EFFECTS OF FEEDING SUBSTRATE ON RETENTION AND TRANSMISSION OF *XYLELLA FASTIDIOSA* STRAINS BY THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
This is a continuation of our three year project designed to study the effect of feeding substrate on the acquisition and retention of *Xylella fastidiosa* (*Xf*) by the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*. We are using two strains of *Xf* that are present in California: a Pierce's disease (PD) strain that infects grape, and an oleander leaf scorch (OLS) strain that infects oleander. Last year we reported that GWSS that acquired the PD strain from grape or the OLS strain from oleander and subsequently confined to chrysanthemum (a non-*Xf* host), retained the bacterium at least seven days after exclusive feeding on the non-host. Thus, *Xf* in the GWSS foregut does not need continual access to host plant xylem. Secondly, we reported that GWSS transmitted PD and OLS strains when they acquired the bacteria from a plant, but they did not transmit either strain when media-grown bacteria were delivered through the cut-stem system. This result forces us to use whole plants as our bacterial source in transmission experiments.

This past year, we focused on objectives 3-5. Studies showed that GWSS given access to PD, followed by OLS, retained both PD and OLS and transmitted PD at a higher rate than OLS. When GWSS were given access to OLS followed by PD, they retained PD at a much higher rate than OLS and transmission to plants was poor. Results suggest that PD may have become established in the foregut better than OLS or that it out-competed the OLS strain. Further analyses showed that sharpshooters which tested positive for only the OLS strain had a higher percentage inoculation rate of the PD strain. Possible explanations for these results are provided. For objective 4, we found that antibiotics effectively killed *Xf* in the GWSS foregut by treating either before or after bacterial acquisition. In our last objective, there was no difference in survival of PD at pH ranging from 4.5 to 9.8.

INTRODUCTION
The GWSS is capable of acquiring and transmitting several strains of *Xf* from a variety of host plants. In this project we are testing the effects of feeding substrate on the acquisition, retention and transmission of *Xf* by GWSS. Two strains of the pathogen present in California are being used in these experiments: a PD strain that infects grapevine, and an OLS strain that infects oleander. These two strains have different host ranges; the PD strain does not infect oleander, and the OLS strain does not infect grape. It is known that both strains are capable of multiplying in GWSS mouthparts, because the insect is capable of retaining and transmitting both strains of the pathogens (Purcell and Hopkins, 1996, Purcell et al. 1999, Costa et al. 2000). It is assumed that *Xf* in the insect mouthparts are surviving on nutrients obtained from the host plant xylem, and would be exposed to other chemical components present in the xylem fluid. Thus, we might expect that the retention and replication of a particular strain of the pathogen in an individual insect would be dependant on the xylem content of the plant host on which it is feeding. For example, it would be expected that the oleander strain would grow better in insects feeding on oleander, the grape strain would grow better in insects feeding on grape, and alternate feeding on both hosts might increase the incidence of retention of both strains in an individual.

The exact mechanism of *Xf* successful attachment and replication in insect mouthparts is unknown. However, a variety of components have been identified as contributing to the initial adhesion and subsequent growth of *Xf* in plants and in media culture. The goal of the last two objectives of this proposal is to alter the feeding substrate with a resultant change in the environment in the insect mouthparts, and examine the subsequent effects on attachment, retention, and transmission of *Xf*. For example, we have used pre-treatments antimicrobials to determine if other microbes present in biofilms of the GWSS mouthparts play a significant role in the successful attachment and transmission of *Xf*. Post-acquisition treatments can identify the types of materials that can successfully kill or stop the growth of the organisms once they are present in insect mouthparts. In addition, if ionic bonding is involved in the initial attachment of *Xf* in insect mouthparts, we can modify substrate pH or vary the amount of free radicals available in substrates, and examine the effect on acquisition rate. The results of these studies will provide information on susceptibility of *Xf* to environmental disruption in insect mouthparts.

OBJECTIVES
1. Compare retention times of *Xf* when infected GWSS are subsequently fed on plants that are either hosts or non-hosts of the strain they carry.
2. Compare acquisition and transmission efficiency of insects fed on infected plants to those fed on media-grown cultures delivered through cut stems.

3. Compare retention times of two strains of *Xf* in GWSS when they are acquired through sequential exposure to infected oleander and grape plants on alternating hosts of each strain.
4. Test the effects of antibacterial materials on acquisition and transmission of *Xf* by GWSS.
5. Test the effects of variation in substrate pH and free ion availability on the acquisition and transmission of *Xf* by GWSS.

RESULTS

Objective 3

In our studies we used clean GWSS from our greenhouse culture. Two treatment groups were established; 1 group was given a 48 hour acquisition access period (AAP) to grape plants infected with PD, followed by a 48 hr. AAP to oleander infected with OLS. The other group was exposed to OLS followed by exposure to PD. After the AAP, insects were individually transferred to grape and oleander test plants, where they were allowed to feed for 24 hrs. The insects then were moved to the alternate host plant for the successive 24 hr period. This serial transmission was repeated every 24 hrs, until the insect died. Once the insects were dead, the heads were processed, DNA was extracted, and PCR was performed with strain-specific primers to verify the bacterial strain infecting them. The test plants were grown in a greenhouse and once per month for a period of three months, tissue was collected and submitted to ELISA testing.

Our studies show that GWSS given access to PD, followed by OLS, retained PD in 9 of 20 cases, OLS in 6 of 20 cases and had both strains in 3 of 20 cases (Table 1). In 2 insects we did not amplify any DNA. These insects transmitted OLS to 8 of 50 oleander test plants (16%) and they transmitted PD to 23 of 57 grape test plants (40.4%). When GWSS were given access to OLS followed by PD, they retained PD in 13 of 20 cases and OLS in 2 of 20 cases. Five of the 20 GWSS retained both strains. Interestingly, transmission to plants was poor, with only 4 OLS positives out of 45 oleander test plants (8.9%) and 3 PD infections out of 44 grape test plants (6.8%). The fact that transmission of PD was substantially greater than OLS transmission, even when insects were given access to OLS after PD, suggests that the PD strain may have become established in the foregut better than the OLS strain or that it out-competed the OLS strain. The converse did not occur when the first access was to the OLS strain, and transmission in this situation was nearly the same between PD and OLS (Table 1).

Table 1. Results from retention and transmission studies where GWSS were given access to PD followed by OLS and to OLS followed by PD.

Order of acquisition (n)	# with PD	# with OLS	# with PD and OLS	PD Inoculation Rate # infected / # tested (%)	OLS Inoculation Rate # infected / # tested (%)
PD – OLS (18)	9	6	3	23 / 57 (40.4%)	8 / 50 (16.0%)
OLS – PD (20)	13	2	5	3 / 44 (6.8%)	4 / 45 (8.9%)

Further analyses were conducted after we categorized the data by *Xf* strain retained by the insects. When PD acquisition was followed by OLS, the GWSS that tested positive for only the PD strain transmitted both strains with 19.4% transmission of PD and 4.8% transmission of OLS (Table 2). The insects that tested positive for only OLS transmitted both strains as well; 28.6% and 14.3% for PD and OLS, respectively. It is interesting that the sharpshooters that tested positive for only the OLS strain had a higher percentage infection of the PD strain. A possible explanation for these results is that during the serial transmissions, there was bacteria of both strains in the insects, thus they inoculated both strains in the test plants. As time progressed, insect lost one of the strains and when they died (the time they were collected for PCR assay) there was only a single strain left in the foregut. It also is possible that interactions between the strains in the foregut played a role in which strain was transmitted and which strain was retained in the foregut until the end of the insect's life. In the OLS followed by PD treatment, only PD was found in 13 of the 20 insects and of these 3 inoculated PD and 1 inoculated OLS. For the GWSS which contained only OLS, there was a single infection of OLS in the serial transmissions.

Table 2. Results from retention and transmission studies where GWSS were given access to PD followed by OLS and to OLS followed by PD, categorized by the strain that was identified in the insect after it died.

Order of acquisition (n)	Strain in Insect (n)	PD Inoculation Rate # infected / # tested (%)	OLS Inoculation Rate # infected/# tested (%)
PD – OLS (18)	PD (9)	12 / 62 (19.4%)	3 / 62 (4.8%)
	OLS (6)	8 / 28 (28.6%)	4 / 28 (14.3%)
	Mixed (3)	2 / 8 (25.0%)	0 / 8 (0%)
	Unknown (2)	1 / 11 (9.1%)	1 / 11 (9.1%)
OLS – PD (20)	PD (13)	3 / 60 (5.0%)	1 / 60 (1.7%)
	OLS (2)	0 / 10 (0%)	1 / 10 (1.0%)
	Mixed (5)	0 / 19 (0%)	2 / 19 (10.5%)

We also learned that both strains were retained by GWSS ("mixed" in Table 2). However, transmission to plants by these multiply-infected GWSS was very low, just 4 of 27 infections (1.5%). In all cases only one strain was transmitted, further suggesting that there is an interaction between strains in the GWSS foregut. The low transmission rate raises questions about the interactions between the two strains when they are in the same insect and the subsequent consequence on transmission of the strains. We will continue addressing these questions in future studies.

Objective 4

Initial studies were conducted to test the effects of an antibiotic treatment on the ability of GWSS to acquire and retain *Xf*. Two experiments were done. In the first, insects were treated with an antibiotic before being given a 24 hr. AAP on grape infected with PD. In the second experiment, insects were treated with the antibiotic after being given a 24 hr. AAP. Our positive control insects were fed on grape plants infected with PD for 24 hrs. Surviving insects were moved to a *Chrysanthemum* stem that was infused with a buffer (0.005 M Phosphate buffer, pH 7.0) in a cut stem delivery system. The negative control consisted of clean adult insects that were fed for 24 hours on a *Chrysanthemum* stem infused with buffer only using the cut stem delivery system. Surviving insects were subsequent fed on clean grape plants for 24 hours. For our post-acquisition treatment, insects were fed on grape plants infected with PD strain of *Xf* for 24 hr. Surviving insects were moved to a *Chrysanthemum* stem that was infused with a 0.01 % solution of oxytetracycline in a weak phosphate buffer (0.005 M phosphate buffer, pH 7.0) using the cut stem delivery system. And the pre-acquisition treatment used insects that were fed for 24 hours on a *Chrysanthemum* stem infused with a 0.01 % solution of oxytetracycline in a weak phosphate buffer (0.005 M phosphate buffer, pH 7.0). Surviving insects were subsequently feed on grape plants infected with PD strain of *Xf* for 24 hours. All insects were subsequently moved to *Chrysanthemum* plants to feed for 48 hours to allow the antibiotic plenty of time to be washed out from the gut. Heads of all insects were cultured on PD3 media, and a sub-sample from the heads was used for PCR to detect the presence of *Xf*. The studies showed that both pre- and post-acquisition treatments effectively reduced survival of *Xf* in the GWSS (Table 3).

Table 3. Effects of antibiotics on the acquisition and transmission of *Xf* by GWSS.

Treatments	Insects				
	Total	Mortality	Survival	PCR	Culture
Positive control grape- buffer	30	8	22	0	4
Positive control buffer-grape	30	15	15	0	1
Negative control grape-buffer	30	3	27	0	0
Negative control buffer-grape	30	6	24	0	0
Post-acquisition, grape-antimicrobial	30	17	13	0	0
Pre-acquisition, antimicrobial-grape	30	6	24	0	0

Objective 5

Insects were fed on infected plants of *Xf* suspended in a series of substrates with pH ranging from 4.8 to 9.8. This range includes a value that is considered optimal for the growth of *Xf* (6.5-6.9, Wells et al. 1987). The conditions of the treatments and control are described in Figure 1.

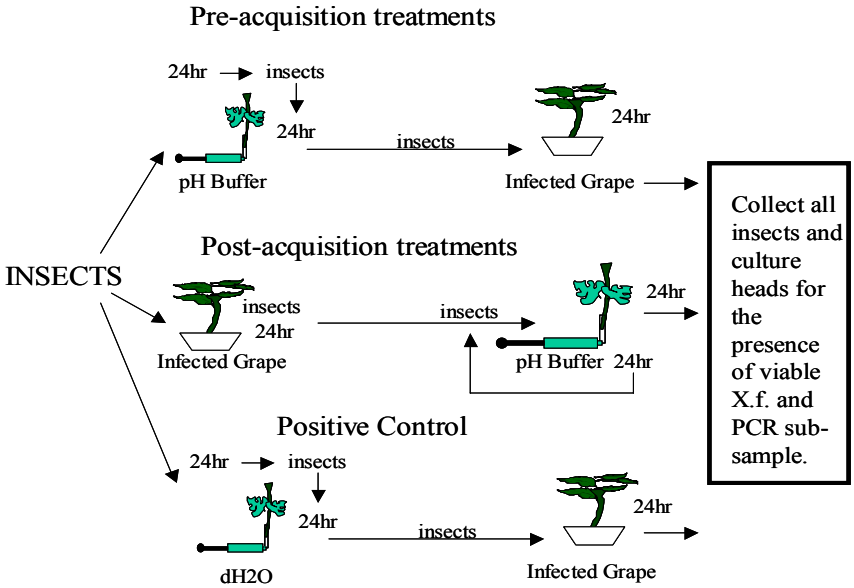

Figure 1. Effects of pH treatments on acquisition and transmission.

In this study, we found that *Xf* survived at all pH ranges tested. It doesn't appear that high pH impacts the survival of bacteria in the foregut of GWSS (Table 4). We are planning transmission experiments to see if these treatments impact acquisition and inoculation of bacteria.

Table 4. Effects of pH on the acquisition and transmission of *Xf* by GWSS.

Treatments		Insects				
		Total	Mortality	Survival	PCR	Culture
Negative control	clean plant	10	0	10	0	0
Positive control	inf. Plant	10	0	10	3	10
pH buffer/infected grape	pH 4.48	5	2	3	0	3
	pH 5.60	5	0	5	0	5
	pH 8.00	5	0	5	3	4
	pH 9.80	5	1	4	0	4
Infected grape/pH buffer	pH 4.48	5	0	5	2	1
	pH 5.6	5	0	5	2	4
	pH 8.00	5	0	5	1	2
	pH 9.80	5	1	4	4	1

CONCLUSIONS

We showed that pH did not influence survival of *Xf*. Antibiotic treatments applied either pre- or post-acquisition effectively kill the bacteria in the foregut of GWSS. Studies on acquisition, transmission, and retention of PD and OLS showed that both strains can be simultaneously acquired and retained in GWSS. Interestingly, the strain that was found at the end of the insect's life did not always coincide with the strain that it transmitted to test plants. We are continuing our investigation into the possible reasons for this result, which may shed light on the interaction of these two strains in GWSS vectors. Our hope is to learn more about bacterial interaction with the insect foregut, and to use this knowledge to reduce transmission of PD and OLS by GWSS.

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