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ORIGINAL ARTICLE

Primers based on the *rpf* gene region provide improved detection of *Xanthomonas axonopodis* pv. *citri* in naturally and artificially infected citrus plants

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Abstract

Aims: To have a PCR-based detection method for *Xanthomonas axonopodis* pv. *citri* (Xac) using primers designed in a specific region of its genome.
Methods and Results: A Xac-specific region was identified inside the *rpf* gene cluster of strain IAPAR 306 in an analysis of its complete genomic sequence. Two primers were designed, Xac01 and Xac02, which, when used in a standard PCR assay, direct the amplification of a 581 bp fragment from DNA of strains belonging to Xac from different regions around the world including unusual American and Asian strains. This product was not observed when DNA from strains of the closely related *X. a. aurantifolli* and *X. a. citrumelo* were used as templates. Extracts prepared from 28 xanthomonads of other species, and epiphytic bacteria isolated from citrus also failed to produce products with these primers. Amplification was obtained from cells grown *in vitro*, from extracts of both fresh and dried citrus canker lesions and from washes of inoculated but asymptomatic leaf surfaces. In sensitivity tests, this PCR technique detected as few as 100 cells.
Conclusions: Primers Xac01 and Xac02 provide specific and sensitive detection of Xac in all citrus tissues where the pathogen is found.
Significance and Impact of the Study: This PCR-based diagnostic test is suitable for monitoring asymptomatic plants in areas where the bacteria is endemic, in plant quarantine and regulatory situations, and also for obtaining an accurate diagnosis in a very short time. These are important characteristics for any assay to be used for the management of citrus canker disease.

Introduction

Xanthomonas axonopodis pv. *citri* (Xac) is the bacterial plant pathogen that causes citrus canker disease (Vauterin *et al.* 1995). Xac was formerly referred to as the 'asiatic' or pathotype 'A' form *of Xanthomonas campestris* pv. *citri* (Stall and Civerolo 1993). Closely related bacteria that infect either only lemons or only limes in South America were referred to as pathotypes 'B' and 'C' (Stall and Civerolo 1993), but are now considered to belong to *Xanthomonas axonopodis* pv. *aurantifolii* (Vauterin *et al.* 1995). Citrus canker affects the majority of the citrus production areas of the world (Goto 1992), including both Florida (Gottwald *et al.* 2001) and Brazil (Leite 2003). Xac is a quarantine organism and is subject to stringent international phytosanitary measures. This restricts markets for citrus from affected areas, and makes accurate identification of the pathogen critically important. Both São Paulo in Brazil and Florida in the USA support eradication programmes in

order to protect against the serious economic consequences of this disease (Gottwald *et al.* 2001; Leite 2003). These programmes depend on the support of the public and legal system to facilitate the prompt elimination of disease foci and control the movement of plant material (Namekata 1991; Gottwald *et al.* 2001). Therefore, because of the economic and legal importance of the disease, the accurate detection of the bacteria in seedlings, budwood and asymptomatic plants, as well as in canker lesions, is critical and is also the best means to prevent further dissemination of the pathogen. Monoclonal and polyclonal antibodies have been previously raised and used for identification of Xac with generally good results. However, the antibodies may cross-react with other *X. axonopodis* pathovars or fail to react with newly described strains (Alvarez *et al.* 1991; Vernière *et al.* 1998).

Methods for nucleic-acid-based detection of plant pathogens have been shown to hold great promise (Henson and French 1993). Previous studies have reported primer sets specifically designed to amplify plasmid-borne genes (Hartung *et al.* 1993; Mavrodieva *et al.* 2004), virulence genes (Mavrodieva *et al.* 2004) or rDNA sequences (Cubero and Graham 2002) of Xac. These allowed greatly improved detection and identification of the bacterium. However, the primer sets were not entirely Xac specific. Amplification products of the predicted size were also obtained with some strains of *X. axonopodis* pv. *aurantifolii* (Hartung *et al.* 1996), the causal agent of citrus cancrosis, and one other xanthomonad (Hartung *et al.* 1993) or other bacteria (Cubero and Graham 2002). Quantitative PCR methods were also described for Xac (Cubero *et al.* 2001; Mavrodieva *et al.* 2004) based on the plasmid sequences discussed above. This technique remains expensive for use in routine diagnosis.

The genomic DNA sequences of Xac and *X. campestris* pv. *campestris* (Xcc) are quite similar, but significant differences are evident. One of these differences is in a region containing genes that regulate the expression of pathogenicity factors, the *rpf* genes (da Silva *et al.* 2002). Two *rpf* genes (*rpf*H and *rpf*I) from the *rpf* cluster present in Xcc (Dow *et al.* 2000) are not present in Xac, with the locus corresponding to *rpf*I in Xac being occupied by a truncated copy of an insertion sequence (da Silva *et al.* 2002). This difference prompted us to develop a new diagnostic test specific for Xac. In this paper, we demonstrate this new PCR assay using DNA purified from different species of *Xanthomonas* and strains of Xac from citrus canker lesions, from asymptomatic leaf surfaces of plants present in naturally infested groves, and in an artificial inoculation experiment.

Materials and methods

Analysis of the *rpf* region in Xac and design of the primers

A Xac-specific genomic region was identified inside the *rpf* gene cluster between *rpf*B and *rec*J of strain IAPAR 306 (da Silva *et al.* 2002) (Fig. 1). Genomic sequences in this region were used for designing primers with PRIMERSELECT software (DNASTAR, Inc.). The primers designated Xac01 (5′-CGC CAT CCC CAC CAC CAC CAC GAC-3′) and Xac02 (5′-AAC CGC TCA ATG CCA TCC ACT TCA-3′) generate a 581 bp amplicon, and were used for these PCR tests.

Bacterial strains and culture conditions

DNA from 83 Xac strains isolated from 19 citrus-producing countries of the world, ten strains of *X. a.* pv. *aurantifolli* (the causative agent of citrus cancrosis B and C) from Argentina, Uruguay and Brazil, and one strain of *X. a. citrumelo* from Florida (Hartung and Civerolo 1991) (Table 1) were tested to evaluate the specificity of the primers. DNA from another 28 strains representing different species or pathovars of *Xanthomonas* (*X. albilineans*, *X. arboricola* pv. *juglandis*, *X. axonopodis* pv. *axonopodis*, *X. axonopodis* pv. *glycines*, *X. axonopodis* pv. *vasculorum*, *X. axonopodis* pv. *vesicatoria*, *X. axonopodis* pv. *vitians*, *X. bromi*, *X. campestris* pv. *armoraciae*, *X. campestris* pv. *campestris*, *X. campestris* pv. *mangiferaeindicae*, *X. campestris* pv. *viticola*, *X. cassavae*, *X. codiaei*, *X. cucurbitae*, *X. cynarae*, *X. hortorum* pv. *hederae*, *X. hyacinthi*, *X. melonis*, *X. oryzae* pv. *oryzae*, *X. pisi*, *X. sacchari*, *X. theicola*, *X. translucens* pv. *graminis*, *X. translucens* pv. *translucens*, *X. vasicola* pv. *holcicola*, *X. vesicatoria*) from different areas of the world were tested as well. These strains were

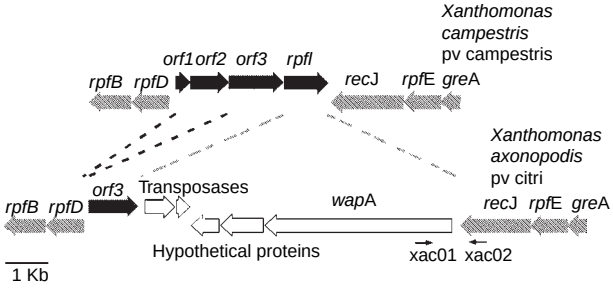

Figure 1 *rpf* region of *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *campetris*. Structural organization of the 3′ end of the *rpf* gene cluster in both xanthomonads. The arrows indicate the orientation of the open reading frames (ORFs). Grey arrows represent ORFs present in both bacteria. Black arrows are ORFs present in Xcc only. White arrows are ORFs that replace *rpfI* in Xac. Arrows indicate the relative positions and orientation of the primers.

Table 1 Strains of *Xanthomonas axonopodis* used to evaluate primers Xac01 and Xac02

Taxon	Isolate designation	Origin	Source
X. axonopodis pv. *citri* (Type A)	306	Brazil	IAPAR*
	1484, 1485,1487, 1491, 283, 316, 982, 1572, 1582, 1593, 1611, 1726, 1763, 1780	Brazil	IBSBF†
	59	Brazil	USDA‡
	1585, 1586, 1587, 1589, 1591	Argentina	IBSBF
	1584, 1603, 1604	Paraguay	IBSBF
	1471, 1547, 1552, 1554, 1555	Bolivia	IBSBF
	81	Uruguay	USDA
	422	Uruguay	IBSBF
	302, 440	USA	USDA
	106, 363, 426	Australia	USDA
	114	China	USDA
	1629, 1644, 1740, 1787, 1799, 1805, 1809, 1813	China	IBSBF
	102	Guam	USDA
	392	Indonesia	USDA
	169, 216	India	USDA
	1625, 1638, 1639	India	IBSBF
	62, 85	Japan	USDA
	1623, 1624, 1631, 1643, 1741, 1790, 1791, 1792, 1794	Japan	IBSBF
	132	Maldive Islands	USDA
	185	Mauritius	USDA
	210	New Zealand	USDA
	1412	New Zealand	IBSBF
	413	Reunion	USDA
	269	Saudi Arabia	USDA
	428	Thailand	USDA
	100	Yemen	USDA
X. axonopodis pv. *citri* (Type A*)	1974, 1609, 1360, 1362	USA	DPI§
X. axonopodis pv. *citri* (Type A^w)	4723, 2082, 2078, 2039, 1844, 1963, 1078, 6149	USA	DPI
X. axonopodis pv. *aurantifolii* (Type B)	392, 409, 1583	Argentina	IBSBF
	96, 64	Argentina	USDA
	423	Uruguay	IBSBF
X. axonopodis pv. *aurantifolii* (Type C)	380, 382, 1353	Brazil	IBSBF
	70	Brazil	USDA
X. axonopodis pv. *citrumelo* (Type E)	1011	USA	IBSBF

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†Phytobacteria Culture Collection of Instituto Biológico, Campinas, Brazil.
‡Fruit Laboratory, Beltsville Agricultural Research Center, USDA.
§Plant Pathology Quarantine Facility, Division of Plant Industry.

obtained from Phytobacteria Culture Collection of the Instituto Biologico, Campinas, São Paulo, Brazil (http://www.biologico.sp.gov.br). In addition to these xanthomonads, five unidentified epiphytic bacteria with different morphologies, which were frequently isolated from canker lesions, were also tested. The strains isolated in our laboratory were grown in 3 ml of nutrient broth medium (phytone 0·5%, beef extract 0·3%, NaCl 0·1%) for 24 h on a rotary shaker (200 rev min^{-1}) at 28°C.

DNA extractions

Two different methods were used for DNA preparation during the course of this work. Method I was used for *Xanthomonas* cells grown *in vitro*, whereas method II was used for the extraction of DNA from canker lesions on leaves and fruit of sweet orange, and also for bacterial pellets from a phosphate-buffered saline (PBS) [20 mmol l^{-1} NaH_2PO_4/Na_2HPO_4, 0·5% NaCl (w/v), pH 6·8] wash of citrus leaves. Each is described in detail below.

Method I: Extraction of DNA from cultured bacteria

For purification of total DNA from purified *Xanthomonas* cells a guanidium isothiocyanate methodology was used (Pitcher *et al.* 1989). The DNA concentration was estimated by visual comparison with known concentrations of lambda DNA in ethidium bromide-stained 0·6% agarose gels.

Method II: Extraction of DNA from lesions and leaf surfaces

Citrus leaves with canker lesions and leaves on the same plant without lesions, but bordering those with lesions, were sampled. Individual canker lesions were cut with a razor blade, put in a 1·5 ml microcentrifuge tube containing 500 μl of TE buffer [50 mmol l^{-1} Tris–HCl, pH 8·0, 5 mmol l^{-1} EDTA, 1% (w/v) SDS] to which 0·15 mg of proteinase K was added. The tube was incubated at 65°C for 30 min and then the lesion was removed using a pipette tip. The DNA was further purified using the Wizard® Genomic purification kit (Promega Corporation, Madison, WI, USA), according to manufacturer's instruction. An aliquot of 10 μl of the supernatant was used for subsequent PCR amplifications. For detection of bacteria present on the leaf surface, the leaf was cut into small pieces (≈1·5 × 1·5 mm), and put in a 50 ml tube containing 15 ml of water. The samples were shaken at room temperature for 2 h at 200 rpm. The suspension was carried to a clean tube and centrifuged at 12 000 ***g*** for 5 min to collect bacteria. The pellet was resuspended in 300 μl of TE buffer and processed with the Wizard® Genomic purification kit as above.

PCR conditions

PCR amplifications were conducted in volumes of 25 μl in a PTC100 thermocycler (Bio-Rad, Hercules, CA, USA). Mg^{2+} and primer concentrations as well as other conditions were optimized to amplify the DNA from Xac strain IAPAR306. The PCR reaction mixtures contained final concentrations of 2·0 mmol l^{-1} $MgCl_2$, 2·5 mmol l^{-1} (each) dNTPs (Invitrogen Corp., San Diego, CA, USA), 3·6 mmol l^{-1} (each) primer (Xac01 and Xac02), 15 ng of template DNA from cultured cells or 10 μl of template DNA solution from leaf washes and lesions, and 1·0 U of *Taq* DNA polymerase (Invitrogen Corp.). An initial denaturation step at 94°C for 3 min was followed by 36 cycles of: denaturation at 94°C for 45 s, annealing at 60°C for 45 s and elongation at 72°C for 45 s, followed by a final extension incubation of 5 min at 72°C. All reactions were prepared using filter-barrier pipette tips. Aliquots of 10 μl from amplification reactions were subjected to electrophoresis through 1·0% (w/v) agarose gels. The gels were stained with ethidium bromide and examined under ultraviolet light.

Infestation of leaves and estimation of the limits of detection of the PCR test

To determine the lower limit of PCR detection, cells of strain IAPAR306 were grown for 24 h in nutrient broth at 28°C and 200 rpm to a concentration of 1 × 10^8 cells ml^{-1}, estimated using a Neubauer chamber. A tenfold dilution series was prepared to the ninth dilution in PBS. Buffer was used as a no template control. For a simple estimation of detection limits using the bacterial suspension, the tenfold dilution series tubes were centrifuged at 12 000 ***g*** for 5 min, the supernatant was discarded and the pellet was resuspended in 300 μl of TE buffer and processed with the Wizard® Genomic purification kit. For estimation of the sensitivity of the method when sampling leaf surfaces, tubes containing bacterial suspensions were centrifuged at 12 000 ***g*** for 5 min and the supernatant was discarded. The pellet was resuspended in 200 μl of Milli-Q water and dropped over the entire surface of a fully expanded citrus leaf. The infested leaf was dried at room temperature and immediately processed for PCR analysis as described for method II.

Results

Unique aspects of the *rpf* cluster region

Computer analysis showed that the *rpf* cluster in Xac is different from the *rpf* cluster present in Xcc (Fig. 1). In Xcc *orf3* is adjacent to *rpf*I, which is absent in Xac. In its place, there is a 6 kb region carrying ORFs of unknown function. On one side of the cluster there is a *wap*A (wall associated protein) homologue that is physically linked to a gene present in both bacteria, *rec*J. This unique aspect of the Xac *rpf* gene cluster prompted us to evaluate the presence of this structure in other xanthomonads. To do this we decided to amplify a region comprising the 5′ end of the *wap*A homologue and the 3′ end of *rec*J (Fig. 1). For strain IAPAR306 of Xac, the reactions were expected to generate fragments of 581 bp.

Specificity of the PCR

The predicted amplification product (581 bp) was obtained for all isolates of Xac from different regions around the world, including the genetic variants A* and A^w, which occur in southwestern Asia and southern Florida, respectively. No amplification product was obtained with *X. a. aurantifolli*, the causative agent of cancrosis 'B' and 'C', or with *X. a. citrumelo*, the causal agent of citrus bacterial spot in Florida nurseries (Fig. 2). DNA from 28 additional non-Xac xanthomonads was used as template for PCR amplification. No amplification product was observed from any non-Xac strains, except for one strain of *X. albelineans*, where a 400 bp fragment was amplified, an amplification product of substantially different size compared to the specific fragment obtained for Xac (581 bp). Five uncharacterized bacteria isolated from

Figure 2 Specific amplification of Xac target by PCR. Lane M, 1-kb DNA ladder (Invitrogen™); lanes 1–6: strains of *Xanthomonas axonopodis* pv. *citri*; lanes 7 and 8: strains of *X. axonopodis* pv. *aurantifolli*, pathotype 'B'; lane 9: *X. axonopodis* pv. *aurantifolli*, pathotype 'C'; lanes 10–20: other xanthomonads; *X. vesicatoria, X. axonopodis* pv. *axonopodis*, *X. sacchari*, *X. cynarae*, *X. axonopodis* pv. *vesicatoria*, *X. cassavae*, *X. bromi*, *X. campestris* pv. *armoraciae*, *X. campestris* pv. *campestris*, *X. cucurbitae*, *X. pisi*, respectively. Arrow indicates the *X. axonopodis* pv. *citri* specific 581-bp fragment.

canker lesions were also assayed using the Xac01/Xac02 primers, and no amplification product was obtained from any of these (data not shown).

Amplification of Xac from canker lesions

Extracts of canker lesions from 'Natal', 'Pera' and 'Valencia' sweet orange varieties field-grown in infected areas showed the expected amplification product, as did asymptomatic leaves bordering diseased leaves (Fig. 3). Amplification products were not observed when extracts of healthy leaves obtained from distant nonsymptomatic plants were used as templates or in reactions without a DNA template (Fig. 3).

Sensitivity of PCR

The sensitivity of the amplification assay for Xac was determined using a tenfold dilution series of cells of Xac strain IAPAR306. The PCR allowed us to detect as few as

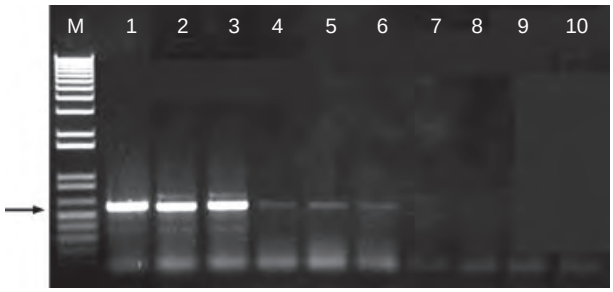

Figure 3 PCR amplification of Xac target from extracts of canker lesions and *Xanthomonas axonopodis* pv. *citri* resident on leaf surfaces. Lane M, 1-kb DNA ladder (Invitrogen™); lanes 1–3: lesions on field-grown leaves of 'Valencia', 'Pera' and 'Natal' sweet orange varieties, respectively; lanes 4–6: nonsymptomatic leaves bordering those with canker lesions; lane 7–9: leaves taken from healthy citrus plants grown distant from diseased plants; lane 10: no DNA template.

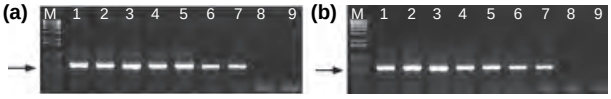

Figure 4 Sensitivity of PCR amplification for *Xanthomonas axonopodis* pv. *citri*: (a) DNA amplification from a tenfold dilution series of cultured cells; (b) DNA amplification using cells removed from leaf surfaces inoculated with the tenfold dilutions. M, 1-kb DNA ladder (Invitrogen™); lanes 1–9: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 cell, respectively.

100 cultured Xac cells using DNA extracts prepared directly from the tenfold dilution series (Fig. 4a). The same result was obtained starting with leaves that had the bacteria spread on their surface (Fig. 4b).

Discussion

In this paper, we describe a PCR-based assay for Xac detection. DNA sequence comparison of the *rpf* region of Xac and Xcc (da Silva *et al.* 2002) allowed us to develop a strategy for the detection of Xac to the exclusion of all other xanthomonads and related species and strains tested. The basis for this Xac-specific PCR assay is the replacement, in Xac, of *rpf*I with a 7·1 kb region containing two transposases, two hypothetical proteins and a *wap*A homologue (Fig. 1). Unlike previously described primers (Hartung *et al.* 1993; Mavrodieva *et al.* 2004), this assay is based on a genomic DNA sequence. We designed primers for this region, which amplify a 581 bp fragment specific to Xac. These primers flank the region between the *wap*A homologue (primer Xac02) and *rec*J (primer Xac01) (Fig. 1). The high specificity of these primers was because of the Xac02 primer designed within the Xac-specific *wapA* homologue insert (Fig. 1). This is because *recJ*, the site of primer Xac01, is conserved, with 84% identity at the nucleotide level between Xac and Xcc. The specificity of the primers was confirmed by the amplification of the expected fragment (581 bp) in all isolates of Xac from countries located in different regions around the world (Australia, Guam, Maldive Islands, Mauritius, New Zealand, Reunion, Saudi Arabia and Yemen) where citrus is grown, including the Americas and Asia. Unusual genetic variants of Xac type A found in Florida, causing citrus canker on Key/Mexican lime (*C. aurantifolia*) (A^W), and in southwest Asia (A^*) (Vernière *et al.* 1998; Sun *et al.* 2004) were also successfully amplified. The amplification of the targeted region in A^* and A^W variants demonstrates the presence of this region in all strains of Xac. Further, the absence of amplification in all other xanthomonads, including *X. axonopodis* pv. *aurantifolii*, demonstrates the high degree of specificity of the designed primers. No amplification was observed with

epiphytic bacteria associated with canker lesions. The specificity of the primers may also have evolutionary significance as it seems that the genomic organization of the *rpf* region is unique for Xac compared to other xanthomonads, suggesting that the insertion of the sequence in the middle of the cluster is a recent evolutionary event.

PCR amplification was successfully used to detect cells of Xac in citrus canker lesions from different varieties of sweet orange. Further, Xac was also detected on adjacent asymptomatic leaves (Fig. 3). Uncharacterized components of plant tissues often inhibit the amplification of nucleic acids by PCR (Wilson 1997). The DNA purification strategy described in this paper was essential to successfully amplify the PCR product from extracts of older canker lesions. Inconsistent results were obtained when using unpurified lesion extracts or leaf washes directly as the source of template DNA (data not shown). No amplification was observed when the Xac concentration was below 100 cells ml^{-1}. Previous reports have indicated that the minimum numbers of Xac cells required to induce citrus canker lesions through infection of wounds and stomata ranged from 10^2 to 10^3 and from 10^4 to 10^5 cells ml^{-1}, respectively (Zubrzycki and Diamante 1987). Xac cell numbers on asymptomatic leaves can range from $<10^1$ to as high as 10^5 CFU per leaf (Stall and Civerolo 1993). Based on our results, the primers Xac01 and Xac02 can be used to detect resident populations of Xac on asymptomatic leaf surfaces to levels around of 100 cells/leaf, which is a biologically significant level. Hartung *et al.* (1996), using a nested-PCR assay, detected 1000 cells of Xac per millilitre of sample. When using immunocapture prior to the PCR, the level of detection attained was ten cells per millilitre. A quantitative PCR methodology for detection of Xac described by Cubero *et al.* (2001) was also able to detect 100 CFU ml^{-1}. In both these reports, the primers used have a gene encoded on a conserved bacterial plasmid as the amplification target. Compared to these more laborious, sophisticated and expensive protocols, we provide a new protocol that enabled us to have a similar sensitivity for Xac detection with higher specificity. These characteristics make the test suitable for Xac diagnosis and together with other diagnostic tests could provide the basis for a formal, clear and rapid identification of the pathogen for regulatory purposes. In addition to regulatory uses, the protocol presented in this paper could be used for routine diagnosis to aid in the study of Xac epidemiology, and to improve the management of canker disease.

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References

Alvarez, A.M., Benedict, A.A., Mizumoto, C.Y., Pollard, L.W. and Civerolo, E.L. (1991) Analysis of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* with monoclonal antibodies. *Phytopathology* **81**, 857–865.

Cubero, J. and Graham, J.H. (2002) Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers. *Appl Environ Microbiol* **68**, 1257–1264.

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.

da Silva, A.C.R., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Sluys, M.A.V. *et al.* (2002) Comparison of the genomes of two *Xanthomonas* pathogens with different host specificities. *Nature* **417**, 459–463.

Dow, J.M., Feng, J.X., Barber, C.E., Tang, J.L. and Daniels, M.J. (2000) Novel genes involved in the regulation of pathogenicity factor production within the *rpf* gene cluster of *Xanthomonas campestris*. *Microbiology* **146**, 885–891.

Goto, M. (1992) Disease of fruits crops. In *Plant Diseases of International Importance* ed. Kumar J., Chaube, H.S., Sing, U.S. and Mukhopadhyay, N.A. pp. 170–208. Englewood Cliffs, NJ: Prentice-Hall.

Gottwald, T.R., Hughes, G., Graham, J.H., Sun, X. and Riley, T. (2001) The Citrus Canker epidemic in Florida: the scientific basis of regulatory eradication policy for an invasive species. *Phytopathology* **91**, 30–34.

Hartung, J.S. and Civerolo, E.L. (1991) Variation among strains of *Xanthomonas campestris* causing citrus bacterial spot. *Plant Dis* **75**, 622–626.

Hartung, J.S., Daniel, J.F. and Pruvost, O.P. (1993) Detection of *Xanthomonas campestris* pv. *citri* by the polymerase chain reaction method. *Appl Environ Microbiol* **59**, 1143–1148.

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 assay. *Phytopathology* **86**, 95–101.

Henson, J. and French, R. (1993) The polymerase chain reaction and plant disease diagnosis. *Annu Rev Phytopathol* **31**, 81–109.

Leite, Jr., R.P. (2003) Surviving with citrus canker in Brazil. In *Proceedings of the 9th Congress of the International Society*

of Citriculture, pp. 890–896. Orlando, FL: International Society of Citriculture.

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.

Namekata, T. (1991) O cancro cítrico. In *Citricultura Brasileira* ed. Rodriguez, O., Viégas, F., Pompeu, J. Jr. and Amaro, A.A. pp. 775–786. Campinas: Fundação Cargill.

Pitcher, D.G., Saunders, N.A. and Owen, R.J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.

Stall, R.E. and Civerolo, E.L. (1993) *Xanthomonas campestris* pv. *citri*: cause of citrus canker. In *Xanthomonas* ed. Swings, J.G. and Civerolo, E.L. pp. 48–51. London: Chapman and Hall.

Sun, X., Stall, R.E., Jones, J.B., Cubero, J., Graham, J.H., Dixon, W.N., Schubert, T.S., Chaloux, P.H. *et al.* (2004) Detection and characterization of a new strain of citrus canker bacteria from Key/Mexican lime and alemow in South Florida. *Plant Dis* **88**, 1179–1188.

Vauterin, L., Hoste, B., Kersters, K. and Swings, J. (1995). Reclassification of *Xanthomonas*. *Int J System Bacteriol* **45**, 472–489.

Vernière, 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**, 477–485.

Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* **63**, 3741–3751.

Zubrzycki, H.M. and Diamante, D.Z.A. (1987) Relationship between the amount of inoculum and the infection caused by *Xanthomonas campestris* pv. *citri* on citrus seedlings through natural infections in the field. In *Proceedings of the Fifth Congress of the International Society of Citriculture* ed. Montenegro, W.S. and Moreira, C.S. pp. 379–382. São Paulo: International Society of Citriculture.