GLASSY-WINGED SHARPSHOOTER FEEDING DOES NOT CAUSE AIR EMBOLISMS IN THE XYLEM OF WELL-WATERED PLANTS

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ABSTRACT
Plant xylem vessels are under negative hydrostatic pressure (tension) as evapotranspiration of water from the leaf surface pulls the column of water in xylem upwards. When xylem fluid flux is under extreme tension, any puncture or breakage of the xylem vessel wall can cause formation of air embolisms that instantaneously empty the length of the xylem vessel (cavitation), disrupting xylem flow. Xylem fluid-ingesting hemipteran insects like the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), penetrate their stylets into xylem cells and imbibe many times their body weight in xylem fluid each day. It has not been known whether GWSS stylet penetration embolizes xylem, however, embolisms from vector feeding have been suggested as one mechanism of xylem blockage in Pierce's disease symptoms. To date, no method has been successful in visualizing xylem during real-time stylet penetration, to determine whether or not air embolism occurs. The present study used videography of live, feeding GWSS under X-ray phase contrast microscopy at the Argonne National Laboratory, to determine whether air was present in stylets or xylem before, during, or after xylem penetration. Air is an excellent contrast agent for X-ray microscopy, and is readily visible in both plant cells and insect stylets. Insects were monitored via electrical penetration graph, to identify when their stylets had penetrated xylem in cowpea stems. After feeding was terminated, stems were cut; subsequent entry of air into xylem was visible in stems under X-ray. X-ray videographs before, during, and after stylet penetration to and inside xylem showed no air present in stylets, and only in cut, not intact, xylem cells. It is hypothesized that salivary sheaths secreted by GWSS during stylet penetration prevent cavitation.

LAYPERSON SUMMARY
Xylem-feeding insects like the glassy-winged sharpshooter (GWSS) penetrate their needle-like mouth parts (stylets) into xylem (water-conducting) cells in plants, and swallow many times their body weight in xylem fluid each day. This is in spite of the fact that plant xylem cells are under extreme hydrostatic suction (tension). Any puncture of the xylem vessel wall can cause formation of explosive air bubbles, disrupting xylem flow. It has not been known whether GWSS stylet penetration causes air bubbles in xylem, however, it has been suggested that air bubbles induced by GWSS feeding might contribute to xylem blockage causing Pierce's disease (PD) symptoms. To date, no method has been successful in directly visualizing xylem real-time during GWSS feeding, to determine whether or not air bubbles develop. The present study video-recorded live, feeding GWSS under an X-ray microscope at the Argonne National Laboratory, to determine whether air was present in stylets or xylem before, during, or after GWSS feeding. Air is an excellent contrast agent for X-ray microscopy, and is visible in both plant cells and insect stylets. X-ray images before, during, and after GWSS feeding inside xylem showed no air present in either stylets or xylem cells. After feeding ended, stems were cut; subsequent air bubble formation in xylem was visible in stems. Thus, air bubble formation probably does not contribute to PD symptoms. Hardened saliva that is secreted by GWSS during feeding may prevent formation of bubbles.

INTRODUCTION
Xylem vessels are under extreme negative hydrostatic pressure (i.e. tension) as evapotranspiration from the leaf surface draws the column of water in xylem upwards. This metastable column of water is highly prone to physical rupture (cavitation) leading to an air embolism that can completely halt xylem flux (i.e. hydraulic failure). Evidence is mounting that Pierce's disease (PD) symptoms in grapevine are the result of a cascade of plant responses (Chatelet et al. 2011) that include xylem embolism in its earliest stages, well before onset of symptoms (Pérez-Donoso et al. 2007, McElrone et al. 2008). Xylem embolism occurs more often in *Xf*-infected than control plants and precedes significant hydraulic failure (McElrone et al. 2008). Therefore, it is likely that air embolism plays an important, albeit incompletely understood, role in *Xylella fastidiosa* (*Xf)* pathogenesis.

Three hypotheses have been proposed for induction of embolisms in *Xf* pathogenesis. First, the earliest embolisms might result from sharpshooter stylet penetration of xylem vessels (Backus, J.M. Labavitch, A. McElrone, A. Pérez-Donoso, pers. comm.; Crews et al. 1998). Second, early embolisms could be directly induced by *Xf* bacteria in xylem vessels, either by reducing water surface tension or by damaging pit membranes (McElrone et al. 2008). Third, xylem plugging by bacteria, gums, and tyloses could exacerbate hydraulic failure via additional cavitation (McElrone et al. 2003). This would not explain air embolisms early in pathogenesis, but could explain the interaction of water stress and air embolisms in *Xf* pathogenesis (McElrone et al. 2003). The present project tested the first of these three hypotheses.

OBJECTIVE

1. Determine whether air occurs in GWSS stylets or xylem before, during, or after vector feeding on a well-watered sunflower plant using real-time videography of live insects, feeding under X-ray phase contrast microscopy.

RESULTS AND DISCUSSION

GWSS, sunflower plants, and electrical penetration graph (EPG) equipment were shipped to the Advanced Photon Source at the Argonne National Laboratory under appropriate USDA APHIS PPQ permits. GWSS's feeding on sunflower stems were simultaneously subjected to X-ray phase contrast microscopy and recorded via EPG, using the following experimental protocol. Insects were tethered to gold wire, then held on sunflower for 1 – 10 hrs, then were starved for 1 – 3 hrs prior to recording. A sunflower plant was placed in a holder in the X-ray room, in the path of the beam. A wired insect was plugged into the head stage amplifier of the EPG monitor, positioned on the vertical stem, and EPG monitoring begun. Lights were turned out and the room was vacated and sealed in anticipation of beam-on; however, to minimize deleterious effects on the insect from X-rays, the beam was not immediately turned on. When the insect's stylets probed the plant, feeding waveforms were digitally recorded and displayed on a Dell Latitude laptop computer, as previously described (Dugravot et al. 2008, Backus et al. 2009). The GWSS was allowed to probe without X-ray exposure until its stylets reached xylem (as indicated by X waves) (Backus et al. 2009) and ingestion had ensued for 15 to 40 min. The X-ray beam was then turned on and video-recording of the plant and insect's proboscis begun. Using The Observer® video acquisition software, EPG waveforms and video output from the X-ray microscope were synchronized and simultaneously displayed on the computer. After a few minutes of recording, the X-ray was turned off, lights turned on, and the room was re-opened and entered. The site of probing was marked and the insect was gently disturbed to artificially terminate the probe. The plant stem was then cut 1 – 3 cm below the mark, allowing air to begin to enter the xylem. The lights were turned off, room vacated, sealed, and the X-ray turned back on, to video-record the progression up the stem of air entering xylem cells (requiring about 10 – 30 min, due to slow evapotranspiration in the cool, dark room). Each experiment lasted 2 – 3 hrs, and was repeated six times, although one insect did not achieve xylem ingestion (see below).

To visualize air inside stylets, three additional sharpshooters were X-rayed and video-recorded while attempting to feed on empty, air-filled Parafilm® sachets, although no EPG was possible due to lack of electrical conductivity of air. Four intact sunflower stems were also X-ray video-recorded for 5 min without sharpshooter feeding, as controls, including two stems that were used several minutes later for experiments, described above. Thus, plants were X-ray imaged before, during and after sharpshooter feeding and stem cutting, and air was clearly visualized both inside insect and plant controls.

X-ray microscopy reveals the interior of biological specimens in unaccustomed ways. First, X-rays have infinite depth of field, so X-ray images are completely flattened. Thus, all visible structures in the interior of a specimen that lie in the cross-sectional area of the beam are superimposed on one another in the same focal plane, regardless of the thickness of the specimen. Second, structures are variably visible based on the strength of contrast agents present in the specimen. Heavy metals such as iodine are excellent contrast agents, but difficult to use with live insects. On the other hand, air is an excellent contrast agent for X-rays, and visible inside both insects and plants. However, degree of visibility depends upon brightness/amount of air relative to the thickness of the subject. We used air as the sole contrast agent in this study.

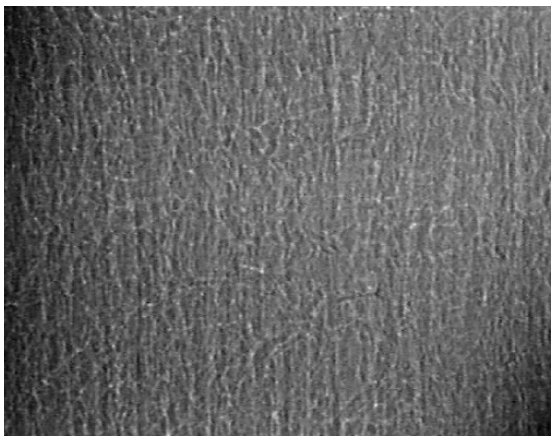

Figure 1. X-ray image of the vascular region of a sunflower stem 10 min after the start of GWSS probing and 5 cm above the probing area. Note bright, reticulated pattern of air in intercellular spaces around parenchyma cell, but absence of xylem cell striation.

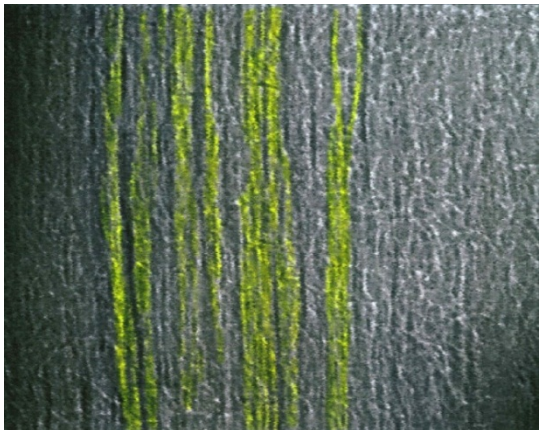

Figure 2. False-colored X-ray image of the vascular region of a sunflower stem 80 min after the start of GWSS probing and 1 cm below the probing area, showing air-filled xylem vessels with superimposed intercellular spaces. Yellow color, here and elsewhere denotes air.

Prior to cutting, sunflower stems were mostly opaque to X-rays, with a tight pattern of reticulation that was probably caused by intercellular air spaces between cells in the ground tissue on the stem periphery (**Figure 1**). No vertical striations were visible. In contrast, obvious, white vertical striations interpreted to be air-filled xylem vessels (**Figure 2**; yellow highlighting) became visible 3 – 5 min after stems were cut, slowly spreading from the cut end of the stem upwards. This occurred in every cut stem, regardless of whether or not the stem had been probed by sharpshooters. The stem in **Figure 2** was imaged 39 min after cutting; air filling of the full length of xylem vessels generally occurred within 90 min of cutting.

Further evidence that white striations represent air-filled xylem is shown in **Figure 3**, displaying a series of X-ray images taken within one minute of each another. We observed two air bubbles in a single xylem vessel, with a narrow strip of fluid between the two bubbles. As we watched, the shape and position of the fluid strip changed (**Figures 3a – c**), and eventually disappeared completely as the two air bubbles suddenly merged (**Figure 3d**). Air bubble movement in this shape and manner could only be explained by air filling of a xylem vessel.

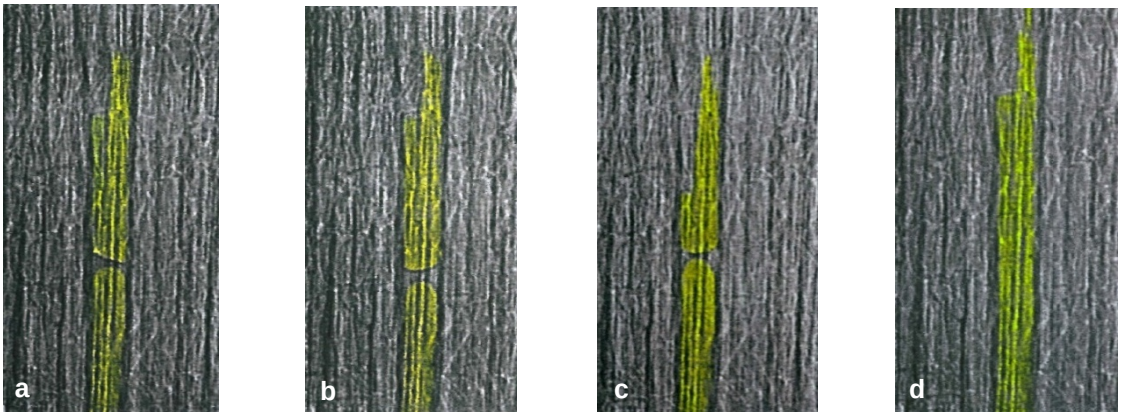

Figure 3. False-colored X-ray images of two air bubbles in a xylem cell, moving and merging. **a.** Two air bubbles in a xylem vessel, 70 min 43 sec after start of stylet probing. **b.** Same vessel, 11 sec later. **c.** Same vessel, 5 sec later. **d.** Same vessel, 43 sec later, after bubbles merged.

GWSS attempting to ingest from an air-filled Parafilm® sachet briefly sucked air into their stylet food canals (**Figure 4**; yellow highlighting), proving that air would be readily visible inside the proboscis (external to the feeding substrate) and stylets (internal to the substrate) using X-ray microscopy. In contrast, sharpshooter stylets inserted into sunflower stems, both before and during xylem ingestion, did not contain air (**Figure 5**). Only a faint outline of the stylets was visible shallowly inserted into the stem, and no air-filled xylem vessels were visible during or after xylem ingestion. The image in **Figure 5** was taken 38 min after the start of stylet probing, during xylem ingestion. Two min later, this insect (no. 1) pulled

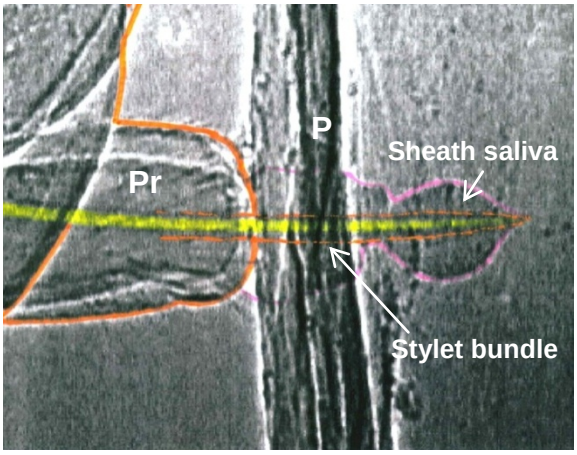

Figure 4. False-colored X-ray image of a GWSS proboscis (Pr) pressed to a Parafilm® (P) feeding sachet, with stylet bundle extended into a recently secreted bead of sheath saliva. Orange, outlined insect structures; pink, outlined saliva.

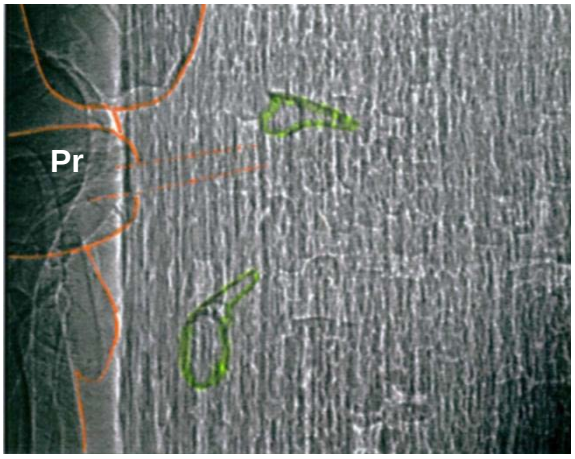

Figure 5. False-colored X-ray micrograph of insect no. 1's proboscis (Pr) pressed to a sunflower stem, during xylem ingestion recorded via EPG. Orange, outlined insect structures; green, outlined trichomes on stem surface. Note intercellular reticulation.

out its stylets and the stem was cut. At no time before, during, or after xylem contact and ingestion was air observed either inside the proboscis, stylets, or xylem vessels. The only time air could be seen inside xylem vessels was after air embolisms were artificially introduced via stem cutting.

After air had completely filled the xylem vessels and intercellular spaces of the first sunflower stem observed (for insect no. 1), an X-ray survey of the entire stem revealed a structure closely resembling a GWSS salivary sheath (**Figure 6a**) inside the stem, in the same location as the marked probe. Thereafter, a whole-stem X-ray survey was performed at the end of each repetition. Similar structures were seen in X-ray images for three out of the six repetitions (**Figure 6b**). One-mm blocks of sunflower tissue were excised from the marked areas of all probed stems, fixed and later prepared for paraffin-sectioning, saffranin-fast green counterstaining, and examination via light microscopy, using previously described methods (Backus et al. 2009). In all six cases, typical GWSS salivary sheaths were later found in probed tissues (**Figure 6c**), demonstrating that the structures seen in X-ray images were sheaths. However, they were only visible when air had filled the stem, and not in all cases. Likelihood of observing salivary sheaths in stems was related to stem thickness; very thick stems did not allow observation, even though salivary sheaths were present.

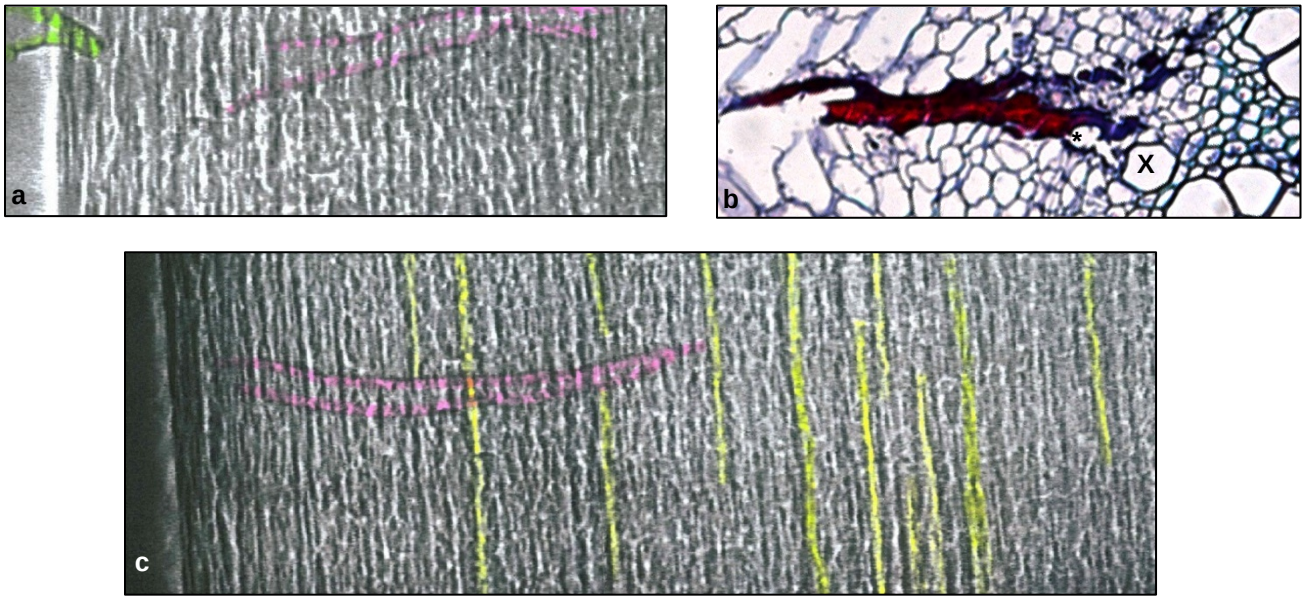

Figure 6. X-ray and light micrographs of GWSS salivary sheaths in probed sunflower stems. **a.** X-ray micrograph (side view, from transversely viewed stem) of sheath from insect no. 1; image was taken before the sheath was observed, hence only part is visible. **b.** Light micrograph of same salivary sheath from insect no. 1 (viewed from above, from cross-sectioned stem). Note thin-walled, hollow, lower sheath branch (*) to large xylem cell (X). The last sheath branch made is always hollow when the probe is artificially terminated. X-ray sheath image in **Figure 6a** corresponds to the red-stained saliva; blue-stained saliva is not visible in X-ray. **c.** X-ray micrograph of salivary sheath from insect no. 2, more clearly showing the full size of the sheath in relation to xylem vessels from multiple, superimposed bundles. Pink, outlined saliva; green, outlined trichome.

Another observation of GWSS saliva is noteworthy, although not related to air embolisms in xylem. **Figure 7** shows a large deposit of presumed watery (digestive enzyme-containing) saliva on the periphery of the stem, immediately below the salivary flange marking the entry point of the saliva. This deposit was left by insect no. 6, the only insect that died from X-ray exposure before its stylets could reach xylem. EPG waveforms indicated that this insect performed pathway activities (formation of the salivary sheath) for 20 min (a very long time) without beginning xylem ingestion. Indeed, later paraffin-sectioning showed that the weakened insect made a large, multi-branched salivary sheath that never arrived at a xylem cell. The sheath was not visible under X-ray, probably due to the thickness of that particular stem. Nonetheless, this insect's probe is remarkable because, in its weakened condition, the insect apparently left a large accumulation of watery saliva that loosened cell walls sufficiently to cause more air-entry than for the surrounding cells. Watery saliva, though hypothesized to be produced by GWSS (Alhaddad et al. 2011, Backus et al. ms. submitted), has never been visualized *in planta*.

Embolism of xylem vessels may be one of the first steps in the cascade of plant responses underlying symptom development in Pierce's disease (PD) (McElrone et al. 2008). However, the present work demonstrates that air embolism cannot be caused by GWSS stylet penetration into a xylem vessel, at least for a well-watered plant, for which these results are most applicable. Water stress due to extreme light, temperature, and/or lack of soil moisture interacts with *Xf* infection in an additive manner to worsen hydraulic failure and PD symptoms (McElrone et al. 2003). It is possible that GWSS feeding under those circumstances might trigger air embolisms. This possibility will need to be examined using X-ray microscopy in future

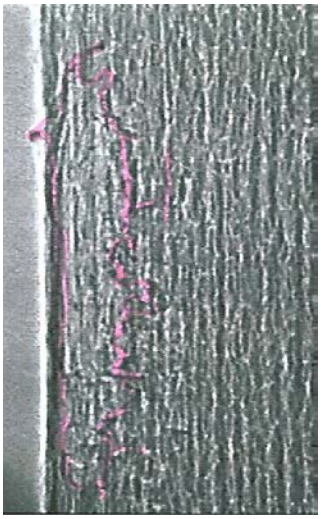

Figure 7. X-ray micrograph of sharpshooter watery saliva deposit from insect no. 6. Round deposit of saliva on the left-outside of stem (F) is the salivary flange, marking the entry point of the stylets. Watery saliva mixed with sheath saliva was injected at this point, flowing downward with gravity. Pink, outlined saliva.

studies. It is interesting to note, however, that once a stem had been cut and xylem cells artificially embolized, GWSS refused to initiate stylet penetration, let alone ingestion. Insects also were very reluctant to probe air-filled Parafilm® sachets. It is possible that presence of too much air in plant tissues, including xylem, could be a deterrent to GWSS feeding.

CONCLUSIONS

Real-time videography of GWSS feeding under X-ray microscopy revealed no air in the proboscis, stylets, or xylem vessels inside well-watered sunflower stems. In contrast, air was visible inside the stylet food canal of insects that had probed an air-filled Parafilm® sachet and briefly sucked up air, as well as in xylem vessels that had been artificially embolized via severing of the stem. The latter controls demonstrate that it would have been possible to detect air in the stylets or xylem if GWSS feeding had triggered air embolisms. We conclude that stylet penetration of a xylem vessel during GWSS feeding does not cause air embolisms in well-watered plants. Continued research will be necessary to determine whether the same is true for water-stressed or *Xf*-infected plants.

Development of novel strategies for PD management depend upon understanding *Xf* pathogenesis. Eliminating the role of vector feeding in onset of xylem embolism will allow researchers to concentrate on the impact of bacterial colonization of pit membranes, production of gums, and plant responses such as tyloses. Because plant responses are under strong genetic control, it is possible that new transgenic mechanisms of resistance to *Xf* could result from such studies.

REFERENCES CITED

Alhaddad, H., T. A. Coudron, E. A. Backus, and F. Schreiber. 2011. Comparative behavioral and protein study of salivary secretions in *Homalodisca* spp. sharpshooters (Hemiptera: Cicadellidae: Cicadellinae). Annals of the Entomological Society of America 104: 543-552.

Backus, E. A., W. J. Holmes, F. Schreiber, B. J. Reardon, and G. P. Walker. 2009. Sharpshooter X wave: Correlation of an electrical penetration graph waveform with xylem penetration supports a hypothesized mechanism for *Xylella fastidiosa* inoculation. Annals of the Entomological Society of America 102: 847-867.

Backus, E. A., K. Andrews, H. J. Shugart, J. M. Labavitch, C. L. Greve, and H. Alhaddad. *ms. submitted*. Saliva containing β-1,4-glucanase is injected into xylem by the glassy-winged sharpshooter, vector of the xylem-limited bacterium *Xylella fastidiosa*. Journal of Insect Physiology.

Chatelet, D. S., C. M. Wistrom, A. H. Purcell, T. L. Rost, and M. A. Matthews. 2011. Xylem structure of four grape varieties and 12 alternative hosts to the xylem-limited bacterium *Xylella fastidiosa*. Annals of Botany 108: 73-85.

Crews, L. J., M. E. McCully, M. J. Canny, C. X. Huang, and L. E. C. Ling. 1998. Xylem feeding by spittlebug nymphs: Some observations by optical and cryo-scanning electron microscopy. American Journal of Botany 85: 449-460.

Dugravot, S., E. A. Backus, B. J. Reardon, and T. A. Miller. 2008. Correlations of cibarial muscle activities of *Homalodisca* spp. sharpshooters (Hemiptera: Cicadellidae) with EPG ingestion waveform and excretion. Journal of Insect Physiology 54: 1467-1478.

McElrone, A. J., J. L. Sherald, and I. N. Forseth. 2003. Interactive effects of water stress and xylem-limited bacterial infection on the water relations of a host vine. Journal of Experimental Botany 54: 419-430.

McElrone, A. J., S. Jackson, and P. Habdas. 2008. Hydraulic disruption and passive migration by a bacterial pathogen in oak tree xylem. Journal of Experimental Botany 59: 2649-2657.

Pérez-Donoso, A. G., L. C. Greve, J. H. Walton, K. A. Shackel, and J. M. Labavitch. 2007. *Xylella fastidiosa* infection and ethylene exposure result in xylem and water movement disruption in grapevine shoots. Plant Physiology 143: 1024-1036.

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