

Improved Sampling Methods for Real-Time Polymerase Chain Reaction Diagnosis of Citrus Canker from Field Samples

Vessela Mavrodieva, Laurene Levy, and Dean W. Gabriel

First and second authors: U.S. Department of Agriculture APHIS, PPQ, CPHST-NPGQC, Beltsville, MD 20705; and third author: Plant Pathology Department, University of Florida, 1453 Fifield Hall, Gainesville 32611.
Current address of V. Mavrodieva: North Carolina State University, Department of Plant Pathology, Raleigh 27695.
Accepted for publication 14 August 2003.

ABSTRACT

Mavrodieva, V., Levy, L., and Gabriel, D. W. 2004. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. *Phytopathology* 94:61-68.

Citrus bacterial canker disease has been introduced at least three times into Florida in the last 15 years and, despite federal and state quarantine and eradication efforts, continues to spread in Florida. Accurate, fast, and reliable detection of the causal agent is of great importance. However, citrus bacterial canker is caused by at least two groups of phylogenetically distinct *Xanthomonas citri* strains, and there is host range variation within both groups. We developed a fast, sensitive and reliable real-time

polymerase chain reaction (PCR) assay using a portable, field-hardened RAPID machine and primers designed to detect all canker-causing strains. Single-lesion sampling methods were developed that required minimal handling and allowed complete real-time PCR diagnosis in a total time of 4 h and with an apparent sensitivity of less than 10 CFU of target cells from diseased lesions. This sensitivity allowed molecular detection for the first time of *X. citri* in a herbarium sample from a 1912 canker outbreak. Sensitivity was improved significantly by the use of CaCO₃ and Silwet L-77, and by either minimizing the amount of citrus lesion tissue sampled or by soaking or swiping but not grinding the lesions. Primer design also was of significant importance in both specificity and sensitivity.

The increasing presence of Asiatic citrus canker in Florida now threatens various aspects of its \$9 billion dollar industry. The disease was first identified in the United States in Florida in 1912 and subsequently was eradicated (31). A second outbreak of Asiatic canker occurred in 1986 (11,30) and was declared eradicated in 1994. In 1995, a third Asiatic citrus canker outbreak was found in urban Miami (12,27). In 1997, a new outbreak of Asiatic citrus canker was found on the west coast of Florida, which possibly was the result of incomplete eradication of the 1986 outbreak (26). Finally, a fifth outbreak, caused by a new strain of citrus canker, termed the "Wellington" strain (A^W), was found in Florida in 1999 (32). A joint state and federal effort to eradicate citrus canker, the Florida Citrus Canker Eradication Program (CCEP), is underway, managed by APHIS and the Florida Department of Agriculture and Consumer Services (FDACS). The cost of the CCEP currently exceeds \$200 million (1). For comprehensive reviews of citrus canker biology, spread, and eradication in Florida, please refer to Schubert et al. (26) and Gottwald et al. (13).

Citrus canker spreads locally and over short distances by wind-driven rain and sometimes by high-pressure sprays. Long-distance spread occurs from the movement of infected plant materials or contaminated equipment. Detection of citrus canker bacteria on equipment, on exposed trees, or in preclinical lesions (less than 3 months old) is difficult. Introduction of the citrus leaf miner, *Phyllocnistis citrella* Stainton, into Florida in 1993 (16) complicates visual canker detection by inspectors because leaf miners cause extensive damage to the foliage. Leaf miners also increase the level of citrus canker inoculum in infected leaves by both wounding leaves and spreading bacterial infection within the

leaf during feeding and formation of extensive galleries within leaves (13).

Despite the best efforts of government and state agencies and the citrus industry, citrus canker still is spreading in Florida, and the infected area under quarantine continues to grow. In 1995, the infected area was 14 mi² (36.3 km²) in a residential area of Miami. In the spring of 2002, the infected area encompassed 657 mi² (1,702 km²) and over 2.1 million infected commercial, residential, or exposed residential trees have been removed. A diagnosis of citrus canker results in the destruction of infected trees and also all "exposed" trees—those within a radius of 1,900 ft (589 m) of any infected tree (an area of ≈0.4 mi² or 1 km²). In addition to the removal of infected and exposed trees, successful control and eradication requires reliable and fast on-site diagnosis of the disease both within the quarantine zones and at ports of entry to prevent new introductions.

Any strain capable of causing cankers (hyperplastic symptoms) on any citrus host is subject to quarantine and eradication; therefore, accurate detection and diagnosis of any and all canker-causing strains, including new ones, regardless of origin or host range, is of extreme importance for successful control and eradication of the disease. Currently, citrus canker is detected visually by identification of disease symptoms on infected trees. Suspect positive samples are sent to diagnostic labs, where the lesions are examined and bacterial cultures are taken. These cultures then are used for reinoculation on citrus and for enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) testing. The need for rapid methods of canker detection to complement and confirm visual detection is obvious. Laboratory tests to confirm visually suspected canker infections are complicated by phylogenetic variation among canker-causing xanthomonads.

Throughout this article, we use *Xanthomonas citri* to refer to any xanthomonad capable of causing hyperplastic cankers on citrus, and nomenclature for its pathovars as proposed by Brunings and Gabriel (2). Citrus canker disease is caused by two phylogenetically distinct, clonal groups of *Xanthomonas* strains, one

Corresponding author: D. W. Gabriel; E-mail address: gabriel@biotech.ufl.edu

Publication no. P-2003-1024-01R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2004.

originating from Asia (*X. citri* pv. *citri* groups) and the other from South America (*X. citri* pv. *aurantifolii* groups); each group has subgroups that differ significantly in host range and, therefore, also are true pathogenic variants (2,8,9). By far the most predominant and widespread subgroup or pathovar is the Asiatic or *X. citri* pv. *citri* A strains, with a host range that includes all citrus cultivars. Much less widespread are the *X. citri* pv. *citri* A* strains from southwest Asia (40). Most recently, a third pathogenic variant strain, apparently of Asiatic origin, designated A^W, was found in one location in Florida (32). The South American citrus canker strains also comprise at least two subgroups or pathovars. Symptoms on susceptible hosts caused by the South American strains are identical to those caused by Asiatic strains, but all South American strains are limited in host range: *X. citri* pv. *aurantifolii* B strains are found primarily on lemon and lime, and *X. citri* pv. *aurantifolii* C strains are limited to Mexican lime.

Identical symptoms induced by phylogenetically distinct groups of strains is indicative of a common pathogenicity factor. Gene *pthA* is essential for *X. citri* pv. *citri* A to elicit cankers on citrus, and *pthA* confers this ability to various *X. campestris* strains (for example, *X. campestris* pvs. *alfalfae* and *citrumelo*) (33,34). Functionally homologous genes (*pthB* and *pthC*) also have been identified and cloned from *X. citri* pv. *aurantifolii* B and C, respectively (7). Both *pthB* and *pthC* are essential for *X. citri* pv. *aurantifolii* B and C strains, respectively, to cause cankers on citrus, and both *pthB* and *pthC* confer this ability to various *X. campestris* strains (7). All three genes, therefore, are functionally interchangeable, and these genes may have been transferred horizontally on plasmids between *X. citri* and *X. citri* pv. *aurantifolii* strains (2). These genes belong to a large family of avirulence or pathogenicity genes found only in the genus *Xanthomonas* (the *avrBs3/pthA* gene family); all members of the *pthA* gene family are long (>3.8 kb) and all have a remarkably high level of DNA sequence identity (>90%) over their entire lengths (6,18). Based on DNA hybridizations, apparently homologous genes are found in all canker-causing strains, and have not been found in non-canker-inducing strains isolated from citrus, such as *X. campestris* pv. *citrumelo* (3,7). Therefore, a single common gene appears to be diagnostic (and necessary) for a *Xanthomonas* strain's ability to induce cankers on citrus (2).

All existing conventional PCR methods for canker detection require gel visualization or utilize primers that do not detect all strains (4,14,15). For example, PCR primers designed by Hartung et al. (14,15) and by Kingsley et al. (17) work very well for detection of *X. citri* pv. *citri* A, but neither are consistent for detection of *X. citri* pv. *aurantifolii* B or C strains. Recently, Cubero and Graham (3) described new PCR primers based on the *pthA* gene sequence that failed to detect only one canker strain tested. There is an urgent need for a rapid assay with universal and highly specific primers able to detect all canker-causing strains. To increase the speed of diagnosis, the assay also needs to (i) be portable, (ii) require minimal sample handling, and (iii) work with suboptimal (old, partially degraded, or stored) field samples.

Real-time PCR technology offers sensitive, reliable, and fast detection and identification of plant pathogens. Compared with conventional PCR, real-time PCR is simpler to perform, less

labor-intensive (no agarose gels required), and much faster. Accurate results can be observed in less than 1 h if the sampling method is reliable. Real-time PCR is becoming increasingly useful for the detection of all plant pathogens: fungi (22,38,39), bacteria (20,24,25,37), and viruses (21,23,28). For a comprehensive review of real-time PCR principles and amplicon detection methods, refer to Mackay et al. (19). We report here a sensitive and reliable SYBR Green real-time PCR assay based on sampling methods performed on field samples using primers designed to amplify conserved regions of a required pathogenicity gene and, therefore, capable of detecting all known strains of citrus canker.

MATERIALS AND METHODS

Citrus canker strains. Citrus canker strains used for developing the assay are listed in Table 1.

Diseased citrus samples. Field samples of citrus leaves and fruit infected with canker in Florida were provided by the FDACS, Division of Plant Industry (DPI) at their Quarantine Greenhouse in Gainesville, FL or were taken directly in the field in Florida by United States Department of Agriculture APHIS personnel. For test development, grapefruit leaves were artificially infected with canker by dipping citrus leaves in *X. citri* pv. *citri* A 3213^T at ≈5,000 CFU/ml in CaCO₃-saturated sterile tap water containing Silwet L-77 (OSI Specialties, Inc., Friendly, WV) at 200 ppm. This resulted in highly uniform inoculations of well-dispersed citrus canker lesions. All such inoculations were performed in the FDACS/DPI quarantine greenhouse. Healthy, uninfected samples were taken from a different (canker-free) greenhouse.

PCR primers. Primers were designed using Primer Express software (version 2.0; Applied Biosystems, Foster City, CA). They were designed specifically for real-time PCR and selected to amplify relatively short fragments from highly conserved regions found in all members in the *pthA* gene family, including: *pthA* (GenBank accession U28802), *pthB* (Gabriel lab, unpublished sequence), *pthC* (Gabriel lab, unpublished sequence), *apl1* (AB021363), *apl2* (AB021364), *apl3* (AB021365), *pthA1*, *pthA2*, *pthA3*, and *pthA4* (the latter four all in GenBank Accession No. AE008925). Three pairs of primers were evaluated and their annealing positions on *pthA* (U28802) are as follows (odd number for forward primers, even for reverse): VM1 (5'-GATCAAAA-GAACCAATCGCCGTAT; 3,388 to 3,411), VM2 (5'-GGGAGT-GGCACTGGAAAAACC; 3,467 to 3,488), VM3 (5'-GCATTT-GATGACGCCATGAC; 3,500 to 3,519), VM4 (5'-TCCCTGAT-GCCTGGAGGATA; 3,631 to 3,650), VM5 (5'-AACAGGAG-AAGATCAAACCGAAGGTT; 1,086 to 1,111), and VM6 (5'-GTTTGCGCACGCCAACGAT; 1,268 to 1,286). Another pair of real-time PCR primers designed by Kingsley et al. (17) also were evaluated and are referred to as Kingsley's primers in the remainder of the article (Kingsley forward, 5'-TCCACTGCATC-CCACATCTG; Kingsley reverse, 5'-CAGGTGTACTGCGCTCT-TCTTG; GenBank Accession No. AF312370). These primers appear to anneal to the *X. citri* pv. *citri* A chromosome (GenBank Accession No. NC_003919) from positions 1,217,824 to 1,217,949, and amplify an apparently noncoding intergeneric region between genes XAC1061 and XAC1062.

TABLE 1. Citrus canker strains used

Strains	Strain description	Host range	Source or reference
<i>Xanthomonas citri</i> pv. <i>citri</i> 3213 ^T	Species type strain, A strain from Florida	All citrus species	Gabriel et al. 1989 (9)
3213Sp ^R Rif ^R	Spontaneous Sp ^R , Rif ^R derivative of Xc 3213 ^T	All citrus species	Gabriel lab strain
205	A* strain from Oman	Lime	Verniere et al. 1998 (40)
0053	A ^W strain from Florida	Lime	Sun et al. 2000 (32)
<i>X. citri</i> pv. <i>aurantifolii</i>			
B69	B strain from South America	Lemon, lime	Gabriel et al. 1988 (9)
C340	C strain from South America	Lime	Gabriel et al. 1988 (9)
<i>X. campestris</i> pv. <i>citrumelo</i> 3048	Bacterial spot strain from Florida	Nursery citrus of many cultivars	Gabriel et al. 1989 (10)

Real-time PCR conditions. Real-time PCR detection of citrus canker was performed on a mobile RAPID 7200 system (Idaho Technology, Salt Lake City, UT) using SYBR Green I (Molecular Probes, Eugene, OR) fluorescent dye detection. Each sample was run in duplicate in glass capillaries tubes (Roche Diagnostics, Indianapolis, IN). Every run included negative (no target DNA) and positive (target DNA) controls. The PCR reactions contained 2 μ l of sample supernatant or purified DNA extracted from culture-grown cells added to 18 μ l of reaction mix. Reaction mix consisted of 1 \times PCR buffer with bovine serum albumin (Idaho Technology), 2 mM MgCl₂, 0.5 μ M each primer, 0.2 mM dNTPs, 2 μ l of 1:3,000 SYBR Green I, and 0.8 units of *Taq* Polymerase (Roche Diagnostics). For hot-start PCR, *Taq* Polymerase was incubated with TaqStart Antibody (Clontech, Palo Alto, CA) 1:28 (vol/vol) for 10 min at room temperature before adding to the reaction mix. The PCR program conditions for the VM3 and 4 primer pair are given as "Program 1"; PCR program conditions using Kingsley's primers were performed according to Kingsley et al. (17) and are given as "Program 2" in Table 2.

PCR assay optimization, crossing point definition, and melting curve analysis. In real-time PCR, the total SYBR Green fluorescence detected after each amplification cycle provides a measure of PCR product formation. The process is monitored in real time and graphically displayed as a semilog curve of the accumulating SYBR Green fluorescence over the number of cycles (Fig. 1A). The crossing point (Cp) of each graphical curve is defined as the cycle in which the level of fluorescence of the particular sample increases above the background. The Cp is used as one criterion to determine target presence or absence and also indicates the concentration of the target. Higher target concentrations will result in lower Cp values, thus allowing reliable quantification of purified DNA (Fig. 1B).

A melting curve analysis was used as a second criterion to determine target presence or absence, because it assists in detecting false-positive PCR product formation. Each PCR product exhibits a characteristic peak at its melting temperature (T_m) maximum. The melting curve analysis was used to identify nontarget PCR product and primer-dimer formation. All results reported are based on both the Cp values and melting curve analyses.

PCR assay optimization was performed using 10 ng of purified DNA from all 5 citrus canker inducing strains and from *X. campestris* pv. *citrumelo*. A water control also was included. All three primer pairs were evaluated at 2, 3, 4, and 5 mM concentrations of Mg and annealing temperatures of 55 and 57°C. Kingsley's primers were run at the recommended conditions (Table 2, program 2). Each sample (10 μ l) was loaded on 2% agarose gels and stained with ethidium bromide to make sure that the Cp and

melting peak observed for each sample in real-time PCR corresponded to a band of expected size on the gel.

Real-time PCR sensitivity and reproducibility. Ten-fold serial dilutions of purified *X. citri* pv. *aurantifolii* B DNA were run six times and the CV (mean coefficient of variation) of the Cp values estimated. DNA (10 to 15 μ g) was used as a positive control in every test run, and these controls always gave Cp values between 17 and 20. Additionally, *X. citri* pv. *citri* A 3213 and *X. citri* pv. *aurantifolii* B69 cells were cultured in peptone, yeast

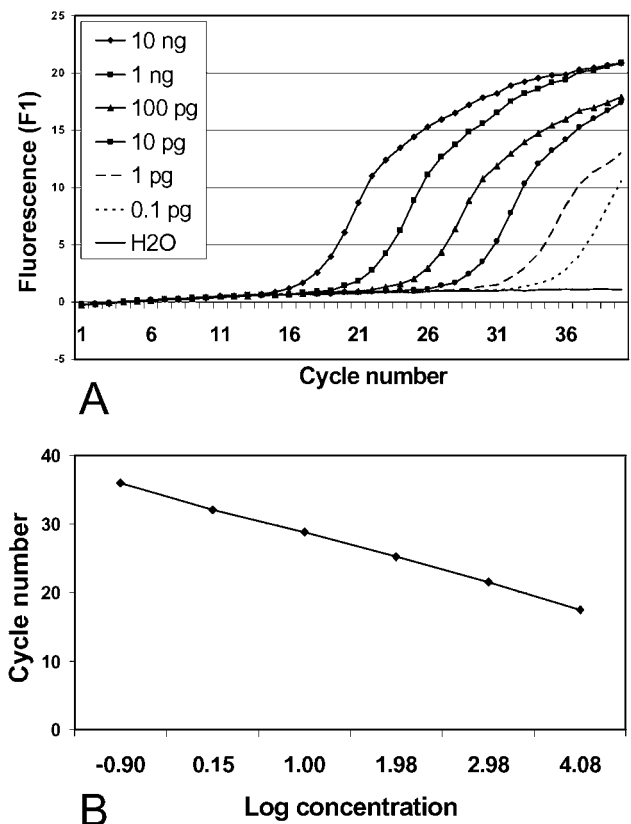


Fig. 1. Real-time polymerase chain reaction assay optimization with the VM3 and 4 primer pair illustrating sensitivity and linearity. **A**, Amplification plot of 10-fold serial dilutions of *Xanthomonas citri* pv. *aurantifolii* B DNA control in a single experiment. **B**, Standard curve for the same experiment, based on the calculated serial dilution points and corresponding crossing point (Cp) values. Standard curve parameters: Slope, -3.739 ; intercept, 32.65 ; error, 0.168 ; and r , -1.00 .

TABLE 2. Real-time polymerase chain reaction program parameters set up^a

Program	Number of cycles	Steps	Temperature (°C)	Hold time (s)	Temperature transition rate (°C/s)	Fluorescence
Program 1						
Denaturation	1	1	95	120	20	None
Amplification	40	1 (denature)	95	5	20	None
	...	2 (anneal)	57	0	20	None
	...	3 (extension)	72	12	2	Single
Melting curve analysis	1	1 (denature)	95	5	20	None
	...	2 (renature)	40	90	20	None
	...	3 (slow melt)	97	0	0.1	Continuous
Program 2						
Denaturation	1	1	95	120	20	None
Amplification	40	1	95	5	20	None
	...	2	54	0	20	None
	...	3	72	15	2	Single
	...	1	95	5	20	None
Melting curve analysis	...	2	40	90	20	None
	...	3	95	0	0.1	Continuous

^a Each program follows the indicated processes of denaturation, amplification, and melting curve analysis; the steps within each process are repeated in the indicated number of cycles. Fluorescence was acquired in channel F1 and the gain setting was 8.

extract, glucose, and MOPS (PYGM) (10) liquid medium to an optical density of 0.5 at 600 nm. Ten-fold serial dilutions of these cultures were made and used for real-time PCR with VM3 and 4 primers. The same dilutions were used for cell count—5 µl of each dilution was plated on PYGM agar, plates were incubated for 18 to 24 h at 30°C, and CFU were counted.

Citrus canker detection in greenhouse and field samples—sample preparation. Single lesions of similar size and condition were cut out of infected citrus leaves using a no. 1 (smallest standard, ≈4 mm in diameter) or no. 3 (≈6 mm in diameter) cork borer. For experiments designed to optimize extraction buffers and protocols, nearly identical, young, single lesions of similar age were placed in Eppendorf tubes containing 100 µl of one of the following solutions: (i) sterile tap water; (ii) phosphate-buffered saline (0.7%) in sterile tap water, pH 7.0 (PBS); (iii) saturated CaCO₃ in sterile tap water; (iv) Silwet L-77 in sterile tap water 1:5,000 (vol/vol); (v) Silwet L-77 in saturated CaCO₃ in sterile tap water 1:5,000 (vol/vol); or (vi) 5% Chelex 100 (Sigma Chemical, St. Louis) in Silwet L-77 in saturated CaCO₃ in sterile tap water 1:5,000 (vol/vol). Some of the samples were crushed with plastic disposable pestles (Kontes Scientific, Vineland, NJ) directly in 1.5-ml Eppendorf tubes after combining with extraction solution.

All samples, whether crushed or not, were shaken vigorously on a reciprocal shaker (250 cycles/min) for 1 h at room temperature and plant debris were pelleted by centrifugation for 5 min at 5,000 rpm. The resulting supernatants were transferred to new tubes and 2 µl of each was used directly for real-time PCR assays. To evaluate assay sensitivity with leaf samples, 10-fold serial dilutions of the supernatants were made and tested by real-time PCR. For viable cell counts, 2-µl droplets of the same dilutions were plated in duplicates on PYGM agar, the plates were incubated at 30°C, and CFU were counted. When artificial inoculations of 3213SpR were used, rifampicin (75 µg/ml) and spectinomycin (35 µg/ml) were added to the agar medium.

In order to compare effectiveness of the different sample preparations from multiple experiments using canker lesions of different age and quality, a scoring scale of 0 to 4 was used to semi-quantify target concentrations in terms of Cp values. A score of 0 indicated no detectable target (no Cp and Tm peak); a score of 4 indicated the highest level of target concentration. A score of 4 was assigned when the Cp occurred before the twentieth PCR cycle (Cp value of <20); scores of 3, 2, and 1 indicated Cp values of 20 to 30, 30 to 35, and 35 to 40, respectively. In all cases except 0, a specific Tm peak was confirmed to be present.

Field sample detection using the IT 1-2-3 RAPID DNA purification kit. The IT 1-2-3 RAPID DNA Purification Kit (the IT kit; Idaho Technology) was used in two double-blind tests. Field samples from freshly discovered canker-infected lesions and also from healthy leaves and fruit were collected from canker-infested zones in Florida. These samples were taken from lesions that were identified unambiguously in the field as canker infected. Leaves and fruit were simply swiped with moistened swabs provided with the kit. The swabs then were placed inside tubes containing ceramic beads suspended in water provided with the

kit, and the samples were encoded and mailed under quarantine permit for lab processing. Samples collected in this manner were stored for up to 1 week at 4°C prior to processing. Samples were processed according to the manufacturer's instructions. Basically, samples were vortexed for 5 min and DNA extraction buffer provided in the kit (guanidine HCL, isopropanol, and sodium acetate) was added. The solution was centrifuged and the supernatant run over a filtration spin column provided. The column was washed once and the DNA was eluted in 100 µl of elution buffer, and used directly for real-time PCR analysis. After performing the PCR tests, results were recorded and sample numbers were decoded.

Herbarium samples. Dry, nonviable herbarium samples collected during the 1912 Florida canker outbreak were received from the FDACS (Gainesville) collection and processed in APHIS labs in Beltsville. Extreme care was exercised to open and process the herbarium samples in lab areas not exposed to canker. Single lesions were cut out of dried citrus leaves with individual razor blades, placed in Eppendorf tubes, and crushed in CaCO₃-Silwet L-77-Chelex solution. The above-described extraction and real-time PCR protocols were applied using the VM3 and 4 pair and with Kingsley's primers. In addition, 20 µl of the crude sample extract supernatant was used for DNA extraction using the IT kit according to the manufacturer's instructions. Precautions were taken to avoid carryover or cross contamination. The expected PCR products were confirmed by DNA sequencing.

RESULTS

Primer specificity. Using extracted total DNA and culture-grown cells, the VM3 and 4 pair of primers was found to work best at 57°C annealing temperature and 2 mM Mg²⁺, consistently and reliably giving the expected 150-bp product for representative strains from all five known citrus canker groups or pathovars from Asia or South America: *X. citri* pv. *citri* A, *X. citri* pv. *aurantifolii* B and C, and *X. citri* pv. *citri* A* and A^w. Under these conditions, all samples had the lowest Cp and highest fluorescence. Melting curve analyses revealed a Tm peak of 90°C (±1°C) for the 150-bp products from all representative strains. No fragments were pro-

TABLE 4. Combined real-time polymerase chain reaction scores using different sample preparation methods for both field and greenhouse samples^a

Sample preparation	No. 3 cork borer	No. 1 cork borer	Total score
Phosphate-buffered saline	1.2	2.6	1.8
CaCO ₃ -H ₂ O	1.9	2.7	2.0
Silwet L-77-H ₂ O	0.9	2.5	1.4
CaCO ₃ -Silwet L-77	2.3	2.9	2.3
CaCO ₃ -Silwet L-77, crushed	1.5	2.3	1.6
CaCO ₃ -Silwet L-77-Chelex, crushed	2.1	3.1	2.7

^a The total score for each preparation represents the average of at least 10 independent experiments using old, dry field samples, relatively fresh field samples, and fresh greenhouse-inoculated samples. Mean score was based on crossing point (Cp) values: 4, Cp < 20 cycles; 3, Cp = 20 to 30; 2, Cp = 30 to 35; 1, Cp = 35 to 40, and 0, no detected Cp or Cp > 40.

TABLE 3. Reproducibility of real-time polymerase chain reaction with the VM3 and 4 primer pair using purified *Xanthomonas citri* pv. *aurantifolii* B DNA in 10-fold dilution series (six independent experiments)^a

<i>X. citri</i> DNA	Cp						Mean	SD	CV
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6			
10 ng	18.8	19.3	17.3	18.7	16.9	17.5	18.08	0.97	5.37
1 ng	21.7	23.7	21.5	23.4	21.8	21.5	22.27	1.01	4.49
100 pg	24.6	27.5	25.3	27.6	25.1	25.2	25.88	1.31	5.06
10 pg	28.1	31.1	29.1	31.2	29.7	29.1	29.72	1.22	4.10
1 pg	32.2	37.0	32.1	36.0	32.8	33.7	33.97	2.07	6.09
0.1 pg	36.8	39.0	36.2	ND	ND	ND	ND	ND	ND
H ₂ O	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00

^a Cp, crossing point; SD, standard deviation; CV, coefficient of variation; ND, not determined.

duced using extracts of healthy citrus leaves. No fragments of expected size were amplified with DNA of *X. campestris* pv. *citrumelo*, or from extracts of *X. campestris* pv. *citrumelo*-infected citrus. The VM1-2 amplified *X. citri* pv. *aurantifolii* C less efficiently and VM5-6 primer pairs failed to react with *X. citri* pv. *aurantifolii* B and C. Nontemplate controls (water controls) in some runs showed Cp of 36 and higher. Melting curve analysis for those samples revealed a peak of $\approx 74^\circ\text{C}$, characteristic for primer-dimer formations. Kingsley's primers gave a prominent band with 3213 (*X. citri* pv. *citri* A) and 205 (*X. citri* pv. *citri* A*) but the reactions with B69 (*X. citri* pv. *aurantifolii* B), C340 (*X. citri* pv. *aurantifolii* C), and 0053 (*X. citri* pv. *citri* A^w) were inconsistent and also gave rise to more primer-dimer products compared with VM3 and 4. The VM3 and 4 primers were selected for further experimentation.

Assay sensitivity and reproducibility. As low as 1 pg of purified total *X. citri* total DNA was reliably detected using the VM3 and 4 primer pair in real-time PCR in repeated experiments; as low as 0.1 pg of purified total *X. citri* DNA was detected in most experiments (Fig. 1A; Table 3). The assay showed quantitative reproducibility over five orders of magnitude of dilutions (Fig. 1B; Table 3). Using *X. citri* pv. *citri* A 3213 or *X. citri* pv. *aurantifolii* B B69 cultured cells, as few as 10 CFU were detected reliably when added directly to the PCR mix as determined by cell count (data not shown).

Citrus canker detection using greenhouse and field samples. After optimizing the PCR assay with DNA and cultured cells, sampling and extraction procedures were optimized. To provide uniformity, single fresh lesions of the same size and age and, preferably, from the same infected leaf were chosen in the greenhouse to evaluate the different sample preparations. Generally, 2- to 3-month-old lesions from greenhouse-infected grapefruit seedlings were used. In addition, actual field samples received by FDACS from recent outbreaks in Florida were tested. The ages of these samples varied and was difficult to estimate, because field samples often were from old infections when they were first recovered.

Real-time PCR samples of lesions cut out with a no. 1 cork borer (4 mm in diameter) had much lower Cp (higher score) and much higher values of total fluorescence compared with those cut out with a no. 3 cork borer (6 mm in diameter), regardless of sample preparation buffer (Table 4). All of the sample preparation buffers performed adequately when using fresh, young, large canker lesions. Samples of crushed lesions had relatively higher Cp values (lower score) and lower total fluorescence than those which had been shaken only (Table 4; data not shown). Adding Chelex to crushed samples resulted in lower Cp values and higher total fluorescence (i.e., improved sensitivity; Table 4). Crushing samples and using the CaCO₃-Silwet-Chelex mixture seemed especially useful for old, small, and dry field lesions (Table 4; data not shown). Detection of citrus canker from field samples usually resulted in Cp values that were higher by at least two cycles com-

pared with the greenhouse samples for the same buffer or extraction procedure. The sensitivity of detection was likely lower due to the age or conditions of the leaves and lesions, and possibly due to the presence of Cu²⁺-containing pesticide spray residues on such leaves.

Ten-fold dilutions of the greenhouse canker-inoculated citrus samples were made in order to determine (i) which sample preparation method or methods resulted in the greatest bacterial cell release, (ii) which preparation methods or methods resulted in the greatest detection sensitivity (lowest Cp value, highest score), and (iii) if there was a correlation between bacterial cell release and detection sensitivity. It is clear (Table 5) that addition of Silwet with CaCO₃ resulted in an $\approx 10\times$ greater increase in CFU count. Adding CaCO₃ to dilute aqueous solutions of *Xanthomonas* cells can prolong survival (data not shown). Importantly, crushing of the samples did not significantly enhance cell release (CFU count) and actually reduced PCR detection sensitivity, unless Chelex was added to the extraction buffer (Table 5). Evidently, crushing of the sample released a component from citrus that was inhibitory to PCR, even at high dilution levels (Table 5). Cell counts of the dilution series revealed that apparently <10 CFU was detected reliably with good reproducibility (Table 5). Overall, the simplest and most effective method involved using the smallest practical citrus leaf lesion sample, using Silwet with CaCO₃ and shaking or soaking to release bacterial cells while minimizing cell damage.

The IT kit also showed excellent accuracy, sensitivity, and reproducibility in two double-blind tests of clearly infected canker field lesion samples (Table 6). Very low PCR scores were obtained with some of the exposed fruit (healthy fruit surrounded by canker-infected leaves). Similar results were obtained in additional greenhouse tests, indicating the potential sensitivity of this method.

Herbarium sample results. Dry, nonviable herbarium samples collected in Florida during the 1912 outbreak were subjected to the described protocols. Citrus canker DNA was detected in some samples using the VM3 and 4 and Kingsley's primers with relatively high Cp (29 and higher) values. *X. citri* DNA was used as positive control. No Cp values were detected for water controls (negative PCR control). Bands of expected size were observed when the real-time PCR products were loaded on agarose gels (Fig. 2). The PCR products were cloned and sequenced, revealing the expected amplification product sequences.

DISCUSSION

Effective control and eradication of citrus canker requires reliable assay specificity. Over a decade ago, *pthA* was identified as essential for *X. citri* to elicit cankers on citrus (33). This gene or its homologues are found in every *X. citri* strain examined (3,7). When expressed in citrus cells (in the absence of any bacterium), *pthA* alone causes the hyperplastic cankers and epidermal cell death that are diagnostic of citrus canker disease (5). Because

TABLE 5. Total viable cells (CFU) extracted and real-time polymerase chain reaction (PCR) crossing point (Cp) values of a dilution series from similar, ideal citrus canker lesions, using different preparation methods^a

Extraction method	Cp in dilution series						CFU/lesion (SD) ^b
	Undiluted	1 × 10 ⁴	1 × 10 ⁵	1 × 10 ⁶	1 × 10 ⁷	1 × 10 ⁸	
Phosphate-buffered saline	15.4	25.5	30.9	35.5	38.0	0.0	6 × 10 ⁶ (1.5)
CaCO ₃	17.8	ND	ND	ND	ND	ND	8 × 10 ⁶ (3.0)
Silwet	18.7	ND	ND	ND	ND	ND	5 × 10 ⁶ (3.6)
CaCO ₃ -Silwet	16.4	26.3	30.6	34.6	38.0	0.0	4 × 10 ⁷ (2.4)
CaCO ₃ -Silwet crushed	20.8	27.1	32.0	35.5	0.0	0.0	9 × 10 ⁷ (15.4)
CaCO ₃ -Silwet-Chelex crushed	16.4	23.4	26.9	30.3	36.5	39.0	2 × 10 ⁷ (2.8)

^a Each value represents the average of three independent experiments of greenhouse-inoculated samples. ND, not determined.

^b CFU/lesion was calculated based on the average of the three independent experiments and recorded on a per lesion basis. The same dilution series was used for real-time PCR and for cell counts. For the last extraction method listed, *Xanthomonas citri* was detected in all three independent experimental runs at the 1 × 10⁷ dilution of 2 × 10⁷ bacteria, allowing a detection sensitivity of an average of <10 CFU in these experiments. SD, standard deviation.

pthA is found in every *X. citri* strain examined and because *pthA* alone causes canker symptoms, there is no reason to suspect that a xanthomonad could cause citrus canker disease without *pthA*. Thus, *pthA* represents an important diagnostic indicator for citrus canker-causing xanthomonads, regardless of their taxonomic

TABLE 6. Double-blind testing of field samples taken in the current Florida citrus canker outbreak, using the IT 1-2-3 RAPID DNA purification kit and real-time polymerase chain reaction (PCR)^a

Experiment, vial no..	Description	PCR score
Experiment 1		
1	6- to 9-month-old lesion	2
2	Healthy	0
3	Healthy	0
4	6- to 9-month-old lesion	3
5	3- to 6-month-old lesion	2
6	6- to 9-month-old lesion	2
7	3- to 6-month-old lesion	3
8	Healthy	0
9	3- to 6-month-old lesion	3
10	Healthy	0
11	3- to 6-month-old lesion	3
12	6- to 9-month-old lesion	3
13	3- to 6-month-old lesion	3
14	6- to 9-month-old lesion	3
15	Healthy	0
Experiment 2		
1	Exposed fruit (2 to 3 months)	1
2	3-month-old leaf lesion	2
3	6-month-old fruit lesion	2
4	Exposed fruit (2 to 3 months)	0
5	6-month-old fruit lesion	1
6	<i>Xanthomonas citri</i> 0053 cultured cells	4
7	Exposed fruit (2 to 3 months)	0
8	<i>X. citri</i> 0053 cultured cells	4
9	6-month-old fruit lesion	2
10	Exposed fruit (2 to 3 months)	1
11	Exposed fruit (2 to 3 months)	0
12	6-month-old fruit lesion	1
13	<i>X. citri</i> 0053 cultured cells	4
14	3-month-old leaf lesion	3
15	6-month-old fruit lesion	2

^a Experiment 1 occurred on 5 February 2002. Only red grapefruit leaves were sampled. Experiment 2 occurred on 12 April 2003. Red grapefruit leaves, fruit, and cultured cells were sampled as indicated.

status. For this reason, we designed primers for citrus canker detection based on the most highly conserved sequences of the *pthA* gene. Primers VM3 and 4 exhibited high specificity and reacted with representative strains of all known canker groups.

Cubero and Graham (3) recently designed a pair of PCR primers named *J-pth1* and 2, also based on the sequence of *pthA*. These primers proved useful in conventional PCR for detecting the target sequence in purified total DNA from 58 *X. citri* strains, including all known citrus canker groups and including strains of different geographical origins. Only one strain (of *X. citri* pv. *aurantifolii* B) did not amplify in their conventional PCR experiments, but sensitivity of these primers was not determined. Because (i) *X. citri* comprises clonal pathogenicity groups; (ii) representative strains of all known groups were detected with VM3 and 4 at a very high level of sensitivity; (iii) VM3 and 4 were designed to amplify a very short and invariant region of *pthA*, and (iv) there is no reason to suspect that a xanthomonad could cause citrus canker disease without *pthA* or a functional homologue, we have no reason to expect that the VM3 and 4 primers would fail to detect any *X. citri* strain.

It should be noted that the *avrBs3/pthA* gene family is widely distributed in the genus *Xanthomonas* (6,18); therefore, the VM3 and 4 primer pair should be able to identify strains of other *Xanthomonas* spp. and pathovars, such as *X. oryzae* (rice blight), *X. phaseoli* (common bean blight), and *X. campestris* pv. *malvacearum* (cotton blight). Indeed, these primers may be equally useful in detecting all strains of any species or pathovar in which a *pthA* homologue is required for pathogenicity (as appears to be the case for rice blight, cotton blight, and common bean blight) (7). Most xanthomonads are highly restricted in host range; therefore, there is little likelihood of a chance cross contamination causing a false positive result; for instance, by a rice blight strain being detected on a citrus sample, or vice versa. However, the sensitivity of the real-time PCR assay reported here means that this possibility would need to be experimentally assessed.

Effective control and eradication of citrus canker also requires reliable assay sensitivity. The assay developed here, including the sampling methods coupled with real-time PCR, enabled reliable detection of what appeared to be <10 CFU of *X. citri* from diseased leaf lesions. By contrast, 10 CFU appeared to be the lower limit of detection of *X. citri* diluted from culture-grown cells. The apparent difference in sensitivity likely is due to the presence of

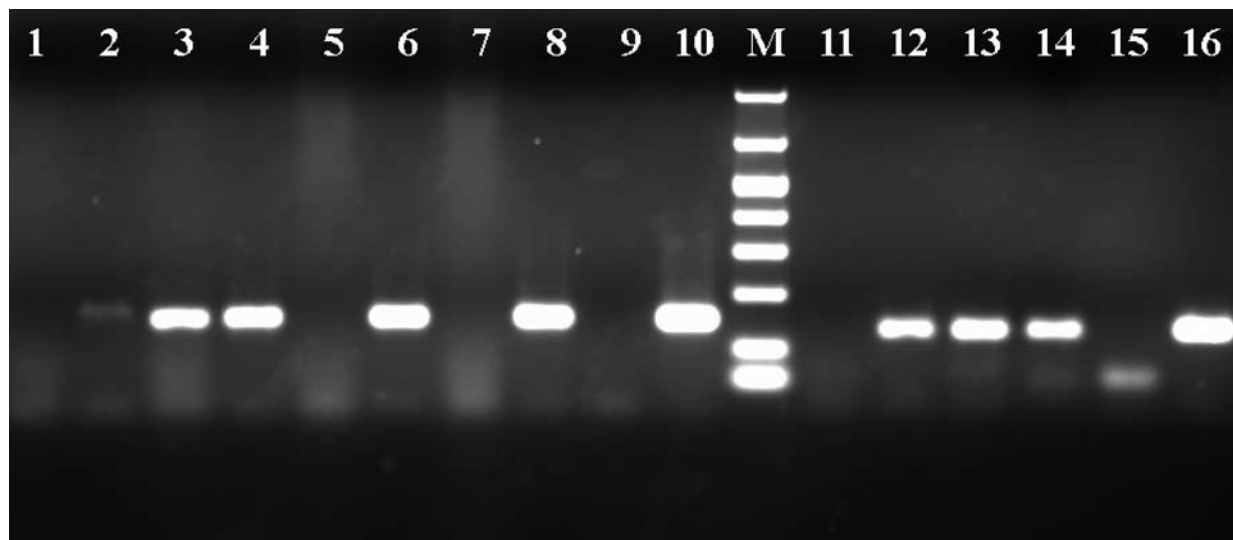


Fig. 2. Real-time polymerase chain reaction (PCR) products of the herbarium samples using direct tissue extracts or IT-purified DNA from the extracts, electrophoresed in 2% agarose gels and stained with ethidium bromide. Lanes 1 to 8: VM3 and 4 primer pair PCR products. 1, A1 extract; 2, A1 DNA; 3, A2 extract; 4, A2 DNA; 5, F3 extract; 6, F3 DNA; 7, F4 extract; 8, F4 DNA; 9, water control; 10, 10 ng of *Xanthomonas citri* 3213 DNA. Lanes 11 to 14: Kingsley's primer pair PCR products. 11, A1 DNA; 12, A2 DNA; 13, F3 DNA; 14, F4 DNA; 15, water control; 16, 10 ng of *X. citri* pv. *citri* A DNA. Lane M, marker DNA (BioVentures Inc., Murfreesboro, TN) of size: 1,000, 700, 525, 500, 400, 300, 200, 100, and 50 bp.

additional *X. citri* DNA present in lesions from dead or nonviable cells; *X. citri* DNA was detected in 90-year-old herbarium citrus canker samples that had no detected living cells.

The ability to detect a lower limit of 10 CFU of diluted culture-grown cells is consistent with the lower limit of detection of 0.1 pg of purified *X. citri* total DNA. The *X. citri* main chromosome is 5.2×10^6 bp in length, meaning on a per cell basis that it carries 5.2×10^6 bp/cell, 1 mole/ 6.02×10^{23} bp, 660 g/mole, 10^{12} pg/g = 0.006 pg of single-copy (chromosomal) DNA per cell. *X. citri* also carries two different multicopy plasmids, one 33 kb and the other 64 kb in size. Assuming that each of the plasmids is estimated to be present at 10 copies per cell, this brings the total to 0.007 pg of DNA per cell. Assuming half of the bacteria are present as clumps or with duplicated genomes due to incomplete cell division, then 10 CFU = 15 bacteria, which should yield 0.105 pg of DNA. This 10-CFU level of sensitivity is due in part to the fact that there are two homologues of *pthA* present on each of the two plasmids, or approximately 40 copies of the target sequence per cell or per 0.007 pg of *X. citri* total DNA.

As previously mentioned, the *X. citri* bacteria detected using this PCR assay need not be viable, because the expected *pthA* sequence from herbarium leaf samples collected in the 1912 were amplified. To our knowledge, this is the first report of any successful molecular confirmation of the presence of *X. citri* from the 1912 canker outbreak in Florida. Repeated attempts by Gabriel (*unpublished data*) to resuscitate *X. citri* from these and other, similar herbarium samples or to detect *X. citri* DNA failed. Confirmation of historical diagnoses may be important if regulatory actions and scientific conclusions are based on the experienced behavior of a pathogen that is erroneously assumed to be *X. citri*. Even as recently as the 1980s, both a fungal disease (*Alternaria limicola*) and a nursery leaf spot disease (*X. campestris* pv. *citrumelo*) were misdiagnosed as causing a "form" of citrus canker disease (8).

Real-time PCR offers a variety of amplicon detection methods; for a discussion of the relative merits of each method, refer Mackey et al. (19). We chose to use SYBR Green dye for fluorescence detection over fluorescent-labeled probes such as Taqman (Applied Biosystems) or hybridization probes because SYBR Green detection is less expensive, generates a stronger PCR signal, and is easy to optimize. Melting curve analyses allows differentiation of specific PCR products from nonspecific ones and from primer-dimers. Moreover, fluorescence after each cycle can be acquired at temperatures higher than the T_m of primer-dimers and just under the T_m of the expected specific fragment, thus eliminating detection of nonspecific fluorescence. Once optimized, a SYBR Green real-time PCR assay can be easily modified for use with other detection formats, such as Taqman or hybridization probes.

Sample preparation was shown to be of paramount importance in sensitivity and reproducibility of this assay. We aimed at user-friendly, rapid sample preparation with minimal handling, preferably without a DNA extraction step. Of seven different sample preparation combinations, the best results were obtained when single lesions were crushed in CaCO_3 -Silwet L-77-Chelex and with the IT kit. Presence of CaCO_3 was important for *Xanthomonas* cell survival because CaCO_3 -containing treatments proved to have the highest overall viable cell counts when plated on PYGM. Silwet L-77 is a surfactant and is useful in bacterial inoculations and in increasing apparent CFU in making cell counts (35).

Lesions cut with the smallest (no. 1) standard cork borer and crushed in CaCO_3 -Silwet L-77-Chelex appeared to release the most bacteria with the least amount of plant material and worked the best for all types and ages of lesion. Citrus sap is strongly inhibitory to PCR reactions (15); therefore, we found that the small cork borer was ideal in cutting just outside the boundary of a typical canker lesion, thus maximizing bacterial yield while minimizing contaminating plant tissue in the sample.

Samples crushed in CaCO_3 -Silwet L-77 without Chelex had relatively higher Cp values and lower total fluorescence, even after extensive dilutions. The inhibition appeared to be due to the inhibitory effect of the crushed citrus. Chelex was used to prevent enzymatic degradation of DNA by chelating heavy metal ions that are needed by many DNases in low-salt conditions (29,36). We found the combination of crushing the samples and including Chelex with the CaCO_3 and Silwet to be most useful for extraction of old lesions or from poor citrus leaf samples.

The IT kit was subjected to only limited testing, but showed excellent accuracy, consistency, and reproducibility in detecting citrus canker from obviously infected field lesions on both leaves and fruit. DNA purification lowers level of inhibitors and minimizes background fluorescence. The sampling procedure using the IT kit is very easy and can be performed directly in the field by personnel with little technical background or training. The samples can be sent to a lab or the entire PCR process could be performed in a mobile field lab. The only equipment needed in addition to a field-hardened RAPID cyclor are a low-speed centrifuge that comes with the instrument and a small vortex machine. Further comparative evaluation of this method is needed, particularly on older or damaged lesions.

The CaCO_3 -Silwet L-77-shaking method appeared to work very well with real field samples despite the fact that field samples very often were from old, dry leaves with some additional fungal or leaf miner contamination or damage. Each sample was set up in duplicate, which provided better quality control, especially for low-titer samples. The assay proved to be highly reproducible—each sampling procedure resulted in consistent scores when comparable samples (lesions of the same age and quality) were used. The assay was quantitative and very sensitive, allowing detection of *X. citri* DNA from dead cells in canker lesions. Using real-time PCR with universal primers, we were able to accomplish fast and reliable detection of all of citrus canker strains with a minimum of labor in a total time of less than 4 h. The assay described here could be very useful for fast and on-site citrus canker diagnosis for large-scale surveys in quarantine zones or ports of entry to prevent new introductions.

ACKNOWLEDGMENTS

This work was performed as part of a USDA-APHIS grant 99-8100-0578-CA to D. W. Gabriel, and is Florida Agriculture Experiment Station Journal Series R-09535. We thank T. Riley, USDA-APHIS, Fort Pierce, FL for collecting swab samples used; T. Schubert and X. Sun of the Division of Plant Industry, Florida Department of Agriculture, Gainesville, for providing citrus canker field samples used in some experiments; and M. Kingsley, Battelle Pacific Northwest National Laboratory, Richland, WA, for republication use of his primers and reaction conditions.

LITERATURE CITED

1. Brown, K. 2001. Florida fights to stop citrus canker. *Science* 292:2275-2276.
2. Brunings, A. M., and Gabriel, D. W. 2003. *Xanthomonas citri*: Breaking the surface. *Mol. Plant Pathol.* 4:141-157.
3. Cubero, J., and Graham, J. H. 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl. Environ. Microbiol.* 68:1257-1264.
4. Cubero, J., Graham, J. H., and Gottwald, T. R. 2001. Quantitative PCR method for diagnosis of citrus bacterial canker. *Appl. Environ. Microbiol.* 67:2849-2852.
5. Duan, Y. P., Castaneda, A., Zhao, G., Ergos, G., and Gabriel, D. W. 1999. Expression of a single, host-specific, bacterial pathogenicity gene in plant cells elicits division, enlargement, and cell death. *Mol. Plant-Microbe Interact.* 12:556-560.
6. Gabriel, D. W. 1997. Targeting of protein signals from *Xanthomonas* to the plant nucleus. *Trends Plant Sci.* 2:204-206.
7. Gabriel, D. W. 1999. The *Xanthomonas avr/pth* gene family. Pages 39-55 in: *Plant-Microbe Interactions*, vol. 4. G. Stacey and N. T. Keen, eds. The American Phytopathological Society, St. Paul, MN.

8. Gabriel, D. W. 2001. Citrus canker. Pages 215-217 in: Encyclopedia of Plant Pathology. O. C. Maloy and T. D. Murray, eds. John Wiley & Sons, New York.
9. Gabriel, D. W., Hunter, J., Kingsley, M., Miller, J., and Lazo, G. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. *Mol. Plant-Microbe Interact.* 1:59-65.
10. Gabriel, D. W., Kingsley, M. T., Hunter, J. E., and Gottwald, T. R. 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) and reclassification of all *X. campestris* pv. *citri* strains. *Int. J. Syst. Bacteriol.* 39:14-22.
11. Gottwald, T. R., Graham, J. H., and Egel, D. S. 1992. Analysis of foci of Asiatic citrus canker in Florida citrus orchard. *Plant Dis.* 76:389-396.
12. Gottwald, T. R., Graham, J. H., and Schubert, T. S. 1997. An epidemiological analysis of the spread of citrus canker in urban Miami, Florida, and synergistic interaction with the Asian citrus leaf miner. *Fruits* 52:371-378.
13. Gottwald, T. R., Graham, J. H., and Schubert, T. S. 2002. Citrus canker: The pathogen and its impact. Online. *Plant Health Progress* doi:10.1094/PHP-2002-0812-01-RV.
14. Hartung, J. S., Daniel, J. F., and Pruvost, O. P. 1993. Detection of *Xanthomonas campestris* pv. *citri* by the polymerase chain reaction. *Appl. Environ. Microbiol.* 59:1143-1148.
15. Hartung, J. S., Pruvost, O. P., Villemot, I., and Alvarez, A. 1996. Rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and nested-polymerase chain reaction. *Phytopathology* 86:95-101.
16. Heppner, J. P. 1993. Citrus leafminer, *Phyllocnistis citrella*, in Florida. *Trop. Lepidoptera* 4:49-64.
17. Kingsley, M. T., and Fritz, L. K. 2000. Identification of the citrus canker pathogen *Xanthomonas axonopodis* pv. *citri* A by fluorescent PCR assays. (Abstr.) *Phytopathology* 90(suppl.):S42.
18. Leach, J. E., and White, F. F. 1996. Bacterial avirulence genes. *Annu. Rev. Phytopathol.* 34:153-179.
19. Mackay, I. M., Arden, K. E., and Nitsche, A. 2002. Real-time PCR in virology. *Nucleic Acids Res.* 30:1292-1305.
20. Mills, D. 2002. Use of real-time PCR and high throughput detection of *Clavibacter michiganensis* subsp. *sepedonicus*. (Abstr.) *Phytopathology* 92(suppl.):S110.
21. Mumford, R. A., Walsh, K., Barker, I., and Boonham, N. 2000. Detection of potato mop top virus and tobacco rattle virus using a multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. *Phytopathology* 90:448-453.
22. Qi, M., and Yang, Y. 2002. Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and northern blot/phosphoimaging analyses. *Phytopathology* 92:870-776.
23. Roberts, C. A., Dietzgen, R. G., Heelan, L. A., and Maclean, D. J. 2000. Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. *J. Virol. Methods* 88:1-8.
24. Schaad, N. W., Berthier-Schaad, Y., Sechler, A., and Knorr, D. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber by BIO-PCR and an automated real-time fluorescent detection system. *Plant Dis.* 83:1095-1100.
25. Schaad, N. W., Opgenorth, D., and Gaush, P. 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. *Phytopathology* 92:721-728.
26. Schubert, T. S., Gottwald, T. R., Rizvi, S. A., Graham, J. H., Sun, X., and Dixon, W. N. 2001. Meeting the challenge of eradicating citrus canker in Florida—again. *Plant Dis.* 85:340-356.
27. Schubert, T. S., Miller, J. W., and Gabriel, D. W. 1996. Another outbreak of bacterial canker on citrus in Florida. *Plant Dis.* 80:1208.
28. Sherman, D. J., Schneider, W. L., Stone, A. L., Damsteegt, V. D., and Frederick, R. D. 2002. Specific detection and quantification of plum pox potyvirus by real-time fluorescent RT-PCR. (Abstr.) *Phytopathology* 92(suppl.):S75.
29. Singer-Sam, J., Tanguay, R. L., and Riggs, A. D. 1989. Use of Chelex to improve the PCR signal from small number of cells. *Amplifications: A Forum for PCR users.* Issue 3:11.
30. Stall, R. E., and Civerolo, E. L. 1991. Research relating to the recent outbreak of citrus canker in Florida. *Annu. Rev. Phytopathol.* 29:339-420.
31. Stevens, H. E. 1915. Studies of citrus canker. *Fla. Agric. Exp. Stn. Bull.* 124:31-53.
32. Sun, X., Stall, R. E., Cubero, J., Gottwald, T. R., Graham, J. H., Dixon, W. N., Schubert, T. S., Peacock, M. E., Dickstein, E. R., and Chaloux, P. H. 2000. Detection of a unique isolate of citrus canker bacterium from Key lime in Wellington and Lake Worth, Florida. (Abstr.) In: *Proc. Int. Citrus Canker Res. Workshop, Ft. Pierce, FL.* Online. Division of Plant Industry. Florida Department of Agriculture and Consumer Services.
33. Swarup, S., De Feyter, R., Brlansky, R. H., and Gabriel, D. W. 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit cankerlike lesions on citrus. *Phytopathology* 81:802-809.
34. Swarup, S., Yang, Y., Kingsley, M. T., and Gabriel, D. W. 1992. A *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. *Mol. Plant-Microbe Interact.* 5:204-213.
35. Tornero, P., and Dangl, J. L. 2001. A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*. *Plant J.* 28:475-481.
36. Walsh, P. S., Metzger, D. A., and Higushi, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10:506-513.
37. Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., and Stead, D. E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Appl. Environ. Microbiol.* 66:2853-2858.
38. Winton, L. M., Stone, J. K., Watrud, L. S., and Hansen, E. M. 2002. Simultaneous one-tune quantification of host and pathogen DNA with real-time polymerase chain reaction. *Phytopathology* 92:112-116.
39. Vandemark, G. J., Baker, B. M., and Gritsenko, M. A. 2002. Quantifying *Aphanomyces euteiches* in alfalfa with fluorescent polymerase chain reaction assay. *Phytopathology* 92:265-272.
40. Verniere, C., Hartung, J. S., Pruvost, O. P., Civerolo, E. L., Alvarez, A. M., Maestri, P., and Luisetti, J. 1998. Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. *Eur. J. Plant Pathol.* 104:477-487.