EVALUATION OF TRANSMISSION OF *XYLELLA FASTIDIOSA* BY *HOMALODISCA VITRIPENNIS* VIA AN ARTIFICIAL DIET SYSTEM

By

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Abstract

Glassy-winged sharpshooters (GWSS), *Homalodisca vitripennis,* are an invasive species brought to California from the Southeastern United States. They are pests that serve as vectors for the bacterium *Xylella fastidiosa* - a pathogen of various plants of great economic importance for California, including grape and citrus. In grapes, *X. fastidiosa* causes Pierce's disease (PD), which famously eradicated over 40% of the Temecula grapevines in the 1980's and continues to cause millions of dollars in losses to viticulture. *In vitro* experiments are essential for evaluating strategies to control GWSS, such as CRISPR. The goal of this experiment is to determine the optimal parameters for *X. fastidiosa* acquisition by GWSS via an artificial diet system and (Periwinkle) inoculation. Using the *in vitro* feeding system that was developed and validated by our lab. The ability of *X. fastidiosa* to colonize the GWSS precibarium and foregut was assessed after 2 and 4 hours of the acquisition-access period. DNA was extracted from GWSS heads and the polymerase-chain reaction (PCR) using specific primers was used to detect the presence of *X. fastidiosa*. The presence of bacteria on GWSS mouthparts was also assessed using fluorescence microscopy. Transgenic Green Fluorescent Protein (GFP) *X. fastidiosa* were injected into the stems of periwinkle plants. The location of GFP fluorescence signals in stem cross-sections at 2, 4 and 6 weeks after GFP-labeled *X. fastidiosa* inoculation was determined. The resulting data will lead to future acquisition/transmission experiments that will assess the ability of a gene-edited GWSS to acquire and transmit *X. fastidiosa.*

Keywords: acquisition rates; artificial diet system; *in planta* inoculation; transmission rates; Pierce's Disease

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how to properly use a pipette to performing DNA extractions and qPCR. I can attribute most if not all of my lab skills to her patience and attention to detail. She forces me to think about why we use a specific technique or what is actually going on when we perform it. She won't just give me the answers, but she will lend a hand in guiding me to the papers that may help. She would often stay much longer than the workday required to ensure I had the time to run my experiments. Aside the professional development, Ina has without a doubt become family (much like the rest of my lab). She has seen me on the brink of tears when something goes wrong after a long day at the lab but always managed to reassure me that science doesn't always work the way we expect it to. She was the first person to make me feel like I can be a researcher. Without her, I genuinely do not know if I would have gained such a passion and understanding for the ups and downs that come with research. Lastly, I would like to thank all of the GWSS that lived and fed for science.

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Introduction

Homaladisca vitripennis, known as the Glassy-winged sharpshooter (GWSS), is an invasive species brought to Southern California about 30 years ago (Stenger et al. 2010). These pests are known to feed on the xylem of over 75 species of plants (Blua et al. 2000). GWSS serve as vectors for the bacterium *Xylella fastidiosa,* causing diseases in host plants such as Pierce's disease (PD) in grapes, Oleander leaf scorch, and almond leaf scorch (Hopkins 1989). Temecula Valley generated about $100 million in revenue for Southern California's wine industry in the decades of 1980 and 1990. However, 40% of their vineyards were destroyed by PD in 1999 (Hopkins 1989). Research into *X. fastidiosa* dramatically increased after 2002 as PD, citrus variegated chlorosis and other serious *X. fastidiosa*-caused diseases affecting crops of economic importance emerged worldwide (Almeida et al. 2005; Lindow 2019). Despite investments from both federal and California agencies to study and develop control strategies for *X. fastidiosa*-associated diseases, approximately $110 million per year is spent in California alone on the prevention, management and control of PD (Lindow 2019; Alston and Serfas 2025).

X. fastidiosa is a xylem colonizer that causes restricted water flow in plants leading to death (Pérez-Donoso et al. 2007; Killiny and Almeida 2009b). This bacterium attaches to and multiplies within the cibarium, precibarium, and foregut of its insect vectors (Hill and Purcell 1995; Almeida and Purcell 2006). As *X. fastidiosa* replicates, a biofilm composed by exopolysaccharides and proteins is formed. Biofilm is essential for the attachment, replication and persistence of *X. fastidiosa* in the vector's mouth parts; however, it is not necessary for bacteria inoculation (Killiny and Almeida 2009b). Finally, bacterial cells are detached from the foregut and transmitted to a host plant during the insect feeding (Almeida et al. 2005; Killiny and Almeida 2009a).

GWSS nymphs and adults can acquire *X. fastidiosa.* However, at the time of molting, nymphs shed their cuticles along with the *X. fastidiosa* that are attached to the cuticle (Almeida and Purcell 2003; Purcell and Finlay 1979). Therefore, for a nymph to reacquire *X. fastidiosa* and transmit PD, it must once again feed on *X. fastidiosa*-infected plants. In contrast, once *X. fastidiosa* acquisition occurs for a GWSS adult, the bacterium will persist in the foregut for the entirety of the vector's lifetime (Almeida and Purcell 2003).

One of the main ways to control *X. fastidiosa*-induced diseases is to control its insect vectors. Currently, insecticides are the main form of control of GWSS; however, insecticides are expensive, not environmentally or ecologically friendly, and insecticide-resistant vector populations are emerging (Brown and Redak 2023). Therefore, new strategies of control are needed (Hashimi et al. 2020; Arora et al. 2018).

Genetic-control strategies have the potential for sustainable and environmentally-friendly management of insect pests. Using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system and CRISPR-associated protein (CRISPR/Cas9) technologies, it is possible to edit an organism's genome, epigenome, or RNA (Mei et al. 2016). CRISPR/Cas technology now offers opportunities to develop new vector-control strategies that are less ecologically destructive and can be designed to interfere with bacterial pathogen transmission. CRISPR-induced gene drive has been used to reduce fertility in malaria-carrying mosquitoes and antipathogenic mosquitoes in Asia. This allows for a reduction in malaria without the adverse effects of pesticides or significant ecological disruption (Ju et al. 2018; Gantz and Akbari 2018).

The GWSS genome has successfully been edited (Pacheco et al. 2022). With this technology developed for GWSS, we are developing novel CRISPR technologies to block the GWSS's ability to transmit *X. fastidiosa.* The first step towards testing the transmission of *X.*

fastidiosa by GWSS lines is to establish the parameters for wild-type GWSS to efficiently acquire and transmit *X. fastidiosa in vitro*.

Although a variety of acquisition/transmission experiments have been conducted with different strains of *X. fastidiosa* and insect vectors, there is no consistency in these rates. One paper notes *X. fastidiosa* transmission rates of *Clastoptera achatina* (spittlebug), *Homalodisca insolita*, and GWSS to *Carya illinoinensis* (pecan) as 11.4, 19.3, and 4%, respectively (Sanderlin and Melanson 2010). Other papers recorded transmission rates of *X .fastidiosa.* subsp. *pauca* grown on XFM media to *C. roseus* (periwinkle) as 14.7% from *Macugonalia leucomelas* and 19.8% from *Bucephalogonia xanthophis* (Esteves et al. 2019). These rates were dependent on the media used for culturing the bacteria. Transmission rates dropped to 3.9% and 5.7% for the sharpshooters *M. leucomelas* and *B. xanthophis,* respectively, when *X .fastidiosa* was grown on XFM+GA media (Esteves et al. 2019). Similarly, acquisition rates were variable and dependent on the media used to grow *X. fastidiosa,* the sharpshooter species, and the system used for *X. fastidiosa* acquisition. For example, Almeida and Purcell (2003) showed that the GWSS had a 20% acquisition rate from infected Vitis vinifera (grapes). Esteves et al. (2019) showed acquisition of *X. fastidiosa* from diets using an artificial feeding system was influenced by the media used to grow the bacteria XFM vs XFM+GA) and the sharpshooter species (*B. xanthophis vs M. leucomelas)* ranging from 44% to 61%.

Both transmission and acquisition rates can be affected by the bacterial population in the infected plant, high vector loads, inoculation access period, body size, as well as vector and host plant used (Hill and Purcell 1997; Almeida and Purcell 2003; Daugherty and Almeida 2009; Bhowmick et al. 2016; Rashed et al. 2011). Due to the large variability of acquisition/transmission rates reported in the literature, we cannot rely on the parameters set by another laboratory. For this

reason, the main goal of my project is to establish the parameters of an *in vitro* feeding system for *X. fastidiosa* acquisition from infected food sources and delivery to non-infected plants by GWSS.

Methods and Materials

Insect and Plant Colony Maintenance

Homalodisca vitripennis (GWSS) colonies were descendants of GWSS originally collected from Bakersfield, California. Colonies were maintained greenhouses in thrips-proof cages with a diverse plant diet composed of basil (*Ocimum basilicum*), sorghum (*Sorghum bicolor*), okra (*Abelmoschus esculentus*), cowpea (*Vigna unguiculata L. Walp*), and sunflower (*Helianthus annuus*) as previously described (Pacheco et al. 2022).

Catharanthus roseus (periwinkle) was grown in UCR soil mix with coarse perlite (1:1 ratio) supplemented with slow-release fertilizer. Plants were grown with a 14-h:10-h light-dark cycle. Humidity was maintained at 35% and temperatures were 76°F (night) and 86°F (day). Plants were fertilized every two weeks starting two weeks after transplantation. Plants were watered when topsoil began to dry.

Bacterial growth

Xylella fastidiosa subsp. fastidiosa (Temecula 1) was obtained from Dr. Caroline Roper (UC Riverside) and maintained on Periwinkle Wilt-Gelrite (PWG) media (Hill and Purcell 1995). For the periwinkle stem inoculation experiments, GFP-labeled *X. fastidiosa* and *X. fastidiosa-GFP* were grown on PWG media for 15 days before use. OD_{600} of the GFP-labeled *X. fastidiosa* cultures used for the periwinkle inoculation was 0.48.

For *X. fastidiosa* acquisition using the artificial stem system and a two-step media growth process as described by Killiny and Almeida (2009b). *X. fastidiosa* was grown using two different media First, *X. fastidiosa* was grown on PWG for 15 days at 28°C; at this time, the bacteria were

transferred to *Xylella fastidiosa* medium (XFM with galacturonic acid) for 12 days (Esteves et al. 2019). This media promotes the optimal biofilm formation and a higher production of afimbrial adhesins (Hxfs) that are important to *X. fastidiosa* adhesion, ultimately enabling *X. fastidiosa* to colonize the mouthparts of its vectors (Killiny and Almeida 2009a). The bacteria from 6 replicate plates were scraped from the solid media and resuspended in 7 mL of sterile artificial diet (L-glutamine, 0.7 mM; L-asparagine, 0.1 mM; sodium citrate, 1 mM; pH 6.4). The optical density at 600 nm (OD_{600}) of the resuspended *X. fastidiosa* culture was measured using a Nanodrop (Thermo Scientific). Previous acquisition/transmission studies used *X. fastidiosa* cultures with OD_{600} of 0.6 was equivalent to 10^8 CFU/ml (Esteves et al. 2019). Despite using similar methods for *X. fastidiosa* growth, the *X. fastidiosa* suspension for the experiments reported here had an OD_{600} of 0.085, seven-fold lower than the optimal inoculation parameters found in the literature.

***X. fastidiosa* acquisition by GWSS: The artificial-stem feeding system**

The artificial stem feeding system used was developed by our lab. My colleague Tim Roose developed the artificial stem and I worked out the optimal conditions for use of this system. Traditionally, feeding sachets are used as artificial feeding systems (Alhaddad et al. 2011). Sachets are constructed by stretching parafilm over the opening of a 50-mL falcon tube, placing insect diet on top of the parafilm, and sealing the diet with another piece of stretched parafilm. We sought to develop a method that more accurately simulates *in vivo* GWSS feeding than the sachet method. When designing a new feeding system, it is important to note that, as GWSSs feed on the stems of host plants, they usually position their heads towards the soil (Leopold et al. 2003). When feeding, the GWSS probe their stylets into the food source and consume xylem fluids from the plant (Ammar and Hall 2012). Considering this, an artificial stem was developed with parafilm thick enough for GWSS to perceive parafilm penetration and feed in their preferred orientation.

An artificial stem was made with a square of parafilm (1 inch x 1 inch) that was cut using scissors, which were sanitized with 70% ethanol. This square was then rolled into a cylinder, and the long edges of the cylinder were sealed using the edge of a credit card. The parafilm cylinder was filled with 400 µL of insect diet with or without *X. fastidiosa* and sealed. This artificial stem and a five- to six-week-old GWSS adult were placed into a plastic tube 3 3/4 inches height and 1 inch diameter. To maintain a humid environment, which was critical for GWSS feeding, the tube was sealed with a moist sponge. Tubes (also called cages) were placed randomly on a plastic grid

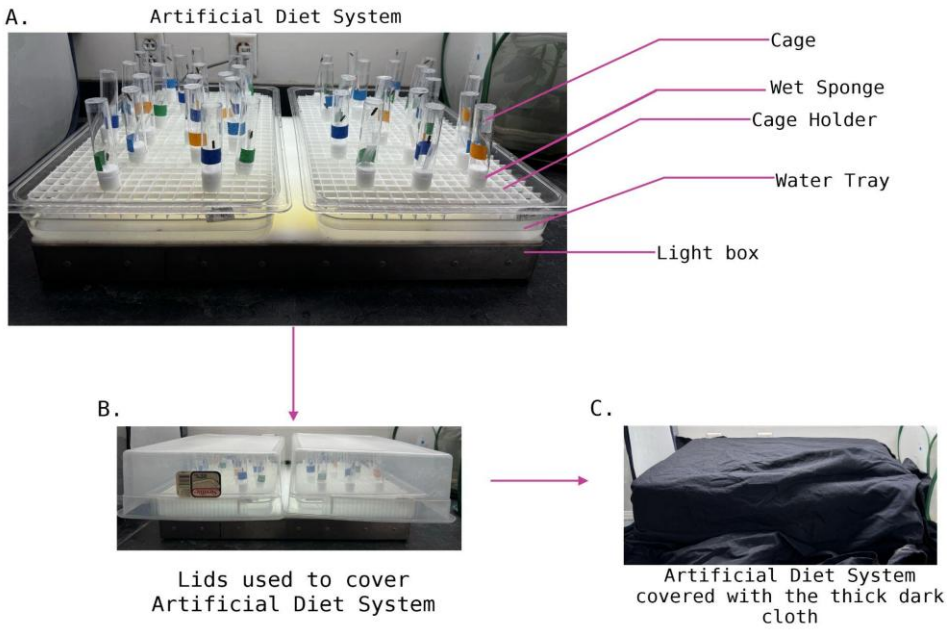

Figure 1: Artificial stem feeding system. A. Tubes (cages) with an artificial stem containing diet with or without *X. fastidiosa* and a single GWSS adult were sealed with a wet sponge. Tubes were randomly arranged on a grid (cage holder) over a tray of water to keep the tubes 6 inches from the light source. **B.** Plastic boxes were used to cover the feeding tubes and tray. **C.** A thick black cloth was draped over for light consistency.

in a tray filled with water (Figure 1).

Feeding experiments were performed at room temperature (29.5 ℃) with continuous light. A light box was placed below the tray of water to provide supplemental light. The tubes, water

reservoir and light box were covered with a plastic box and a thick black cloth to prevent extraneous light or temperature fluctuations (Figure 1). Insects were allowed to feed for 2 or 4 h on an artificial diet with or without *X. fastidiosa*; ten insects were used for each treatment. After the acquisition period, seven insects from each treatment were moved to *Helianthus annuus* (sunflower) for 24 h. During this feeding time, the *X. fastidiosa* that did not adhere to GWSS surfaces were flushed out. The remaining three GWSS from each trial were frozen at -80°C for future analysis. After the 24-h *in planta* feeding period, the seven insects from each treatment were collected and frozen at -80°C until use. After the GWSS feeding, the sunflower plants were cut at the base of the stem, and the plants and pots were frozen at -80°C. The frozen plant material and soil containing the roots were discarded in biological waste and autoclaved.

Detection of *X. fastidiosa* acquisition by GWSS

After the artificial-stem acquisition assay, all insects were removed from the -80°C freezer and immediately decapitated with a sterile scalpel. Individual heads were placed in DNAse/RNAse-free 1.5-mL microfuge tubes submerged in liquid nitrogen. GWSS heads were ground in liquid nitrogen using a DNAse/RNAse-free pestle. DNA was isolated using a DNeasy Blood and Tissue DNA isolation Kit (QIAGEN) following the manufacturer's instructions. DNA was resuspended into 50 µL of nuclease-free water. The quantity and quality of the extracted DNA was assessed using a Nanodrop (Thermo Scientific) to measure the concentration of DNA, as well as the 260/280 and 260/230 ratios indicating purity. *X. fastidiosa* Plasmids (80 ng/µL) containing *X. fastidiosa 16S rRNA* or *ITS* genes were used as positive controls.

Frozen DNA samples were thawed on ice. All GWSS DNA samples were diluted with water to 80 ng/µL. To determine the frequency of *X. fastidiosa* acquisition by GWSS, the presence of *X. fastidiosa* DNA was detected using the Polymerase-Chain Reaction (PCR) with *X. fastidiosa*

16S rRNA and 16S-23S internal transcribed spacer (ITS) specific primers. The primer sets used are shown in Table 1. Primer efficiency and specificity were also confirmed by members of our lab.

Table 1. *X. fastidiosa* PCR primers				
Gene	**Primer pairs**	**PCR product size**	**Annealing Temperature**	**Reference**
16S-23S ITS	XfF1: 5'-AAAAATCGCCAACATAAACCCA-3' XfR1: 5'-CCAGGCGTCCTCACAAGTTAC-3'	74 bp	66°C	(Schaad et al. 2002)
16S rRNA	Xf-F-fwd: 5'-TCAACGTGCAGAGATCAGAGG-3' Xf-F-rev: 5'-CGCAACTACATCAGTCAAGCG-3'	97 bp	60°C	(Burbank and Ortega 2018)

Each 25-µL reaction was set up using the Q5 high-fidelity DNA polymerase Kit (NEB) following the manufacturer's instructions and 1 µL of 80 ng/µL GWSS DNA using a T100 thermal cycler (BioRad). The template was denatured at 95ºC for 3 min. There were 34 cycles of 95ºC for 20 sec; a 20-sec primer-template annealing period; and a 40-sec extension period at 72ºC. This was followed with a final extension at 72ºC for 3 min s. The gene-specific annealing temperatures are in Table 1. Amplified fragments were fractionated on a 1% agarose gel, and PCR products were visualized under UV light. *X. fastidiosa* plasmid DNA samples were used as a positive control (80 ng/µl) and reaction mix with 1 µl of RNase/DNase water (no template) served as the negative control.

X. fastidiosa symptom induction in periwinkle (_Catharanthus roseus_)

The literature indicates that periwinkle (*Catharanthus roseus*) is an excellent host for *X. fastidiosa* with initial symptoms of deformed plant leaves and size reduction typically being revealed two months post inoculation (Monteiro et al. 2001). Symptoms increase in severity and include marginal and chlorotic zones along the veins, most frequently in the older leaves 4 months

post-inoculation and highly stunted growth six months post-inoculation (Monteiro et al. 2001). To establish periwinkle as a host plant for future *X. fastidiosa* acquisition and transmission studies in our lab, we assessed *X. fastidiosa* symptom development and *X. fastidiosa* presence in plant stems. Transgenic *X. fastidiosa* Temecula 1 expressing the Green Fluorescent Protein (GFP) were obtained from Dr. Caroline Roper (UC Riverside). Bacteria were grown for 40 days on PWG media at 28°C. After this period, the bacteria were scraped from the solid media and resuspended in 3 mL of Phosphate-Buffered Saline (PBS [pH 7.4]; 2 mM K_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, 2 mM KCl) (Killiny and Almeida 2009b). The OD600 of the bacterial suspensions was determined using the Nanodrop. Based on Esteves et al. (2019) and Monteiro et al. (2001) an OD_{600} of 0.6 corresponds to approximately 10^8 CFU/mL In my experiment, the OD_{600} of the GFP-labeled *X. fastidiosa* cultures used for the periwinkle inoculation was 0.48.

Ten five-to-seven-week-old periwinkle plants (7-9 cm tall) were used in this experiment. Five periwinkle plants served as negative controls and were inoculated with PBS. Five plants were inoculated with GFP-labeled *X. fastidiosa.* Bacterial inoculation was performed by puncturing the stem of the plant between the first and second node with a 22-gauge sterile needle. Ten µL of the resuspended GFP-labeled *X. fastidiosa* were infiltrated into the puncture site, which was rapidly taken up by the plant vascular system.

Plant stem cross sections

Plants inoculated with GFP-labeled *X. fastidiosa* were collected at 2, 4 and 6 weeks post inoculation. Plants were photographed and their nodes were counted. A sterile razor blade was used to manually cut thin slices of the stem in between each node. The stem cross-sections were analyzed for GFP fluorescence using a fluorescent microscope (Leica M165 FC). Images were

captured using bright field, as well as images using filters to visualize cyan, mCherry (red), and green fluorescence.

Results and discussion

Establishment of methods for GWSS acquisition of *X. fastidiosa* using an artificial stem

To provide GWSS with an artificial feeding system that more realistically reflects their behaviors on plant stems, an artificial stem parafilm feeding system was developed as described in Materials and Methods and shown in Figure 2. The artificial stem allows the GWSS to feed in an orientation similar to that used when feeding on plant stems. Adult GWSS were placed in a tube with an artificial stem filled with a diet and the tube was sealed (Figure 2). GWSS fed on diet with or without *X. fastidiosa* 2 or 4 h (the acquisition period). Afterward, GWSS were moved to sunflower plant to feed for 24 h (n=7) (clearing period) or immediately frozen for future analysis

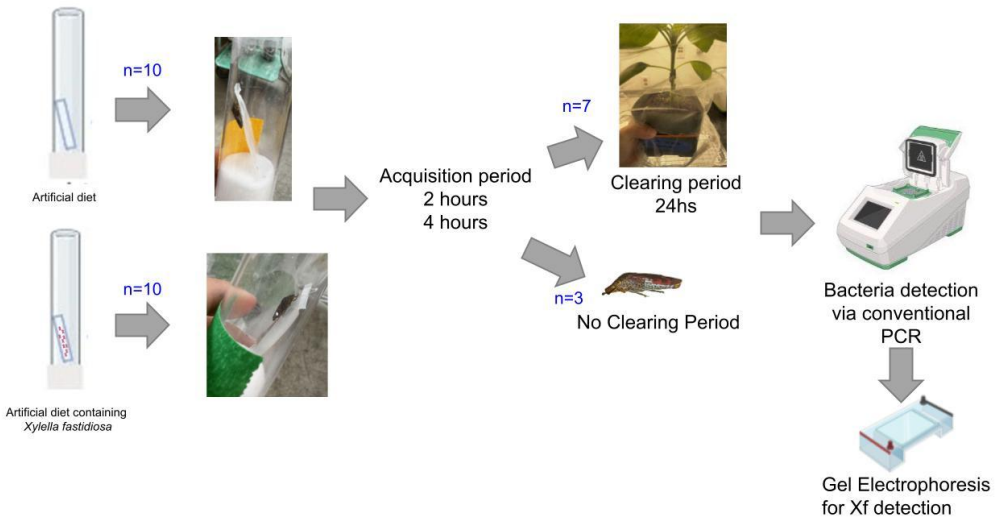

Figure 2. Experimental scheme for *X. fastidiosa* acquisition experiments. GWSS adults (n=10) were allowed to feed on diets with or without *X. fastidiosa* for 2 or 4 h using our *in vitro* stem feeding system. After the acquisition period, three GWSS were frozen to assess *X. fastidiosa* consumption. The remaining insects from each time point (n=7) fed on *Helianthus annuus* (sunflower) for a 24-h clearing period to remove bacteria that were not strongly adhered to the GWSS feeding structures. At 24 h, GWSS were frozen. GWSS heads were excised, genomic DNA isolated and PCR was performed using *X. fastidiosa* ITS region and 16S-23S ribosomal RNA primers to detect *X. fastidiosa* in GWSS.

(n=3). Feeding on sunflower plants removes the GWSS that were poorly adhered to the GWSS precibarium, cibarium and foregut. This allows us to most accurately measure *X. fastidiosa* acquisition.

After the 2-h or 4-h acquisition period, 100% insects were alive, indicating that the artificial stem system was sufficient to maintain GWSS (Table 2). When we removed the black cloth and plastic lids from the system at the end of the 2-h or 4-h acquisition periods, many of the GWSSs were not feeding on the diets but were instead flying throughout the tubes. Only 10% of the insects were feeding on an artificial stem with a diet containing *X. fastidiosa* after 2 h and 30% after a 4 h of feeding (Table 3). At present, it is unclear whether removing the cloth and plastic box disrupted GWSS feeding behaviors or whether another aspect of the feeding system used was not optimal for continuous GWSS feeding.

Table 2: Survival of the GWSS insects subjected to *X. fastidiosa* acquisition experiment

Acquisition Time	Diet Type	# of insects	# survived	% of survival
2 h	No Xf	10	10	100
2 h	Xf	10	10	100
4 h	No Xf	10	10	100
4 h	Xf	10	10	100

Table 3: GWSS insects seen to be feeding on artificial diet after acquisition period

Time Trial	Diet Type	# of insects	# of insects on an artificial stem	% of insects on an artificial stem
2 h	No Xf	10	1	10%
2 h	Xf	10	1	10%
4 h	No Xf	10	3	30%
4 h	Xf	10	0	0%

To determine if *X. fastidiosa* was acquired by GWSS after 2 or 4 h of feeding on the artificial stem feeding system and if *X. fastidiosa* is retained in GWSS feeding organs after 24 h of feeding on sunflower plants, we determined the amount of *X. fastidiosa* present in GWSS after the four treatments. DNA was extracted from GWSS heads, which contain the three GWSS feeding organs (the precibarium, cibarium and foregut). The DNAs recovered represent GWSS genomic and mitochondrial DNAs, as well as DNA from any *X. fastidiosa* that were present or retained in GWSS feeding organs. We detected the presence of *X. fastidiosa* in GWSS using PCR and gene-specific primers for the 16S-23S Internal Transcribed Spacer *ITS* region and the *16S rRNA*. The ITS primers produce a 74-bp product, while the 16S rRNA primers produce a 97-bp product (Burbank and Ortega 2018; Schaad et al. 2002).

A 97-bp PCR product was detected after the PCR using the 16S rRNA primers with the *X. fastidiosa* positive plasmid control, indicating that the 16S rRNA region primers were functional (Figure 3-C). The negative control samples (no DNA template) did not produce PCR products, confirming the absence of contamination in the PCR reactions (Figure 3-A). Additionally, no PCR products were observed in samples from insects that fed on diets without bacteria, indicating no cross-contamination during the acquisition experiment (Figure 3-A-C). A similar result was observed when using the *ITS* primer set, which produced a 74-bp PCR product (Figure 4).

Of the 20 GWSS that fed on diets with *X. fastidiosa*, only one insect (Xf-7) produced a PCR product using both the *ITS* and *16S rRNA* gene primers (Figure 3A, Figure 4A). *Xf-7* was one of the ten insects that fed on diet with *X. fastidiosa* for 2 h and was one of seven that fed on sunflower for 24 h (Figures 3-4).

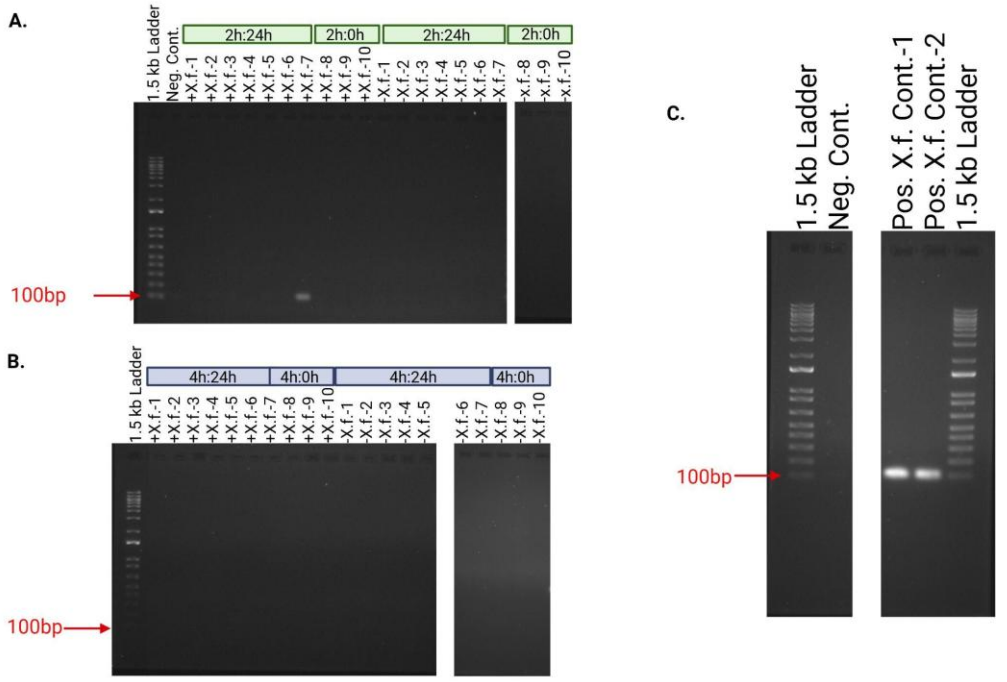

Figure 3: Detection of *X. fastidiosa* 16S rRNA fragment in insects exposed to 2-h and 4-acquisition period. PCR products (97-bp) were size fractionation on agarose gels and stained with ethidium bromide. **A.** PCR products from insects in 2-h acquisition treatment. **B.** PCR products from insects in 4-h acquisition treatment. **C.** PCR products from negative control sample containing no DNA template (Neg. Cont.) and samples containing *X. fastidiosa* 16S rRNA plasmid DNA as a positive control (Pos. X.f. Cont.). **+X.f.:** insects that fed on an artificial diet containing X. fastidiosa; -X.f.: insects that fed on an artificial diet without X. fastidiosa. (2h:0h) and (4h:0h): correspond to insects that were frozen immediately after the acquisition period. **(2h:24h)** and **(2h:24h)**: correspond to insects feeding on sunflower plants for 24 h.

While this frequency of acquisition and retention is lower than anticipated (14%), this value is not far from the 20-64% acquisition observed in the literature (Almeida and Purcell 2003; Esteves et al. 2019). More than that, this insect had fed on sunflower for 24 h to remove weakly adhered *X. fastidiosa* suggesting that *X. fastidiosa* not only entered the GWSS system, but also properly attached itself to the mouthparts to colonize. We attribute the low % acquisition due to the lower amounts of *X. fastidiosa* in the feeding system. Despite the fact that we used 7-fold lower amounts of *X. fastidiosa* in the artificial diet than is recommended by the literature, we were able

to detect *X. fastidiosa* presence of GWSS heads, indicating that the artificial stem has the potential to become a reliable in vitro feeding system in the future.

Only one GWSS was PCR-positive for *X. fastidiosa*. Therefore, the PCR methods used in my study may not have been sensitive enough to detect *X. fastidiosa* in our samples. This is based on the fact that the PCR performed is a qualitative test, rather than a quantitative one (Dagher et al. 2004; Baldi and La Porta 2017). To increase our ability to detect *X. fastidiosa* and thereby increase the % acquisition, the sensitive and quantitative polymerase-chain reaction (qPCR) will

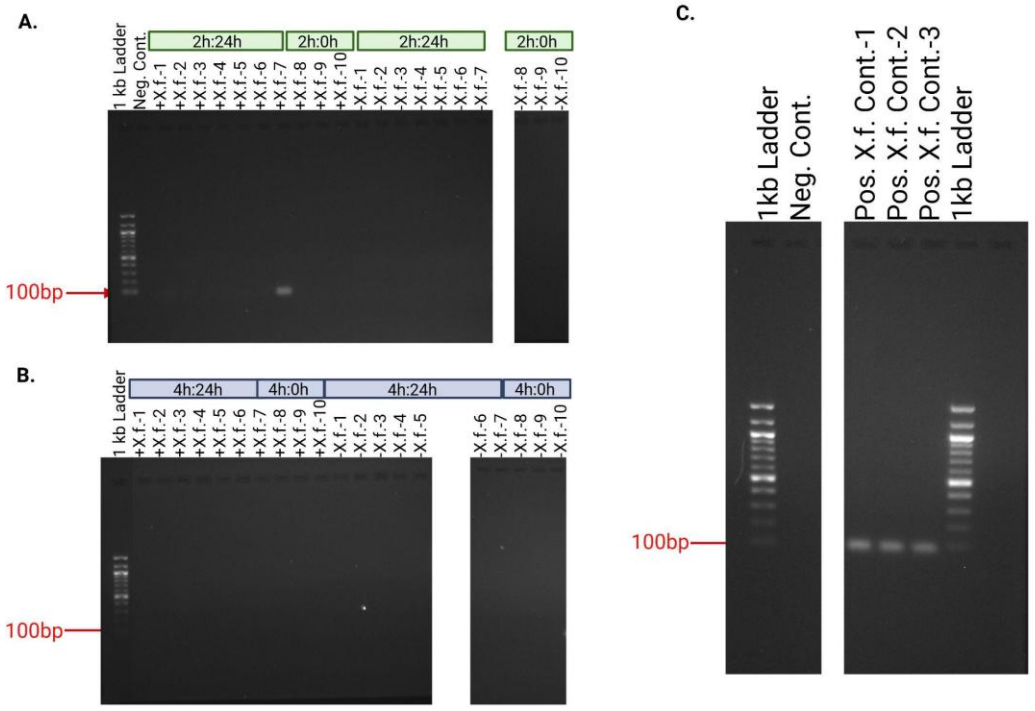

Figure 4: Detection of *X. fastidiosa ITS* fragment in insects exposed to 2-h and 4-acquisition period. PCR products (74-bp) were size fractionated on agarose gels and stained with ethidium bromide. **A.** PCR products from insects in 2-h acquisition treatment. **B.** PCR products from insects in 4-h acquisition treatment. **C.** PCR products from negative control sample containing no DNA template (Neg. Cont.) and samples containing *X. fastidiosa* 16S rRNA plasmid DNA as a positive control (Pos. X.f. Cont.). **+X.f.:** insects that fed on an artificial diet containing *X. fastidiosa;* **-X.f.:** insects that fed on an artificial diet without *X. fastidiosa.* **(2h:0h)** and **(4h:0h)**: correspond to insects that were frozen immediately after the acquisition period. **(2h:24h)** and **(2h:24h)**: correspond to insects feeding on sunflower plants for 24 h

be used in the future to determine if lower levels of *X. fastidiosa* DNA is present in other GWSS that fed on *X. fastidiosa* diets.

Although it was clear (based on 100% survival of GWSS from all treatments), that enough diet was consumed to avoid dehydration and death (Table 2). It is also important to consider the possibility that GWSS did not actually feed on the artificial diet system for the full acquisition periods and therefore did not have sufficient time to acquire *X. fastidiosa* (Table 3).

Due to the high variability in acquisition rates and the factors that contribute to them in the literature, there is much to consider when refining the experiment. First, the acquisition time can be extended to promote longer feeding periods and increase the likelihood of GWSS uptake. Second, GWSS could be starved for a time before initiating feeding on diet to ensure they will be 'hungry' and will want to consume diet from the artificial stems. Third, diets with higher concentrations of *X. fastidiosa* should be tested, as we used concentrations 7-fold lower than those recommended in the literature. This would mean refining *X. fastidiosa* plate growth times or the number of bacterial plates used in our experiments. Fourth, the *X. fastidiosa* concentration in the diet could be increased by resuspending the bacteria from plates in a liquid medium, centrifuging to pellet them and resuspending the cells in a smaller volume of medium. Alternatively, we can grow *X. fastidiosa* on PWG for a longer time before XFM transfer. Finally, as suggested above, we can use qPCR to enhance PCR product detection and perform more PCR cycles. While our positive plasmid controls yielded positive PCR results, there was a large amount of plasmid template in each reaction. Therefore, I should test serially diluted plasmids to determine the sensitivity of my PCR assay. This currently needs to be optimized.

Symptoms observed after *X. fastidiosa* inoculation of periwinkle stems.

Five-to-six-week-old periwinkle plants were stem inoculated with *X. fastidiosa-GFP* (n= 5) or PBS buffer (the negative control) (n=5). Plants were allowed to grow for 6 weeks. While most of *X. fastidiosa*-induced symptoms develop after 8 weeks of infection, plants were inspected every two weeks. Plants were examined based on non-quantitative visual assessments, symptoms such as deformed plant leaves and size reduction, chlorotic zones, and stunted growth were looked for (Monteiro et al. 2001).

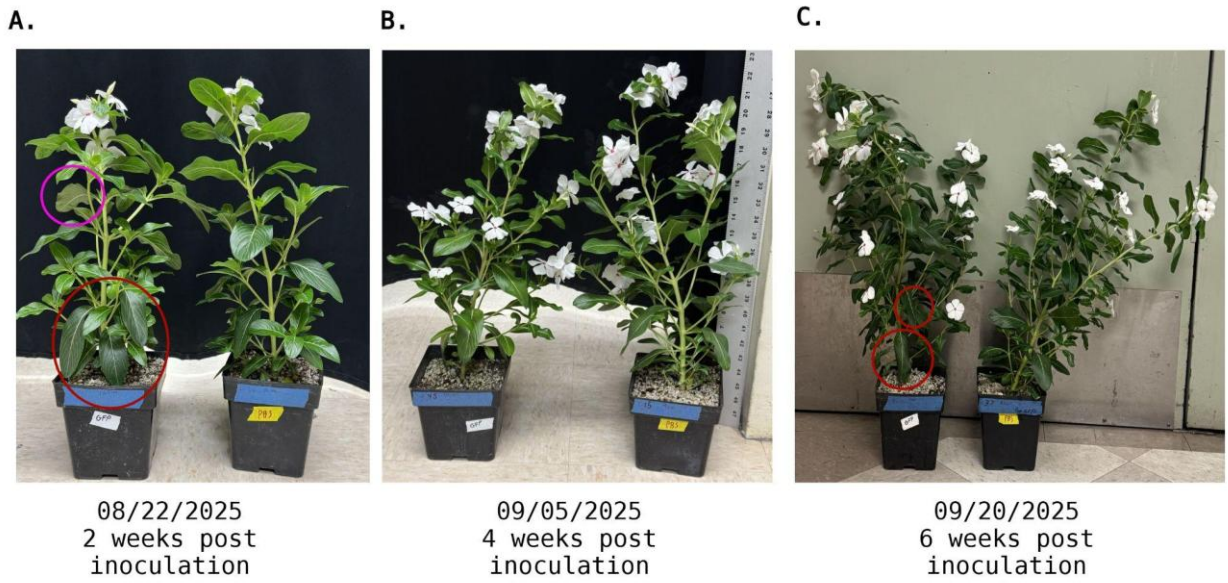

Figure 5: Periwinkle growth and symptoms 2, 4, and 6 weeks after inoculation with *X. fastidiosa-GFP*. **A.** Visual assessment 2 weeks after inoculation. Possible signs of wilting are indicated by the red circle and possible discoloration of leaves are indicated by the purple circle. **B.** Visual assessment 4 weeks after inoculation. **C.** Visual assessment 6 weeks after inoculation. Possible signs of wilting are indicated by the red circle.

While plants were of similar stature at two weeks post inoculation, the *X. fastidiosa* -inoculated plants had wilted lower leaves and a slight browning of leaves in the middle of the plant compared to its PBS counterpart (Figure 5A.). I do not think these signs of stress were caused by

X. fastidiosa-GFP because symptoms usually do not appear until after 8 weeks (Monteiro et al. 2001); the symptoms are likely due to water-deficit stress. In addition, these stress signs were absent in plants examined at 4 weeks (Figure 5B). While not rigorously quantitified, the plants at 2 and 4 weeks after inoculation appeared to have similar numbers of leaves and flowers, had dark green leaf pigmentation, and similar amounts of branching (Figure 5A-B). Six-week-old plants had a similar height with dark green leaves and abundant flowers (Figure 5C). The plants showed signs of wilting from top to bottom of both the *X. fastidiosa-GFP*- and PBS-inoculated periwinkle (Figure 5C).

Although wilting is indicative of a diseased plant, the fact that PBS-inoculated plants displayed the same symptoms suggests the wilting was not due to the bacterial infection, but rather growing conditions (Monteiro et al. 2001). One explanation is that the pots were relatively small compared to the size of the periwinkle plant. Another consideration was that as the plants grew taller and bushier, it became apparent that their interactions might be affecting one another's growth. While dead flowers were removed from plants every other day, it is possible that an abundance of dead, wet flowers clinging onto the leaves of neighboring plants influenced plant health. Towards the end of the collection period, an undesired amount of moisture and fungus was noted on many of the periwinkle plants. This was likely due to overcrowding in our limited growth space.

To detect the presence of *X. fastidiosa* in periwinkle plants at 2, 4, and 6 weeks post- *X. fastidiosa-GFP* inoculation, stem cross sections from *X. fastidiosa-GFP*- and PBS-inoculated plants were made manually (Figures 6-7). Three cross-sections were taken in between every node of each plant, resulting in 21, 27, and 30 cross-sections of the X. fastidiosa-GFP-inoculated periwinkle and 24, 27, and 33 for the PBS control plants for collection weeks 2, 4, and 6,

respectively. Fluorescent microscopy imaging of the periwinkle cross sections revealed no detectable GFP fluorescence in any of the control (PBS-inoculated) plants. *X. fastidiosa-GFP* green fluorescence was not detected in any of the 78 *X. fastidiosa-GFP*-inoculated periwinkle. Most surprisingly, *X. fastidiosa-GFP* was not detected at the site of inoculation.

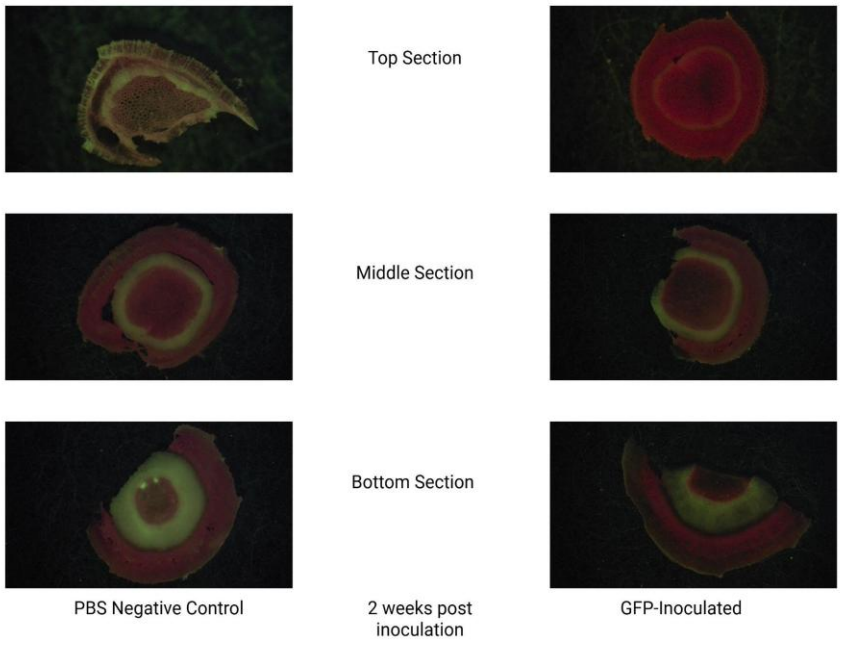

Figure 6: Periwinkle stem cross sections at 2 weeks *X. fastidiosa-GFP* inoculation. Manual cross sections were made at all nodes of *X. fastidiosa* and PBS inoculated periwinkle plants. Representative sections taken from the bottom (closest to inoculation site), middle, and top sections of plants are shown.

These data suggest that the inoculation method was insufficient to deliver *X. fastidiosa-GFP* into the xylem of periwinkle plants. It is possible that during inoculation, the 22-gauge needle was pushed beyond the xylem layer and into the pith, depositing GFP-labeled *X. fastidiosa* into a location that did not facilitate transfer to the xylem. During the secondary growth stage of a plant, the xylem becomes incompressible, and the stem continually increases in circumference, forcing the tissue inside and outside the cambium to adapt to the larger circumference and ultimately

leading to a woody-like structure (Schweingruber 2008). Periwinkle stems were challenging to inoculate and to cross-section as they aged and their stems became more woody. This made preparing high-quality cross sections close to the initial inoculation point almost impossible, as early as 2 weeks post-inoculation.

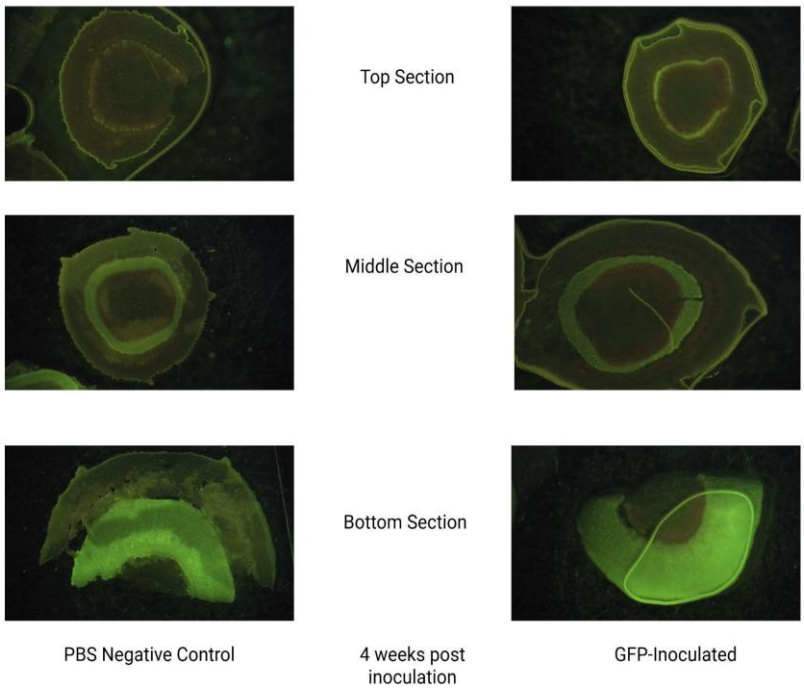

Figure 7: Periwinkle stem cross sections at 4 weeks *X. fastidiosa-GFP* inoculation. Manual cross sections were made at all nodes of *X. fastidiosa*- and PBS-inoculated periwinkle plants. Representative sections taken from the bottom (closest to inoculation site), middle, and top sections of plants are shown.

Due to the required time to cross section periwinkle stems (6 h for 2 stems), I became concerned that the time to section may have caused problems in *X. fastidiosa-GFP* viability or created stress in the isolated stem sections. For a small number of tissue sections, there were minute GFP-positive signals (Figure 6). Unfortunately, none were localized to xylem, which is the cell type that *X. fastidiosa* colonizes. Therefore, it is prudent to conclude that the green fluorescent

specks were likely due to the plant's natural defense response. There were no signs of GFP fluorescence or any identifiable constricted vascularized tissue in the cross-sections retrieved (Figures 6 and 7).

When my results from the *in vitro* feeding studies and the stem inoculation studies are viewed collectively, the low *in vitro* acquisition rates (14%) by GWSS and inoculation rates (0%) by periwinkle could be due to many factors. Acquisition rates could have been affected by acquisition time, concentration of the *X. fastidiosa* inoculum, absence of GWSS, or issues with PCR sensitivity (as described above). For the stem-inoculation studies, rates could have been affected by needle puncturing the xylem layer and entering the pith, or by failed colonization by *X. fastidiosa*-GFP, which grows less robustly than non-transgenic *X. fastidiosa* (Newman et al. 2003). To mitigate these issues in the future, GWSS can be starved for five hours before being placed on the artificial diet, higher inocula of *X. fastidiosa* can be used for both experiments, and qPCR can be used to detect *X. fastidiosa* in both experiments. For the stem-inoculation experiment, the delivery of *X. fastidiosa* into the xylem at the inoculation site should be validated by microscopy and qPCR. This should be done immediately and 24 h after inoculation. Finally, while the growth of *X. fastidiosa-GFP* is known to be less robust than that of *X. fastidiosa* on bacterial media (Newman et al. 2003), it is not clear whether there is a difference in their success in periwinkle plants; this is also a good experiment to pursue in the future.

Reference

Alhaddad H, Coudron TA, Backus EA, Schreiber F (2011) Comparative behavioral and protein study of salivary secretions in Homalodisca spp. sharpshooters (Hemiptera: Cicadellidae: Cicadellinae). Annals of the Entomological Society of America 104 (3):543–552. doi:10.1603/an10137

Almeida RPP, Blua MJ, Lopes JRS, Purcell AH (2005) Vector transmission of *Xylella fastidiosa*: Applying fundamental knowledge to generate disease management strategies. Annals of the Entomological Society of America 98 (6):775–786. doi:10.1603/0013-8746(2005)098[0775:Vtoxfa]2.0.Co;2

Almeida RPP, Purcell AH (2003) Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera : Cicadellidae). Journal of Economic Entomology 96 (2):264–271. doi:10.1603/0022-0493-96.2.264

Almeida RPP, Purcell AH (2006) Patterns of Xylolella fastidiosa colonization on the precibarium of sharpshooter vectors relative to transmission to plants. Ann Entomol Soc Am 99 (5):884–890

Alston JM, Serfas D (2025) The costs of Pierce's disease in the California grape and wine industry: Summary report. https://www.cdfa.ca.gov/pdcp/Documents/2025/Cost_of_PD_in_CA_Grape_and_Wine_Industry.pdf.

Ammar E-D, Hall DG (2012) New and simple methods for studying hemipteran stylets, bacteriomes, and salivary sheaths in host plants. Annals of the Entomological Society of America 105 (5):731–739. doi:10.1603/an12056

Arora AK, Pesko KN, Quintero-Hernandez V, Possani LD, Miller TA, Durvasula RV (2018) A paratransgenic strategy to block transmission of *Xylella fastidiosa* from the glassy-winged sharpshooter *Homalodisca vitripennis*. BMC Biotechnology 18. doi:10.1186/s12896-018-0460-z

Baldi P, La Porta N (2017) *Xylella fastidiosa:* Host range and advance in molecular identification techniques. Frontiers in plant science Volume 8 - 2017. doi:10.3389/fpls.2017.00944

Bhowmick TS, Das M, Heinz KM, Krauter PC, Gonzalez CF (2016) Transmission of phage by glassy-winged sharpshooters, a vector of Xylella fastidiosa. Bacteriophage 6 (3):e1218411. doi:10.1080/21597081.2016.1218411

Blua MJ, Phillips PA, Redak RA (2000) A new sharpshooter threatens both crops and ornamentals. Plant Health Progress 1 (1):4. doi:10.1094/php-2000-0627-01-rs

Brown DJ, Redak RA (2023) Fitness costs associated with insecticide resistance in populations of Homalodisca vitripennis Germar (Hemiptera: Cicadellidae). Journal of Economic Entomology 116 (2, Sp. Iss. SI):560–564. doi:10.1093/jee/toad009

Burbank LP, Ortega BC (2018) Novel amplification targets for rapid detection and differentiation of *Xylella fastidiosa* subspecies fastidiosa and multiplex in plant and insect tissues. Journal of Microbiological Methods 155:8–18. doi:https://doi.org/10.1016/j.mimet.2018.11.002

Dagher H, Donninger H, Hutchinson P, Ghildyal R, Bardin P (2004) Rhinovirus detection: comparison of real-time and conventional PCR. Journal of Virological Methods 117 (2):113–121. doi:https://doi.org/10.1016/j.jviromet.2004.01.003

Daugherty MP, Almeida RPP (2009) Estimating *Xylella fastidiosa* transmission parameters: decoupling sharpshooter number and feeding period. Entomologia Experimentalis Et Applicata 132 (1):84–92. doi:10.1111/j.1570-7458.2009.00868.x

Esteves MB, Kleina HT, Sales TD, Oliveira TP, de Lara IAR, Almeida RPP, Coletta HD, Lopes JRS (2019) Transmission efficiency of *Xylella fastidiosa* subsp. *pauca* sequence types by sharpshooter vectors after in vitro acquisition. Phytopathology 109 (2):286–293. doi:10.1094/phyto-07-18-0254-fi

Gantz VM, Akbari OS (2018) Gene editing technologies and applications for insects. Curr Opin Insect Sci 28:66–72. doi:10.1016/j.cois.2018.05.006

Hashimi MH, Hashimi R, Ryan Q (2020) Toxic effects of pesticides on humans, plants, animals, pollinators and beneficial organisms. Asian Plant Research Journal 5 (4):37–47. doi:10.9734/aprj/2020/v5i430114

Hill BL, Purcell AH (1995) Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathology 85:1368–1372

Hill BL, Purcell AH (1997) Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. Phytopathology 87 (12):1197–1201. doi:10.1094/phyto.1997.87.12.1197

Hopkins DL (1989) *Xylella fastidiosa:* Xylem-limited bacterial pathogen of plants. Annual Review of Phytopathology 27 (Volume 27, 1989):271–290. doi:https://doi.org/10.1146/annurev.py.27.090189.001415

Ju X-D, Xu J, Sun ZS (2018) CRISPR editing in biological and biomedical investigation. Journal of Cellular Biochemistry 119 (1):52–61. doi:https://doi.org/10.1002/jcb.26154

Killiny N, Almeida RPP (2009a) Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. Proceedings of the National Academy of Sciences of the United States of America 106 (52):22416–22420. doi:10.1073/pnas.0908562106

Killiny N, Almeida RPP (2009b) *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. Applied and environmental microbiology 75 (2):521–528. doi:10.1128/AEM.01921-08

Leopold RA, Freeman TP, Buckner JS, Nelson DR (2003) Mouthpart morphology and stylet penetration of host plants by the glassy-winged sharpshooter, *Homalodisca coagulata*, (Homoptera: Cicadellidae). Arthropod Structure & Development 32 (2):189–199. doi:https://doi.org/10.1016/S1467-8039(03)00047-1

Lindow S (2019) Money matters: Fueling rapid recent insight into *Xylella fastidiosa*—An important and expanding global pathogen. Phytopathology® 109 (2):210–212. doi:10.1094/phyto-09-18-0325-per

Mei Y, Wang Y, Chen H, Sun ZS, Ju X-D (2016) Recent progress in CRISPR/Cas9 technology. Journal of Genetics and Genomics 43 (2):63–75. doi:https://doi.org/10.1016/j.jgg.2016.01.001

Monteiro PB, Renaudin J, Jagoueix-Eveillard S, Ayres AJ, Garnier M, Bové JM (2001) *Catharanthus roseus*, an experimental host plant for the citrus strain of *Xylella fastidiosa*. Plant Disease 85 (3):246–251. doi:10.1094/pdis.2001.85.3.246

Newman KL, Almeida RPP, Purcell AH, Lindow SE (2003) Use of a green fluorescent strain for analysis of Xylella fastidiosa colonization of Vitis vinifera. Applied and environmental microbiology 69 (12):7319–7327. doi:10.1128/aem.69.12.7319-7327.2003

Pacheco IdS, Doss A-LA, Vindiola BG, Brown DJ, Ettinger CL, Stajich JE, Redak RA, Walling LL, Atkinson PW (2022) Efficient CRISPR/Cas9-mediated genome modification of the glassy-winged sharpshooter Homalodisca vitripennis (Germar). Scientific reports 12 (1):6428. doi:10.1038/s41598-022-09990-4

Pérez-Donoso AG, Greve LC, Walton JH, Shackel KA, Labavitch JM (2007) *Xylella fastidiosa* infection and ethylene exposure result in xylem and water movement disruption in grapevine shoots. Plant Physiology 143 (2):1024–1036. doi:10.1104/pp.106.087023

Purcell AH, Finlay A (1979) Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. . Phytopathology 69:393–395

Rashed A, Daugherty MP, Almeida RPP (2011) Grapevine genotype susceptibility *to Xylella fastidiosa* does not predict vector transmission success. Environmental Entomology 40 (5):1192–1199. doi:10.1603/en11108

Sanderlin RS, Melanson RA (2010) Insect transmission of *Xylella fastidiosa* to pecan. Plant Disease 94 (4):465–470. doi:10.1094/pdis-94-4-0465

Schaad NW, Opgenorth D, Gaush P (2002) Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. Phytopathology 92 (7):721–728. doi:10.1094/phyto.2002.92.7.721

Schweingruber FH (2008) Secondary growth: Advantages and risks. In: Schweingruber FH, Borner A, Schulze ED (eds) Atlas of Woody Plant Stems Evolution, Structure, and Environmental Modifications. Springer, pp 39–67

Stenger DC, Sisterson MS, French R (2010) Population genetics of *Homalodisca vitripennis* reovirus validates timing and limited introduction to California of its invasive insect host,

the glassy-winged sharpshooter. Virology 407 (1):53–59.

doi:https://doi.org/10.1016/j.virol.2010.08.002