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Plant Pathology Fact Sheet

Real-Time PCR Detection of *Xylella fastidiosa* is Independent of Sample Storage Time and Temperature

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Abstract Leaf petioles collected for isolation of *Xylella fastidiosa* are usually processed within 12 hours of collection in order to optimize culturing the fastidious bacterium. However, for detection by other means, leaf samples from symptomatic shade trees, shrubs, and grapes are often sent to our diagnostic laboratory several days after being collected. ELISA-positive samples with weak to moderate ELISA scores sometimes yield a weak positive reaction by PCR, suggesting the possibility that sample handling conditions may have detrimental effects on detection of *X. fastidiosa*. This study suggests that neither storage temperature (RT, 4ºC, -20ºC, or -80ºC) nor duration of storage (≤24 hours or 6 days) negatively affects detectability of *Xylella fastidiosa* by real-time PCR in petioles. The use of ELISA sample extract as substrate for *X. fastidiosa* DNA extraction reduced the amount of time and effort required to conduct PCR detection in bacterial leaf scorch suspects, compared to "TE bacterial release" (pulverized infected tissue resuspended in TE buffer and used directly in PCR reactions without DNA extraction) or total DNA purification by QIAamp® DNA Stool Kit.

Introduction

The xylem-limited bacterium *Xylella fastidiosa*, first associated with Pierce's disease of grapevines and alfalfa dwarf disease in 1973 (4) continues to be an economically important pathogen of several commercial crops. It also causes bacterial leaf scorch in urban shade trees such as sycamore, oaks, maples, mulberry, and elm (5). The usual course of action, in an effort to control the spread of this pathogen by insect vectors (9), is to prune out infected branches and vines or to rogue infected plants. Therefore, timely testing of suspect hosts is important.

Leaf samples showing symptoms are typically tested via ELISA and/or Taqman® PCR for the presence of *X. fastidiosa* several days after being collected. Assessing samples for *X. fastidiosa* by PCR requires several steps during which delays can, and often do, occur. These delays are often

due to the distances separating the collection location and the assay lab. Samples may be delayed at their origin prior to shipping, or for the sake of efficiency in the assay lab itself, in order to have several samples to test simultaneously. At each of these delays, samples are sometimes stored as desiccating twigs or branches, or as leaves placed in plastic bags; also, they are sometimes either kept at room temperature on a bench or placed at 4ºC. In our experience with these samples, ELISA scores ranging from negative to very strong positives often appear to be independent of the intensity of symptoms seen on the leaf margins. ELISA-positive samples with weak to moderate ELISA scores sometimes yield a weak positive reaction by PCR. Since PCR has become an accepted method of detection of *X. fastidiosa* due to its much greater detection sensitivity compared to ELISA (12), weak PCR-positive values in stored ELISA-positive samples suggested the possibility that sample storage conditions may have had detrimental effects on detection of *X. fastidiosa* by PCR.

The objectives of this study were: (a) to investigate whether sample storage conditions and duration affected detectability of *X. fastidiosa* by real-time PCR, and (b) to evaluate DNA extraction methods for use on host tissues infected by *X. fastidiosa* to determine the quickest method without impacting detectability of the bacterium.

Effects of Sample Storage Time and Temperature

Shoots from suspect trees or shrubs were collected and taken directly to the laboratory where processing was begun on the day of collection. Excised petioles were surface-sterilized twice for 2 min in 1% sodium hypochlorite, twice for 2 min in 70% ethanol, rinsed twice for one min in two changes of sterile reverse-osmosis (RO) water, and allowed to dry.

In order to test sample handling/storage parameters, surface-sterilized petioles were arbitrarily allocated among two storage times (≤24 h and 6 d) and storage temperatures (RT, 4ºC, -20ºC, or -80ºC). Samples consisted of field-collected shoots of *Acer griseum, Acer platanoides*, *Acer saccharum*, *Chionanthus virginicus, Clematis* sp., *Clethera* sp., *Fraxinus americana, Gallum odoratum, Kerria* sp., *Morus alba, Platanus occidentalis, Quercus palustris, Quercus rubra, Quercus shumardii, Stewartia* sp., *and Vitis vinifera.* Two data sets were available for this analysis: one consisting of 11 samples for which total sample DNA was quantified (see methods below), and 3 samples for which total sample DNA was unquantified; for the latter, all PCR reactions received a uniform volume of sample extract.

Surface-sterilized petioles were tested for the presence of *X. fastidiosa* by ELISA (AgDia® PathoScreen® Xf, cat# PSP34501, http://www.agdia.com/) within 24 hours of collection. Ten to 14 petioles (2007) or three petioles (2008 and 2009) per sample were ground in an AgDia® mesh sample bag along with a 10X volume (v/wt) of AgDia® ELISA general extraction buffer. Tissue was disrupted using a hammer to break petiole ends and then mashed with an AgDia® circular-bearing Tissue Homogenizer (cat# ACC00900) attached to a drill press to complete tissue disruption. One hundred µl of crude extract was added to the ELISA plate for antibody detection of the bacterium. The remaining crude extract was used for DNA extraction (hereafter referred to as "eDNA") using the DNeasy® Plant Mini Kit (Qiagen, http://www1.qiagen.com/, cat# 69104) as described below.

The PCR master mix consisted of (final concentrations, reaction volume=25 µl): 1X Epicentre® Biotechnologies FailSafe™ Probes Pre-mix #6 (cat# FSP51206), 500 nM each of primers XfF1 and XfR1 and 200 nM Taqman® probe XfP1 (11) with FAM and BHQ1 as the reporter dye and quencher, respectively; and 2.5 units of Failsafe™ Enzyme Blend (cat# FSE51100). For most samples, DNA was added as 10 ng extracted total DNA. For certain samples, DNA concentration was too low to achieve a 10-ng aliquot in the reaction tube; in those cases, the

maximum allowable volume of 8 µl was added to the PCR reaction. Negative and positive controls were 2 µl of molecular-grade water and 2 µl of known *X. fastidiosa* genomic DNA (American Type Culture Collection # 35881D), respectively. For each sample, a parallel control was tested and consisted of a sample reaction spiked with 2 µl of known *X. fastidiosa* genomic DNA in order to test for PCR inhibition (12). Reactions were amplified on a SmartCycler® II thermocycler (Cepheid, http://www.cepheid.com) with the following thermocycling conditions: a 95ºC hold for 1 min, followed by 40 cycles at 95ºC for 1 sec and 60ºC for 20 sec. In the parallel control, a failure to amplify was taken to be indicative of inhibition. For samples where PCR inhibition occurred, the sample was serially diluted tenfold until inhibition was overcome. Quantification of genomic DNA of *X. fastidiosa* in DNA extracts of samples were estimated against a standard curve of C_t vs. DNA concentration generated using known *X. fastidiosa* genomic DNA (ATCC #35881D). DNA concentrations were determined using Quant-It® dsDNA HS Assay Kit (cat# Q32851) from Invitrogen and the Invitrogen™ Qubit® fluorometer (cat# 32857). Estimates of amounts of genomic DNA of *X. fastidiosa* were expressed on a basis of pg per ng of sample total DNA or pg per µl of sample total DNA.

Data from samples subjected to the temperature (4 levels) and time (2 levels) conditions described above were subjected to analysis of variance following a 4X2 factorial design within a randomized complete block design, where plant samples were considered blocks. Because missing data created an unbalanced design, Type II sums of squares were used to evaluate treatment effects (10).

Because the storage temperature X duration interaction was non-significant (P=0.96, Tables 1 & 2), evaluation of main effects was possible. Storage temperature had no effect ($P \geq 0.15$) on the detectability of *X. fastidiosa* by PCR in petioles of shade trees and shrubs (Tables 1 & 2). Processing samples within 24 h did not result in improved detectability as compared to holding samples for six days. Indeed, in one of the two datasets (Table 1), detection was significantly better (P=0.059) after six days than 24 h, although we postulate that this could be an anomalous result. In both datasets, variability was substantial, as reflected in high standard errors (Tables 1 & 2) and coefficients of variation of 111% and 126% in datasets 1 and 2, respectively. In any case, our data suggest that among-petiole variability, as reported in sampling studies of grape petioles for detection of *X. fastidiosa* (6), is at least as important a factor in detectability of *X. fastidiosa* as the sample handling parameters included in this study. This suggests that it would be advisable to pool small subsamples of several petioles before DNA extraction and PCR.

Evaluation of Methods for Preparing PCR Template

TE bacterial release: Following the method of Chen et al.(3), surface-sterilized, excised petioles (~100 mg per sample) were finely chopped, placed into a Mini-BeadBeater tube, flash-frozen in liquid nitrogen, and pulverized as described above. To this pulverized tissue was added 500 µl of sterile elution buffer taken from a Qiagen DNA extraction kit (a Tris/EDTA buffer, pH 9.0) (1) and allowed to soak at RT for 15 min, vortexed for 10 sec, and centrifuged for 10 sec at 24,000 x g. A series of tenfold dilutions was conducted using sterile RO water. A 5-µl aliquot of this was used directly in the PCR reaction.

For all TE bacterial release samples, in order to overcome PCR inhibition, dilutions ranging from 1/100 to 1/10,000, were necessary (Table 3). In spite of the ease of disrupting suspect tissue directly in TE buffer and using that supernatant directly in the PCR reaction, the broad range of dilutions required to overcome PCR inhibition renders this an inefficient method due to the number of times the PCR had to be repeated until there was no longer evidence of PCR inhibition. Furthermore, excessive dilution runs the risk of a false negative for samples with very low pathogen titers.

Evaluation of ELISA Buffer Extract as a Source of DNA for Extraction: After removing a 100-µl aliquot for ELISA testing the remaining crude extract from the ELISA extraction was immediately transferred into one or two 1.5 ml microcentrifuge tubes and centrifuged at maximum speed for 15 min at RT in order to precipitate plant debris and any bacteria. After discarding the supernatant, each pellet was resuspended in 400 µl of AP1 Buffer from the Qiagen's DNeasy® Plant Mini Kit (cat# 69104) plus 4 µl RNase A by vortexing. DNA extraction was accomplished by following the DNeasy® protocol with the exception of incubation at 85°C for 5 min at step 8 of the procedure, instead of incubation at 65°C for 10 min. DNA was eluted with 100 µl AE elution buffer. After extraction, the DNA was stored at -20ºC until testing was completed.

eDNA was extracted from 23 samples, eight of which were also processed using the QIAamp® Stool kit, permitting a direct comparison using a paired *t*-test (8). Qiagen's QIAamp® DNA Stool Mini Kit (cat# 51504) was used (7) to extract total DNA from surface-sterilized subsamples of the samples also processed via eDNA/DNeasy® extraction. One hundred mg of finely chopped petiole (~1-2 mm) was placed into a Mini-BeadBeater 3110BX (BioSpec Products) tube without buffer, flash-frozen in liquid nitrogen, and pulverized at 2500 rpm at repeated 30-sec intervals, with flash-freezing between each beating. Immediately after pulverization, the DNA was extracted following kit instructions with the exception of elution in 100 µl volumes, and stored at -20ºC until testing was completed.

Our study validates the approach of Buzombo et al. (2) in that we found ELISA buffer extract provided amplifiable DNA template (Table 4). This is based on the observations that: (a) a *t*-test indicated no significant difference ($P>0.1$) in the quantity of *X. fastidiosa* DNA recovered from the eight samples processed by both the eDNA/DNeasy® method and the QIAamp® Stool kit method (Table 4); and (b) no PCR inhibition was observed in any sample DNA obtained by the eDNA/DNeasy® method, including 15 samples extracted only by the eDNA technique (*data not shown*). Using ELISA-buffer extract for both ELISA and for DNA extraction for PCR tests speeded sample processing substantially over QIAamp® Stool kit DNA extraction alone, which require approximately one additional hour for completion. Furthermore, using the same host tissue fragments for both ELISA and PCR addresses discrepancies that may be caused by non-uniform distribution of the pathogen in the host (6).

In summary, this study shows that bacterial leaf scorch suspect samples may remain at ambient temperature for up to six days after collection without adversely affecting detectability of *X. fastidiosa*. It also verifies that the use of ELISA extract remaining from the antibody test can successfully be used as a source of bacterial DNA for PCR and reduces preparation time and effort.

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Table 1. Mean concentration of *X. fastidiosa* genomic DNA obtained from plant samples stored at various times and temperatures, using eleven sample extracts having quantified total sample DNA.

Timing of DNA extraction	**RT[a]**	**4°C**	**-20°C**	**-80°C**
Within 24 h of collection	0.39 *(0.39)*[b]	1.1 *(0.39)*	0.52 *(0.38)*	0.44 *(0.38)*
Six days after collection	0.97 *(0.39)*	1.4 *(0.41)*	0.85 *(0.42)*	0.92 *(0.41)*

ANOVA source	df	MS[c]	*F*-value	*P*
Model	17	4.232	4.80	<0.0001
Plant sample (=blocking variable)	10	6.284	7.13	<0.0001
Temperature (RT, 4°, -20° & -80°C)	3	1.422	1.61	0.1956
Time (≤24 h v. 6 d)	1	3.264	3.70	0.0590
Time X Temp	3	0.088	0.10	0.9598
Error	60	0.881	-	-

[a]RT - ambient room temperature
[b]Mean genomic DNA content of *X. fastidiosa* in pg/ng total sample DNA extract (italicized values are standard errors), determined using quantitative real-time PCR (11). Least-square means generated using SAS PROC GLM (10).
[c]Mean squares for treatment factors are Type II mean squares (10).

Table 2. Mean concentration of *X. fastidiosa* genomic DNA obtained from plant samples stored at various times and temperatures, using three sample extracts for which quantitation of total sample DNA was unavailable[a].

Timing of DNA extraction	**RT[b]**	**4°C**	**-20°C**	**-80°C**
Within 24 h of collection	16 *(56)*[c]	17 *(56)*	45 *(62)*	103 *(56)*
Six days after collection	29 *(59)*	10 *(62)*	78 *(56)*	96 *(62)*

ANOVA source	df	MS[d]	*F*-value	*P*
Model	9	12549	3.31	0.0326
Plant sample (=blocking variable)	2	40412	10.67	0.0027
Temperature (RT, 4°, -20° & -80°C)	3	8232	2.17	0.1487
Time (≤24 h v. 6 d)	1	210.9	0.06	0.8178
Time X Temp	3	391.0	0.10	0.9564
Error	11	3787	-	-

[a]Uniform volumes of sample total DNA extract were added to PCR reactions.
[b]RT - ambient room temperature
[c]Mean genomic DNA content of *X. fastidiosa* in pg/ng total sample DNA extract (italicized values are standard errors), determined using quantitative real-time PCR (11). Least-square means generated using SAS PROC GLM (10).
[d]Mean squares for treatment factors are Type II mean squares (10).

Table 3. Dilutions of TE-released *X. fastidiosa* required to overcome PCR inhibition.

Host	**Sample ID**	**Dilution needed to overcome inhibition[a]**
Vitis vinifera	1685-09	1/100
Quercus sp	1702-09	1/10,000
Platanus occidentalis	1718-09	1/10,000
Stewartia sp	1744-09	1/100
Acer saccharum	1768-09	1/10,000
Quercus sp.	1770-09	1/10,000
Clethera sp.	1779-09	1/1000
Clematis sp.	1780-09	1/1000
Gallum odoratum	1781-09	1/1000
Kerria sp.	1782-09	1/1000
Kerria sp.	1783-09	1/1000
Kerria japonica, variegated	not numbered	1/1000

[a]Inhibition was considered to be overcome by dilution if the Ct value of the parallel control was greater than 0.

Table 4. Evaluation of the suitability of using ELISA buffer sample extract as a source of DNA for PCR amplification

Sample ID	Host plant	ELISA value [a]	Amount (pg) Xf [b] DNA per µl of "eDNA" [c]	Amount (pg) Xf DNA per ng total DNA extracted using QIAamp® Stool DNA kit
41BA09w[e]	*Acer griseum*	++	17.4	17.05
41BA09st[e]	*Acer griseum*	+++	58.1	16.47
76BA09 early[e]	*Quercus rubra*	nd	10.0	5.84
76BA09w[e]	*Quercus rubra*	+ weak	6.2	3.15
76BA09st[e]	*Quercus rubra*	+ weak	25.6	0
93BA09w[e]	*Acer platanoides*	Neg	0	0
106H09w[e]	*Chionanthus virginicus*	+ weak	0	0
106H09st[e]	*Chionanthus virginicus*	+ weak	0	0

[a]ELISA values are subjectively determined based on color reaction intensity
[b]Xf =*Xylella fastidiosa*
[c]ELISA extract was used as source of sample materials for DNA extraction using Qiagen DNeasy® Plant Kit. .
[d]"-" - not tested
[e]Samples used in *t*-test comparison of yields of eDNA vs. DNA extracted via the QIAamp® Stool Kit.

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