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Quantitative PCR Method for Diagnosis of Citrus Bacterial Canker†

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For diagnosis of citrus bacterial canker by PCR, an internal standard is employed to ensure the quality of the DNA extraction and that proper requisites exist for the amplification reaction. The ratio of PCR products from the internal standard and bacterial target is used to estimate the initial bacterial concentration in citrus tissues with lesions.

Citrus bacterial canker (CBC), caused by *Xanthomonas axonopodis* pv. *citri*, is a serious disease of most citrus species and cultivars in many citrus-producing areas worldwide (1). In Florida, CBC is currently under eradication because this disease threatens to reduce fruit quality, to cause premature leaf and fruit abscission, and to restrict foreign and domestic markets by regulatory measures that prevent disease spread (1, 13). When CBC-like symptoms are detected in a new location, the disease is confirmed by the isolation of the putative *X. axonopodis* pv. *citri* population from the lesion and inoculation of leaves of a grapefruit seedling to reproduce the symptoms. Although CBC causes characteristic leaf and fruit symptoms, rapid, specific, and reliable methods for pathogen identification are critical for disease diagnosis in quarantine programs.

PCR is a principal tool for plant disease diagnosis (9). However, routine application of PCR for the detection of pathogens in plant tissue is restricted by the presence of plant factors that inhibit the amplification of nucleic acids (16). This condition can result in a false-negative diagnosis when the pathogen is actually present. Another limitation of the traditional PCR methods is their inability to quantify the initial amount of target sequence present in the tissue. Use of competitive PCR avoids these two obstacles through simultaneous coamplification of an internal standard with the specific target sequence in one reaction (17).

Internal standard construction. An internal standard plasmid was constructed by following an approach similar to that described for *Agrobacterium tumefaciens* (11). Primers CiH2 and CiH3 (Fig. 1) containing 5′ termini identical to those of primers designed to amplify a fragment inside a plasmid in *X. axonopodis* pv. *citri* (7) and 3′ termini homologous to a sequence from Figwort mosaic virus (FMV) were used for PCR using FMV DNA as a target (Fig. 2). PCR was carried out with a 50-μl volume containing 1× *Taq* buffer, 3 mM $MgCl_2$, 0.1 μM each primer, 0.2 mM each deoxynucleoside triphosphate, and 1 U of *Taq* polymerase (Promega, Madison, Wis.). The amplified product of 400 bp was cloned into the pGEM-T vector (Promega) to create plasmid pGXIS, and competent *Escherichia coli* (strain JM109) cells were transformed. Adjusted concentrations of purified pGXIS were added and used as internal standards for PCRs. To optimize the concentration of plasmid pGXIS in the reactions, without creating an appreciable decrease in the sensitivity of the PCR assay for detection of the bacterium, a titration of the internal standard was performed. An extract from an asymptomatic leaf or fruit from a mature grapefruit tree was amended with a suspension of *X. axonopodis* pv. *citri* to achieve bacterial con-

TABLE 1. Quantification of *X. axonopodis* pv. *citri* in lesions from naturally infected plants by PCR using pGXIS as the competitive element

Sample	Species[a]	Sample type	% of pixels that were from the target sequence[b] with a pGXIS concn (pg/μl) of:			Avg from different pGXIS concns (log_{10} CFU/ml)[c]
			1.5	0.15	0.015	
S1a	Grapefruit	Leaf	34.2	46.4	100	5.8 ± 0.2
S1b	Grapefruit	Leaf	0	13.6	89.1	5.0 ± 0.3
5W	Lemon	Fruit	10.9	33.3	100	4.9 ± 0.6
5B	Lemon	Fruit	22.1	45.2	96.7	5.5 ± 0.4
S2W	Grapefruit	Leaf	39.1	49.9	100	6.0 ± 0.2
S3B	Grapefruit	Leaf	28.1	41.4	90.8	5.5 ± 0.2
8W	Grapefruit	Leaf	0	0	51.1	4.3
7B	Lemon	Leaf	25.6	36.7	93.0	5.4 ± 0.2
X00-00048	Persian lime	Leaf	0	12.8	85.8	5.0 ± 0.3
XI00-00080	Grapefruit	Fruit	0	0	0	<2.7
XI00-00075	Grapefruit	Fruit	0	0	0	<2.7
X00-12906	Mexican lime	Leaf	11.3	33.0	100	4.9 ± 0.6
XI00-143a	Sweet orange	Leaf	0	0	63.6	4.6
XI00-143b	Sweet orange	Leaf	0	0	70.1	4.8
X00-13042	Mexican Lime	Leaf	38.4	52.5	100	6.1 ± 0.2
X00-13043	Grapefruit	Leaf	34.5	37.1	100	5.6 ± 0.1
X00-13044	Lemon	Leaf	0	13.4	100	4.7

[a] Grapefruit, *Citrus paradisi* (Macf.); lemon, *C. limon* (L) Burm. f.; Persian lime, *C. latifolia* Tan.; Mexican lime, *C. aurantifolia* (Christm.) Swing; sweet orange, *C. sinensis* (L.) Osbeck.

[b] Percentage of the pixels from PCR product obtained from the target sequence (222 bp) relative to the total number of pixels generated from target and pGXIS (400-bp) PCR products using three concentrations of pGXIS in PCRs.

[c] Arithmetic mean and standard deviation obtained with the three bacterial concentrations calculated from the equation that resulted from each pGXIS concentration.

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2: CAC GGG TGC AAA AAA TCT TC

3: TGG TGT CGT CGC TTG TAT GG

CiH2: CAC GGG TGC AAA AAA TCT TC**T ATA TAC TGC AA**

CiH3: TGG TGT CGT CGC TTG TAT GG**A GTA CAG GAA GG**

FIG. 1. Sequences 2 and 3 are primers designed to amplify a fragment of the *X. axonopodis* pv. *citri* plasmid (7). CiH2 and CiH3 are the primers designed for internal standard construction. Fragments underlined are those corresponding to FMV DNA.

centrations ranging from 0 to 10^8 CFU/ml. DNA was extracted using a protocol previously described (2, 10) with an improvement of the DNA precipitation step by the use of Pellet Paint coprecipitant (Novagen, Darmstadt, Germany) (14). Amplifications were carried out with 25-μl volumes containing 1× *Taq* buffer, 3 mM $MgCl_2$, 0.2 μM concentrations of primers 2 and

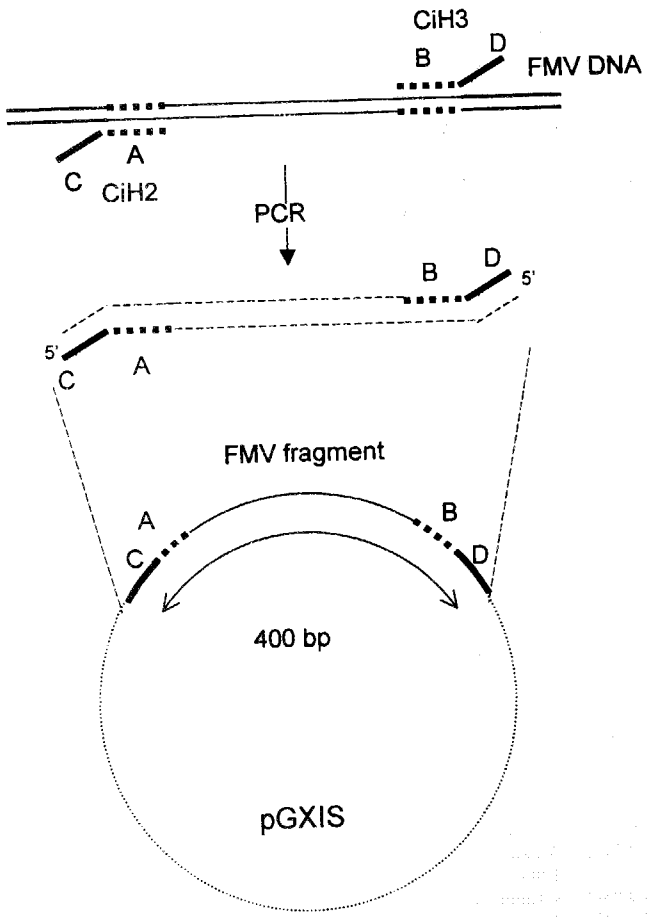

FIG. 2. pGXIS construction. A and B are sequences in FMV DNA that were incorporated into the 3′ termini of primers CiH2 and CiH3; C and D are the sequences from primers 2 and 3 that specifically amplify a fragment in the *X. axonopodis* pv. *citri* plasmid (7) and are placed at the 5′ termini in primers CiH2 and CiH3. CiH2 (C plus A) and CiH3 (D plus B) were used to amplify the fragments from FMV DNA. The PCR product (400 bp) was cloned into the pGEM-T vector to result in pGXIS. In quantitative PCR, primers 2 (C) and 3 (D) were used for amplifications.

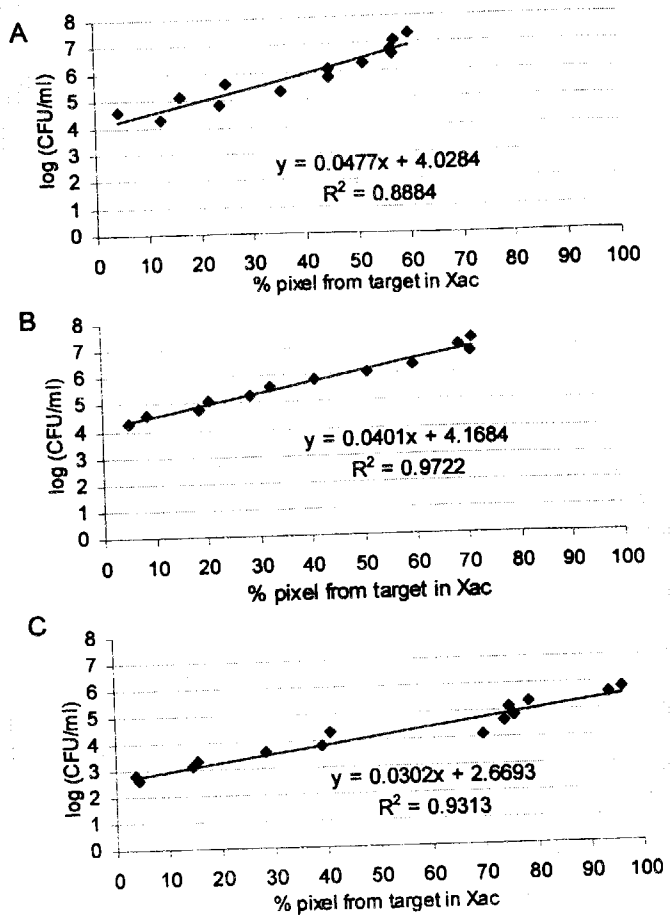

FIG. 3. Linear regressions for calibration of PCR products generated from the target sequence in serial dilutions of *X. axonopodis* pv. *citri* (Xac) in grapefruit leaves and from pGXIS at concentrations of 1.5 (A), 0.15 (B), and 0.015 (C) pg/μl. Each point represents a measure of the percentage of the pixel density from the PCR product obtained from the target sequence (222 bp) relative to the total pixel density generated from the target and pGXIS (400 bp) PCR products. Also shown are equations that resulted from a linear-regression analysis.

3 (7), 0.2 mM each deoxynucleoside triphosphate, and 1 U of *Taq* polymerase (Promega) with a profile of 93°C (30 s), 58°C (30 s), and 72°C (45 s) for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min. PCR products were visualized in agarose gels. Reactions were performed in the presence of pGXIS concentrations of 0.015, 0.15, 1.5, and 15 pg/μl. Serial dilutions of the plant extracts amended with *X. axonopodis* pv. *citri* were also plated on kasugamycin-cephalexin-Bravo-amended medium (4). After 72 h, colonies that appeared were counted to check initial concentrations of *X. axonopodis* pv. *citri* in the suspensions.

Amplification from the internal standard resulted in a 400-bp DNA fragment, whereas amplification from target *X. axonopodis* pv. *citri* yielded a 222-bp fragment. The two fragments were easily differentiated in agarose gels. pGXIS at a concentration of 0.015 pg/μl provided a sensitivity for detection of *X. axonopodis* pv. *citri* in plant material comparable to that obtained without the addition of this plasmid in the PCR. At 0.015 pg of pGXIS per μl, *X. axonopodis* pv. *citri* was de-

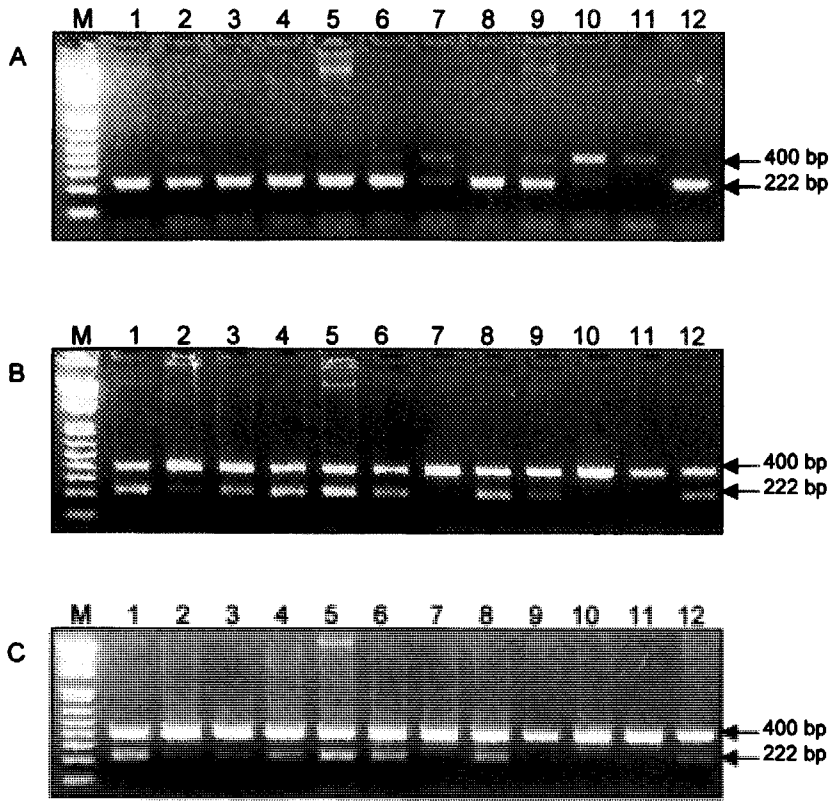

FIG. 4. PCR products generated after coamplification from the target sequences in *X. axonopodis* pv. *citri* (222 bp) and pGXIS (400 bp) at different concentrations: 0.015 pg/μl (A), 0.15 pg/μl (B), and 1.5 pg/μl (C). Lane M, 1-kb Plus DNA ladder (Life Technologies); lanes 1 (S1a), 2 (S1b), 5 (S2W), 6 (S3B), and 7 (8W), lesions on leaves of a grapefruit tree; lanes 3 (5W) and 4 (5B), lesions on fruits of a lemon tree; lane 8 (7B), lesions on a leaf of a lemon tree; lane 9 (X00-00048), lesions on a leaf of a Persian lime tree; lanes 10 (XI00-00080) and 11 (XI00-00075), lesions on a fruit of a grapefruit tree; and lane 12 (X00-13044), lesion on a leaf of a Mexican lime tree.

tected when the initial concentration was 10^2 CFU/ml, whereas at 0.15 and 1.5 pg of pGXIS per μl, detection was possible only when the initial concentration was above 10^4 and 10^5 CFU/ml, respectively. At concentrations of bacteria greater than 10^9 CFU/ml, only the 222-bp fragment from the target sequence in *X. axonopodis* pv. *citri* was amplified, whereas at concentrations of bacteria below 10^2 CFU/ml, only the 400-bp fragment from the internal standard was detected. The sensitivity of the PCR with pGXIS is similar to that previously described for this bacterium using the same primers before the addition of an internal standard (7) and is adequate for detection of endophytic and epiphytic populations of *X. axonopodis* pv. *citri* from diseased plants (3, 5, 6, 12). Moreover, this PCR assay is sufficiently sensitive to detect bacteria in initial ages of infection before visible symptoms develop or to detect epiphytic populations in asymptomatic trees (1, 15). Although the sensitivity (10^2 CFU/ml) of our quantitative method is slightly lower than that described by other authors using nested PCR (8), our method does not require a second round of PCR. A one-step PCR minimizes false-positive results and facilitates the analysis for quantification purposes.

Quantification of PCR products and constructions of calibration curves. Images from visualized PCR products were recorded by a video camera and were processed with an Alphaimager (Alpha Innotech Corporation, San Leandro, Calif.). Their densities were quantified as average pixel densities. Peaks from an internal standard and target sequences were compared, and their density ratios were used to estimate initial bacterial concentrations relative to the calibration curves. These calibrations were established by comparing the densities of PCR products obtained from serial dilutions of known bacterial concentrations in plant material and known concentrations of pGXIS. The density ratio was defined as the ratio of amplified product generated from the target sequence (222 bp) in *X. axonopodis* pv. *citri* to the product of the internal standard (400 bp). This ratio was calculated for each concentration of pGXIS (0.015, 0.15, and 1.5 pg/μl) employed in the PCR. Linear-regression equations were derived for each concentration of pGXIS to relate the initial bacterial concentration (log CFU per milliliter) to the density ratio. For each concentration of pGXIS, at least 12 measurements were taken from at least two different DNA extractions and PCRs (Fig. 3).

Based on the regression curve derived from an internal standard at 0.015 pg/μl, bacterial DNA concentration was related to bacterial populations ranging from 10^3 to 10^6 CFU/ml. At 0.15 pg of pGXIS per μl, quantification of *X. axonopodis* pv. *citri* was possible from 10^4 to 10^8 CFU/ml, and at 1.5 pg of pGXIS per μl, quantification was possible from 10^4 to almost 10^9 CFU/ml. Bacteria in extracts from fruit gave results similar to those obtained from leaf extracts (data not shown).

Quantification of *X. axonopodis* pv. *citri* in naturally infected samples. DNA extraction from 17 samples of lesions suspected

to be caused by *X. axonopodis* pv. *citri* in grapefruit, Mexican lime, lemon, Persian lime, and sweet orange tree leaves and grapefruit and lemon fruits (Table 1) were examined in a competitive PCR utilizing pGXIS at concentrations of 0.015, 0.15, and 1.5 pg/μl. Coamplification of pGXIS and DNA from *X. axonopodis* pv. *citri* was obtained from lesions of CBCs on naturally infected leaves and fruits of different citrus species using three concentrations of the internal standard (Fig. 4). All samples contained bacterial concentrations ranging from 10^4 to 10^6 CFU/ml, and results required the use of only one pGXIS concentration and one standard curve (Table 1). In many cases, averages and standard deviations of bacterial concentrations were derived from two or three pGXIS standard curves. In only one of the positive samples (XI00-00080) was the quantitative-PCR method not sensitive enough to accurately quantify a low bacterial population. Although the target sequence for *X. axonopodis* pv. *citri* was detected in this sample, the 222-bp band at the lowest pGXIS concentration was too faint to accurately estimate the population. In another sample (XI00-00075), the conditions for PCR were sufficient and the band from pGXIS was obtained but bacterial detection was not possible because the concentration of bacteria was below the detection limit of 10^2 CFU/ml.

The addition of the internal control in PCRs confirms the CBC diagnosis by ensuring the attainment of at least one PCR product, allows the quantification of bacterial concentration in plant samples, and thereby aids in the study of the pathogen in quarantine situations.

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