Improved ELISA detection of *Xylella fastidiosa* in woody plant tissue using sap extracted by a pressure chamber

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Abstract

Xylella fastidiosa is a xylem-limited, fastidious bacterium which causes scorch and dwarfing diseases in many plant species. We recently reported *X. fastidiosa* in chitalpa, an ornamental landscape tree. In evaluating chitalpa trees, we observed that some trees tested positive by PCR, but consistently tested negative or borderline using ELISA, which is preferred for ease of use and cost effectiveness compared with PCR. This inconsistency may be due to significant within-plant and plant-to-plant variability in bacterial titer, thereby making detection using small amounts of tissue and ELISA more difficult. We evaluated the use of four extraction techniques, mortar and pestle, hammer, mini-bead beater, and pressure chamber, for their ability to extract *X. fastidiosa* from chitalpa and grape, and compared the results from ELISA with those from PCR. For each extraction technique, except the pressure chamber, 0.3g of tissue was ground in 3 ml buffer. For the pressure chamber, a branch sample (0.5-1 cm in diameter) with leaves attached was used and sap was extracted at 20–40 bars of pressure. Results indicated that all of these sap-extraction techniques work well for some trees; however only sap from the pressure chamber could consistently match detection using PCR. This method increases the volume of tissue tested, thereby reducing the potential problem of within-plant variability in bacterial titer in certain trees.

Introduction

Xylella fastidiosa is a gram negative, xylem-limited, fastidious bacterium which causes scorch and dwarfing diseases in many plant species. *X. Fastidiosa* is vectored exclusively by xylem feeding insects and is the causal agent of Pierce's Disease, Pecan Bacterial Leaf Scorch, Citrus Variegated Chlorosis, Oleander Leaf Scorch and a variety of other diseases (1). Fastidious organisms like *X. fastidiosa* are incredibly difficult to culture which makes traditional methods for identification of bacteria ineffective. Rapid diagnostic techniques like Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) have been developed to aid in identification of these organisms. ELISA which is preferred over PCR for its ease of use and cost effectiveness offers rapid diagnosis with little or no specialty equipment needed. However, detection using ELISA is not perfect and results using this method do not always match results obtained by PCR which is a much more sensitive test. *X. Fastidiosa* is known to be highly variable in the plant and bacterial titer varies dramatically from plant to plant (2). As such, detection using small amounts of plant tissue is problematic. In order to find a means to inexpensively and accurately detect *X. fastidiosa*, we investigated the use of several different sap extraction methods.

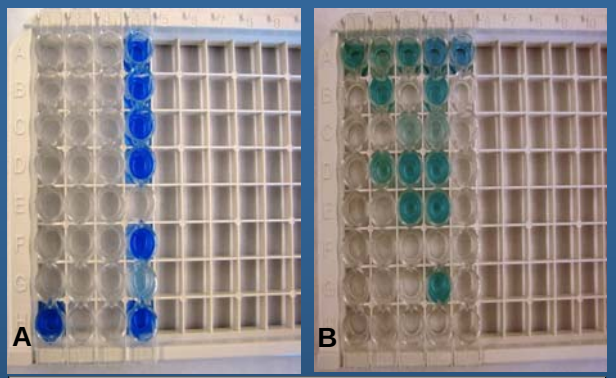
Methods and Materials

Collection of chitalpa and grape samples.
Samples were collected from chitalpa and grape that had produced variable results when previously tested using ELISA and PCR. The variability of these results was presumed to be related to variability of the bacterial titer within the plant, or overall low bacterial titer in the plant. Samples consisted of branches or vines 5 to 7 mm in diameter with the leaves attached. The samples were then placed in individual plastic bags which were labeled and stored at 4°C.

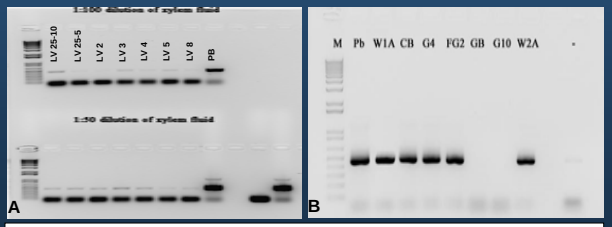
Extraction techniques and ELISA for *Xylella fastidiosa*.
The presence of *X. fastidiosa* was tested by ELISA using four different extraction methods: a hammer, a pressure chamber, a mortar and pestle, and a mini-bead beater. For the first method, 0.3 grams of leaf petioles and the mid-veins were placed in plastic samples bags with 3 ml of general extraction buffer 3 (Agdia, Inc. Elkhart IN) and the tissue was crushed with the use of a hammer at room temperature. In the second method, the sap was extracted from plant tissue branches using a pressure chamber (Soil moisture Equipment, Santa Barbara CA) pressurized with compressed nitrogen gas. Sap was obtained between 20 and 40 bars of pressure. The third extraction method involved grinding 0.3 grams of leaf petioles and the mid-veins in liquid nitrogen with a mortar and pestle. The ground plant tissue was then placed into a plastic sample bag with 3 ml of general extraction buffer 3. The forth and final extraction method used 0.3 grams of leaf petioles and mid-veins that were crushed using the mini-bead beater 96+ (Biospec products inc.) with 3 ml of general extraction buffer 3 at room temperature. To complete the ELISA test, 100 ul of the extracted sap and buffer solution were loaded into microtiter plates pre-coated with *X. fastidiosa* specific antibodies (Xf. PathoScreen Kit, AgDia, Inc.). Sap extracted using the pressure chamber was mixed 50:50 with general extraction buffer 3 and 100 ul of the mixture was loaded in the microtiter plate. The microtiter plates were process according to the manufacturer's instructions (Agdia Inc.). Results were analyzed for the presence of color and using a plate reader (Bio-Tek KC4 v.3.1) at 620 nm. All test plates included at least 1 method control and negative control (buffer only) and samples were considered positive at two times the background of the negative control.

PCR Procedure for detection of Xylella fastidiosa.
Total DNA expressed in xylem fluid (diluted 1:100 or 1:50) obtained from the pressure chamber was used for PCR analysis. The components to the PCR reaction included 1X PCR Buffer (100mM Tris-HCl, 500mM KCl, pH 8.3), 1.5mM MgCl2, 0.2mM dNTP's, 0.1 ng of each primer(272-1 and 272-2 external and internal primers), two units of Taq Polymerase and 1ul sap. The reaction conditions were as follows: an initial denaturation step of 95°C for 2 minutes, thirty-five cycles of the following: 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 2 minutes, with a final elongation step of 72°C for 5 minutes. The products were then separated on a 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light with the Kodak 2000R Station.

Chitalpa trees in Southwest New Mexico. Although these trees look similar the tree on the top (A) produces inconsistent ELISA results and the tree on the bottom (B) always produces a strong positive result. (C) Typical leaf symptoms on chitalpa trees – some leaves test positive for *Xylella fastidiosa* while others test negative.



ELISA plate results evaluating *Xylella fastidiosa* extraction techniques from grape (A) and chitalpa (B). Columns 1 – 4 are different extraction techniques: 1) mini-bead beater, 2) mortar and pestle, 3) hammer, and 4) pressure chamber. The grape samples were randomly selected from a *Xylella* infected vineyard. The chitalpa samples were collected from landscapes in southwest New Mexico and from nursery stock.



Amplified products of grape and chitalpa samples. *X. fastidiosa* nested PCR products were amplified from grapes (A) and chitalpa trees (B) were separated on a 1% agarose ethidium bromide stained gel. In gel B the sample labeled W1A was a 1:50 dilution of xylem fluid, the remaining samples were all 1:100 dilution. PCR was determined to be positive or negative by the presence of a product the correct size on the agarose gel.

Chitalpa	No. ELISA	Result	%	PCR	Culture
PB	50	+	80	+	+
	11	+/-	18		
	1	-	2		
W1A	18	+	90	+	-
	2	-	10		
CB	17	+	24	+	+
	3	+/-	4		
	50	-	72		
W2A	6	+	50	+	+
	6	-	50	-	
FG2	24	+	77	+	-
	7	-	23	-	
GUB	2	+	19	+	dnp
	9	-	81		
GU4	1	+	17	+	dnp
	2	+/-	33		
	3	-	50		
GU10	1	+	14	-	dnp
	1	+/-	14		
	5	-	72		

Grape	No ELISA	Result	%	PCR	Culture
	1	+	8	+	+
	1	+/-	8		
LV-2	11	-	84		
	2	+	16	+	-
LV-3	11	-	84		
	2	+	17	+	-
	1	+/-	8	-	
LV-4	9	-	75		
	1	+	9	+	-
LV-5	10	-	91		
	2	+	18	+	-
	1	+/-	9		
LV-8	8	-	73		
	1	+	20	+	-
LV-25-5	4	-	80	-	
	1	+	20	+	-
LV-25-10	4	-	80	-	

Summary of chitalpa and grape results from June 2006 to present. The table displays the plant designation, how many times each plant was tested by ELISA, the result obtained, the percentage of time each result was observed, and the PCR and culture outcomes. Several plants were never plated for culture which is designated by dnp.

Results and Discussion

Results from these experiments indicate that all of the sap-extraction techniques tested work well for some chitalpa trees; however only sap extracted using the pressure chamber could consistently match detection using PCR. In this experiment the pressure chamber matched the PCR results 100% of the time, the hammer matched 75% of the time, the mortar and pestle matched 62.5% and the mini bead beater 37.5%.

For grapevines, sap extraction using the pressure chamber matched detection using PCR 87% of the time. The pressure chamber is the only extraction method that detected *Xylella* in grapes.

We hypothesize that the inconsistency in results is due to within-plant and/or plant-to-plant variability in bacterial titer. These problems can be minimized by using a pressure chamber to extract xylem fluid containing the bacteria. By using the pressure chamber, the amount of tissue tested is increased, thereby reducing the plant-to-plant and within-plant variability.

Future Directions

- We are currently using quantitative polymerase chain reaction (Q-PCR) data to aid in identifying the variability of *X. fastidiosa* in plants.
- We are evaluating the use of the pressure chamber as an extraction technique for other plant species.

References

1. Hopkins, D. L. 1999. *Xylella fastidiosa*: Xylem-limited bacterial pathogen of plants. Ann. Rev. Phytopathol. 27:271-290.
2. Krell, R. K., Perring, T. M., Park, C. A., Park, Y-L., and Gispert, C. 2006. Intraplant sampling of grapevines for Pierce's disease diagnosis. Plant Dis. 90:351-357.

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