IMPACTS OF AN ORANGE OIL SOLVENT AND STICKEM® ON THE DETECTION OF *XYLELLA FASTIDIOSA* DNA IN GLASSY-WINGED SHARPSHOOTERS, *HOMALODISCA VITRIPENNIS* (HEMIPTERA: CICADELLIDAE)

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Abstract

Xylella fastidiosa is a plant pathogenic bacterium that causes many economically important agricultural diseases and is transmitted by the glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Efficient detection of *X. fastidiosa* in field collected *H. vitripennis* in an area-wide management program can contribute to risk assessment associated with insect presence in vineyards. Prior to conducting molecular assays for detection of *X. fastidiosa* in individual insects, *H. vitripennis* must be removed from yellow sticky traps with a solvent such as orange oil. In this study, we determined the effect of orange oil concentration on extraction of individual *H. vitripennis* following trap removal on detection of *X. fastidiosa* by qRT-PCR. In a ten-fold dilution series of orange oil, increasing amounts of orange oil caused decreasing levels of *X. fastidiosa* detection in standardized positive samples. Additionally, tests on the effects of Stickem® brand trap adhesive on qRT-PCR and development of methods which lowered the concentration of orange oil often present in field samples determined the point where detection of *X. fastidiosa* was negatively impacted. These results benefit the monitoring and screening for *Xylella fastidiosa* from leafhoppers collected on sticky cards used in regulatory area-wide management.

Key Words: orange oil, *Xylella fastidiosa*, glassy-winged sharpshooter, *Homalodisca vitripennis*, PCR

Resumen

Xylella fastidiosa es una bacteria patogénica de plantas que causa muchas enfermedades agrícolas economicamente importantes y es transmitida por el salta hojas, *Homalodisca vitripennis* (Hemiptera: Cicadellidae). La detección eficiente de *X. fastidiosa* de *H. vitripennis* recolectados en el campo en un programa de manejo de largas áreas puede contribuir a la determinación del riesgo asociado con la presencia del insecto en los viñeros.Antes de realizar los ensayos moleculares para la detección de *X. fastidiosa* en individuos del insecto, *H. vitripennis* debe ser recolectados de trampas pegajosas amarillas con un solvente como el aceite de naranja. En este estudio, determinamos el efecto de la concentración del aceite de naranja sobre la extracción de individuos de *H. vitripennis* después de que fueron quitados de las trampas para la detección de *X. fastidiosa* usando qRT-RPC. En una serie con el aceite de naranja diluido 10 veces, un aumento de la cantidad de aceite de naranja causo una disminución en los niveles de *X. fastidiosa* detectados en muestras positivas estandarizadas. En adición, las pruebas sobre los efectos del adhesivo de trampa de la marca Stickem® sobre qRT-RPC y el desarrollo de métodos que bajan la concentración del aceite de naranja en muestras de campo determinaron el punto donde la detección de *X. fastidiosa* fue impactado negativamente. Estos resultados benefician el monitoreo y la detección de *Xylella fastidiosa* de los salta hojas recolectadas en tarjetas pegajosas usadas en un programa regulatorio de manejo por todo el área.

The glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae), is a major vector of *Xylella fastidiosa* (Wells et al. 1987) in the southern USA (Adlerz 1980; Blua et al. 2003). The plant pathogenic bacterium, *X. fastidiosa*, has caused economic losses to several agricultural industries in North, Central, and South America. *Xylella*-related complications and diseases in agriculture are well understood; the vector model is well known and the epidemiology of the disease is well documented (Hopkins et al. 2002; Mizell et al. 2008). *Xylella fastidiosa* is the causative agent of Pierce's disease in grapevines. The introduction of *H. vitripennis* into new areas is directly related to increased occurrence of Pierce's disease in vineyards (Perring et al. 2001). Therefore, the management and control of Pierce's disease depends heavily on the ability to closely and accurately monitor its leafhopper vectors, especially *H. vitripennis*.

In area-wide management programs in California and Texas, *H. vitripennis* typically are col-

lected on yellow sticky cards. The method is useful for monitoring population numbers and identifying insect species that occur in the field; however, the adhesive (Stickem®, Seabright Laboratories, Emeryville, CA) that coats these cards can be problematic when applying molecular techniques (amplification of DNA and RNA studies) to insects removed from the traps. A process involving application of a strong organic solvent to dissolve the adhesive on the insects must be performed. Downstream molecular assays require DNA extraction and PCR, both of which involve heating of samples; therefore, a solvent with a high flashpoint is essential.

Orange oil (Florida Chemical Co. Inc., Winter Haven, FL) has the highest flashpoint of any organic solvent on the market, 118°F/48°C. In addition to its low volatility, 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 adhered to traps (Bextine et al. 2004). Because all specimens come in direct contact with the solvent as well as the adhesive, they often absorb unknown amounts of both. Our speculation was that orange oil or Stickem® retained by *H. vitripennis* specimens might inhibit the extraction of DNA, the amplification of target *X. fastidiosa* DNA during PCR, or the fluorescence signal emitted during quantitative Real-Time PCR (qRT-PCR). In this study, a 10-fold dilution series of different volumes of orange oil and Stickem® mixed with positive *X. fastidiosa* control specimens were analyzed by qRT-PCR to determine the amount of interference caused by the solvent or the adhesive. We determined individually the amount of orange oil and Stickem® contained in a typical extracted leafhopper sample and discussed the potential effect this may have on *X. fastidiosa* detection in field samples.

MATERIALS AND METHODS

Sample Collection

Insect samples were collected with sticky adhesive-based traps. Traps were either standard double-sided traps (Seabright Laboratories, Emeryville, CA), each 23 × 14 cm, bright yellow in color and coated with Stickem Special® glue (2004 to date) or sheets of yellow sticky strips sold by Great Lakes IPM (Vestaburg, MI) and cut to fit the trap frame in 2003 and 2004. These samples were analyzed at the University of Texas at Tyler, TX. *Homalodisca vitripennis* were individually recovered by placing traps in 4.43-L plastic containers (Tupperware Brands Corp., Orlando, FL) with 2 L orange oil (Citrus Depot, St. Petersburg, FL) for 5 min per two-sided card. Insects were removed from the traps via forceps and placed into

1.5-mL centrifuge tubes labeled according to trap location and corresponding vineyard.

DNA Extraction

Homalodisca vitripennis heads were removed and placed in 96-well plates, 1 head per well containing 200 µL PBS buffer (Bextine et al. 2004). The plates were placed under vacuum for 2 min 5 times consecutively. The heads were removed, and 200 µL of Lysis Buffer L6 (Boom et. al. 1990) added to each well. The plates were centrifuged at 4,295 g for 5 min, and 300 µL were transferred from the supernatant in each well to a corresponding micro-centrifuge tube. Next 53 µL of silica slurry (Boom et al. 1990) were mixed into the 300 µL-solution, and samples were incubated at room temperature for 5 min and centrifuged at 687 g for another 5 min. Afterward, the supernatant was discarded, and 200 µL of silica wash buffer added. Samples were then centrifuged at 687 g for 5 min, and the supernatant was aspirated and discarded. This washing step was repeated twice. The samples were dried at 60°C for 10 min, or until the silica was dry. DNA was then eluted with 100 µL TE buffer and centrifuged at 2000 rpm for 5 min. Lastly, 70 µL of the supernatant were transferred to a sterile 1.5-mL centrifuge tube in preparation for qRT-PCR.

qRT-PCR

PCR was conducted in 10-µL reactions, including 2 *X. fastidiosa* positive controls and 2 No Template Controls (NTCs). Each sample reaction, a total of 10 µL, included 5 µL iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 µL SYBR® Green nucleic acid gel stain (Invitrogen Molecular Probes™, Eugene, OR), 1.7 µL nanopure water, 0.4 µL of 10 µM primer INF2 (GTTTGATTGATGAACGTGGTG), 0.4 µL of 10 µM primer INR1 (CATTGTTTCTTGGTAGGCATCA) (Sigma-Aldrich, St. Louis, MO) (Bextine & Child 2007), and 2 µL-sample DNA. Each *X. fastidiosa* positive control contained 1 µL DNA, and each NTC contained 10 µL master mix. Each reaction was carried out in 0.1-mL 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 screened for *X. fastidiosa* DNA.

Orange Oil or Stickem® Retained

In order to create an applicable orange oil dilution series, the average amount of orange oil retained in each *H. vitripennis* body extracted from a sticky trap was determined. The average mass of the centrifuge tube + retained orange oil was 965.84mg. The average mass of a micro-centri-

fuge tube containing retained orange oil minus the average mass of an empty microcentrifuge tube was determined to be the average mass of retained orange oil in an *H. vitripennis* head (965.84 mg - 965.7 mg = 0.14 mg). Stickem® retained was calculated similarly.

Dilution Series

The inhibitory effects on fluorescence of 2 orange oil and 2 Stickem® adhesive dilution series were examined by qRT-PCR. The first was a tenfold dilution series. Triplicates containing 8 µL master mix, 1 µL *X. fastidiosa* DNA, and 1 µL of either 100%, 10%, or 1% orange oil or Stickem® were analyzed by qRT-PCR. With the information gained from the initial ten-fold dilution series, a more precise dilution series was developed. Other non-inhibitory dilutions were ruled out. The resulting dilution series was a 1:1-1:10 dilution of orange oil or Stickem®. The same PCR reaction volumes were used and the same procedure was followed.

PCR and Gel Electrophoresis

In order to determine whether or not complete inhibition of *Xylella* DNA amplification was occurring, a separate traditional PCR reaction was performed. A dilution series containing orange oil and another containing Stickem® were subjected to traditional PCR followed by electrophoresis in a 1% agarose gel suspended in TE buffer for 60 min at 400 amperes and 85 volts. The ladder used was TrackIt™ 1Kb Plus DNA Ladder, (Invitrogen Molecular Probes™, Eugene, OR, cat. no. 10488-085), These dilution series were constructed identically to the series used in the qRT-PCR test. Positive samples (123,457 copies/µL) were visualized WITH the Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London).

RESULTS

Inhibitory Effect of Orange Oil

The average volume of orange oil retained by an individual *H. vitripennis* head (V_{Ret}) was 0.178 µL, respectively. This figure was reached by first determining the average mass of 5 sterile, empty 1.5-mL microcentrifuge tubes (M_{MCT} = 965.7 mg, SD = 12.77 mg). Next, the average mass of 5 microcentrifuge tubes containing retained orange oil was determined (M_{Oil} = 965.84 mg, SD = 11.33 mg). The average mass of a centrifuge tube containing retained orange oil minus the average mass of an empty centrifuge tube was the average mass of retained orange oil in an *H. vitripeninis* head ($M_{Ret} = M_{Oil} - M_{MCT}$ = 965.84 mg - 965.7 mg = 0.14 mg). The average mass of 5 micro-centrifuge tubes containing 100 µL orange oil (M_{MCT100} =

1044.5 mg, SD = 12.58 mg) minus the average mass of an empty microcentrifuge tube equaled the average mass of 100 µL of orange oil (M_{100oil} = $M_{MCT100} - M_{MCT}$ = 1044.5 mg - 965.7 mg = 78.8 mg). Since Mass1/Mass2 = Volume1/Volume2, the average mass of orange oil retained divided by the average mass of 100 µL of orange oil was equal to the volume of orange oil retained divided by 100 µL orange oil (M_{Ret}/M_{100oil} = 0.14 mg/78.8 mg = V_{Ret}/100 µL). The average volume of orange oil retained by an individual *H. vitripennis* head was 0.178 µL ($V_{Ret} = M_{Ret}/M_{100oil}$ x 100 µL = 0.14 mg/78.8 mg × 100 µL = 0.178 µL).

The average volume of orange oil retained by an individual *H. vitripennis* head, 0.178 µL, was used to determine the range of an orange oil dilution series. Analysis of qRT-PCR data revealed that 1 µL of 100% orange oil as well as 1 µL of 10% orange oil in a 10 µL-reaction completely inhibited fluorescence of *X. fastidiosa* DNA, and 1% orange oil had no effect. Analysis of qRT-PCR data collected in a second, narrower 1:1-1:10 dilution of orange oil produced similar results (Fig. 1). The result was a strong deviation from fluorescence expected under normal conditions (samples con-
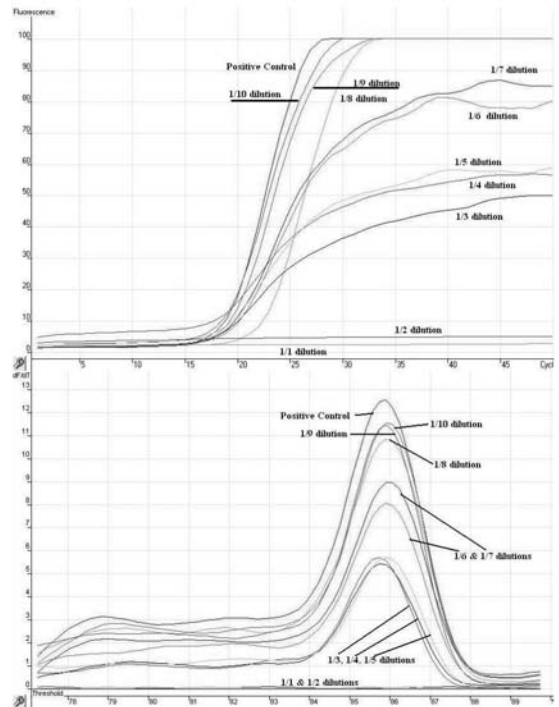

Fig. 1. Cycling and melting curve graphical analysis of *Xylella* DNA fluorescence during qRT-PCR. Notice an inverse proportion of Orange Oil to *Xylella* DNA fluorescence. The positive *Xylella* control sample containing no Orange Oil fluoresces the highest, while the sample containing 1µL of Orange Oil shows no fluorescence at all. Values are averages.

taining *X. fastidiosa* DNA and PCR reaction reagents) in samples containing between 1 µL and 0.1 µL of orange oil. Further statistical analysis of qRT-PCR data yielded an inverse proportion of orange oil concentration to fluorescence (Fig. 2).

Effects of Stickem®

The average mass of Stickem® retained by an individual *H. vitripennis* head was 0.0077 g, respectively. This figure was reached by first determining the average mass of 10 sterile, empty 1.5-mL micro-centrifuge tubes (M_{MCT} = .9632 g, SD = .0054 g). Next, 10 GWSS bodies were placed on a sticky trap, removed, and decapitated. Afterwards, the average mass of 10 GWSS heads was determined (M_{Head} = .0099 g, SD = .0037 g). These 10 GWSS heads were placed into their corresponding sterile, pre-weighed centrifuge tubes to determine the average mass of a microcentrifuge tube containing a GWSS head with Stickem® ($M_{StickyHead+MCT}$ = .9808 g, SD = .0091 g). The average mass of a microcentrifuge tube containing GWSS head + Stickem® ($M_{StickyHead+MCT}$ = .9808 g) minus the average mass of a GWSS head (M_{Head} = .0099 g) minus the average mass of a sterile, empty micro-centrifuge tube (M_{MCT} = .9632 g) equals the average mass of Stickem® retained by a GWSS head removed from a yellow, sticky trap (M_{Sticky} = $M_{StickyHead+MCT}$ - M_{Head} - M_{MCT} = .0077 g).

The average mass of Stickem® retained by an individual *H. vitripennis* head, .0077 g, was used to determine the range of a Stickem® dilution series (Fig. 3). Statistical analysis of qRT-PCR data from a Stickem® dilution series showed an inverse proportion of fluorescence to Stickem® concentration (Fig. 4).

Mode of Fluorescence Inhibition in qRT-PCR

Following qRT-PCR, gel electrophoresis was performed on orange oil and Stickem® dilution series qRT-PCR products. Despite variation in qRT-PCR, endpoint analysis by gel electrophoresis yielded no complete inhibition of *Xylella* DNA amplification by either orange oil or Stickem® (Fig. 5). However, according to data collected, both orange oil and Stickem® are fluorescence inhibitors.

DISCUSSION

The presence of orange oil and Stickem® can inhibit the ability to detect *X. fastidiosa* DNA by qRT-PCR. The root of this inhibition was not known but presence of strong bands by gel electrophoresis following DNA extraction ruled out this step being the root of the problem. Therefore, inhibition had to be the result of either poor amplification of target DNA during PCR or interfer-

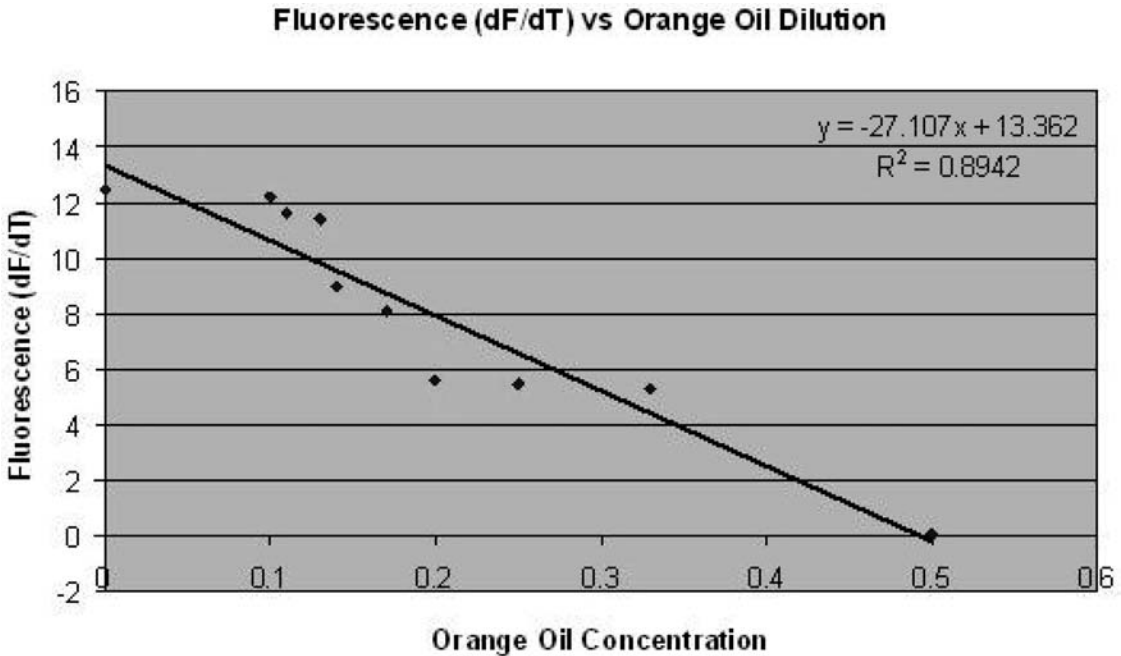## Fluorescence (dF/dT) vs Orange Oil Dilution

Fig. 2. Orange Oil dilution vs. rate of change in fluorescence/rate of change in temperature of *Xylella* DNA. Note a negative correlation in *Xylella* DNA fluorescence with respect to an increase in Orange Oil concentration. Also note that the R^2 value of the trend line is 0.8942.
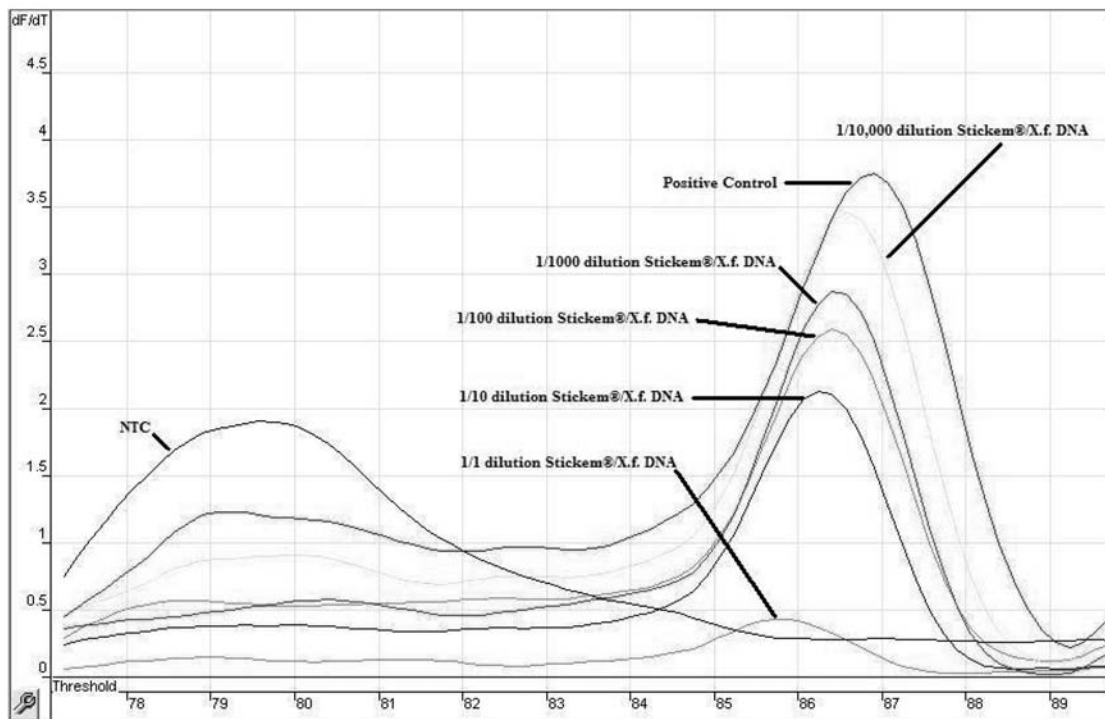
Fig. 3. Cycling and melting curve graphical analysis of *Xylella* DNA fluorescence during qRT-PCR. Notice an inverse proportion of Stickem® concentration to fluorescence of *Xylella* DNA during qRT-PCR. The positive *Xylella* control containing no Stickem® fluoresces the highest, while the sample containing 1μL of Stickem® fluoresces the lowest.

ence with SYBR green fluorescence. End point analysis of qRT-PCR product by gel electrophoresis yielded no affect on *Xylella* DNA amplification (i.e., amplicons were seen on gels when qRT PCR results were negative). Further studies may benefit from the determination of the exact cause of fluorescence inhibition in qRT-PCR by orange oil and Stickem®.

Practically speaking, we did not find levels of either compound in our field samples to be above amounts that resulted in inhibition that produced false-negatives. The average volume of retained orange oil per *H. vitripennis* head was determined to be 0.178 μL which is within the range calculated to interfere with qRT-PCR; however, *H. vitripennis* samples collected and extracted prior to this study did not contain a level considered critical.

Precautions were taken in collection and extraction of *H. vitripennis* samples that allowed contamination to be controlled and closely monitored. These precautions or modifications to the collection and extraction procedures include the use of a squeeze bottle that can directly apply

small amounts of orange oil to each individual insect instead of soaking an entire trap. Also, prompt transfer of each insect into a 70% ethanol wash, followed by a DI water wash, before being placed in a sterile micro-centrifuge tube is recommended. Concentrated orange oil may also be diluted. Other solvents such as turpentine, hexanes, or ethers have flashpoints lower than the denaturing temperature needed for qRT-PCR and may be hazardous to use.

End point analysis of qRT-PCR product by gel electrophoresis yielded no affect on *Xylella* DNA amplification. This determination that orange oil and Stickem® are non-inhibitors of DNA amplification in qRT-PCR is essential in further examining the mode of fluorescence inhibition during qRT-PCR.

The results of this experiment are crucial to the successful monitoring of the spread and occurrence of Xylella in area-wide management programs. The management and control of Pierce's disease depends heavily on the ability to closely and accurately monitor its vectors. These results impact the successful analysis by

Fluorescence (dF/dT) vs Stickem® Dilution

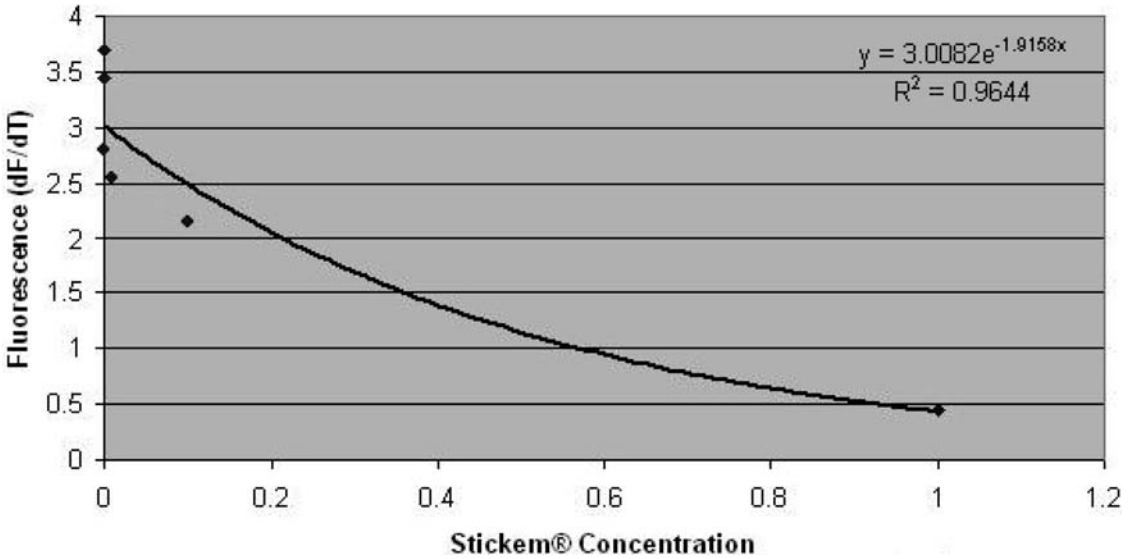$$y = 3.0082e^{-1.9158x}$$
$$R^2 = 0.9644$$

Fig. 4. Stickem® dilution vs rate of change in fluorescence/rate of change in temperature of *Xylella* DNA. Notice a negative correlation in *Xylella* DNA fluorescence with respect to an increase in Orange Oil concentration. Also note that the R²value of the trend line is 0.9644.
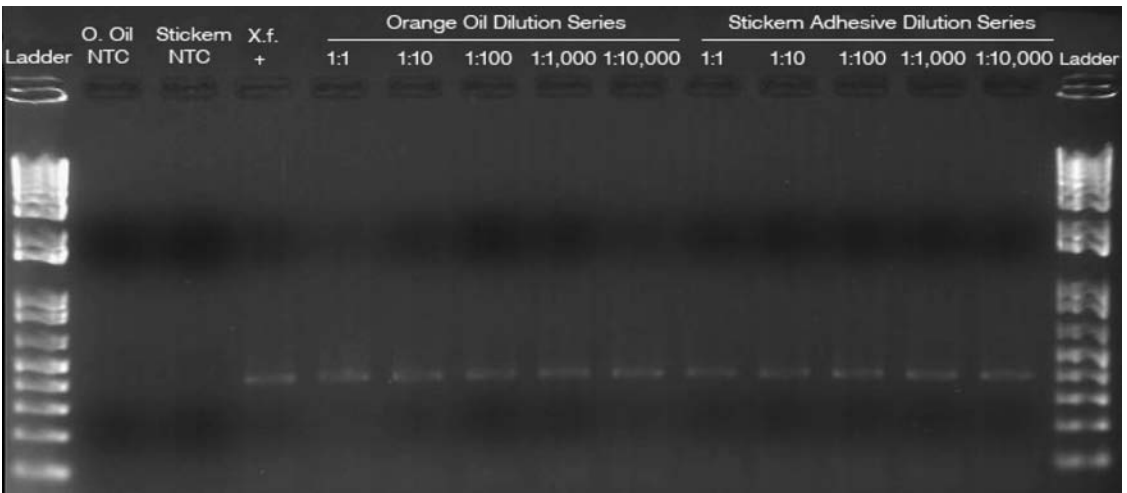

Fig. 5. Shown is a 1% agarose gel after being suspended in TE buffer for 60 min at 400 amperes and 85 volts. The ladder used was TrackIt™ 1Kb Plus DNA Ladder, (Invitrogen Molecular Probes™, Eugene, OR, cat. no. 10488-085. Positive samples (123,457 copies/µL) were visualized with the Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London). This figure shows no complete inhibition of *Xylella fastidiosa* DNA amplification with varying amounts of Orange Oil and Stickem®. Note that no visible effect on *Xylella* DNA amplification by Orange Oil or Stickem® suggests a direct effect on fluorescence of *Xylella* DNA in qRT-PCR.

PCR needed for any insect being removed from sticky cards involving organic solvents, and processed for nucleic acid extraction, a prominent practice in the fields of regulation and entomology.

age by Danny McDonald, Texas AgriLife Research, Stephenville, TX 76401.

REFERENCES CITED

ADLERZ, W. C. 1980. Ecological observations on two leafhoppers that transmit the Pierce's disease bacterium. Proc. Florida State Hort. Soc. 93:115-120.

BEXTINE, B. R., AND CHILD, B. 2007. *Xylella fastidiosa* genotype differentiation by SYBR Green-based QRT-PCR. FEMS Microbiol. Lett. 276(1): 48-54.

BEXTINE, B. R., BLUA, MATTHEW J., AND REDAK, R. 2004. Developing a method to detect *Xylella fastidiosa* in the glassy-winged sharpshooter. Pierce's Dis. Res. Symp.

BEXTINE, B. R., SHAIKH, H., TUAN, S., BLUA, M. J., AND MILLER, T. A. 2004. Evaluation of methods for extracting *Xylella fastidiosa* DNA from the glassy-winged sharpshooter. J. Econ. Entomol. 97: 757-763.

BEXTINE, B. R., BLUA, M. J., AND MILLER, T. A. 2005. A SYBR green-based real-time polymerase chain reaction protocol and novel DNA extraction technique to detect *Xylella fastidiosa* in *Homalodisca coagulata*. J. Econ. Entomol. 98(3): 67-72.

BLUA, M. J., AND MORGAN, D. J. W. 2003. Dispersion of *Homalodisca coagulata* (Hemiptera: Cicadellidae), a Vector of *Xylella fastidiosa*, into Vineyards in Southern California. J. Econ. Entomol. 96(5): 1369-1374.

BOOM, R., SOL, C. J., SALIMANS, M. M., JANSEN, C. L., WERTHEIM-VAN DILLEN, P. M., AND VAN DER NOORDA, J. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28(3): 495-503.

HOPKINS, D. L., AND PURCELL, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's Disease of Grapevines and Other Emergent Diseases. American Phytopath. Soc. 86(10): 1056-1064.

REDAK, R. A., PURCELL, A. H., LOPES, J. R. S., BLUA, M. J., MIZELL III, R. F., AND ANDERSEN, P. C. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. Annu. Rev. Entomol. 49: 243-270.

WELLS, JOHN M., RAJU, BOLIGALA C., HUNG, HSUEH-YUN, WEISBURG, WILLIAM G., MANDELCO-PAUL, LINDA, AND BRENNER, DON J. 1987. *Xylella fastidiosa*: Gram-Negative, Xylem-Limited, Fastidious Plant Bacteria Related to Xanthomonas. Intl. J. Syst. Bacteriol. 37: 136-143.