Quantification of *Xylella fastidiosa* from Citrus Trees by Real-Time Polymerase Chain Reaction Assay

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ABSTRACT

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Xylella fastidiosa is the causal agent of citrus variegated chlorosis (CVC), a destructive disease of sweet orange cultivars in Brazil. Polymerase chain reaction (PCR)-based assays constitute the principal diagnostic method for detection of these bacteria. In this work, we established a real-time quantitative PCR (QPCR) assay to quantify *X. fastidiosa* in naturally and artificially infected citrus. The *X. fastidiosa* cell number detected in the leaves increased according to the age of the leaf, and

bacteria were not detected in the upper midrib section in young leaves, indicating temporal and spatial distribution patterns of bacteria, respectively. In addition, the *X. fastidiosa* cell number quantified in leaves of 'Pera' orange and 'Murcott' tangor reflected the susceptible and resistant status of these citrus cultivars. None of the 12 endophytic citrus bacteria or the four strains of *X. fastidiosa* nonpathogenic to citrus that were tested showed an increase in the fluorescence signal during QPCR. In contrast, all 10 CVC-causing strains exhibited an increase in fluorescence signal, thus indicating the specificity of this QPCR assay. Our QPCR provides a powerful tool for studies of different aspects of the *Xylella*-citrus interactions, and can be incorporated into breeding programs in order to select CVC-resistant plants more quickly.

Xylella fastidiosa is a gram-negative, nonflagellate, xylem-limited bacterial pathogen, measuring 0.1 to 0.3 × 1.5 µm (52). This bacterium belongs to the family *Lysobacteriaceae*, and it was the first plant pathogen to have its genome completely sequenced (48). Pathotypes of *X. fastidiosa* cause a wide range of diseases in many agriculturally important plants, including alfalfa, almond, grapevine, lucerne, peach, and plum, and in several noneconomically important plants (21,52). Chang et al. (7) definitively associated a strain of *X. fastidiosa* with citrus variegated chlorosis (CVC), a destructive disease of all commercial sweet orange cultivars (*Citrus sinensis* (L.) Osbeck). CVC is considered the main problem faced by Brazilian citriculture and most sweet orange cultivars are susceptible to it (27). CVC's symptoms include mottled variegations on older leaves, with chlorotic areas on the upper side and corresponding light-brown lesions on the lower side. The fruits from the affected trees are small, exhibiting early ripening, and, therefore, having no commercial value (10). In Brazil, 34% of the citrus trees are affected by CVC, indicating the alarming spread of the disease in the field (14). *X. fastidiosa* is vectored by at least 11 sucking insects that feed on xylem-sap, such as sharpshooter leafhoppers (*Homoptera*, *Cicadellidae*) (14). Transmission also can occur through grafting in commercial nurseries (10).

The symptoms of plant bacterial diseases are not always reliable for diagnosis because they can vary according to environmental conditions (43). Limiting an outbreak of CVC requires a sensitive diagnostic procedure to prevent the spreading of *X.*

fastidiosa into mother plants and budwoods. Thus, diagnostic methods that allow the early detection of bacteria in symptomless citrus trees were developed, including microscopy analysis (6), serological tests such as enzyme-linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA), western blotting (7, 20,25,32), immunofluorescence (35), and polymerase chain reaction (PCR)-based assays (4,12,17,34,42). So far, the PCR-based method is considered the quickest and most efficient method of detecting *X. fastidiosa* in symptomless CVC-infected trees (4), and has been used routinely for this purpose. The PCR-based method described by Pooler and Hartung (42) is useful for detecting only CVC-causing strains, and allows the detection of 10 to 100 bacteria per amplification reaction. However, this PCR approach provides only qualitative detection.

Competitive PCR-based quantification of plant pathogenic fungi in infected plants has been reported (36,39,47). Hyman et al. (23) described the first PCR-based quantitative method for a bacterial plant pathogen, which was *Erwinia carotovora* on potato tubers. However, the competitive PCR method is labor intensive and has a high risk of carryover contamination (9). Recent developments in PCR technology have improved the quantification of phytopathogenic bacteria. The 5'-nuclease quantitative PCR assay (18) represents a significant advance for measuring input target nucleic acid templates quantitatively. The principle of this real-time PCR has been described elsewhere (18,28,41). Briefly, this method requires the inclusion in the reaction mix of a dual-labeled fluorescent nonextendable probe that anneals specifically to the target DNA between the forward and reverse primer sites (18). The fluorescent emission from the sample occurs because the reporter fluorophore is released due to the endogenous 5'–3' exonuclease activity of the DNA polymerase. Unlike other quantitative PCR methods, such as competitive PCR, the 5'-nuclease quantitative PCR assay (i) does not require post-PCR sample handling, thus

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preventing potential PCR product carry-over contamination; (ii) is less labor intensive, and (iii) does not have a plateau effect because the assay is not based on an endpoint measurement (18).

A real-time quantitative PCR (QPCR) assay for *X. fastidiosa* using the 5'-nuclease PCR was assessed in assays designed to (i) examine temporal and spatial distributions of the bacterium in xylem vessels of experimentally infected citrus leaves and (ii) evaluate the differential multiplication of *X. fastidiosa* in xylem vessels of leaves from CVC-resistant and -susceptible cultivars of citrus and their hybrids. We were able to quantify *X. fastidiosa* and determine temporal and spatial patterns of translocation and differential accumulation of the bacterium in inoculated citrus cultivars.

MATERIALS AND METHODS

***X. fastidiosa* culture and DNA extraction.** *X. fastidiosa* strain 9a5c, whose complete genome has been sequenced (48), was grown on supplemented periwinkle wilt-agar medium (16) at 28°C and subcultured every 14 days. Bacterial cells were used for the establishment of standard curves and the artificial infection of citrus trees. Dilution plating on buffered charcoal yeast extract (10 10-fold dilutions) as described by Wells et al. (53) was used to estimate the number of viable bacterial cells (CFU).

In order to extract DNA under conditions similar to those encountered in field samples, we mixed a bacterial suspension containing 1.63×10^6 CFU of *X. fastidiosa* with 150 mg of a freeze-ground xylem tissue from leaves of a healthy Pera sweet orange tree, then performed the extraction of DNA on the cell-spiked material as described by Murray and Thompson (38) and modified by Machado et al. (31). The DNA resulting from the extraction process was resuspended in 15 µl of Tris-HCl, 1 mM, pH 7.5, and EDTA, 0.1 mM, pH 8.00, and the concentration was adjusted to 100 ng/µl, so that each microliter contained the amount of DNA found in 1.09×10^5 CFU of *X. fastidiosa*. DNA concentration and purity were assessed by comparison with known DNA standards in an ethidium bromide-stained 1% agarose gel (45) and by spectrophotometry at 260 nm. The mixture of *X. fastidiosa* and citrus genomic DNA was stored at -20°C.

Real-time QPCR primers and probe. The *X. fastidiosa* nucleotide sequence used for the design of the primers and TaqMan probe was first described by Pooler and Hartung (42) (GenBank Accession No. AE003946). The sequence for the forward primer CVC-1 (5'-AGA TGA AAA CAA TCA TGC AAA-3'; 424–404 bp) was obtained from Pooler and Hartung (42), and the reverse primer CCSM-1 (5'-GCG CAT GCC AAG TCC ATA TTT-3'; 306–286 bp) was designed for this work based on the target sequence. The TaqMan probe TAQCVC (5'-(6FAM)AAC CGC AGC AGA AGC CGC TCA TC(TAMRA)p-3'; 335–313 bp) also was designed based on the target sequence and carries a fluorescent reporter dye (6-carboxy-fluorescein, $\lambda_{em} = 518$) added to the 5' end and a fluorescent quencher dye (6-carboxytetramethylrhodamine, $\lambda_{em} = 582$) and a phosphate group (p) added to the 3' end. Both primers and the TAQCVC probe were synthesized by Applied Biosystems (Foster City, CA). The CCSM-1 primer and the probe were designed using the Primer Express software (version 3.1; Applied Biosystems).

Real-time QPCR assay reaction conditions. The amplification was performed in a 25-µl reaction. The reaction mix was slightly modified from that given in the ABI PRISM 7700 User's Manual: 12.5 µl of TaqMan Universal PCR Master Mix, 525 nM of each primer (CVC-1 and CCSM-1), 500 nM TAQCVC probe, and the different DNA concentrations used throughout this work. The master mix contained a passive reference dye, ROX, which is used to standardize the reaction (18). In all experiments, appropriate negative controls containing no template DNA were subjected to the same procedure. Each sample was tested in triplicate. For amplification, the recommended universal thermal cycler pro-

ocol was used: a 50°C hold for 2 min (uracil-N-glycosylase [UNG] digestion, in an effort to prevent PCR product carryover), a 95°C hold for 10 min (activation of AmpliTaq Gold DNA polymerase and denaturation of UNG and the matrix DNA), and then 40 cycles of 15 s at 95°C and 1 min at 60°C (annealing and polymerization steps were combined). Applied Biosystems supplied all consumables. Amplification and data analysis were carried out in an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Data were analyzed using the Sequence Detector Software 1.6 software as described by the manufacturer. All reactions were analyzed by agarose gel electrophoresis to confirm that only the expected PCR product was amplified.

Real-time QPCR of *X. fastidiosa* from artificially infected citrus. Eight trees each of 'Pera' sweet orange (*Citrus sinensis* (L.) Osbeck) and 'Murcott' tangor (*Citrus reticulata* Blanco $\times$ *C. sinensis* (L.) Osbeck) cultivars and one plant of each of the hybrids H-19, H-31, H-55, H-61, H-80, H-129, H-214, and H-243 (belonging to the Germoplasm Active Bank of the Sylvio Moreira Citriculture Center, Agronomic Institute of Campinas, Cordeirópolis, SP, Brazil) were artificially infected with 20 µl of a suspension containing *X. fastidiosa* strain 9a5c at 10^8 cells per ml in PBS (sodium phosphate 100 mM; NaCl 0.85%). Artificial infection was carried out by injecting a droplet of the bacterial suspension into the xylem using a fine needle as described by Hill and Purcell (20). Control plants were inoculated with sterile PBS saline solution. In addition, five 'Pera' sweet orange trees were artificially infected by grafting as described by Medina et al. (33). In summary, the CVC-symptomatic branch of 'Valencia' sweet orange (*C. sinensis* (L.) Osb.) was grafted onto a 'Rangpur' lime rootstock (*C. limonia* L.). The resulting grafted plants were subsequently grafted onto the rootstock (Rangpur lime) of healthy Pera plants. The control plants were grafted only with the CVC-free Rangpur lime.

DNA from xylem tissue used for QPCR assays was extracted from three sections of infected leaves as follows: (i) petioles collected from 'Murcott' tangor, 'Pera' sweet orange, and the eight hybrids, 5 months after the artificial inoculation; (ii) petioles plus the initial two-thirds of the midrib; and (iii) the final one-third of the midrib. Samples ii and iii were collected from leaves of three different ages: 2- and 8-month-old leaves (symptomless samples), and 12-month-old leaves (symptomatic samples), from 'Pera' sweet orange trees infected with *X. fastidiosa* by grafting. Sample number i was obtained from 16 leaves. Samples ii and iii were collected from 16 leaves, previously inoculated with *X. fastidiosa*. Each sample type was ground in liquid nitrogen until a fine powder was obtained. DNA was extracted from 150 mg of the ground samples according to a method modified by Machado et al. (31). The concentration of each DNA sample was adjusted to 100 ng/µl and its purity was determined as described previously (45). Each DNA sample was tested in triplicate in QPCR assays.

The number of *X. fastidiosa* cells in each sample was estimated from a standard curve constructed by plotting the mean C_T values (cycle threshold, calculated as the first cycle PCR at which a statistically significant increase in the reporter fluorescence can first be detected) for each template DNA dilution (y axis) against the $\log_{10}$ of the initial quantity of template DNA (x axis) for each run.

RESULTS

Specificity of *X. fastidiosa* real-time QPCR assay. Pooler and Hartung (42) found a DNA sequence specific for CVC-causing strains of *X. fastidiosa*. An extensive search of the GenBank database indicated that this DNA sequence (GenBank Accession No. AE003946) (i) shared no significant identity with other known nucleotide sequences; (ii) encodes a hypothetical protein, according to the GenBank Accession No. NC_002488-1; and (iii) constitutes a unique genomic region, without repetitions in the genome of the CVC-causing strain 9a5c of *X. fastidiosa* (48). The TAQCVC

probe and CCSM-1 reverse primer were designed based on this region's sequence. There is only a single copy of this DNA sequence in *X. fastidiosa* strain 9a5c; therefore, there should be only one template per cell. Known amounts of *X. fastidiosa* genomic DNA corresponding to *X. fastidiosa* populations from 1.09×10^0 to 1.09×10^5 CFU were used in the construction of standard curves. In this assay, an increase in fluorescent signal above a preset threshold within 40 PCR cycles was considered positive (i.e., $C_T < 40$). The amplification plot (fluorescent emission for each reaction versus the number of PCR cycles) and a standard curve from these data are shown in Figure 1A and B, respectively. With increasing amounts of *X. fastidiosa* genomic DNA the respective C_T values ranged from 21.97 to 39.71 (Fig. 1A). Under the PCR conditions tested, the standard curve obtained was linear over five orders of magnitude, from 10.9 to 109,000 initial copies of template DNA per reaction, with the corresponding linear regression coefficient of $r^2 = 0.967$ [$y = -3.7887x \ln(X. fastidiosa \text{ genomic DNA copy number}) + 41.901$] (Fig. 1B). The PCR-amplification produced a single expected band of 137 bp as confirmed by gel electrophoresis. After 40 rounds of amplification, no PCR products were detected in any of the negative control samples that contained all reagents except the *X. fastidiosa* genomic DNA (data not shown).

The reliability and reproducibility of the real-time QPCR assays also were assessed. According to Locatelli et al. (30), the standard curves for QPCR require high repeatability (when repeated measurements are taken with the same material in a single assay) and reproducibility (repeated measurements are taken in different assays). The *X. fastidiosa* real-time QPCR appeared to be highly reproducible, because the coefficient of variation (CV) ranged from 0.54 to 1.98% in intraassay variability and 0.81 to 2.51% in interassay measurements (Table 1). No statistical difference was found between the intraassay and interassay CV ($P > 0.29$, t test) determined from three standard curves performed on different days and starting from new dilutions of the DNA mixture citrus-*X. fastidiosa*. Standard curves revealed high precision and reproducibility, as indicated by correlation coefficients no lower than $r = 0.993$.

The specificity of the *X. fastidiosa* real-time QPCR assay was tested using genomic DNA from 12 citrus endophytic bacteria, four strains of *X. fastidiosa* responsible for causing disease on other plants, and 9 other CVC-causing strains of *X. fastidiosa* isolated from a wide range of citrus cultivars and from a number of different geographical locations (2,3) (Table 2). Neither the endophytic bacteria nor the strains of *X. fastidiosa* associated with diseases of other plants showed an increase in fluorescence signal

Fig. 1. Real-time quantitative polymerase chain reaction (PCR) of CFU of *Xylella fastidiosa* using the CVC-1 and CCSM-1 primers and TAQCVF fluorescence probe. **A**, Amplification plot of the *X. fastidiosa* standards. Amplification plot of normalized fluorescence intensity change (ΔR_n) versus PCR cycle number. The y axis (ΔR_n) is plotted in log scale. The experiment, done in triplicate, consisted of serial 10-fold dilutions of *X. fastidiosa* genomic DNA, corresponding to 1.09×10^0 to 1.09×10^5 CFU of *X. fastidiosa*, which were used in the construction of standard curves. This is a computerized evaluation of the data obtained using the ABI PRISM 7700 Sequence Detection System. The x axis indicates the PCR cycle, where a, b, c, d, and e are samples with 1.09×10^5 to 1.09×10^1 copies of *X. fastidiosa* genomic DNA, respectively. Fluorescence emission for samples with 1.09×10^0 copies of *X. fastidiosa* genomic DNA was not recorded ($C_T \geq 40$). **B**, *X. fastidiosa* standard curve. C_T values are plotted against the log number of *X. fastidiosa* cells in samples of 10-fold dilutions of template DNA. The straight line is calculated by linear regression ($y = -3.7887x \ln[\text{number of DNA copies}] + 41.901$). The linear regression coefficient (r^2) is 0.9678. A C_T value is defined as the PCR cycle where the increase in fluorescence first goes above the baseline. Each point on the curve represents the mean value from three separate PCR amplifications for each dilution.

during PCR ($\Delta R_n < 0.2$ after 40 cycles of amplification; data not shown), indicating the lack of target DNA for the CVC-1 and CCSM-1 primer set. In contrast, all CVC-causing strains exhibited the expected 137-bp band.

Detection and quantification of *X. fastidiosa* from artificially infected citrus. We decided to use the 5'-nuclease PCR assay to evaluate the movement of the bacterium into vessels of the xylem and its differential multiplication in leaves of CVC-resistant and -susceptible cultivars of citrus. The number of *X. fastidiosa* cells present in citrus tissue samples was calculated by extrapolating the mean C_T value for the test sample into the standard curve generated with *X. fastidiosa* cells. The number of *X. fastidiosa* cells detected in different xylem regions of 2- and 8-month-old (symptomless), and 12-month-old (symptomatic) leaves of 'Pera' sweet orange are presented in Figure 2. The mean number of *X. fastidiosa* cells detected in the leaf xylem ranged from 0 to 324 *X. fastidiosa* cells per mg of tissue. Bacterial populations increased according to the age of the leaf as described by the linear regression $y = -140.34x \ln(X. fastidiosa \text{ genomic DNA copy number}) + 113.73$ ($r^2 = 0.95$), therefore suggesting a temporal accumulation of *X. fastidiosa* in citrus leaf xylem as leaves get older.

X. fastidiosa cells were detected in all samples from petioles plus the initial two-thirds of the midrib regardless of their age. In contrast, cells of *X. fastidiosa* were detected only in samples from the upper section of the midrib from 8- and 12-month-old leaves (Fig. 2). These results indicate a spatial distribution of *X. fastidi-*

osa in the xylem of citrus leaves (i.e., movement of the bacterium from the petiole to the upper midrib).

The number of *X. fastidiosa* cells detected in the leaf petioles of 'Murcott' tangor, 'Pera' sweet orange, and the eight hybrids are presented in Figure 3. The number of cells varied from 0 to 487 *X. fastidiosa* cells per mg of tissue. Pera sweet orange, very susceptible to CVC (24), had 109.2 and 487 *X. fastidiosa* cells per mg of tissue when infected by the needle procedure and natural inoculation, respectively. The bacterium was not detected in 'Murcott' tangor, a resistant-citrus cultivar ($C_T \geq 40$) (Fig. 3).

DISCUSSION

CVC is a destructive citrus disease in Brazil (26). This article describes the development of a simple, rapid, and reproducible real-time QPCR-based assay for *X. fastidiosa* detection and its

Fig. 2. Temporal-spatial distribution of *Xylella fastidiosa* cells in leaves of 'Pera' sweet orange quantified by real-time quantitative polymerase chain reaction. The x axis indicates the age of the leaves: 1 = 2 months old, 2 = 8 months old, and 3 = 12 months old. Vertical columns in white and black indicate the number of *X. fastidiosa* cells detected in the petioles plus the initial two-thirds of the midrib and in the upper section of the midrib, respectively. Each sample was tested in triplicate. Vertical bars represent standard errors.

TABLE 1. Coefficient of variation (%) of the standard curves for *Xylella fastidiosa* quantification by real-time quantitative polymerase chain reaction^a

Input DNA copy number	Intraassay	Interassay
1.09×10^5	0.54	0.96
1.09×10^4	1.71	1.73
1.09×10^3	0.3	0.81
1.09×10^2	1	2.51
1.09×10^1	1.98	2.09
1.09×10^0	... ^b	... ^b

^a Intraassay and interassay variability were estimated by computing the coefficient of variation (ratio between the standard deviation and the mean of repeated measurements).

^b No fluorescence above threshold was recorded ($C_T \geq 40$).

TABLE 2. Endophytic citrus bacterial strains and other strains of *Xylella fastidiosa* used in this study

Identification	Species or strain	Host	Geographic source	QPCR assay result ^a
PF4/9	<i>Pantoea agglomerans</i>	<i>Citrus reticulata</i>	Frutal/SP	–
PR1.5/8	<i>Enterobacter cloacae</i>	<i>C. reticulata</i>	Novais/SP	–
PF1/2	<i>E. cloacae</i>	<i>C. reticulata</i>	Novais/SP	–
CT 03	<i>Bacillus</i> sp.	<i>Citrange</i>	Cordeirópolis/SP	–
PF1/8	<i>E. cloacae</i>	<i>C. reticulata</i>	Novais/SP	–
ER1.6/4	<i>Methylobacterium mesophilicum</i>	<i>C. sinensis</i>	Novais/SP	–
SR1.6/17	<i>Curtobacterium flaccumfaciens</i>	<i>C. sinensis</i>	Novais/SP	–
SR5/3	<i>M. fujisawaense</i>	<i>C. sinensis</i>	Colina/SP	–
TS49	<i>P. agglomerans</i>	<i>C. sunki</i>	Cordeirópolis/SP	–
CT04	<i>Alcaligenes</i> sp.	<i>P. trifoliata</i>	Cordeirópolis/SP	–
ER2/3	<i>E. cloacae</i>	<i>C. sinensis</i>	Elisiário/SP	–
LD29	<i>Pseudomonas</i> sp.	<i>C. sinensis</i>	Cordeirópolis/SP	–
11347	<i>Xylella fastidiosa</i>	<i>C. sinensis</i>	Paraná	+
11067	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Paraná	+
11834	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Paraná	+
11380	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Santa Catarina	+
11067	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Londrina/PR	+
11775	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Rio Grande do Sul	+
M2-1	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Matão/SP	+
Ita D	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Itapetininga/SP	+
SL4	<i>X. fastidiosa</i>	<i>C. sinensis</i>	Sete Lagoas/MG	+
8935	<i>X. fastidiosa</i>	Grape	Paraná	–
12319	<i>X. fastidiosa</i>	Periwinkle	Botucatu/SP	–
9746	<i>X. fastidiosa</i>	Plum	Ponta Grossa/PR	–
C13	<i>X. fastidiosa</i>	Coffee	Cordeirópolis/SP	–

^a Absence and presence of signal in the real-time quantitative polymerase chain reaction (QPCR) assay are indicated by – and +, respectively.

successful use for quantification of the bacterial cells in citrus tissues. In phytopathological studies, real-time QPCR has been employed to quantify virus (11,37,44,46), fungi (5,55), and bacteria (51). To our knowledge, this is the first report of a quantitative PCR detection method for xylem-limited bacteria, as well as the first report of quantification of a citrus pathogen using the 5'-nuclease PCR assay. Ubiquitous pathogens such as *X. fastidiosa* are present in healthy plants and often survive in the host tissue without inducing symptoms (19). The increased accuracy of detection and quantification obtained with this QPCR assay should facilitate studies of how these pathogens multiply and move in plant tissue (1).

We were able to quantify *X. fastidiosa* from serial dilutions over 5 log units (1.09×10^1 to 1.09×10^5 CFU) showing a strong linear relationship between the log of the starting CFU number and the C_T value. The lack of amplification products or fluorescent signal in reactions containing genomic DNA of bacteria other than CVC-causing strains of *X. fastidiosa* confirmed the specificity of this QPCR assay.

According to Pooler and Hartung (42), the PCR-based method for diagnosis of CVC-causing *X. fastidiosa* strains allows detection of as few as 10 to 100 bacteria per amplification reaction. The genomic content of a citrus cell is ≈ 0.6 pg/haploid DNA (15) and the real-time QPCR assay can detect 10 copies of *X. fastidiosa* DNA in citrus DNA at 15 ng/ μ l; therefore, the lower limit of detection of this assay is 1 bacterium in 1,250 citrus cells or 2.47 fg of bacterial DNA in 1.5 ng citrus DNA. This level of detection already has been reported for *Campylobacter jejuni* (54) and *Phytophthora infestans* (5).

The quantification of *X. fastidiosa* in the xylem of 'Pera' sweet orange leaves of different ages indicated that the *X. fastidiosa* concentration is closely associated with the progress of the CVC infection. These results agree with a study in which Machado et al. (32) showed a higher concentration of bacteria in old leaves infected with CVC by western blotting. Fry and Milholland (13) and Hopkins (21) observed that the intensity of symptoms of Pierce's disease on grape in response to infection by *X. fastidiosa* is related to the bacterial concentration.

In addition, Nunes (40) observed that, during the summer, sweet orange cultivars had high, severe CVC symptoms on leaves formed the previous spring. Our real-time QPCR supports this observation, indicating that bacterial colonization had possibly occurred initially in the xylem of symptomless young leaves formed during the spring followed by an increase in the population over time

and, consequently, causing the appearance of foliar symptoms during the summer.

The quantification of *X. fastidiosa* throughout the xylem of 'Pera' sweet orange leaves suggested a pattern of spatial distribution of these bacteria. When the leaves were just 2 months old, the bacteria had not reached the upper midrib ($C_T \geq 40$). By the time they were 8 and 12 months old, bacteria were now present in the xylem region (324 and 242 *X. fastidiosa* cells per mg of tissue, respectively). Taking into consideration that the trees used in this work were artificially inoculated through grafting and kept in a sharpshooter-free greenhouse, the colonization of new leaves was caused only by bacteria initially present on those plants. Later on, bacteria moved to petioles, progressively colonizing the midrib until they reached the upper midrib. These results are in agreement with those of Fry and Milholland (13), who observed that the *X. fastidiosa* FC strain first colonized grape leaves closest to the inoculation point. Until now, systemic distribution of CVC-causing strains of *X. fastidiosa* had been determined only through conventional PCR in the roots (29), stems, and branches (8) of citrus plants. The TaqMan quantitative PCR assay also has been used to study the spatial distribution of the fish pathogen *Pseudomonas plecoglossicida* in tissues of fish (49) and for planktonic microbial groups (50).

Quantification of *X. fastidiosa* cells in petioles of 'Pera' sweet orange leaves (109.2 and 487 *X. fastidiosa* cells per mg of tissue) and 'Murcott' tangor (none) determined by our real-time QPCR assay confirmed the susceptible and resistant status of these citrus cultivars (24). Hopkins (22) pointed out that the ability of *X. fastidiosa* strains to move through xylem vessels is indicative of their pathogenicity. Our results are in agreement with previously published data where the multiplication and spread of *X. fastidiosa* occurs faster within the more susceptible cultivars and species of grapevine (20). In the *Xylella*-grapevine pathosystem, there is a close association between the degree of intervessel migration of the bacteria within the petiole and the amount of resistance observed (13).

In summary, the real-time QPCR method described in this report has been used to quantify *X. fastidiosa* in citrus tissues and was found to be straightforward, sensitive, and reproducible. It provides a convenient tool that can be used to study different aspects of CVC development on citrus. The use of resistant or tolerant cultivars is the most effective CVC control method; therefore, research must first identify sources of resistance or tolerance (26). This novel real-time QPCR assay should be very useful in such breeding programs.

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Fig. 3. Number of *Xylella fastidiosa* cells detected in healthy and in naturally or artificially (by using a needle) infected plants of 'Murcott' tangor, 'Pera' sweet orange, and eight hybrids as determined by real-time quantitative polymerase chain reaction. 1 = 'Murcott' (needle inoculation), 2 = hybrids (DNA of the hybrids H-19, H-31, H-55, H-61, H-80, H-129, H-214, and H-243 was bulked) (needle inoculation), 3 = 'Pera' (needle inoculation), 4 = Healthy 'Pera', and 5 = 'Pera' (naturally infected). Each sample was tested in triplicate. Vertical bars represent standard errors.

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