USING AN ORANGE OIL SOLVENT TO REMOVE INSECTS FROM YELLOW STICKY TRAPS: IMPACTS ON THE DETECTION OF *XYLELLA FASTIDIOSA* DNA IN GLASSY-WINGED SHARPSHOOTER SAMPLES

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ABSTRACT

Xylella fastidiosa (Xf) Wells is a plant pathogenic bacterium that causes many economically important agricultural diseases and is transmitted by the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Effective detection of *Xf* in field collected GWSS in an area-wide management program can contribute to the assessment of risk associated with insect presence in vineyards. Prior to conducting molecular assays for detection of *Xf* in individual insects, GWSS must be removed from yellow sticky traps using a solvent. In this study, we determined the effect of orange oil concentration in individual GWSS on detection of *Xf* by QRT-PCR. In a ten-fold dilution series of orange oil, increased amounts of orange oil caused decreased levels of *Xf* detection in standardized positive samples. Additionally, we determined methods for lowering the concentration of orange oil found in processed field samples below the point where detection of *Xf* is negatively impacted.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Hemiptera: Cicadellidae), is the major vector of *Xylella fastidiosa (Xf)* Wells in the southern USA (Adlerz 1980; Blua et al., 1999). The plant pathogenic bacterium, *Xf*, has caused economic losses to a several agricultural industries in North, Central, and South America. Pierce's disease (PD) of grapevine has become a well understood *Xylella*-related disease; the vector complement is well known and the epidemiology of the disease is well documented (Hopkins et al., 2002). The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management and control of PD depends heavily on the ability to closely and accurately monitor its vectors, especially GWSS.

In area-wide management studies in California and Texas, GWSS are collected using yellow sticky cards such as the Trece Inc. adhesive trap T3306 (Trécé, Inc., Adair, OK). This method works very well for monitoring population numbers and identifying species that occur in the field; however, the adhesive that coats the yellow sticky card can be problematic when applying molecular techniques (DNA and RNA studies) to these samples. Therefore, the sticky adhesive on the yellow sticky cards must be removed. This specimen extraction process involves application of a strong organic solvent to remove the adhesive. Because the downstream molecular assays involving DNA extraction and PCR both require the heating of samples, a solvent with a low flashpoint must be used. Orange oil has the lowest flashpoint of any organic solvent on the market, 118˚F/48˚C (Florida Chemical Co. Inc., Winter Haven, FL). In addition to its non-volatile composition, this product is inexpensive, nontoxic and effective at removing sticky adhesives. Orange oil is the most common product used in studies involving the removal of insects from yellow sticky cards and is usually applied directly to insects on the adhesive traps. Because all specimens come in direct contact with the solvent, they often absorb unknown amounts of the orange oil. As a result of this, concern was expressed as to whether or not orange oil retained in the insect bodies interfered with quantitative real-time polymerase chain reaction (QRT-PCR). It was speculated that the presence of orange oil in an GWSS specimen containing *Xf* would inhibit either the extraction of DNA, the amplification of target DNA during PCR, or the fluorescence signal emitted during QRT-PCR. In this study, a 10-fold dilution series of different volumes of orange oil mixed with positive *Xf* control specimens were analyzed by QRT-PCR to determine the amount of interference caused by the solvent. We also determined the amount of orange oil contained in a typical extracted sample and discussed the potential effect this will have on *Xf* detection in field samples.

MATERIALS AND METHODS

Sample Collection. Samples were collected using sticky adhesive-based double-sided traps (Seabright Laboratories, Emeryville, CA), each 23 x 14 cm in size, bright yellow in color (Pantone® Matching System (PMS) and coated with Stikem Special® glue, stored at -4˚C, and transported to the University of Texas at Tyler in Tyler, TX. Traps were processed one at a time by individually marking GWSS that were to be recovered and then placing traps in plastic containers and soaking them in orange oil (Citrus Depot, St. Petersburg, FL) for five minutes per two-sided card. Then, using tweezers, the insects were removed individually from the traps, and placed into micro centrifuge tubes (MCTs). Each MCT was labeled according to its corresponding trap with its vineyard's location and its individual location within said vineyard.

DNA Extraction. Once extracted from sticky traps, GWSS bodies were separated from their heads and placed back in their original MCTs (Bextine et al. 2004). Briefly, the heads were then placed in 96 well plates, with one head per well, and covered with 200µL PBS buffer. The plates were placed under vacuum suction for two minutes a total of five consecutive times. The heads were removed, and 200µL of Lysis Buffer L6 was added to each well. The plates were centrifuged at 5,000rpm for 5 minutes, and 300µL of the supernatant in each well was transferred into a corresponding MCT. Afterward, 53µl of silica slurry was mixed into the 300µL solution, and the MCTs were incubated at room temperature for five minutes, and centrifuged at 2000rpm for another five minutes. Afterward, the supernatant was drawn off and discarded, and 200µL of wash buffer was added to each MCT. The MCTs were then centrifuged at 2,000rpm for five minutes, and the supernatant was drawn off and discarded. This washing step was repeated twice for a total of three washes. The MCTs were dried at 60˚C with their caps open for ten minutes, or until the silica was dry. One hundred µL of TE buffer was gently mixed with the silica in each MCT, and the MCTs were incubated at 60˚C for another five minutes, and centrifuged at 5,000rpm for another five minutes. Seventy µL of the supernatant was transferred into a sterile MCT, without picking up any silica, and the MCT's were labeled and placed in the freezer.

QRT-PCR. The PCR hood was left under UV light for a minimum of thirty minutes prior to use. Once UV light was turned off, the station was sterilized using 10% bleach. All samples to be run were placed in a cold block, and the master mix reagents were allowed to defrost. PCR was conducted in 10µL reactions, including two *Xf* positive controls and two No Template Controls (NTCs). Each sample reaction, a total of 10µL, included 5µL iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5µL SYBR® Green nucleic acid gel stain (Molecular Probes™, Eugene, OR), 1.7µL nanopure water, 0.4µL forward primer, 0.4µL reverse, and 2µL sample DNA. Each *Xf* positive control contained only 1µL DNA, and each NTC contained only 10µL master mix (every reagent except DNA). Each reaction was carried out in 0.1mL PCR tubes (Corbett Research, St. Neots, Cambridgeshire, UK). The prepared samples were placed into a Rotor-Gene RG-3000 QRT-PCR machine (Corbett Research, St. Neots, Cambridgeshire, UK) and run to determine if they contained *Xf* positive DNA.

Determining Orange Oil Retained. In order to create an applicable orange oil dilution series, the average amount of orange oil retained in each GWSS body was first determined. Ten empty MCTs were weighed and logged. The average MCT mass was found to be 965.7mg. The same ten MCTs were each filled with 100µL orange oil and weighed once more. The average MCT + 100µL orange oil mass was found to be 1044.5mg. Ten GWSS bodies were soaked in orange oil for five minutes, following the trap removal protocol. The heads were removed, placed in ten MCTs, and centrifuged at maximum rpm (14,000rpm) for one minute. The heads were then removed and the MCTs containing retained orange oil were weighed. The average mass of the MCTs + retained orange oil was 965.84mg. The average mass of an MCT containing retained orange oil minus the average mass of an empty MCT was found to be the average mass of retained orange oil in an GWSS head (965.84mg - 965.7mg = 0.14mg).

Dilution Series. Two orange oil dilution series were run through QRT-PCR in this test. The first was a ten-fold dilution series. Triplicates of samples containing 8µL master mix, 1µL positive *Xf* DNA, and 1µL of either 100%, 10%, or 1% orange oil were run through QRT-PCR. With the information gained from the ten-fold dilution series, a more precise dilution series was developed. This dilution series was a 1/1 – 1/10 dilution of orange oil. The same reaction volumes were used, and the same procedure was followed.

RESULTS AND DISCUSSION

Determining Orange Oil Retained. The average mass of an MCT containing retained orange oil minus the average mass of an empty MCT was the average mass of retained orange oil in an GWSS head (965.84mg - 965.7mg = 0.14mg). The average mass of an MCT containing 100µL orange oil minus the average mass of an empty MCT equaled the average mass of 100µL of orange oil (1044.5mg - 965.7mg = 78.8mg). Since M1/M2 = V1/V2, the average mass of orange oil retained divided by the average mass of 100µL of orange oil was equal to the volume of orange oil retained divided by 100µL orange oil (0.14mg/78.8mg = V1/100µL). The average volume of orange oil retained by an individual GWSS head was 0.178µL (0.14mg/78.8mg x 100µL). This figure was used to determine the range of an orange oil dilution series.

Dilution Series. From the QRT-PCR data collected (**Figure 1**), 1µL of 100% as well as 1µL of 10% orange oil in a 10µL reaction completely inhibited binding of fluorescent binding proteins to *Xf* DNA, and 1% orange oil had no effect on binding.

Following the first dilution series, a more precise dilution series was developed. This dilution series was a 1/1 – 1/10 dilution of orange oil. The same reaction volumes were used, and the same procedure was followed. The result was a strong deviation from fluorescence expected under normal conditions in samples containing between 1µL and 0.1µL of orange oil. The average volume of retained orange oil per GWSS head, 0.178µL, is within this range, proving that the orange oil present in GWSS heads interferes with *Xf* DNA fluorescence in QRT-PCR.

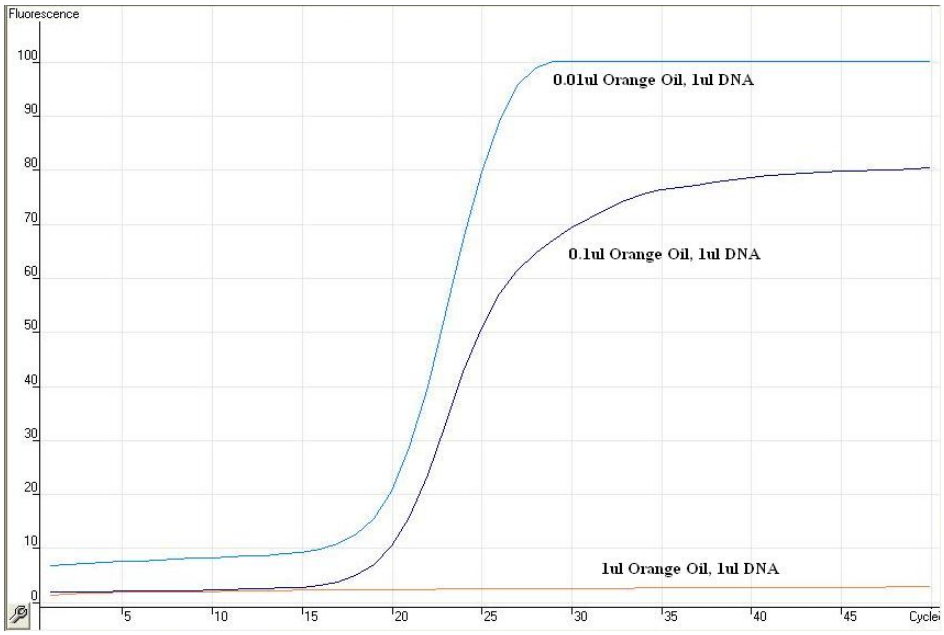

Figure 1. Dilution Series 1, Cycling Curve Analysis. QRT-PCR with SYBR green®, 10ul reactions. 1ul oil, 1ul *X.f.* DNA (Orange). 0.1ul oil, 1ul *X.f.* DNA (Purple). 0.01ul oil, 1ul *X.f.* DNA (Blue).

CONCLUSIONS

The presence of orange oil does inhibit the ability to detect *Xf* DNA by QRT-PCR by inhibiting the extraction of *Xylella* DNA, the amplification of target DNA during PCR, or the fluorescence signal emitted by fluorescent binding proteins during QRT-PCR. In our protocol, care was taken to avoid an overabundance of orange oil in samples. Primarily, we were extremely selective and conservative with the amount of orange oil used during insect extraction. Instead of soaking an entire trap, our procedure calls for a squeeze bottle that can directly apply small amounts of orange oil to each individual insect. Another step is the prompt transfer of each insect into a 70% ethanol wash, followed by a DI water wash, before being placed in a sterile MCT. This step ensures that each insect has been thoroughly washed of enough orange oil that contamination is no longer a concern. Seventy percent ethanol also cleanses each insect of many other contaminants as well. Concentrated orange oil can also be diluted, but loses its solvent strength the more it is diluted. We use concentrated orange oil in our trap extractions because it dissolves the sticky trap adhesive faster and more efficiently. Again, other solvents may be more efficient and less contaminating, but we must use orange oil in our extractions due to its high flashpoint. Other strong organic solvents, such as turpentine, hexanes, or ethers have flashpoints too low to be used in silica-based DNA extraction or QRT-PCR. The temperatures reached in these processes are too high for other organic solvents except orange oil.

Another factor in the inhibition of QRT-PCR performance by *Xylella* DNA may be the sticky adhesive that is dissolved in orange oil during the trap extraction process. We believe that this does not have an inhibiting effect due to the adhesive's inability to absorb into the bodies of GWSS. However, our follow up experiment will test this theory using the same methods discussed in this test.

The results of this experiment are crucial in further understanding the insect vector, GWSS. The management and control of Pierce's disease depends heavily on the ability to closely and accurately monitor its vectors. This experiment impacts any insect extraction and trap removal procedure involving organic solvents, a prominent practice in entomology across the globe.

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