DEVELOPING A METHOD TO DETECT *XYLELLA FASTIDIOSA* IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
Dependable detection of *Xylella fastidiosa* (*Xf*) in glassy-winged sharpshooters (GWSS) is imperative for understanding *Xf* epidemiology and optimizing grapevine protection strategies. In this study, we have developed methods for extracting *Xf* DNA from GWSS vectors and optimized a SYBR green I based real-time PCR detection protocol that is fast, consistent, and inexpensive. The Qiagen DNeasy Tissue kit (Qiagen Inc., Hercules, CA, USA) was the most efficient kit tested in our studies, having a lower detection limit of 500 cells in the presence of insect tissue. The considerably faster pre-extraction method of repeatedly flushing the foregut with lytic buffer with vacuum pressure prior to extraction using Qiagen DNeasy Tissue kit was not significantly different than whole-tissue maceration. Storage of GWSS samples at -4°C did not compromise *Xf*-detection capabilities.

INTRODUCTION
New epidemics of Pierce's disease of grapevine induced by *Xylella fastidiosa* (*Xf*) in southern California are associated with the invasive vector species *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS). These epidemics have stimulated investigations of plant protection tactics that depend on reducing the interaction between infectious GWSS and grapevines. Deployment of these tactics would be optimized by the ability to monitor the dispersion of *Xf* in infectious GWSS. The ability to detect *Xf* in GWSS vectors would also allow us to describe its movement in time and space at both local and regional levels.

PCR protocols have been developed to detect *Xylella fastidiosa* (*Xf*) in plants (Minsavage et al. 1993, Oliveira et al. 2002, Schaad et al. 2002). Unfortunately, when methods are adapted for insects they tend to be inconsistent. Extraction of DNA from *Xf* in insect vectors may be difficult for several reasons. First, *Xf* exists at low titers in vectors (Nome et al. 1980). The large amount of insect DNA present in the extract could interfere with extraction of *Xf* DNA by saturating the capacity of the extracting process, thus diluting the DNA of interest. Second, the bacterium adheres to sites in the foregut of the insect. This region of the insect's alimentary canal is chitonized and may be difficult to expose to DNA extraction. Third, potential DNA inhibitors are commonly found in insects. It has been suggested that pigments contained in the insect's eyes might inhibit PCR, and removal of the eyes prior to extraction has improved detection (Gispert, unpublished data). Removal of the eyes is tedious and time consuming and therefore not suitable for extracting DNA from large numbers of GWSS samples.

The selection of a DNA extraction protocol depends on the system in which it is being applied. Because no one extraction protocol fits every detection system, factors such as performance of the kit, price, time investment, and maximum number of samples extracted must be considered. In our recent studies, we compared three standard methods and 11 commercially available kits for relative efficiency of *Xf* DNA extraction in the presence of insect tissue. All of the protocols tested were proficient at extracting DNA from pure bacterial culture (1x105 cells) and all but one protocol successfully extracted sufficient bacterial DNA when in the presence of insect tissue. DNA extraction kits were found to be amenable to large sample sizes by being relatively inexpensive and time-efficient.

OBJECTIVES
The over-all goal of this project is to develop a method of detecting *Xf* in GWSS that would allow us to conduct epidemiological studies and optimize plant protection. To this end, our objectives are:
1. Develop a DNA extraction protocol that is optimal for *Xf* DNA recovery from GWSS.
2. Develop a real-time PCR-based detection system that is sensitive, rapid, and cost-efficient.

RESULTS
Extraction kit comparison
The Qiagen DNeasy Tissue kit detected *Xf* 50 cells without insect tissue present (Table 1). When the sample dilution series was tested with insect tissue, 500 cells was the lowest concentration detected (a 10-fold decrease). Although the immunomagnetic separation method returned similar results without insect tissue, it only allowed detection of *Xf* at $\geq$1600 cells with insect tissue (Table 1). The Fermentus DNA Purification kit only detected the concentration of 1600 cells without insect tissue and did not detect any dilution with insect tissue present.

Table 1. Comparison of three DNA extraction methods by PCR results from samples spikes with dilutions of *X. fastidiosa* culture with or without insect background. n=5 for each kit and sample combination

cells/ml	DNeasy Tissue kit (Qiagen Inc.)		Immunomagnetic separation[a]		Genomic DNA purification kit (Fermentus Inc.)	
	With GWSS[b]	Without GWSS[c]	With GWSS[b]	Without GWSS[c]	With GWSS[b]	Without GWSS[c]
5000	++++	++++	+++	+++++	-	++
1600	++	+++	+	++++	-	L
500	L	++	-	++	-	-
50	-	L	-	L	-	-
0	-	-	-	-	-	-
PCR + control	++++		++++		++++	
PCR – control	-		-		-	

*The density of the PCR products on the agarose gels was related to the relative intensities of the bands compared to one another. The number of + illustrated the relative brightness of the band, L indicates a very faint band, and - indicates no PCR product.

[a]Immunomagenetic separation (Dynal Biotech and Agdia Inc.).

[b]Extract of one GWSS head added to sample.

[c]No insect background in sample.

Real-time (RT) PCR (Sybr Green I)

Real-time PCR was performed in a Rotor Gene 3000 (Corbett Research, Australia) using iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA) in 20µl reactions with 5µl of extracted DNA in H_2O. For detection of *Xf*, we used the *Xf*-ITS primer set (Schaad et al. 2002), which was originally designed to by used with a molecular probe system; XfF1 (5' AAA AAT CGC CAA CAT AAA CCC A 3') AND XfR1 (5' CCA GGC GTC CTC ACA AGT TAC 3'). After an initial denaturing step of 3m at 95°C, the reactions were cycled 40 times under the following parameters: 95°C for 20s, 53°C for 20s, 72°C for 20s. At the end of the PCR, temperature was increased from 72 to 99°C at a rate of 1°C/45s, and fluorescence was measured every 45s to construct the melting curve. PCR product produced in positive samples had a specific melting temperature of 86.1° compared to the melting temperature of the primer/dimmer (80.9°), so the *Xf*-positive samples were easy to separate from negative samples (Figure 1). A non-template control (NTC) was run with each assay and the negative control.

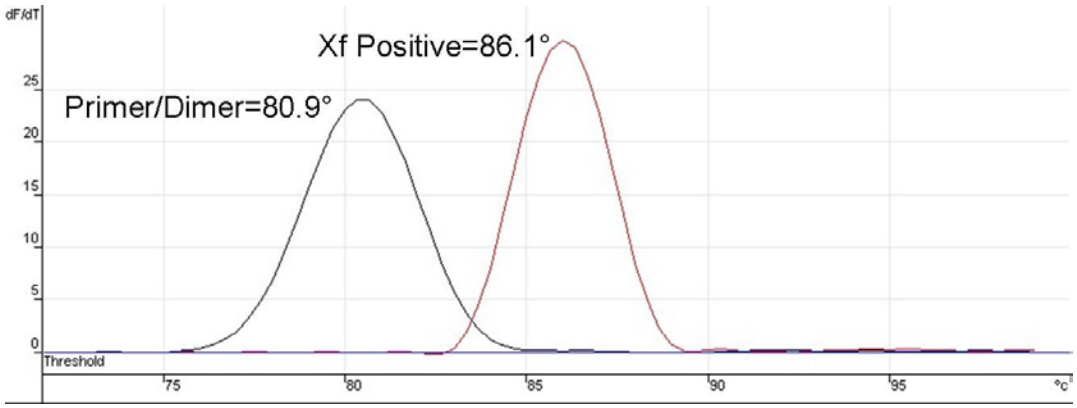

Figure 1. Melting curve analysis with two amplicons produced by *Xf* positive or primer/dimmer. The melting peaks separate by size similar to bands on an electrophoresis gel.

Vacuum infiltration as a pre-extraction method for detecting Xf in GWSS. We developed a method of extruding *Xf* from GWSS that does not require maceration of insect tissue, the most tedious step in the DNA extraction process. Using a method of repeatedly flushing the foregut with lytic buffer by changing vacuum pressure, *Xf* DNA was sufficiently collected for detection by RT-PCR. In a direct comparison, the proportion of *Xf*-positive GWSS was not significantly different using the vacuum extraction method (71.2%, n=139), compared to whole-tissue maceration (76.3, n=139) (chi-square=0.91). We believe this method works well for several reasons. First, because insect tissue is not macerated, less insect DNA and PCR inhibitors should be released. Second, by repeatedly applying vacuum pressure and releasing it, lytic buffer is continually flushed through the foregut of the insect allowing for better recovery of *Xf*. Vacuum extraction was amenable to large numbers of samples. Using a 96-well plate, 96 samples can be processed at the same time, compared to one sample at a time with maceration.

Effects of freezing on detection of Xf from GWSS

Storing GWSS samples at -4° for 10 d prior to extraction and RT-PCR detection did not affect *Xf* detection (ANOVA, LSD, p=0.001). In a direct comparison, 85.1% of GWSS having DNA extracted immediately after removal from *Xf*-infected plants (n=48) tested positive, compared to 77.1% of GWSS stored at -4° for 10 d and 81.3% of GWSS that were stored in mineral oil at -4° for 10 d.

CONCLUSIONS

We have made substantial progress in our goal of developing an efficient and sensitive method to detect *Xf* in GWSS vectors. First, we have successfully used a commercially available DNA extraction kit to detect a lower detection limit of 50 *Xf* cells without a GWSS head background and 500 *Xf* cells with a background. This kit is consistent with our goal of making our method cost-efficient and amenable to a large sample size. Second, we have developed the used of RT PCR technique to simultaneously detect and quantify *Xf* DNA. Third, we have developed a means of rapidly extracting multiple samples simultaneously.

Our future research directions will focus on lowering detection limits by examining additional primer sets, and crude dissections of GWSS head to reduce interfering DNA and PCR inhibitors. After optimizing our detection method we will examine the relationship between grapevine inoculation by individual GWSS and the ability to detect *Xf* in them. Understanding that relationship will allow us to study details of *Xf* epidemiology and optimize plant protection.

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