DEVELOPMENT OF QRT-PCR PROTOCOLS FOR RAPID *XYLELLA FASTIDIOSA* SUBSPECIES DIAGNOSTICS

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ABSTRACT
Xylella fastidiosa (Xf) is a plant pathogenic bacterium that is transmitted between hosts by the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)*. Multiple subspecies of *Xf* occur and are host specific. *Xf* subsp. *fastidiosa* is the causal agent of Pierce's disease (PD). *Xf* subsp. *multiplex* and *Xf* subsp. *sandyi* are commonly found in North America but do not cause PD. Rapid diagnostics to determine presence of *Xf* and differentiation of these subspecies is necessary for effective management of PD. In this study, three methods by which the subspecies can be distinguished by QRT-PCR were compared. SYBR green, Eva Green®, and Takara SYBR Green® melt curve analysis of partial gyraseB amplicons, Zot gene amplicons, and five TonB amplicons were evaluated for consistency and quality. Multiple melts were performed to find the ideal conditions for distinguishing between the subspecies. Emphasis was placed on a Ragweed insertion in the *Zot* gene, for which a probe was designed to increase the reliability of rapid diagnostics and differentiation of *Xf* subspecies. These new methods provide a more reliable protocol by which the subspecies of *Xf* can be determined.

LAYPERSON SUMMARY
Detection of the pathogen in an agronomic disease system is an important component of a management strategy. This is especially true in cases where incredibly mobile insects (like the glassy-winged sharpshooter) are the primary vectors of a pathogen (like *Xylella fastidiosa; Xf)*. In short, it is important to know which plants are infected with the pathogen and which insects in the vineyards are carrying the pathogen. To further confuse our understanding of the system, three subspecies of *Xf* exist in our grape growing areas which are currently detectible by standard assays; however, only one of these subspecies will lead to Pierce's disease if it is in a vine. So in this study, we have developed a detection assay that is inexpensive and more sensitive than any detection protocols available. Furthermore, we have designed the protocol to distinguish between strains of *Xf.*

INTRODUCTION
Xylella fastidiosa (Xf) is a plant pathogenic bacterium residing in the xylem vessels and is the causal agent of many plant diseases including Pierce's disease (PD) of the grapevine (Hopkins and Purcell, 2002; Bextine et al, 2007). In the southwestern region of the United States, there are three common strains of *Xf*: *Xf* subsp. *fastidiosa* (PD), *Xf* subsp. *multiplex* (RW) (Schaad et al, 2004), and *Xf* subsp. *sandyi* (OLS) (Hernandez-Martinez et al, 2007). Previous methods of distinguishing *Xf* subspecies are slow and more prone to error. Initially, these previous methods, using SYBR Green, were evaluated and compared to new methods which used Takara SYBR Green® and Eva Green®. Melt analysis was used to analyze the strains. The *gyraseB* gene was used in the evaluation of subspecies determination protocols. In the second part of the experiment, new primers were designed for the *Zot* and *TonB* genes in order to attempt differentiation of the species based on melting temperature analysis. In the *Zot* gene of the Ragweed strain an insertion from base pair 379-426 (**Figure 1**) was the main focus of the *Zot* primers.

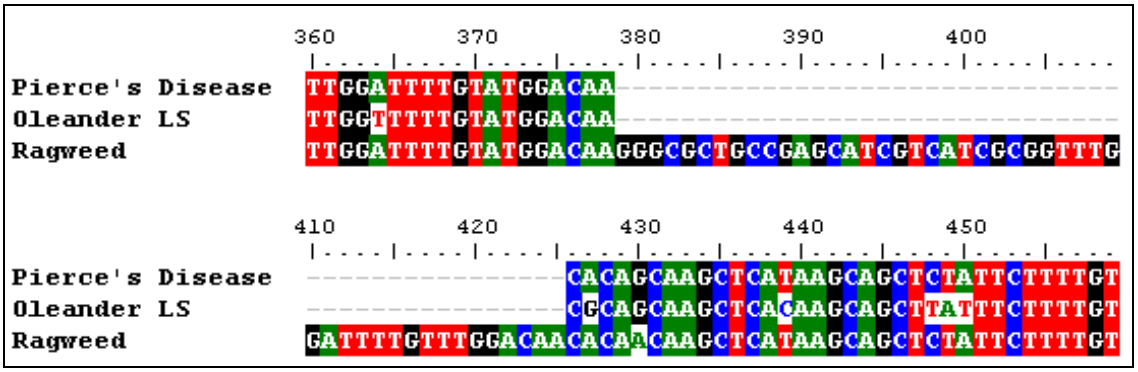

Figure 1: Section of Zot gene in PD, OLS, and RW *Xf* subspecies showing Ragweed insertion.

OBJECTIVES

1. Determine accuracy of currently used QRT-PCR methods for identification of *Xf*.
2. Compare to new methods using Eva Green and Takara SYBR Green.
3. Develop rapid and more reliable methods for identification of *Xf* subspecies.

MATERIALS AND METHODS

DNA Extraction. *Xf* colonies were collected from agar plates using a sterilized metal loop and placed in 100µl of PBS buffer in a MCT. The bacteria were vortexed until mixed into the solution and 100µl Lysis Buffer L6 was added to each MCT. The samples were centrifuged at 5000 rpm for five minutes. Next 53µl of silica slurry was added to each MCT and vortexed. The samples were incubated at room temperature for five minutes, followed by five minutes of centrifugation at 2000 rpm. The supernatant was drawn off and discarded and 200µl of wash buffer was added to each MCT. The MCTs were centrifuged at 2000 rpm for five minutes and the wash step was repeated three times for a total of four washes. The MCTs were then placed in an incubator at 60° C until the silica was dry, approximately 10 minutes. After the silica dried, 100µl of TE Buffer was added to each sample and mixed with the silica, followed by incubation for another five minutes at 60° C. The MCTs were centrifuged for five minutes at 5000 rpm. Afterward 70µl of the supernatant was drawn off, without any silica, and placed into a sterile MCT, labeled, and stored in the freezer.

QRT-PCR. Prior to use the, PCR hood was subjected to a minimum of 30 minutes of UV light and sterilization with 10% bleach. All sample MCTs were placed in a cold block during preparation and all master mix reagents were placed on ice. QRT-PCR was conducted in 10µl reactions consisting of 5µl iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.4µl forward primes, 0.4µl reverse primes, 1.0µl nanopure water, 1.0µl SYBR® Green nucleic acid gel stain (Molecular Probes™, Eugene, OR) and 2µl sample DNA. The sample DNA consisted of RW, OLS, and PD *Xf* strains. Two No Template Controls (NTCs) were included each time QRT-PCR was conducted and consisted of 10µl master mix. Eva Green (Phenix Research Products, NC) and Takara SYBR Green (Takara Bio USA, WI) were each switched with SYBR® Green nucleic acid gel stain for comparison. QRT-PCR reactions were carried out in 0.1mL PCR tubes and consisted of an initial denaturing step at 95 C for ten minutes, followed by 40 cycles of 30 seconds denature, 30 seconds anneal, and one minute elongation at varying temperatures. Melt analysis was performed after a final elongation step and consisted of ramping the temperature a varying number of degrees per second.

QRT-PCR Probe Prior to use the, PCR hood was subjected to a minimum of 30 minutes of UV light and sterilization with 10% bleach. A 96-well plate was placed on ice along with all reagents used during preparation. QRT-PCR was conducted in 10µl reactions consisting of 5µl iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 1µl forward primes, 1µl reverse primes, 0.6µl nanopure water, 0.4µl probe and 2µl sample DNA. Only the PD and RW strains of *Xf* were used with the probe. A minimum of two No Template Controls, consisting of 10µl master mix, and no positive controls were used. QRT-PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Analysis of current QRT-PCR Protocols. Current QRT-PCR protocols consisted of using SYBR® Green to measure fluorescence of PCR products. After comparison of SYBR® Green to Eva Green and Takara SYBR Green, it was found that the Takara SYBR green provided the most consistently reliable results of the three during melt analysis (**Table 1**). The results from the best QRT-PCR run/melt from each method were used in the comparison, in order to compare the best possible outcome from each method.

Table 1: Average Tm & SD for *Xf* subspecies using each SYBR Green method under ideal melt conditions		
SYBR Green Type & *Xf* Subspecies	**Avg. T_m**	**Avg SD**
SYBR® Green		
PD	85.42° C	0.3869
RW	85.66° C	0.0520
OLS	85.89° C	0.0346
Eva Green®		
PD	86.58° C	0.0458
RW	86.43° C	0.0346
OLS	86.87° C	0.0458
Takara SYBR Green®		
PD	84.90° C	0.0346
RW	85.30° C	0.0346
OLS	85.57° C	0.0346

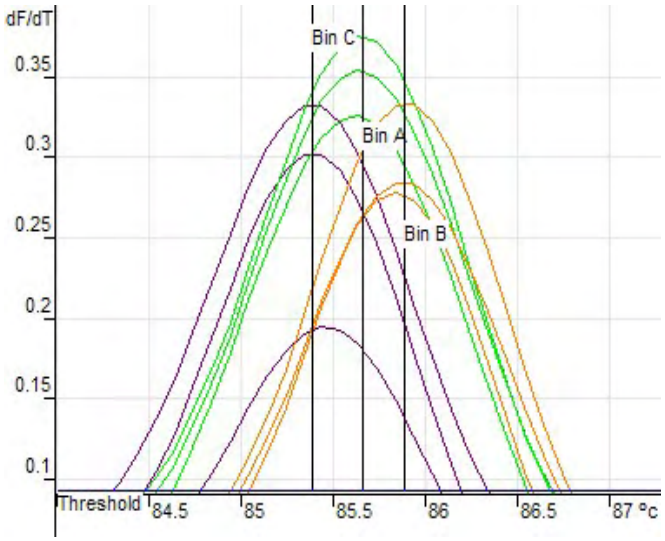

Figure 2. Melt analysis of OLS (Bin A), RW (Bin B), & PD (Bin C) using set6 primer set and Takara SYBR Green® melted at a rate of 0.3° C every five seconds.

Zot and TonB gene regions. The primers designed in the *TonB* gene region were better at differentiating between the different *Xf* subspecies using melt analysis then Sybr Green. The primers that did yield some seemingly useful differentiation of the strains were found to have standard deviations of equal or larger magnitude than the difference in melting temperature. Primers in the *Zot* gene region were designed in the Ragweed insertion sequence as well as bridging over the insertion site. Melt analysis after QRT-PCR found considerable differentiation of the *Xf* subspecies.

Zot Probe. Although primers in the *Zot* gene region provided a relatively reliable method to differentiate *Xf* strains, the melting temperatures did not separate far enough to conclude the identity of an experimental unknown. Dual-labeled probes were designed in the Ragweed insertion sequence and in the grape sequence around where the insertion would be. Using QRT-PCR and the Ragweed insertion probe, the Ragweed strain was successfully isolated from the grape strain.

Using the bridge probe, the PD strain was differentiated from the Ragweed strain, but not necessarily isolated as with the ragweed insertion probe (**Figures 2-4**).

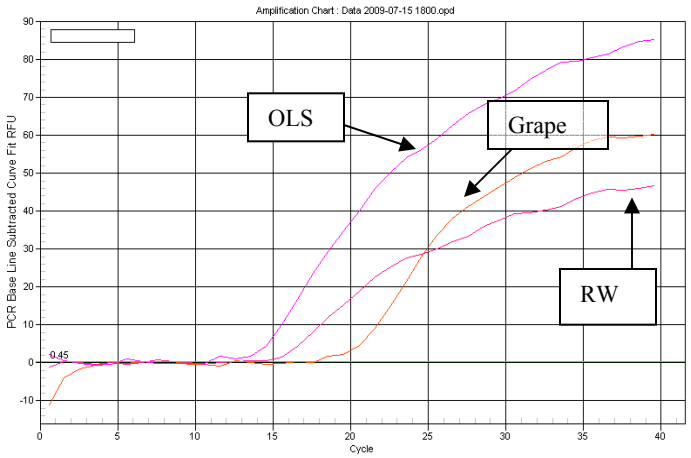

Figure 3. QRT-PCR using Grape bridge probe.

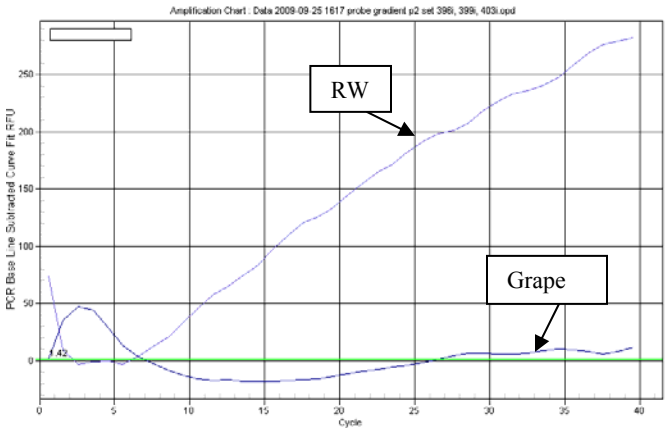

Figure 4. QRT-PCR using Ragweed insertion probe.

CONCLUSIONS

For QRT-PCR in general, Takara SYBR Green provides a statistically more accurate and consistent method of measuring fluorescence. Although more accurate, only using Takara SYBR Green instead of the previously used SYBR Green provides no significant advantage in identification of *Xf* subspecies in an unknown sample. Takara SYBR Green paired with primers

designed to take advantage of the insertion in the ragweed sequence provides a decent method of identifying strains, although the results were not consistent enough to come to conclude the *Xf* strain present based solely on QRT-PCR fluorescence or melt analysis.

The probe designed the using a bridge ragweed insertion provided an identification method while the Ragweed insertion provided a reliable and unmistakably accurate method of identifying between Grape and Ragweed strains. Utilizing the set6 primer set initially used in the comparison of the three SYBR green reagents with the Ragweed insertion probe provides a fast, reliable and accurate protocol by which strains of *Xf* can be identified using only QRT-PCR.

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