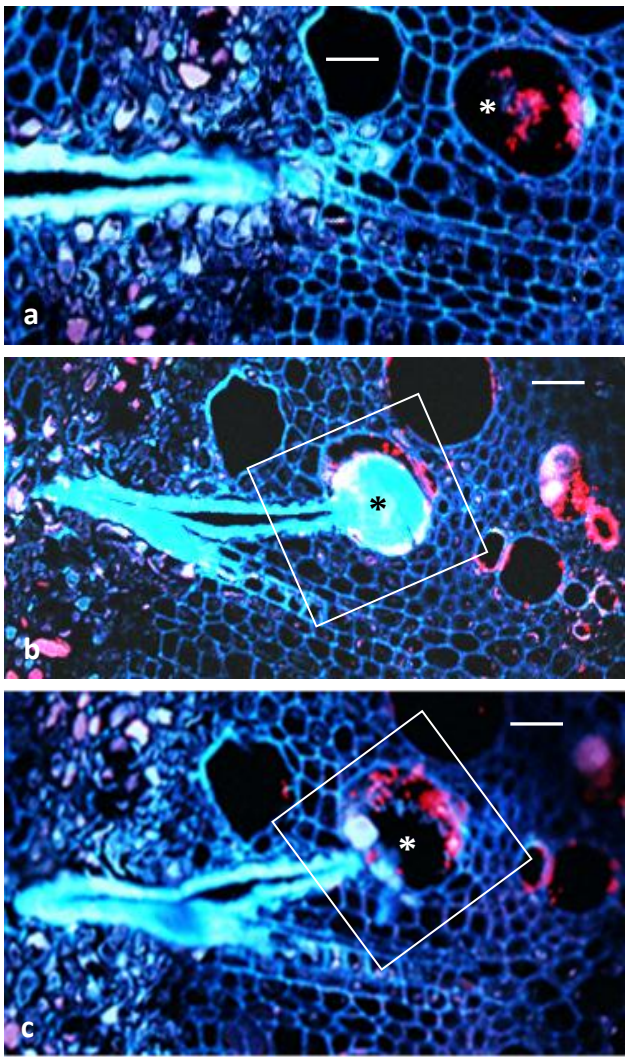

Figure 3. Three contiguous sections containing a salivary sheath with two branches. **a.** Side section of all but the tip of main branch, approaching a xylem cell with large amount of red-stained *Xf* (*). **b.** Section of the central part of main branch, showing saliva bolus inside same xylem cell (*). **c.** Other side section of main branch, showing smaller amount of saliva, more Xf. Colors same as Figure 2. Scale bars 25 µm.

Six of the ten salivary sheaths entered xylem cells that were later inoculated with *Xf*. Four of these were not close to needle punctures, so the intersected xylem cells were inoculated from distant needle punctures. Images from one representative sheath are displayed in **Figure 3**. This sheath was notable because it had a bolus of sheath saliva that was injected into its terminal xylem cell, probably after a bout of ingestion (sucking) (Backus et al. 2005) (**Figure 3b)**. It is common for GWSS sheaths to terminate in a bolus of saliva, but usually the bolus has been pulled some distance away from the site of stylet penetration into the xylem cell (Backus and Labavitch 2007). The sheath's terminal xylem cell has a large accumulation of red-staining *Xf* bacteria (**Figure 3a, c**) as do several other, nearby xylem cells (**Figure 3b**, right side). In the cell with the saliva bolus, most bacteria are clearly outside the sheath, in fact, located directly surrounding the saliva bolus. However, a small number are inside the saliva near the edge of the bolus (**Figure 4a)**.

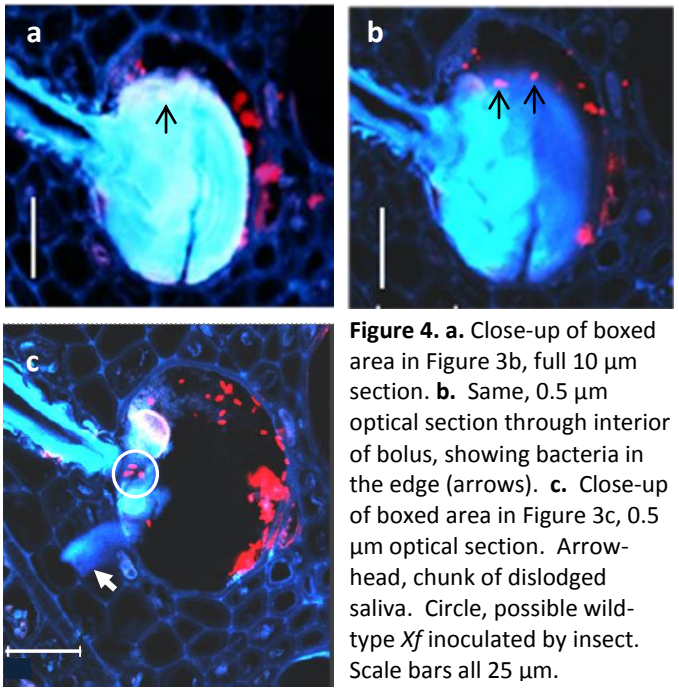

Figure 4. a. Close-up of boxed area in Figure 3b, full 10 µm section. **b.** Same, 0.5 µm optical section through interior of bolus, showing bacteria in the edge (arrows). **c.** Close-up of boxed area in Figure 3c, 0.5 µm optical section. Arrowhead, chunk of dislodged saliva. Circle, possible wild-type *Xf* inoculated by insect. Scale bars all 25 µm.

The large saliva bolus in the latter images has a crack running through its center (**Figure 4a, b**, previous page). This disruption of the hardened saliva may have occurred from pressure on surrounding tissues during needle puncturing, even though there was no apparent damage to cell walls. Although large accumulations of bacteria were transported into this xylem cell, none were found near or inside the crack in the salivary bolus. If bacteria had entered the saliva by mechanical damage during puncturing, we would expect them to be near or in this crack. This suggests that the few bacteria inside the saliva entered by some other means, perhaps via enzyme-mediated dissolving and penetrating. Because salivary sheaths of sharpshooters and other hemipterans are primarily lipoproteinaceous (Miles 1999, Alhaddad et al. *ms. submitted*), this suggests action of a protease.

The salivary sheath in **Figures 3** and **4** was also notable because three discrete bacterial cells were located at the apparent entry point of the stylet tips, on one side of the saliva bolus (**Figure 4c**, previous page, circle). It is possible that these bacteria could have penetrated into the saliva from outside. However, it is also possible, given their position compared with that of other bacteria (surrounding the salivary bolus, on the periphery of the cell) that these bacteria were wild-type *Xf* from the insect, inoculated during probing of the xylem cell. The immunostain would bind equally to any *Xf*, i.e. both naturally insect-inoculated as well as needle-inoculated GFP *Xf*. The innoculativity status of the colony GWSS used for this test was not known, however, some *Xf* contamination is likely. Clearly, however, the vast majority of bacteria found in xylem were needle-inoculated, because bacterial accumulations were only found in regions of the stem that had been punctured.

Of the six sheaths that entered xylem cells later inoculated with *Xf*, two were directly intersected by needle punctures at some point along their length. Yet other portions of both sheaths also entered xylem cells inoculated by a distant puncture. One of these two salivary sheaths is pictured in **Figures 5** and **6**. **Figure 5** shows a section that nicked the side of this two-branched sheath, thus only portions of sheath material are visible. But they clearly show the trajectory of the two branches (**Figure 5**, line 1), and how they were both intersected by a needle puncture (**Figure 5**, line 2) at nearly a right angle to the sheath trunk.

The structure of the left-hand sheath branch (as viewed in the image, **Figure 6, next page**, which shows the main section that contains most of the salivary sheath) supports the following interpretation. During feeding, the insect's stylets entered and salivated into a xylem cell (labeled by an asterisk in **Figure 5**). Saliva is indicated by the blue-white ring in the xylem cell (**Figure 6, next page**). However, the insect apparently abandoned that cell, because the sheath branch was extended further. This behavior occurs commonly during sharpshooter feeding (Backus et al. 2005, Backus et al. 2009). The upper (as viewed, **Figure 5** and **6a**) extension of the left branch was later intersected by a needle puncture and associated *Xf* bacteria (red).

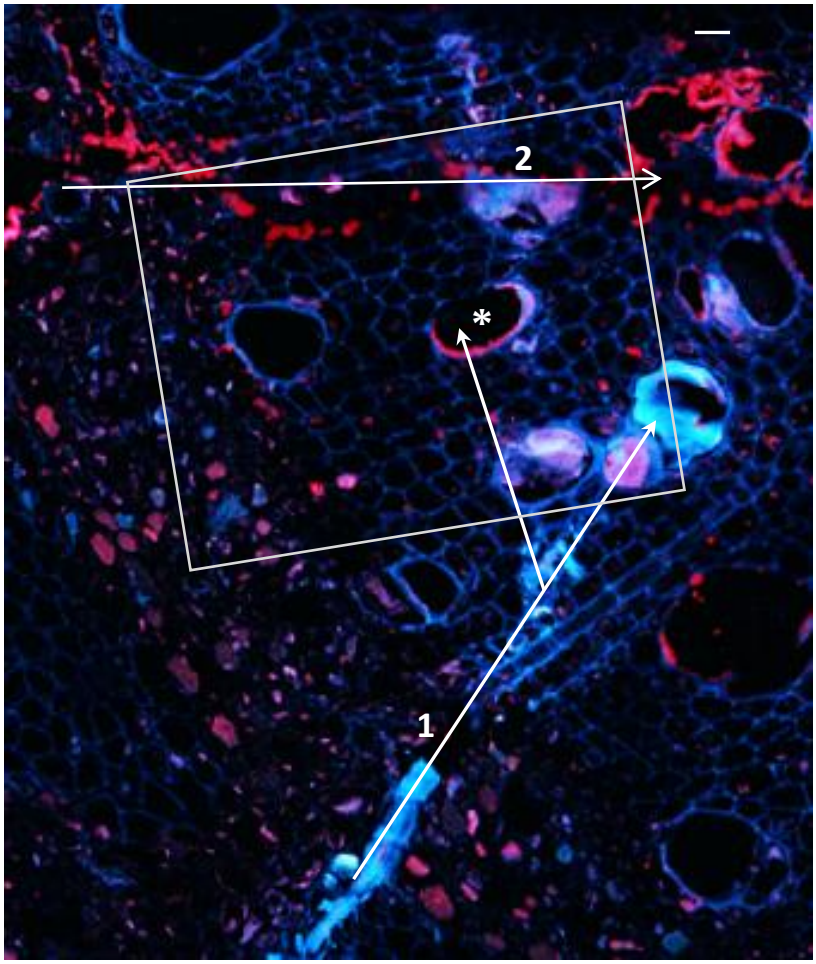

Figure 5. Side-section, showing portions of a large, two-branched salivary sheath (line 1) that was intersected by an *Xf* inoculation puncture (line 2). Box shows outline of similar image in Figure 6. Cell with asterisk is same cell as the one boxed in Figure 6. Colors as in Figure 2. Scale bar 25 µm.

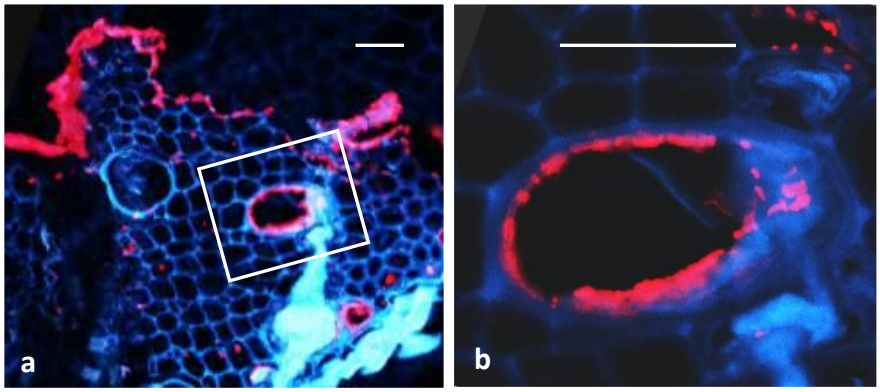

Figure 6. a. Two branches of the salivary sheath in Fig. 5, the left branch of which was intersected by a needle puncture and associated *Xf* bacteria (red). Colors as in Figure 2. Full 10 µm-thick section. **b.** 0.5 µm optical section through interior of physical section boxed in part a. Scale bars 25 µm.

In contrast, the lower portion of the left branch that had entered the abandoned xylem cell was undamaged by the needle puncture (**Figure 6a, b**). This xylem cell was inoculated by a distant puncture, not the nearby one, because its cell wall was unbroken and bacteria were not connected to the puncture in this or any adjoining section. A thick bacterial biofilm developed in this cell during the 30 – 60 minutes after needle-inoculation. It formed a circular, near-continuous lining of the cell wall that was also seen in adjoining sections (**Figure 5**). Optical sectioning through the sheath material in the xylem cell shows bacteria inside the saliva, in the same, near-continuous configuration as bacteria outside the saliva (**Figure 6b**).

In summary, the following evidence supports that *Xf* bacteria can penetrate into the portion of hardened saliva that is left by feeding GWSS inside xylem cells. 1) All six of the existing, hardened salivary sheaths that were later encountered by *Xf* needle-inoculated into xylem were found to contain small to large amounts of bacteria. 2) The *Xf* cells were embedded only in the portion of the sheath found inside the inoculated xylem cell. Therefore, the hardened saliva was not a conduit through which later-inoculated *Xf* could migrate into any plant cell. 3) *Xf* probably was not forced into salivary sheaths by the mechanical action of needle-puncturing, because: a) *Xf* was often found in undamaged xylem cells or sheaths, and b) in one sheath that was slightly torn, bacteria did not enter the sheath through the tear, but instead were found elsewhere in the sheath.

It is not known whether *Xf* penetration of GWSS sheath saliva occurs via: 1) hydrostatic pressure from transpiration or 2) cavitation propelling the bacteria forcefully into the saliva, or 3) secretion of enzymes, allowing bacteria to dissolve their way into the saliva. In any case, our findings suggest that even hardened saliva is not perfectly solid, but may be composed of a network of microfibrils with some porosity, similar to sclerotized, proteinaceous insect cuticle. Bacterial penetration into hardened saliva also suggests that *Xf* should be able to either actively or passively move out of newly-secreted, soft saliva. This finding provides indirect support for the egestion-salivation hypothesis for vector inoculation of *Xf*.

CONCLUSIONS

Results from this study show that *Xf* bacteria in grape xylem cells can penetrate GWSS saliva deposited therein, even when the saliva is 1 – 24 hours old and completely hardened. This supports that sheath saliva is a porous network of microfibrils similar to insect cuticle that, at minimum, does not present an impediment to passive bacterial movement via the transpiration flow. Additionally, it is possible that *Xf* bacteria can actively penetrate into or out of GWSS saliva, perhaps via secretion of bacterial proteases. Results of this test support the egestion-salivation hypothesis for the *Xf* inoculation mechanism, which proposes that bacteria are carried into a xylem cell by a mixture of plant fluid and saliva that is ejected into the xylem before sucking (ingestion) commences. Bacteria encased within secreted saliva can probably penetrate out into the xylem transpiration flow. Tests to definitively prove this inoculation mechanism are underway. If proven, this mechanism will support use of salivary antagonists for novel genetic approaches to PD management. Also, because both egestion and salivation into xylem have been correlated with a unique Electrical Penetration Graph (EPG) waveform (Backus et al. 2009), definitive proof of the egestion-salivation hypothesis will facilitate rapid testing of transgenic or classically bred grape plants, or plants inoculated with a benign strain of *Xf*, to determine their effects on vector inoculation behavior.

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