Journal of Phytopathology and Pest Management 2(3): 55-66, 2015
pISSN:2356-8577 eISSN: 2356-6507
Journal homepage: http://ppmj.net/

Detection of viable *Xanthomonas citri* pv. *citri*, the causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods

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Abstract

Xanthomonas citri* pv. *citri* (*Xcc*), the causal agent of citrus canker, is a quarantine pest in many citrus-growing countries and is strictly regulated by the international phytosanitary programs. Citrus fruits with canker-like symptoms imported from India and Pakistan were collected from Riyadh supermarkets, Saudi Arabia. Different diagnostic detection methods, including culturing, direct PCR, BIO-PCR and bioassays were applied on the sampled citrus fruits. The direct culturing on KCB medium succeeded in detecting living cells of *Xcc* in 49 out of the 217 lesions observed on the sampled citrus fruits. However, only 5.55 and 24.77% from lesion washates of Pakistani and Indian fruits, respectively, caused citrus canker lesions on the inoculated grapefruit and/or Mexican lime leaves compared with 100% from pure cultures. The application of 3 diagnostic primer sets (2/3, J-pth1/2 and Xac01/Xac02) confirmed the bacterial etiology of 83 out of the 217 lesions. Based on BIO-PCR assay, 57 out of 217 lesions were *Xcc* positive. Significant differences were observed between direct PCR and BIO-PCR results. Overall, this study shows that chemical and physical fruit treatments prior citrus fruit exportation is not 100% efficient in killing all *Xcc* populations. Moreover, it is essential to integrate different diagnostic techniques to improve the accuracy of detection of quarantine bacteria and consequently prevent their introduction and dissemination.

Key words: citrus canker, imported fruits, diagnostic methods.

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Introduction

Citrus bacterial canker (CBC) caused by *Xanthomonas citri* pv. *citri* (*Xcc*) is one of the most destructive disease of citrus crops and creates serious phytosanitary problems worldwide (Das, 2003). It was probably introduced to the Arabian Peninsula, including Saudi Arabia and United Arab Emirates, in the early 1980s (Civerolo, 1988). There are three types of CBC, namely CBC-A, CBC-B, and CBC-C, which exhibit different host range compatibilities within citrus species. The causative agent for type A which originated in Asia is *Xcc*, while *X. fuscans* subsp. *aurantifolii* causes types B and C in South America (Das, 2003). Based on host range, several groups within pathotype A have been identified including *Xcc*-A* and *Xcc*-A^{w}. *Xcc*-A strains have a wide host range of plant species belonging to family Rutaceae, e.g. *Citrus aurantifolia* and *C. reticulate* (Gottwald & Graham, 1992). *Xcc*-A* is the common *Xcc* strain in Saudi Arabia with restricted host range to Mexican lime. Copper treatments are traditionally used to manage CBC disease and their effectiveness is often measured by the absence of bacterial growth on a solid medium. However, the failure to produce a visible colony may not necessarily mean that the bacterial cell is dead. Several plant-pathogenic bacteria including *Xcc* are induced to enter a viable but nonculturable state (VBNC) after copper treatment, losing their culturability on nonselective solid media but remaining viable and virulent (Del Campo et al., 2009). In addition, in commercial citrus fruits, the bacterial cells in the lesions could also lose their culturability due to the stresses of different fruit treatments, e.g. washing, disinfection, and storage at low temperatures. These stresses may decrease bacterial multiplication ability, leading to reduction in the sensitivity of different detection methods. In Saudi Arabia, in addition to the local production of citrus, more than 331,958 tons of fresh citrus fruits are imported annually from several countries where CBC is present (Ministry of Agriculture, Central Administration of Economic Studies and Statistics, 2012). The Movement of contaminated plant materials, mainly through human activities, is the main cause for long distance spread of CBC (Roberts et al., 2005). In fact, it is evident that CBC was introduced into different countries, e.g. USA, South Africa and Australia, via infected citrus plants (Das, 2003; Graham et al., 2004). Importing infected citrus fruits by CBC agents can act as an inoculum source for the disease epidemic in citrus canker-free countries. Many countries enforce strict quarantine measurements to restrict the dissemination of citrus canker by exporting /importing only free-CBC citrus fruits (Rybak et al., 2009). Consequently, only the disinfected and asymptomatic citrus fruits are allowed to be exported (Golmohammadi et al., 2007). Moreover, for a completely CBC eradication system, the asymptomatic citrus fruits should be also treated because these fruits may harbor *Xcc*. Several methods, e.g. cultural, serological and molecular, have been developed for CBC detection (Anon, 2005). Culturing is a very slow and inconsistent method, and requires pathogenicity testing for its validation. In addition, colonies of *Xcc* cannot be distinguished by morphological charact-

eristics from other *Xanthomonas* strains, e.g. *X. campestris* pv. *campestris* and *X. citri. citrumelo* (EPPO, 2005). Serological methods, including immune-fluorescence (IF) and enzyme-linked immunosorbent assay (ELISA), are available, but they are not very sensitive and cross-reactions may often result in false-positives. Although PCR-based methods are very sensitive to detect *Xcc* (Golmohammadi et al., 2007), there is difficulties to discriminate dead cells, which have no biological significance, from viable ones, which could be potentially virulent. Consequently, the main objective of this study was to evaluate different diagnostic CBC detection methods, including culturing, direct PCR, BIO-PCR and bioassays. The selected diagnostic CBC detection methods were applied on sampled citrus fruits imported from Pakistan and India, with and without CBC lesions, collected from markets distributed in Riyadh city, Saudi Arabia.

Materials and methods

Isolation of *Xcc* from symptomatic imported citrus fruits: A total of 80 cankered Mexican lime (*Citrus aurantifolia*) and mandarin (*C.reticulate*) fruits imported from India and Pakistan, respectively, were collected from several markets in Riyadh city, Saudi Arabia. The fruits showing canker-like lesions were sampled on different times with one to fifteen fruits per collection time. From each sampled fruit, one canker-like lesion with an area of 2 mm around it was peeled, cut into pieces, and then placed in a vial containing 3 ml sterile distilled water (SDW). Each vial was vortexed for 5 s, then incubated for 10 min to allow bacteria to exude from lesions and continued to do so for many times, before being vortexed after each incubation time (Bock et al., 2005). Plant tissue was removed from the vial and the lesion washate was; 1) plated onto plates of KCB (kasugamycin-cephalexin-Bravo) semi-selective medium (Graham & Gottwald, 1990), 2) injected into the bioassay plants and 3) used for DNA extraction.

Isolation of *Xcc* from asymptomatic imported citrus fruits: To verify the presence of *Xcc* on the surface of asymptomatic Mexican lime and mandarin fruits, twenty samples with 3 to 5 fruits/each were collected from the same batches containing symptomatic cankered fruits. Each fruit was placed in 40 ml SDW and vortexed for 5 s. Fruit wash was diluted and 100 µl were placed on the semi-selective medium as previously described.

Culture of *Xcc*: The lesion washates (0.1-ml aliquots) and fruit washes were placed on KCB semi-selective plates (Graham & Gottwald 1990). Tween 80 (10 ml/l) was added to the KCB medium to aid the detection of Xanthomonads (McGuire et al., 1986). After 3-5 days, *Xcc*-like colonies were selected and purified for further analyses. Single colonies were sub-cultured on yeast peptone glucose agar (YPGA) plates for 24 h. These subcultures were used for bioassays where bacterial suspensions were infiltrated into grapefruits leaves (*Citrus grandis* var. Duncan).

Bioassays: To confirm the isolation of viable and infective *Xcc* strains from

various groups of symptomatic and apparently healthy fruits, bioassays were carried out by injection-infiltration of inocula from both purified *Xcc* colonies and lesion washates into attached grapefruit leaves cv. Duncan (Graham & Leite, 2004). Seedlings were grown in greenhouse until they had flush with 3/4 to just fully expanded leaves, but not cutinized. The washates or bacterial suspension of 10^8 colony forming unit (CFU)/ml were injection-infiltrated into a single expanding leaf using two 0.1-ml aliquots (the same volume as plated, to ensure direct comparison) through a needleless syringe, one 0.1-ml aliquot being injection-infiltrated for each side of the midrib (Graham & Leite, 2004). A fresh sterile syringe was used for each sample to avoid any cross contamination. Each injection-infiltrated leaf was labeled with the lesion source. The seedlings were maintained in a greenhouse at 27°C under natural light, watered daily and the development of lesions on leaves (presence or absence) was assessed at 40 days after inoculation (Gottwald et al., 2009).

Figure 1: Citrus fresh fruits showing canker-like symptoms imported from India (Mexican lime) and Pakistan (mandarin), respectively (panel a). The Citrus fruits were collected from Riyadh supermarkets, Saudi Arabia. Panel (b) represents a close up of a lesion showing a cracking center with a crusty material inside resembling brown sugar, middle part corky in texture and a volcano or pimple like point.

Table 1: Primer pairs used in this study.

Primer pairs	Sequences (5'-3')	Size of amplicons (bp)	Location of the target sequence*	Function
2/3	(CACGGGTGCAAAAAATCT**)** (TGGTGTCGTCGCTTGTAT**)**	222	P	Unknown
J-pth1/2	(CTTCAACTCAAACGCCGGA) (CATCGCGCTGTTCGGGAG**)**	197	P	*pth*A
Xac01/Xac02	(CGCCATCCCCACCACCACCACGAC**)** (AACCGCTCAATGCCATCCACTTCA**)**	581	C	*rpf*

*P= plasmid and C= chromosome.

Molecular detection: Total DNA was extracted from pure bacterial suspensions and lesions collected from citrus fruits according to Llop et al. (1999). DNA preparations were stored at -20°C until further use for PCR assays. Direct PCR amplifications were carried out in a final volume of 50 μl containing 3 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dNTPs, 1 μmol l^{-1} of each primer, 2 µl of each DNA preparation and 1 U of DNA polymerase for primers designed by Cubero and Graham (2002) and Hartung et al. (1993) (Table 1). Primers based on Coletta-Filho et al. (2006) employed 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dNTPs and 2.5 μmol l^{-1} of primers and 1 U of DNA polymerase. BIO-PCR was employed in an attempt to increase the sensitivity of the direct PCR assays (Schaad et al., 1995). The lesion washate (0.1-ml aliquots) was placed on KCB plates. The inoculated plates were then incubated at 28°C for 48 h. Grown bacteria were collected by washing their colonies with 1 ml of SDW. The 3 mL pooled sample was processed, as described for classical PCR, except that 35 µl of cell suspension was used in 50 µl total volume per reaction. Fifteen µl of PCR products were run on 2% (w/v) agarose gels stained with ethidium bromide and visualized under ultraviolet light. Negative samples (healthy fruits) were included along different steps of the procedure to check for possible contaminations. In addition, water as negative control and *Xcc* strains (GenBank accession number JQ890095-A* and JQ890094-A) as positive control were used along PCR assays.

Statistical analysis: Analysis were performed using SAS software system (SAS Inc., Cary, NC, USA, 2003). Data from the samples were subjected to analysis of variance and the means determined using Fisher's least significant difference (LSD) at $P < 0.05$.

Results

To compare the efficiency of the detection of *Xcc* in citrus fruits imported from India and Pakistan to Saudi Arabia during 2013-2015 years, 80 samples (27 Mexican lime and 53 Mandarin fruits imported from India and Pakistan, respectively) with almost 3 lesions per fruit, along with asymptomatic ones, were tested and analyzed by different diagnostic methods including culturing on NA-KCB medium, conducting bioassays tests on both Mexican lime and grapefruit leaves, and performing direct and BIO-PCR reactions (Table 2). The average lesion size on Mexican lime fruits ranged from 1.3 to 1.4 mm in

diameter while on mandarin fruits was ranged from 4.2 to 4.8 mm (Table 2). The isolation process resulted in 49 putatively *Xcc* colonies (Tables 3 and 4).

Table 2: List of sampled fruits; sampling date, origin, host and total number of samples.

Sampling date	Origin	Host plant	Samples total number	No. lesions per sample	Average of lesion diameter (mm)
January 2013	India	Mexican lime	15	58	1.4±0.2
March 2013	Pakistan	Mandarin	12	18	4.8±0.6
January 2014	Pakistan	Mandarin	11	16	4.2±0.6
March 2014	India	Mexican lime	12	51	1.2±0.3
January 2015	Pakistan	Mandarin	16	32	4.2±0.5
March 2015	Pakistan	Mandarin	14	42	4.2±0.5

Table 3: *Xanthomonas citri* pv. *citri* detection by isolation on KCB medium, pathogenicity tests, direct and BIO-PCR in Mandarin fruits imported from Pakistan collected from Riyadh supermarkets during 2013-2015.

Sampling date	Isolation on KCB	Bioassay test from cultures on grapefruit leaves	Bioassay test from cultures on Mexican lime leaves	Bioassay test from lesion washate	Direct-PCR from lesion washate	BIO-PCR
March 2013	2/18	2/2	2/2	0/18	8/18	4/18
January 2014	2/16	2/2	2/2	1/16	5/16	3/16
January 2015	3/32	3/3	3/3	2/32	11/32	5/32
March 2015	6/42	6/6	6/6	7/42	12/42	7/42

These bacterial colonies were developed on NA-KCB medium through 3-5 days and showed typical *Xcc* characteristics, where colonies were mucoid, convex and yellow in color (Fig. 1). In some cases, the putative *Xcc* colonies appeared 7 days post inoculation. Results from preliminary virulence studies prompted a more detailed characterization of *Xcc* strains recovered from sampled citrus fruits. Out of 108 lesions observed on mandarin fruits imported from Pakistan, thirteen colonies produced pathogenic reactions identical to those developed by *Xcc*-A JQ 89004 strain. However, the 36 colonies recovered from 109 lesions observed on Mexican lime imported from India displayed pathogenic reactions resembling *Xcc*-A* JQ 890095 strain. The injection-infiltration bio-assays into attached cv. Duncan grapefruit and Mexican lime leaves confirmed the identity, viability and infectivity of the isolated *Xcc* strains from sampled imported citrus fruits. All isolates recovered on KCB and washates of lesions from Pakistan fruits induced typical CBC-A lesions on grapefruit and Mexican lime that were raised and erumpent, with callus-like tissues, narrow water-soaked margins, and light yellow chlorotic haloes (Table 3). However, the isolates and washates from Indian fruits gave typical CBC-A lesions on Mexican lime, whereas on grapefruit, they only gave water-soaked lesions (Table 4). In the case of Pakistani samples, 12.03% produced viable *Xcc*

cultures on KCB medium, and 100% from pure culture caused typical CBC symptoms on grapefruit and Mexican lime leaves but only 9.25% from washates lesions produced symptoms on leaves (Fig. 2a) and 33.02 and 30.27% from pure culture and lesion washes produced typical symptoms of CBC on Mexican lime and water-soaked on grapefruit leaves (Fig. 2b). Un-expectedly, the bioassay test from washate of lesion for the Mandarin fruits from Pakistan collected in March 2015 gave one more positive samples than isolation on KCB medium (Table 3).

Table 5: *Xanthomonas citri* pv. *citri* detection by isolation, pathogenicity tests, direct and BIO-PCR in asymptomatic mandarin and Mexican lime fruits imported from Pakistan and India collected from Riyadh supermarkets during 2013-2015.

Sampling date	Isolation on KCB	Bioassay test from cultures on grapefruit leaves	Bioassay test from cultures on Mexican lime leaves	Bioassay test from lesion washate	Direct-PCR from lesion washate	BIO-PCR
Pakistan	0/41	0/41	0/41	0/41	0/41	0/41
India	8/48	0/48	0/48	0/48	0/48	0/48

Table 4: *Xanthomonas citri* pv. *citri* detection by isolation, pathogenicity tests, direct and BIO-PCR in Mexican lime fruits imported from India collected from Riyadh supermarkets during 2013-2015.

Sampling date	Isolation on KCB	Bioassay test from cultures on grapefruit leaves	Bioassay test from cultures on Mexican lime leaves	Bioassay test from lesion washate	Direct-PCR from lesion washate	BIO-PCR
January 2013	20/58	0/58	20/58	15/58	24/58	21/58
March 2014	16/51	0/51	16/51	12/51	23/51	17/51

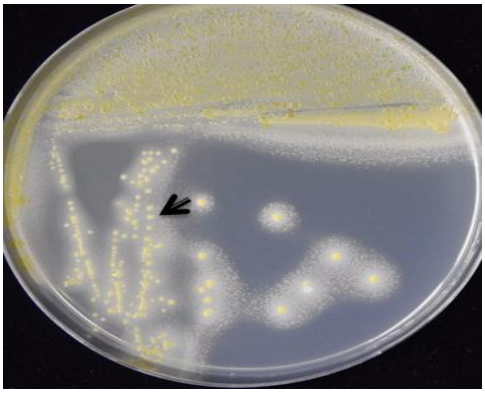

Figure 2: Typical *Xcc* mucoid, convex and yellow colonies on NA-KCB medium. Black arrow points out to a typical *Xcc* colony surrounded by white halo.

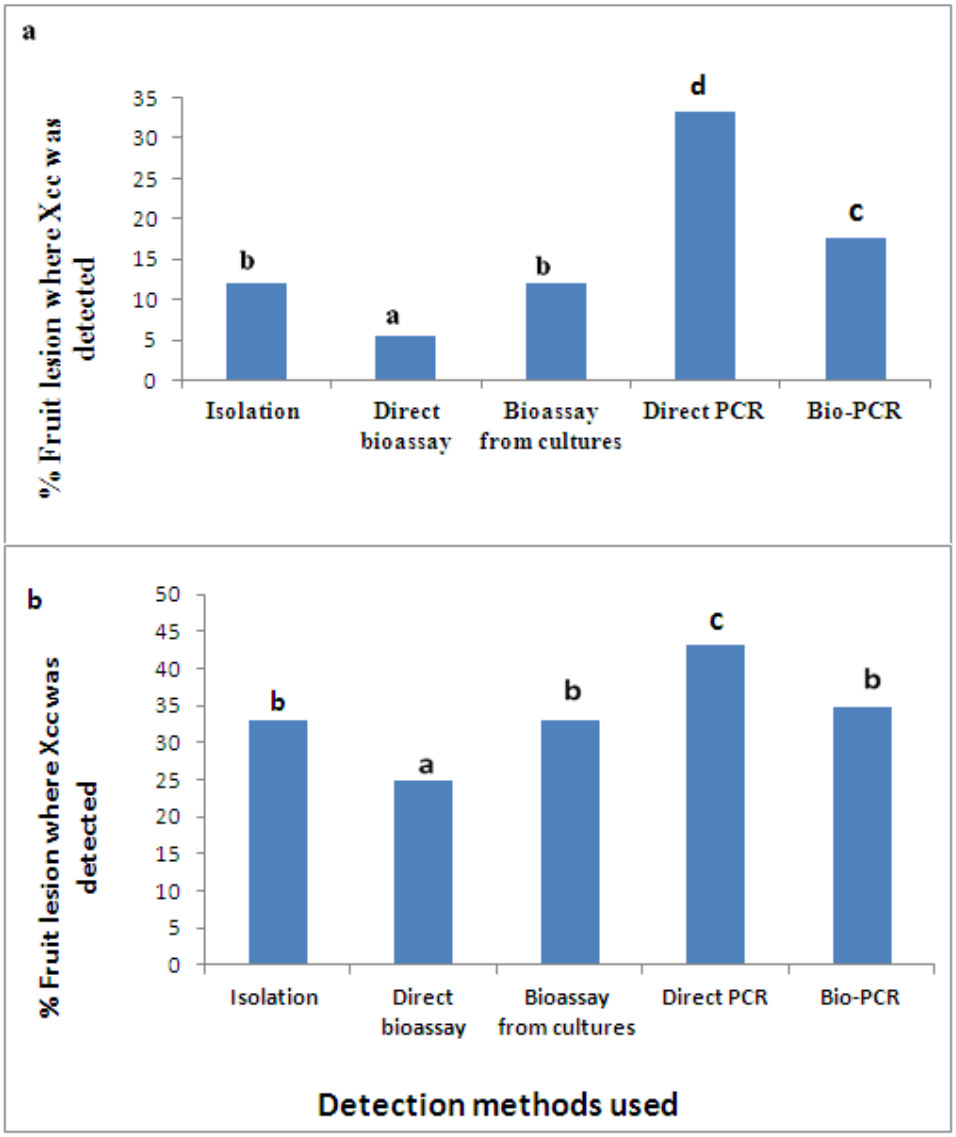

Figure 3: Percentage of *Xcc* detection in fruit lesions based on different detection methods including: bacterial isolation on NA-KCB medium, direct bioassays from lesion washate, bioassays from cultures which grown on KCB medium, direct PCR from lesion washate and BIO-PCR. Panel A represents data collected from sampled Mandarin fruits imported from Pakistan, whereas panel B represents data collected from sampled Mexican lime fruits imported from India. Detection methods with the same letters are not significantly different at $p < 0.05$.

Discussion

Since *Xcc* is considered as a quarantine pathogen and different policies are needed to manage CBC disease depending on the type of strain (narrow vs wide host), developing rapid and sensitive techniques are essential to differentiate citrus canker strains. Several of the available protocols were compared in two types of samples, pure cultures as well as citrus fruits lesion washate. In this study, the direct isolation of bacteria on KCB medium

succeeded in detecting living cells of *Xcc* in only 49 out of 217 analyzed lesions. The results showed that the number of isolates obtained from the imported Mexican lime fruits from India was 3 times the isolates obtained from the imported mandarin fruits from Pakistan, despite the number of lesions was almost equal and the lesion diameter in Pakistani samples was nearly three times the Indian ones. One of the explanations of the previous results could be that citrus fruits from Pakistan were chemically and/or physically treated to kill bacteria, but the fruits from Indian were either not efficiently treated or not treated at all. The fact that isolation was successful in 49 of 217 analyzed lesions is rather significant and this means that the disinfection protocols were not 100% effective. The long time required, in some cases, for the appearance of *Xcc* colonies on KCB medium (up to 7 days), suggests that the bacterial cells could be stressed but not dead in the lesions after the fruit treatments (washing, disinfection, copper treatments, transport, and storage at low temperatures for variable periods of time). These stresses may decrease the bacterial multiplication ability, and delay the appearance of colonies. Ghezzi and Steck (1999) showed that stress factors, e.g. copper compounds and low temperature, can induce VBNC state in bacteria. Similar results were obtained from tests on citrus shipments from Argentina and Uruguay to Spain, where *Xcc* colonies were recovered from 11 out of 15 tested patches (Golmohammadi et al., 2007). In addition, the imported citrus fruits from which *Xcc* was isolated had been treated with chlorine or sodium orthophenylphenate bactericides (Golmohammadi et al., 2007). The occurrence of saprophytes on the citrus fruits with similar morphology to that of *Xcc* on KCB medium was observed in our analyses, complicating the accurate diagnosis using isolation technique, but such colonies did not give positive results with subsequent bioassays and PCR analysis. Bock et al. (2014) demonstrated that isolation technique is a reliable and straightforward method to detect and quantify *Xcc* compared with injection–infiltration bioassay, particularly when the bacterial concentration is $\leq 10^3$ CFU per ml. These experiments have demonstrated that culture of lesion washes on a semiselective medium is effective way for determining whether a lesion contains viable *Xcc*, although final identification of colonies may still require further confirmation through pathogenicity and/or PCR tests. In this study, the injection–infiltration bioassays on attached leaves of cv. Duncan grapefruit and Mexican lime confirmed the identity of the isolated viable and infective *Xcc* strains from imported symptomatic fruits. Only 5.55 and 24.77% from lesion washates of Pakistani and Indian fruits, respectively, caused citrus canker lesions on the inoculated grapefruit and/or Mexican lime leaves compared with 100% from pure cultures. There was a slight loss of washate volume associated with the leakage around the end of the syringe during injection–infiltration. This was not taken into account in this study, and it might explain a difference between the bioassay using pure culture and washate lesions. Only a small volume was injected into each leaf (equal to the volume cultured, 0.1 ml). If a greater volume of lesion washate would been

injection–infiltrated, better data may be obtained. In case of Indian samples, injection-infiltration bioassay did not appear to be affected by the presence of other microbes washed from the lesion sample. In this work, the 8 positive samples obtained by the isolation from Indian asymptomatic fruits were negative with bioassays and PCR tests. The cause of this negative result may be due to the epiphytic yellow and mucoid *Xanthomonas* colonies grown on KCB media that were visually indistinguishable from *Xcc* colonies by morphological characteristics. Similarly, researchers with many years of experience get numerous yellow, mucoid bacterial colonies on KCB media but were serologically and pathogenically negative based on monoclonal immunostrip tests and/or bioassays on grapefruit leaves (Gottwald et al., 2009). This finding indicates that asymptomatic produced-commercially fruits are not likely a pathway for *Xcc* transmission. Moreover, more than one technique is always advised for detection of any quarantine bacterium. For *Xcc* detection, conventional PCR protocols using primers targeting different genes have been developed (Coletta-Filho et al., 2006; Cubero & Graham 2002; Hartung et al., 1993). We have not detected any significant differences among the previous diagnostic primers. Generally, the application of the 3 primer sets confirmed the bacterial etiology of 83 out of 217 lesions observed on sampled citrus fruits. Detection ability of several molecular methods has been compared using known bacterial concentrations in either suspension or injection–infiltration bioassays (Golmohammadi et al., 2012). Depending on the PCR method, detection is possible from 10 to 10^3 CFU per ml, while limits of bioassay in some tests reach 10^2, but as little as one bacterium injection–infiltrated into a grapefruit leaf has been shown to cause disease (Gottwald & Graham, 1992). The routine application of PCR for detecting pathogens in plant tissues is restricted by the presence of inhibitors that may interfere with the amplification of the target sequence (Henson & Fench, 1993). Another limitation of the direct PCR methods is how to distinguish between positive results that are generated from viable versus dead cells. To avoid these problems, BIO-PCR was used in this study. Based on this test, 57 out of 217 lesions were *Xcc* positive. In the case of Indian samples there was a little difference between plating on KCB media and BIO-PCR, indicating that the fruits were not chemically or physically treated. However, in Pakistani fruits, there was no significant difference between plating on KCB media and BIO-PCR. Additional BIO-PCR positives would be obtained if a larger volume of lesion washates was used (only 0.1 ml was used). Also, as shown in Tables 3 and 4, negative results occurred in BIO-PCR when high numbers of yellow saprophytic bacteria were present. But BIO-PCR still offers major advantages over agar plating. Results do not need to be verified by pathogenicity tests and are available five days sooner. In addition, BIO-PCR has advantages over serology and direct PCR, including a much greater sensitivity and the detection of only viable cells. The lower specificity of direct PCR protocols is probably due to the disinfection fruits treatments, which would reduce bacterial population and

induce VBNC state. These advantages are very important for a highly regulated quarantined pathogen such as *Xcc*. A major disadvantage of BIO-PCR over direct PCR is the additional three days required because of the slow growth of *Xcc* especially from treated commercial fruits. The presence of *Xcc* living bacteria, that remain pathogenic, constitutes a risk of dissemination of CBC disease through symptomatic fruits. The pathogenicity assays on grapefruit and/or Mexican lime leaves confirmed the fact that the *Xcc* cells produce CBC symptoms, even after chemical and physical treatments on the commercial fruits. An integrated approach for *Xcc* diagnosis in commercial citrus fruits should combine the following detection methods: isolation, BIO-PCR and bioassays. This combination of complementary tests should improve the accuracy of the detection of quarantine bacteria.

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