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# Transmission of phage by glassy-winged sharpshooters, a vector of *Xylella fastidiosa*

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**ABSTRACT**

*Xylella fastidiosa* subsp. *fastidiosa* (*Xff*) is the causal agent of Pierce's Disease (PD) of grapevines and is vectored by the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*). Previously we have reported the development of a bacteriophage (phage) based biocontrol system for PD, but no information on insect transmission of phages has been reported. Here we communicate that laboratory reared GWSSs fed on cowpea plants (*Vigna unguiculata* subsp. *unguiculata*) harboring the virulent phage Paz were able to uptake of phage efficiently when the phage was present in high concentration, but were inefficient in transfer to plants.

## Introduction

*Xylella fastidiosa* (*Xf*) are xylem limited, gram-negative, gamma-proteobacteria that cause several agriculturally significant plant diseases, such as Pierce's Disease (PD) of grape, coffee leaf scorch, almond leaf scorch and citrus variegated chlorosis.[1] *Xf* species are transmitted between plants by sap-feeding vectors, including sharpshooter leafhoppers (Hemiptera: Cicadellidae), spittlebugs (Hemiptera: Cercopidae) and cicadas (Hemiptera: Cicadidae).[2] *Xf* has a distinctive relationship with its xylem-feeding leafhopper vector, the glassy-winged sharpshooter (GWSS, *Homalodisca vitripennis*), in that it is foregut borne, reproduces in the insect vector without circulating in the hemolymph and forms a biofilm in the foregut of GWSS.[2] If the sharpshooter acquires *Xf* as an adult, it remains infective for the remainder of its life.[3] Upon introduction by an insect vector, the bacterium multiplies in a plant's xylem and eventually blocks the movement of water, which results in cell death in grapevines.[4] GWSSs are endemic to the grape growing regions of Southern California and Texas,[5] and due to their polyphagous feeding on a variety of plants and its ability to fly great distances [6] the insect is a major vector in spread of PD.[7]

All cultivars of European grapevines, *Vitis vinifera*, are susceptible to *X. fastidiosa* subsp. *fastidiosa* (*Xff*)

infection.[3] The most widely used control strategies for PD depend on the use of systemic insecticides such as neonicotinoids that are absorbed by plants and transferred throughout the grapevine vascular system for vector control.[4,5,8] Other strategies include certification programs, locating vineyards away from riparian areas or the rouging of infected grapevines.[9] However, recent concerns of the role of neonicotinoids in honey bee colony collapse disorder[8,10] has resulted in a 2 year ban of 3 neonicotinoid insecticides (clothianidin, imidacloprid and thiamethoxam) by the European Commission because of the acute and chronic effects on bee colony survival and development.[8,9,10] In 2013, the US Environmental Protection Agency issued a notification to registrants on registered pesticides containing neonicotinoid advising of strengthen pollinator protective labeling on neonicotinoid products.[8,11]

Our laboratory has reported on the development of a phage based therapy system that may be used in conjunction with other control measures or as an alternative treatment for the control PD.[8] Previous studies have focused on the role of the GWSS in the transfer of *Xf*, however there are no studies, to our knowledge, that address the transfer of phages by GWSS. Considering the potential practical application and the relationship between the GWSS and *Xf*, it was of interest to

determine interaction between the phage and the vector. To our knowledge this is the first report that addresses the uptake and transfer of phage by GWSSs.

## Results

### Uptake of phage by bean plants and acquisition by GWSSs

Standard quantitative real time PCR (qRT-PCR) line plots obtained for phage Paz had an $R^2$ value greater than 0.9 and efficiency of 123%. Stems immersed in phage suspensions showed an average level of phage of $(3 \pm 0.3) \times 10^8$ plaque forming units (PFU)/gram of plant tissue (gpt) and $(2 \pm 0.3) \times 10^8$ PFU/gpt after 4h and 48h, respectively, in 2 independent experiments. GWSSs allowed to feed on stems immersed in a phage Paz solution showed efficient acquisition of phage, exhibiting $(2 \pm 0.4) \times 10^6$ PFU/GWSS $(1 \pm 0.4 \times 10^8$ PFU/gm of GWSS tissue [gGWt]) phage after feeding for 48h, in 2 independent experiments. No phages were detected in GWSSs or bean plants immersed in sterile distilled water (SDW) in both experiments.

### Phage transmission by GWSSs

Once the ability of GWSSs to acquire phage from stems was established, it was of interest to determine if the phage harboring GWSSs could transfer phage to plants. GWSSs were allowed to feed on stems harboring an average of $(2 \pm 0.3) \times 10^8$ PFU/gpt. GWSSs assayed after feeding for 48h harbored an average of $(2 \pm 0.4) \times 10^6$ PFU/GWSS $(1 \pm 0.4 \times 10^8$ PFU/gGWt). Cowpea stems, on which the phage harboring GWSSs were allowed to feed for a 48h period, showed an average of $(3 \pm 0.3) \times 10^2$ PFU/gpt, and assayed GWSSs contained $(3 \pm 0.3) \times 10^3$ PFU/GWSS $(1 \pm 0.3 \times 10^5$ PFU/gGWt), respectively. No phages were detected in GWSSs or stems from control set of SDW.

## Discussion

Sharpshooter leafhoppers (Hemiptera: Cicadellidae) and spittlebugs (Hemiptera: Cercopidae), both xylem fluid-feeding leafhoppers are the primary vectors for dispersal of Xf.[12-14] The recent establishment of the GWSS (Homalodisca vitripennis) in California now threatens the major portion of wine and table grape production in the US.[15] Previous vector studies with GWSSs have focused on the acquisition and transmission of Xf.[14,16] In these studies, the GWSSs were

allowed to feed on infected tissue and then allowed to feed on non-infected tissue to qualitatively determine acquisition and the transfer efficiency. Almeida [16] found that adult GWSSs acquired and transferred Xf within less than one hour of access time on grapevine tissue, but observed that acquisition efficiency did not increase beyond 6h access and that estimated inoculation efficiency per GWSS decreased over a 4 day period. Bextine [17] developed an artificial system using parafilm to study leafhopper-borne pathogen acquisition and transmission of Xff. Using this model, he found that a lower transmission rate to chrysanthemum stems was associated with longer times of inoculation access period. We have previously reported [18] that weed and plant extracts yielded 10-10^5 PFU/gpt of Xylella phages without detection of Xf by either direct plating or by qRT-PCR, and noted the Xanthomonas isolates that could also act as hosts were isolated from the extracts. Our experience with the isolation of phages from petioles obtained from field samples indicates that phages are present at very low levels in vines, since we were not able to detect phages by direct plating, but were able to enrich samples for Xf phages. It has been documented that Xf is transferred by the polyphagous feeding activity of the GWSS,[14] and we speculate that phages present in weeds and other host plant reservoirs are also transferred by the same activity. We have presented quantitative data for the uptake of phage Paz by immersed plant stems and the efficient acquisition of the phage by GWSSs from the phage harboring stems. We have expressed the uptake in terms of PFU/gpt and on average plant uptake was $(3 \pm 0.3) \times 10^8$ PFU/gpt and $(2 \pm 0.3) \times 10^8$ PFU/gpt after 4h and 48h, respectively. When GWSSs were allowed to feed on the plant tissue, acquisition was quantitated at $(2 \pm 0.4) \times 10^6$ PFU/GWSS $(1 \pm 0.4 \times 10^8$ PFU/gGWt) indicating high efficiency of acquisition. However, when the phage harboring GWSSs were transferred to bean stems immersed in SDW and allowed to feed over a 48h period the phage PFU decreased 1000-fold. The observed reduction in the PFU is most likely the result of feeding activity, since GWSSs ingest 100 to 300 times their dry body weight in xylem fluid per day that is excreted.[19]

During this same period, transfer was quantified to bean stems at an average of $(3 \pm 0.3) \times 10^2$ PFU/gpt indicating that while the uptake of phage is highly efficient when the phage is present in high concentration,

there is an apparent dilution effect due to feeding activity resulting in low transfer of phage by GWSSs. The results correlate with our experience of not being able to isolate phages from direct plating of extracts from field trapped GWSSs, but being able to enrich for *Xf* phages from the same samples using a permissive host. This indicates that phages are present in field GWSSs at very low levels ($<\sim$4 PFU/gGWt), and are therefore not efficiently transferred by GWSSs.

As phages are implemented as biocontrol agents we must understand their potential for transfer by vectors. In greenhouses studies, we have reported that phages persist in vines for up to 12 weeks in the absence of a host and increase in the presence of a host.[8] We have also reported that *Xff* phages have low adsorption constants for their host of $(4.33 \pm 0.28) \times 10^{-12}$ ml cell$^{-1}$ min$^{-1}$, which is the lowest reported for a phage with its plaque-permissive host.[18,20] The adsorption rate is affected by both phage and host concentrations because the rate of phage particle disappearance is defined as $dP/dt = -kBP$, where $B$ is the concentration of bacteria, $P$ is the concentration of free phage at any time ($t$), and $k$ is the adsorption rate constant in ml cell$^{-1}$ min$^{-1}$.[21] As we have observed in our greenhouse studies, *Xff* and phage can exist in the vascular tissue of a vine at low levels. The implementation of phage therapy shifts the equilibrium through the introduction of high numbers of phage, which reduces the susceptible pathogen population below a critical level, and disease is not observed.

Phage-based biocontrol system using lytic phages of *Xff* is an alternative strategy to methods currently being used for control of PD. Phages are clearly ubiquitous in nature[22] and our studies show that *Xff* phages population vary from habitat to habitat. More research is needed to address the phage-vector-pathogen interaction and to understand the role that lytic phages may play in the ecology of *Xf*.

## Materials and methods

### Bacterial strains, phages and inoculum preparation

*X. fastidiosa* subsp. *fastidiosa* strain Temecula was used as propagating host for phage Paz.[18] The high-titer lysate of phage Paz [$10^{12}$ PFU/ml] was prepared, titered and stored as described by Ahern et al.[18] Phage suspensions used in GWSS experiments were diluted in SDW for uptake studies.

### GWSS

Insects used in experiments were laboratory-reared, young adults (<3 week old), which were initially obtained from the rearing facility at the California Department of Food Agriculture (CDFA) Field Station, Arvin, CA. The laboratory colony was originally collected mainly from citrus orchards in Ventura County, CA. At the CDFA rearing facility, the laboratory colonies of *H. vitripennis* were reared from egg stage on multiple host plants under greenhouse conditions at $31 \pm 4°C$, under high-intensity sodium lighting with a photoperiod of 16:8 (L:D) h. The CDFA *H. vitripennis* colonies were maintained on several host plants including cowpea *Vigna unguiculata* [L.] Walp), sunflower (*Helianthus annuus* L.), Japanese euonymus (*Euonymus japonica* Thunb.), and sorghum (*Sorghum bicolor* [L.] Moench). The *H. vitripennis* laboratory colony was replenished by introducing eggs laid by field-collected females as needed. Adult insects were overnight express shipped from Arvin, CA to College Station, TX. The GWSSs used in this study were young adults with an approximate sex ratio of 55% males.

In College Station, Cowpea (*Vigna unguiculata* subsp. *unguiculata*) plants were used as host for GWSSs. Cowpea seeds were planted using 101 Sunshine Mix 1 (Sun Gro Horticulture, Vancouver, British Columbia, Canada). Cowpea were grown in a growth chamber on a 16h light (26°C, 300–400$\mu$Em-2 s-1) / 8h dark (18°C) cycle supplemented with illumination from sodium vapor lamps. Cowpea were watered every other day with tap water and fertilized with Peter's General Purpose 20-20-20 fertilizer and micronutrients every 15 days.

The GWSSs were housed in the Biological Control Facility, Department of Entomology at Texas A&M University. After receiving, insects were fed on cowpea plants, maintained at 24°C to 29°C (16 and 8h of light and dark, respectively), for 2 days to allow for adaption to chamber conditions. Prior to use, 3 GWSSs from each lot were processed to insure the absence of *Xff* and phage using qRT-PCR, as described below.

### Experimental unit design

Each experimental unit (i.e., cage, Fig. 1A) consisted of a 3 liter polyethylene terephthalate container with 2 ports (2 cm in diameter; seal with corks), to allow for introduction and removal of GWSSs, and two 6 × 6 cm windows covered by fine mess to allow for air exchange. A sterile 50 ml centrifuge tube was hot
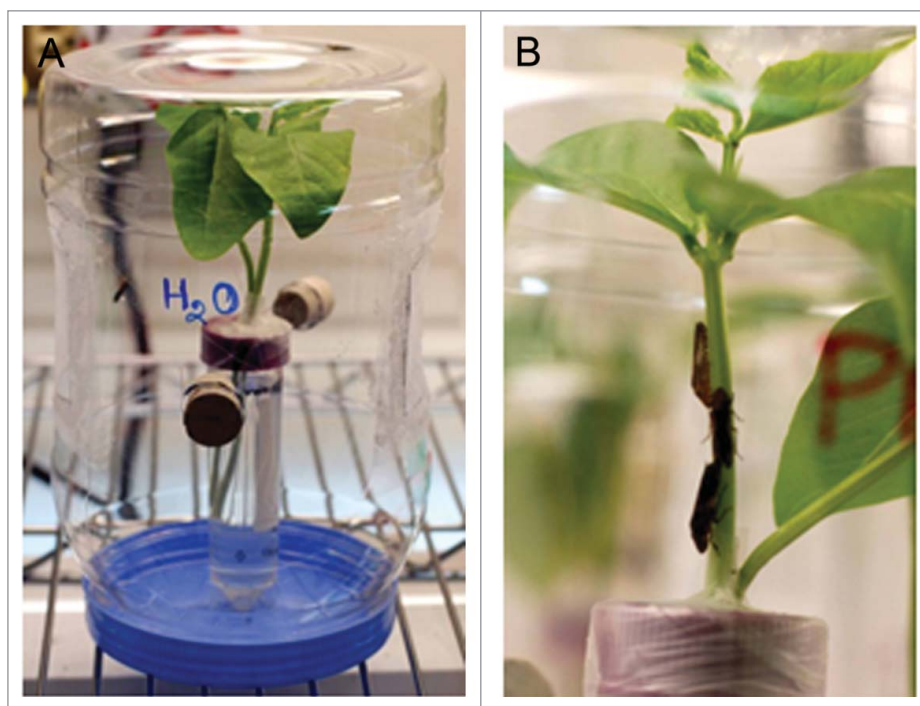
**Figure 1.** Experimental units and feeding of GWSSs. (A) Each experimental unit (i.e., cage) contained two 15 cm-long stems of bean plants at 3-4 leaf stage and a 50 ml flat bottom tube with a 50 ml suspension (phage or SDW, as appropriate). (B) GWSSs (3 GWSSs/plant/cage) were placed in cages and allowed to feed, as appropriate.

glued to the bottom of each container lid and filled with 50 ml of the appropriate suspension, i.e. phage suspension or SDW. Two 15 cm-long plant stems without roots at the 3-4 leaf stage were placed through a hole in the tube cap, with the lid screwed on the container and then sealed with parafilm.

### Plant uptake of phage and acquisition by GWSSs

To determine uptake of phage by plants, stems (2 stems/cage) were immersed in tubes containing a 50 ml suspension of phage Paz ($2 \times 10^{10}$ PFU/ml) for 4h in 6 separate cages. Plants immersed in SDW served as control in 6 separate cages. After 4h, stems that were either phage or SDW immersed (3 each) were collected by removing the stem at the top of the tube with a sterile razor blade to avoid contamination from immersed stem surface. The stems and leaves were weighed and then macerated in 15 ml of P-buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 8 mM MgSO$_4$) using a sterile mortar and pestle, filtered through sterile cheesecloth to remove the plant debris, centrifuged (10,000 × g at 4°C for 15 min) and filter sterilized (0.22μm). DNA was extracted as described by Das et al. (2015) [8] to determine the PFU/gpt. Quantification of PFU/gpt was determined by qRT-PCR using specific primers for phage Paz (See below).

To determine acquisition of phage from stems, 3 GWSSs per cage were introduced into the 3 remaining individual cages (triplicate) containing 2 stems/cage immersed in phage suspension or SDW, and allowed to feed. After 48h, GWSSs and stems from each treatment replicate were removed from the cages and assayed individually for phage by qRT-PCR (See below).

### Transmission of phage Paz by GWSSs

To conduct transmission studies it was first necessary to determine uptake of phage Paz by GWSSs. Eighteen stems (2 stems/cage; 9 cages) were placed in 50 ml tubes filled with phage Paz ($2 \times 10^{10}$ PFU/ml) suspension. Control stems (2 stems/cage; 9 cages) were immersed in SDW. After 4h, to determine the phage levels in stems, 3 sets of stems from each experimental set (6 each) that had been allowed to uptake phage Paz or SDW were assayed by qRT-PCR (See below). GWSSs were then introduced into the remaining 6 cages (3 GWSSs/cage; Fig. 1B) of each experimental set and allowed to feed on stems immersed in the phage suspension or SDW. At 48h, GWSSs from 3 cages (9 each) of each experimental set that had fed on the phage harboring stems or stems immersed in

SDW (6 each) were assayed for phage by qRT-PCR (See below). GWSSs from the remaining 3 cages (9 GWSSs) of the phage experimental set were transferred to cages (3 GWSSs/cage) that contain stems (2/cage) immersed in SDW, and allowed to feed. Control GWSSs (9 each; 3 cages) that had fed on stems immersed in SDW were also allowed to continue to feed. After 48h, the stems (6 each) and GWSSs (9 each) from the phage or SDW set were assayed for phage by qRT-PCR (See below).

### Phage levels in GWSSs

To determine the levels of phage Paz, GWSSs were sacrificed by exposure to −20°C for 5 min. GWSS from each cage (3 GWSSs/cage) were weighed (average 39mg/GWSS) and homogenized separately. Stems and GWSSs were processed in 1 ml and 0.5 ml of P-buffer, respectively, and filtered through sterile cheesecloth (Fisher Scientific, USA) to remove tissue debris. The filtrate was centrifuged (10,000 × $g$ at 4°C for 15 min). The supernatant was filter sterilized and used for extraction of phage DNA using the Wizard DNA Clean-up system (Promega, Wisconsin, USA) with modifications as described by Summer et al.[20] and PFU/gpt determined by qRT-PCR.

### qRT-PCR

The stem and GWSS extracts were assayed for phage by qRT-PCR. The SYBR-green based qRT-PCR protocols were conducted as described by Das et al.[8] using the phage specific primers for phage Paz as reported by Ahern et al.[18] qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, California, USA). Standard curves were constructed by plotting cycle threshold ($Ct$) values generated from qRT-PCR against phage DNA concentrations (Log DNA conc. /$\mu$l as determined by $A_{260}$). The efficiency (E) was calculated as follows: $E = 10^{-1/slope} - 1$.

### Abbreviations

| | |
|---|---|
| CDFA | California Department of Food Agriculture |
| gGWt | gm of GWSS tissue |
| gpt | gm plant tissue |
| GWSS | Glassy-winged Sharpshooter |
| PD | Pierce's Disease |
| PFU | plaque forming units |
| qRT-PCR | quantitative real time PCR |
| SDW | sterile distilled water |
| Xf | *Xylella fastidiosa* |
| Xff | *Xylella fastidiosa* subsp. *fastidiosa* |

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