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2004-011: Draft Annex to ISPM 27:2006 – Xanthomonas citri subsp. citri
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[1]   **DRAFT ANNEX to ISPM 27:2006 – *Xanthomonas citri* subsp. *citri* (2004-011)**

[2]   **Draft history**

[3]

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Discipline leads history	2006-07 SC Lum KENG-YEANG (MY) 2011-05 SC Robert TAYLOR (AU)
Consultation on technical level	The first draft of this protocol was written by: • Enrique VERDIER (General Direction of Agricultural Services, Biological Laboratories Department, Montevideo, Uruguay) • Rita LANFRANCHI (Plant Pests and Diseases Laboratory, National Service of Agrifood Health and Quality (SENASA), Capital Federal, Argentina) • Maria M. LÓPEZ (Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Spain). The following expert also contributed to the preparation of the draft: • Jaime CUBERO (Instituto Nacional de Investigación v Tecnologia Agraria y Alimentaria (INIA), Spain).
Main discussion points during development of the diagnostic protocol	-
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[4]   **1. Pest Information**

[5]   *Xanthomonas citri* subsp. *citri* (Xcc) is the causal agent of citrus bacterial canker. It causes severe damage to many cultivated species of *Rutaceae* (EPPO, 1979) – primarily *Citrus* spp., *Fortunella* spp. and *Poncirus* spp. – grown under the tropical and subtropical conditions that are prevalent in many countries in Asia, South America, Oceania and Africa as well as in Florida, USA (CABI, 2006; EPPO, 2006). Atypical strains of Xcc with a restricted host range have been identified and are designated as strains A* and A^w (Sun *et al*., 2004; Vernière *et al*., 1998). These strains affect only *Citrus aurantiifolia* (Mexican lime) and *Citrus macrophylla* Webster (Alemow) in Florida, USA (Cubero & Graham, 2002, 2004).

[6]   Citrus bacterial canker typically occurs on seedlings and young trees in which there is a flush of actively growing shoots and leaves from late summer through to autumn. Canker lesions are formed on the leaves,

shoots, twigs and fruits of susceptible hosts. Attacks of *Phyllocnistis citrella*, the citrus leaf miner, can increase the susceptibility of leaves to citrus canker (Hall *et al.*, 2010).

[7]   Xcc can survive in diseased plant tissues, as an epiphyte on host and non-host plants, and as a saprophyte on straw mulch or in soil. However, overwintering lesions, particularly those formed on angular shoots, are the most important source of inoculum for the following season. The main mechanisms of short distance dispersal are wind-driven rain and splashing of water within and between plants: the bacteria are disseminated by rainwater running over the surface of lesions and then splashing onto healthy shoots (CABI, 2006). The movement of infected plant material, including budwood, rootstock seedlings and budded trees, has been implicated in long distance dispersal. There is no evidence that this pathogen is seed-borne (CABI, 2006).

[8]   **2. Taxonomic Information**

[9]   **Name:** *Xanthomonas citri* subsp. *citri* (Hasse) Gabriel *et al.*, 1989

[10]   **Synonyms:** *Xanthomonas axonopodis* pv. *citri* (Hasse) Vauterin *et al.*, 1995

[11]   *Pseudomonas citri* Hasse, 1915

[12]   *Xanthomonas citri* (Hasse, 1915) Gabriel *et al.*, 1989

[13]   *Xanthomonas citri* f.sp. *aurantifoliae* Namekata & Oliveira, 1972

[14]   *Xanthomonas campestris* pv. *citri* (Hasse) Dye, 1978

[15]   *Xanthomonas citri* (ex Hasse) nom. rev. Gabriel *et al.*, 1989

[16]   *Xanthomonas campestris* pv. *aurantifolii* Gabriel *et al.*, 1989

[17]   **Taxonomic position:** Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae

[18]   **Common names:** citrus canker, citrus bacterial canker

[19]   **Note:** Xcc has been recently reclassified from the A pathotype *X. axonopodis* pv. *citri*. The nomenclature of Gabriel *et al.* (1989) has been reinstated and the accepted name for the citrus bacterial canker pathogen is now *X. citri* subsp. *citri* (Bull *et al.*, 2010; Schaad *et al.*, 2006). The B and C pathotypes of *X. axonopodis* pv. *citri* have been reclassified as *X. fuscans* subsp. *aurantifolii* (Schaad *et al.*, 2006).

[20]   **3. Detection**

[21]   **3.1 Detection in symptomatic plants**

[22]   Diagnosis of citrus canker can be achieved by observing morphological characteristics on nutrient media, serological testing (by immunofluorescence (IF)), molecular testing (by polymerase chain reaction (PCR)), bioassay of leaf discs or detached leaves, and pathogenicity testing. Positive and negative controls must be included for all tests (see section 4 for reference controls).

[23]   **3.1.1 Symptoms**

[24]   The disease characteristically causes scabs or crater-like lesions on the rind of the fruits and on leaves, stems and shoots. Symptoms of citrus canker can occur on seedlings in any season and on young trees from late summer through to autumn, when a flush of abundant growth of angular shoots occurs (CABI, 2006) (Figures 1–4). The disease becomes sporadic as trees reach full fruiting development, because fewer angular shoots are produced and older leaf tissue and mature fruit are more resistant to citrus canker infection under natural conditions. Disease severity also depends on the susceptibility of the host plant species and cultivars (Goto, 1992).

[25]   *Symptoms on fruits.* Crater-like lesions develop on the surface of the fruit; they may be scattered singly over the fruit or several lesions may occur together with an irregular pattern. Exudation of resinous substances may be observed on young infected fruits. The lesions never extend through the rind.

[26]   *Symptoms on branches.* In dry conditions, the canker spot is corky or spongy, is raised, and has a ruptured surface. In moist conditions, the lesion enlarges rapidly, and the surface remains unruptured and is oily at the margin. In the more resistant cultivars, a callus layer may form between the diseased and healthy tissues. The scar of a canker may be identified by scraping the rough surface with a knife to remove the outer corky layer, revealing light to dark brown lesions in the healthy green bark tissues. The discoloured area can vary in shape and in size from 5 to 10 mm, depending on the susceptibility of the host plant.

[27]   *Symptoms on leaves.* Bright yellow spots are first apparent on the underside of leaves, followed by erumpent brownish lesions on both sides of the leaves, which become rough, cracked and corky. The canker may be surrounded by a water-soaked yellow halo margin.

[28]   Confusion may occur between citrus canker and scab or leaf spot-like symptoms caused by other plant pathogenic bacteria and fungi or by physiological disorders. Other bacteria on citrus that can cause citrus canker-like symptoms are *X. alfalfa* subsp. *citrumelonis* and *X. fuscans* subsp. *aurantifolii*. Both these bacteria have a limited host range, cause less aggressive symptoms, and rarely produce lesions on fruit (Timmer *et al.*, 2000). Citrus scab caused by the fungus *Elsinoë fawcettii* has been reported to have symptoms similar to citrus canker, especially on varieties that exhibit resistance to citrus scab (Taylor *et al.*, 2002; Timmer *et al.*, 2000), but in general, its scab lesions are drier and more irregular than those of citrus canker and sometimes lack the characteristic yellow halo. Citrus scab can be differentiated from citrus canker by the lack of bacterial ooze.

[29]   **3.1.2 Sample isolation**

[30]   Freshly prepared sample extracts are essential for successful isolation of Xcc from symptomatic plant material. However, when symptoms are very advanced or when environmental conditions are not favourable, the number of Xcc culturable cells can be very low and isolation can result in plates being overcrowded with competing saprophytic or antagonistic bacteria. Particular care should be taken to not confuse Xcc colonies with *Pantoea agglomerans*, which is also commonly isolated from canker lesions and produces yellow colonies on standard bacteriological media.

[31]   Isolation of the causal organism can be performed by streaking lesion extracts onto plates of suitable media, on which colonies of Xcc have a characteristic appearance. There are as yet no exclusively selective media available for Xcc.

[32]   Lesions are macerated in 0.5–1.0 ml saline (distilled sterile water with NaCl to 0.85%, pH 7.0), and when required they may be disinfected with 1% NaClO for 1 min, rinsed three times with sterile distilled water, and comminuted. An aliquot of the extract is streaked on nutrient media. Suitable general isolation media are nutrient agar supplemented with 0.1% glucose (NGA), yeast peptone glucose agar (YPGA) (yeast extract, 5 g; Bacto™ Peptone, 5 g; glucose, 10 g; agar, 20 g; distilled water, 1 litre; pH 7) and Wakimoto medium : potato broth (250 ml; sucrose, 15 g; peptone, 5 g; $Na_2HPO_4.12H_2O$, 0.8 g; $Ca(NO_3)_2 \cdot 7\ H_2O$, 0.5 g; Bacto™ Agar, 20 g; distilled water, 1 litre; pH 7.2). Filter-sterilized cycloheximide (100 mg/litre) can be added when necessary after autoclaving the media. The colony morphology on all three media is round, convex and smooth-edged and the colony is mucoid and creamy yellow. Growth is evaluated after incubation at 25–28 ºC for three to five days. In commercial fruit samples, the bacteria can be stressed and may have difficulty growing on the plates; therefore, more incubation days may be required or bioassays can be used to recover the bacteria from the samples.

[33]   **3.1.3 Serological detection – immunofluorescence**

[34]   For serological detection on bacterial cells, a loopful of fresh culture is collected from the plate and resuspended in 1 ml phosphate-buffered saline (PBS) (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water to 1 litre; pH 7.2) to make approximately 10^8 colony-forming units (c.f.u.)/ml. The suspension is centrifuged at 10 000 *g* for 2 min, and then the supernatant is discarded and the cells are resuspended in 100 ml coating buffer and applied to the serological test.

[35]   For serological detection on plant tissue, samples with symptoms – shoots, twigs, leaves and fruits, all with necrotic lesions, or tissue from cankers on twigs, branches, the trunk or the collar – should be chosen. Plant material should be analysed as soon as possible after collection; it may be stored at 4–8 ºC for up to two weeks until processing. The samples should be processed following the general procedure recommended for the specific serological test to be used. Generally, plant tissue is ground in freshly prepared antioxidant maceration buffer (polyvinylpyrrolidone (PVP-10), 20 g; mannitol, 10 g; ascorbic acid, 1.76 g; reduced glutathione, 3 g; PBS, 10 mM, 1 litre; pH 7.2) sterilized by filtration or PBS (NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water to 1 litre; pH 7.2) before use in serological tests.

[36]   Aliquots of 25 µl of each bacterial preparation or plant sample to be tested are pipetted onto a plastic-coated multi-window microscope slide, allowed to air dry and then gently heat-fixed over a flame. Separate slides are set up for each test bacterium, and also for positive and negative controls as are used for enzyme-linked immunosorbent assay (ELISA). Commercially available antiserum is diluted with PBS (pH 7.2) and appropriate dilutions are added to the windows of each slide. Negative controls can consist of normal (pre-immune) serum at one dilution and PBS. Slides are incubated in a humid chamber at room temperature for 30 min. The droplets are shaken off the slides and they are rinsed with PBS and then washed three times for 5 min each in PBS. The slides are gently blotted dry before 25 µl goat anti-rabbit gamma globulin-fluorescein isothiocyanate conjugate (FITC) at the appropriate dilution is pipetted into each window. The slides are incubated in the darkat room temperature for 30 min, rinsed, washed and blotted dry. Finally, 10 µl of 0.1 mmol/litre phosphate-buffered glycerine (pH 7.6) with an anti-fading agent is added to each window, which is then covered with a coverslip.

[37]   The slides are examined under immersion oil with a fluorescence microscope at 600✕ or 1 000✕ magnification. FITC fluoresces bright green under the ultraviolet light of the microscope. If the positive control with known bacterium shows fluorescent rod-shaped bacterial cells and the negative controls of normal serum and PBS do not, the sample windows are examined for bacterial cell wall fluorescence, looking for the cells with the size and form of Xcc. This method permits detection in the order of 10^3 cells/ml.

[38]   **3.1.4 Molecular detection**

[39]   **3.1.4.1 Controls for molecular testing**

[40]   For a reliable test result to be obtained, appropriate controls – which will depend on the type of test used and the level of certainty required – are essential. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template control) should be used as a minimum. These and other controls that should be considered for each series of nucleic acid isolation, target pest amplification or target nucleic acid amplification are described below.

[41]   **Positive nucleic acid control** Pre-prepared (stored) nucleic acid, whole genome amplified DNA or a synthetic control (e.g. a cloned PCR product) may be used as a control to monitor the efficiency of the test method (apart from the extraction) and the amplification of the PCR.

[42]   **Internal control**

[43]   For conventional and real-time PCR, a plant housekeeping gene (HKG) such as COX (Weller *et al.*, 2000) or 16S ribosomal (r)DNA (Weisberg *et al.,* 1991) should be incorporated into the PCR protocol as a control to eliminate the possibility of false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors.

[44]   **Negative amplification control (no template control)** For conventional and real-time PCR, PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage to rule out false positives due to contamination during preparation of the reaction mixture.

[45]   **Positive extraction control** This control is used to ensure that nucleic acid from the target is of sufficient quantity and quality for PCR amplification and that the target is detected. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target.

[46]   The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that the sequence can be readily compared to the sequence obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared to PCR amplicons of the correct size.

[47]   **Negative extraction control** This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue, and it requires nucleic acid extraction and subsequent amplification of uninfected host tissue. Multiple controls are recommended when large numbers of positive samples are expected.

[48]   **3.1.4.2 DNA extraction from infected citrus tissue**

[49]   DNA extraction from infected citrus tissue was originally performed by Hartung *et al.* (1993) with a hexadecyltrimethylammonium bromide (CTAB) protocol, but there are commercial methods and an isopropanol protocol (not requiring phenol) that have been extensively evaluated (Llop *et al.*, 1999). In the isopropanol protocol, lesions or plant material suspected to be infected are cut into small pieces, covered with PBS and shaken in a rotary shaker for 20 min at room temperature. The supernatant is filtered (to remove plant material) and then centrifuged for 20 min at 10 000 *g*. The pellet is resuspended in 1 ml PBS: 500 µl is saved for further analysis or for direct isolation on agar plates, and 500 µl is centrifuged at 10 000 *g* for 10 min. The pellet is resuspended in 500 µl extraction buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM ethylenediaminetetraacetic (EDTA); 0.5% sodium dodecyl sulphate (SDS); 2% polyvinylpyrrolidone (PVP)), vortexed and left for 1 h at room temperature with continuous shaking. The suspension is then centrifuged at 5 000 *g* for 5 min, after which 450 µl of the supernatant isis transferred to a new tube and mixed with 450 µl isopropanol. The suspension is mixed gently and left at room temperature for 1 h. Precipitation can be improved by the use of Pellet Paint[®] co-precipitant (Cubero *et al.,* 2001). The suspension is centrifuged at 13 000 *g* for 10 min, the supernatant is discarded, and the pellet is dried. The pellet is resuspended in 100 µl water. A 5 µl sample is used in a 50 µl PCR reaction. The conventional PCR method allows detection of 10^3 c.f.u./ml (Hartung *et al.*, 1993).

[50]   **3.1.4.3 Conventional PCR**

[51]   Several primer pairs are available for diagnosis of Xcc. Hartung *et al.* (1993) primers 2 and 3 target a *Bam*HI restriction fragment length polymorphic DNA fragment specific to Xcc and are the most frequently used in assays on plant material because of their good specificity and sensitivity (approximately 10^2 c.f.u./ml). Primers J-pth1 and J-pth2 target a 197 base pair (bp) fragment of the nuclear localization signal in the virulence gene *pthA* in *Xanthomonas* strains that cause citrus canker symptoms. These strains include Xcc, *X. fuscans* subsp. *aurantifolii* (formerly citrus canker pathotype strains B and C) and the atypical Xcc strains A[*] and A[w] detected in Florida (Cubero & Graham, 2002). The primers are universal, but they have lower sensitivity (10^4 c.f.u./ml in plant material) than the Hartung *et al.* (1993) primers. However, the Hartung primers do not detect the atypical Xcc strains A[*] and A[w] or *X. fuscans* subsp. *aurantifolii*. In situations where the presence of atypical Xcc strains A[*] and A[w] are suspected – for example, where citrus canker symptoms are observed on the hosts *C. aurantiifolia* (Mexican lime) and *C. macrophylla* Webster (Alemow) – both primer sets should be used.

[52]   • **PCR protocol of Hartung *et al.* (1993)**

[53]   The primers are:

[54]   • 2 (Reverse): 5′-CAC GGG TGC AAA AAA TCT-3′

[55]   • 3 (Forward) : 5′-TGG TGT CGT CGC TTG TAT-3′.

[56]   The PCR mixture is prepared in a sterile vial and consists of PCR buffer (50 mM Tris-HCl, pH 9; 20 mM NaCl; 1% Triton™ X-100; 0.1% gelatin; 3 mM MgCl$_2$), 1 µM of each primer 2 and 3, 0.2 mM of each deoxynucleotide triphosphate (dNTPs) and 1.25 U Taq DNA polymerase. Extracted DNA sample volume of 5 µl is added to 45 µl of the PCR mixture to give a total of 50 µl per reaction. The reaction conditions are an initial denaturation step of 95 ⁰C for 2 min followed by 35 cycles of 95 ⁰C for 60 s, 58 ⁰C for 70 s and 72 ⁰C for 75 s, and a final elongation step of 72 ⁰C for 10 min. The amplicon size is 222 bp.

[57]   • **PCR protocol of Cubero and Graham (2002)**

[58]   The primers are:

[59]   • *J-pth1(*Forward*):* 5′-CTT CAA CTC AAA CGCC GGA C-3′

[60]   • *J-pth2 (*Reverse*):* 5′-CAT CGC GCT GTT CGG GAG-3′.

[61]   The PCR mixture is prepared in a sterile vial and consists of 1× Taq buffer, 3 mM MgCl$_2$, 1 µM of each primer *J-pth1* and *J-pth2*, 0.2 mM of each dNTPs and 1 U Taq DNA polymerase. Extracted DNA sample volume of 2.5 µl is added to 22.5 µl of the PCR mixture to give a total of 25 µl per reaction. The reaction conditions are an initial denaturation step of 94 ⁰C for 5 min followed by 40 cycles of 93 ⁰C for 30 s, 58 ⁰C for 30 s and 72 ⁰C for 45 s, and a final elongation step of 72 ⁰C for 10 min. The amplicon size is 197 bp.

[62]   Nested PCR, immunocapture and colorimetric detection of nested PCR products for direct and sensitive detection of Xcc in plants have also been developed (Hartung *et al.*,1993). A review of the comparative sensitivity of the different protocols and primers in pure culture and fruit extracts has been reported (Golmohammadi *et al.*, 2007).

[63]   **3.1.4.4 Real-time PCR**

[64]   After obtaining DNA from plant material by using the protocol previously described by Llop *et al.* (1999), the pellet is resuspended in 100 µl sterile ultrapure water and stored at –20 °C until further use.

[65]   A set of primers, *J-pth3* (5'-ACC GTC CCC TAC TTC AAC TCA A-3') and *J-pth4* (5'-CGC ACC TCG AAC GAT TGC-3'), and the corresponding TaqMan® probe (*J-Taqpth2*) (5'-ATG CGC CCA GCC CAA CGC-3') labelled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with tetramethylrhodamine were designed based on sequences of the *pth* gene, a major virulence gene used in other studies specifically to detect Xcc strains (Cubero & Graham, 2005). These strains include Xcc, *X. fuscans* subsp. *aurantifolii* (formerly citrus canker pathotype strains B and C) and the atypical Xcc strains A[*] and A[w] detected in Florida.

[66]   Real-time PCR is carried out by adding 2 µl template DNA to a reaction mixture containing 12.5 µl QuantiMix Easy Kit, which comprises QuantiMix Easy Master Mix[1] and MgCl$_2$ (50 mM), 1 µl of 10 µM forward primer (*J-RTpth3*), 1 µl of 10 µM reverse primer (*J-RTpth4*) and 0.5 µl of 10 µM TaqMan® probe (*J-Taqpth2*) and made up to a final reaction volume of 25 µl with sterile distilled water. The real-time PCR is completed in an ABI[2] PRISM® 7000 Sequence Detection System. Amplification conditions for all primers and probes are an initial activation step of 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

[67]   The real-time PCR provides similar specificity to the *pth* gene primers used in the conventional PCR method (Cubero & Graham, 2002, 2005) and enables reliable detection of approximately 10 c.f.u. of Xcc from diseased leaf lesions and from a dilution of cultured cells (Mavrodieva *et al.*, 2004). This method has recently been compared with standard and nested PCR (Golmohammadi *et al.*, 2007) and the sensitivity obtained is very good (10 c.f.u./ml).

[68]   **3.1.5 Interpretation of results from conventional and real-time PCR**

[69]   **Conventional PCR**

[70]   The pathogen-specific PCR will be considered valid only if:

[71]   • the positive control produces the correct size product for the bacterium; and

[72]   • no bands of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

[73]   If 16S rDNA internal control primers are also used, then the negative (healthy plant tissue) control (if used), positive control, and each of the test samples must produce a 1.6 kilobase (kb) band (16S rDNA). Note that synthetic and plasmid positive controls will not produce a 1.6 kb band. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.

[74]   A sample will be considered positive if it produces an amplicon of the correct size.

[75]   **Real-time PCR**

[76]   The real-time PCR will be considered valid only if:

[77]   • the positive control produces an amplification curve with the pathogen-specific primers; and

[78]   • no amplification curve is seen (i.e. cycle threshold (Ct) value is 40) with the negative extraction control and the negative amplification control.

[79]   If the COX internal control primers are also used, then the negative control (if used), positive control, and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the nucleic acid has degraded.

[80]   A sample will be considered positive if it produces a typical amplification curve. The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

[81]   **3.1.6 Bioassays**

[82]   **3.1.6.1 Inoculation test in leaf discs**

[83]   In this test, citrus leaf tissue susceptible to Xcc is inoculated with diseased sample extracts and incubated under appropriate conditions for bacterial multiplication and development of incipient pustules of the disease.

[84]   The procedure for this bioassay begins by sterilizing ELISA plates for 15 min in a microwave oven and adding to their wells 200 µl of 1.5% agar in sterile water in a laminar flow chamber at room temperature. Young *Citrus paradisi* var. Duncan (grapefruit) leaves are surface-disinfected for 1 min with 1% NAClO. The leaves are rinsed three times with sterile distilled water and then surface dried in a laminar flow chamber at room temperature. The leaf discs, obtained with a hole punch (disinfected with 96% ethanol), are placed back up in each well with the agar-water. Fifty microlitres of macerated citrus canker lesions (four replicates for each sample) are added.

[85]   An Xcc suspension of 10^5 c.f.u./ml is used as a positive control and sterile saline as a negative control (four replicates each). Plates are sealed with Parafilm®, achieving a relative humidity of almost 100%, and incubated at 28 ºC for 12 days under constant light. The formation of incipient whitish pustules in each of the leaf discs is evaluated from the third day using stereoscopic microscopy and isolation techniques for Xcc as described in section 3.1.2. The symptomless discs can be further analysed for the presence of living bacteria by isolation onto semi-selective media (Verdier *et al.*, 2008). After 12 days, if Xcc is present, the bacterial cells have multiplied on the plant tissue and can be isolated onto media in higher numbers. This bioassay is a very specific and sensitive (10^2 c.f.u./ml) diagnostic method (Verdier *et al.*, 2008).

[86]   **3.1.6.2 Detached leaf enrichment**

[87]   Xcc can also be selectively enriched in wounded detached leaves of *Citrus paradisi* var. Duncan (grapefruit). Young terminal leaves from glasshouse-grown plants are washed for 10 min in running tap water, surface-disinfected in 1% NAClO for 1 min, and aseptically rinsed thoroughly with sterile distilled water. The lower surface of each leaf is aseptically wounded by puncturing it with a needle or by making small cuts with a scalpel, and the whole leaves are placed onto 1% agar in sterile water in the wells of ELISA plates with their lower surface up. Droplets of 10–20 µl of macerated citrus canker lesions are added. Positive and negative controls as for the leaf disc bioassay are used. After 7–12 days at 25 ºC in a lighted incubator, pustule development is evaluated and Xcc is isolated as above (EPPO, 1998).

[88]   **3.2 Detection in asymptomatic plants**

[89]   Isolation of Xcc from asymptomatic plants on semi-selective media can be achieved by washing the leaf or fruit samples in peptone buffer, concentrating the supernatant, and then plating onto the media (Verdier *et al.*, 2008). Ten leaves or one fruit constitute a sample.

[90]   Samples are shaken for 20 min at room temperature in 50 ml peptone buffer (NaCl, 8.5 g; peptone, 1 g; Tween® 20, 250 µl; distilled water, 1 litre; pH 7.2). For bulked samples, 100 leaves in 200 ml peptone buffer can be used. Individual fruits are shaken for 20 min at room temperature in sterile bags containing 50 ml peptone buffer.

[91]   The suspension is then centrifuged at 6 000 *g* for 20 min. The supernatant is decanted and the pellet resuspended in 10 ml of 0.85% saline. Aliquots (100 µl) of 1:100 and 1:1000 dilutions of each suspension are streaked in triplicate onto XOS semi-selective medium (sucrose, 20 g; peptone, 2 g; monosodium glutamate, 5 g; $Ca(NO_3)_2$, 0.3 g; K_2HPO_4, 2 g; EDTA-Fe, 1 mg; cycloheximide, 100 mg; cephalexine, 20 mg; kasugamycine, 20 mg; methyl violet 2B, 0.3 mg; Bacto™ Agar, 17 g; distilled water, 1 litre; pH 7.0) (Monier, 1992). After incubation at 28 ºC for 5–6 days, growth as well as colony type and morphology are evaluated (see section 3.1.2).

[92]   **4. Identification**

[93]   Identification of presumptive Xcc colonies should be verified by several techniques because other species of *Xanthomonas*, such as *X. fuscans* subsp. *aurantifolii* and *X. alfalfa* subsp. *Citrumelonis*, can be isolated from citrus. Techniques include observing morphological characteristics on nutrient media, serological testing, molecular testing, bioassay of leaf discs or detached leaves, and pathogenicity testing.

[94]   The minimum requirements for identification are isolation of the bacterium and a positive result from each of the three techniques: (1) PCR using two sets of primers (see section 4.1); (2) double antibody sandwich (DAS)-ELISA or indirect ELISA using specific monoclonal antibodies (see sections 4.2 and 4.2.1); and (3) pathogenicity testing by inoculation of citrus hosts to fulfil the requirements of Koch's postulates (see sections 4.3 and 3.1.5). Additional tests (see sections 4.4 and 4.5) may be done to further characterize the strain present. In all tests, positive and negative controls must be included. The recommended techniques are described in the following sections.

[95]   The following collections, among others, can provide Xcc reference strains (the Xcc isolates recommended for use as positive controls are given):

[96]   • NCPPB 3234 from National Collection of Plant Pathogenic Bacteria, Central Science Laboratory,

York, UK

[97] • CFPB 2911 from Collection Française de Bactéries Phytopathogènes, INRA Station Phytobactériologie, Angers, France

[98] • ICMP 24 from International Collection of Microorganisms from Plants, Landcare Research (Manaaki Whenua) New Zealand Ltd, Auckland, New Zealand

[99] • ATTC 49118 from American Type Culture Collection, Manassas, VA, USA

[100] • IBSBF 1594from Biological Institute Culture Collection of Phytopathogenic Bacteria, Centro Experimental Central do Instituto Biológico - Laboratório de Bacteriologia Vegetal, Campinas, Brazil.

[101] The authenticity of the strains can be guaranteed only if obtained directly from the culture collections.

[102] **4.1 PCR methods**

[103] Cubero and Graham (2002) developed PCR primers for the *pthA* gene involved in virulence (all citrus canker strains) and for the intergenic transcribed spacer (ITS) regions of 16S and 23S rDNAs specific to Xcc. Variation in the ITS sequences allowed the design of specific primers for Xcc and these primers detect the atypical strains A* and A^w (Cubero & Graham, 2002). The primers are:

[104] • *J-Rxg:* 5′-GCGTTGAGGCTGAGACATG-3′

[105] • *J-RXc2:* 5′-CAAGTTGCCTCGGAGCTATC-3′.

[106] PCR is carried out in 25 µl reaction mixtures containing 1× Taq buffer, 1.5 mM MgCl$_2$, 0.04 µM primer *J-RXg*, 0.04 µM primer *J-RXc2*, 0.2 mM each dNTP and 1 U Taq DNA polymerase. The PCR amplification conditions are the same as those used with the *pthA* primers described in section 3.1.4.1.

[107] It is recommended that in addition to the PCR protocol described in section 3.1.4.2, the identification of pure cultures of suspect strains are confirmed by using two sets of primers, based on rDNA and the pthA gene (Cubero & Graham, 2002). The DNA extraction procedure, primer description and PCR method are as described in section 3.1.4.2. Identification can be further confirmed by sequencing the resulting PCR amplicons and comparing their sequences with those of Xcc strains deposited in the NCBI GenBank database.

[108] **4.2 DAS-ELISA**

[109] For the DAS-ELISA, microtitre plates are coated with 200 µl/well carbonate coating buffer (Na$_2$CO$_3$, 1.59 g; NaHCO$_3$, 2.93 g; NaN$_3$, 0.2 g; distilled water, 1 litre; pH 9.6) containing appropriately diluted anti-Xcc immunoglobulins (IgG) and incubated overnight at 4 ⁰C. After washing the plates three times with PBS-Tween (NaCl, 8 g; KH$_2$PO$_4$, 0.2 g; Na$_2$HPO$_4$·12H$_2$O, 2.9 g; KCl, 0.2 g; NaN$_3$, 0.2 g; Tween® 20, 0.25 ml; distilled water, 1 litre; pH 7.4), test sample, negative control (healthy plant material) or positive control (reference strain of Xcc) is added (200 µl/well). The plates are incubated for 2 h at 37 ⁰C. After washing, anti-Xcc IgG conjugated with alkaline phosphatase at the appropriate dilution in PBS-Tween is added (200 µl/well) and the plates are incubated for 2 h at 37 °C. After washing, p-nitrophenyl phosphate substrate buffer (1 mg/ml) is added (200 µl/well) and the plates are incubated for 30–60 min at room temperature. The absorbances are measured using a spectrophotometer equipped with a 405 nm filter. The criterion for determination of a sample as positive is two times the optical density (OD) value of the healthy plant material control. The detection limit of DAS-ELISA is 10^4–10^5 c.f.u./ml (Civerolo & Fan, 1982). This method is not recommended for direct detection in plant tissue.

[110] Monoclonal antibodies are available for ELISA, but are advised to be used only for identification of pure cultures because of their low sensitivity of detection in plant tissue. Commercial kits for detection of Xcc by ELISA are available (e.g. from Agdia, Inc.). For specificity data, refer to the technical information provided by

the manufacturer. Some monoclonal antibodies have been reported to cross-react with *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas campestris* pv. *zinnea*, *Xanthomonas citromelo* and *Xanthomonas hortorum* pv. *Pelargonii*; however, these pathovars are unlikely to be present on citrus.

[111] **4.2.1 Indirect ELISA**

[112] Indirect ELISA with monoclonal antibodies described by Alvarez *et al.* (1991) can be used for culture identification. ELISA kits containing all the necessary components for the identification of Xcc are available commercially (e.g. Agdia, Inc.[3]). In theory, all Xcc strains can be identified, but it has been reported that some phenotypically distinct strains isolated in South-West Asia do not react with the available monoclonal antibodies (Vernière *et al.*, 1998).

[113] Pure culture suspensions are centrifuged at approximately 10 000 *g* for 2 min and the supernatant is discarded. One millilitre of 1× PBS is added and the cells are resuspended by vortexing. The operation is repeated twice more. After the third wash, the cells are resuspended in coating buffer. The bacterial concentration is adjusted spectrophotometrically to OD_{600} 0.01 (approximately 2.5×10^7 c.f.u./ml). Aliquots of the samples are loaded onto microtitre plates (two wells per sample, 100 µl/well). A positive control (a reference culture or sample provided by the manufacturer) and negative buffer control with another bacteria should be included. The plates are incubated overnight at 37 °C until they are dry. Blocking solution (5% non-fat dried milk powder in PBS) is added (200 µl/well). The plates are incubated for 30 min at room temperature and then washed twice with 1× PBS-Tween. Primary antibody at the appropriate dilution in 2.5% dried milk powder in PBS-Tween is added (100 µl/well). The plates are incubated for 1 h at room temperature and then washed five times with 1× PBS-Tween. Enzyme conjugate at the appropriate dilution in 2.5% dried milk powder in PBS-Tween is added (100 µl/well). The plates are incubated for 1 h at room temperature and then washed five times with 1× PBS-Tween. Freshly prepared substrate solution containing 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer (pH 9.8) is added (100 µl/well). The plates are incubated for 30–60 min at room temperature. The OD is measured using a spectrophotometer with a 405 nm filter. Positive samples are determined as for DAS-ELISA.

[114] **4.3 Pathogenicity testing**

[115] Xcc and its pathotypes should be identified by pathogenicity on a panel of indicator hosts such as *Citrus paradisi* var. Duncan (grapefruit), *Citrus sinensis* (Valencia sweet orange) or *Citrus aurantiifolia* (Mexican lime) for confirmation of the diagnosis.

[116] Leaf assays by infiltration with a syringe with or without needle on susceptible cultivars of *Citrus* hosts allow demonstration of pathogenicity of bacterial colonies. Lesions develop 7–14 days after inoculation of intact leaves or detached leaves (Francis *et al.*, 2010; Koizumi, 1971) after incubation at 25 ºC in high humidity. With these assays, the eruptive callus-like reaction of Xcc can readily be distinguished. Bacteria grown in liquid media or colonies from a freshly streaked agar plate are resuspended in sterile distilled water and the concentration is adjusted to 10^6–10^8 c.f.u./ml for inoculation into hosts. A negative and a positive control should always be included. Plants inoculated with the positive control strain should be kept apart from test plants.

[117] **4.4 Description and biochemical characteristics**

[118] Xcc is a Gram-negative, straight, rod-shaped bacterium measuring 1.5–2.0 × 0.5–0.75 µm. It is motile by means of a single polar flagellum. It shares many physiological and biochemical properties with other members of the genus *Xanthomonas*. It is chemoorganotrophic and obligatorily aerobic with an oxidative metabolism of glucose. The yellow pigment is xanthomonadin. Some of the biochemical characteristics that identify Xcc are listed in Table 1.

[119]

Table 1. Key biochemical characteristics of *Xanthomonas citri* subsp. *citri*

	X.c. subsp. *citri*
Catalase	+
Oxidase	- or weak
Nitrate reduction	-
Hydrolysis of:	
starch	+
casein	+
Tween 80	+
aesculin	+
Gelatin liquefaction	+
Pectate gel liquefied	+
Utilization of asparagine	-
Growth requires:	
methionine	+
cysteine	+
0.02% TTC (w/v)	-

[120]   For further information on the bacteriological and biochemical properties of Xcc see Goto (1992).

[121]   **4.5 Molecular identification**

[122]   Features of citrus-attacking xanthomonads including Xcc and the genus *Xanthomonas* as a whole have been characterized at the molecular level to develop quick and accurate methods for reclassification and identification. The procedures include DNA–DNA hybridization (Vauterin *et al.*, 1995), genomic fingerprinting (Lazo *et al.*, 1987), multilocus sequence analysis (Young *et al.*, 2008) and rep-PCR (Cubero & Graham, 2002, 2004).

[123]   **4.5.1 Rep-PCR fingerprinting**

[124]   Rep-PCR fingerprinting using primers designed from repetitive extragenic palindromic (REP) elements – enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX element (Louws *et al.*, 1994) – can be used for strain identification and characterization under specific PCR conditions (Cubero & Graham, 2002).

[125]   DNA can be extracted from bacterial suspensions (absorbance at 600 nm from 0.2 to 0.5) in a single step with phenol-chloroform-isoamyl alcohol, precipitated in ethanol, and resuspended in ultrapure water. DNA is stored at −20 °C until use. The DNA extraction procedure described in section 3.1.4.1 can also be used.

[126]   BOX PCR is carried out in 25 µl reaction mixtures containing 1× Taq buffer, 6 mM $MgCl_2$, 2.4 µM primer BOX1R (5′-CTACG-GCAAGGCGACGCTGCAG-3′)(Louws et al., 1994), 0.2 mM each dNTP, 2 U Taq polymerase and 5 µl DNA extracted from xanthomonad strains. The reaction conditions are an initial step of 94 °C for 5 min followed by 40 cycles of 94 °C (30 s), 48 °C (30 s) and 72 °C (1 min), and a final step of 72 °C for 10 min. PCR products are analysed in 3% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer (40 mmol/litre Tris-acetate; 1 mmol/litre EDTA; pH 8.0) for 2 h at 110 V and stained with ethidium bromide.

[127]   ERIC PCR is carried out in 25 µl reaction mixtures containing 1× Taq buffer, 3 mM $MgCl_2$, 1.2 µM primer ERIC1R (5′-ATGTAAGCTCCT-GGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACT-GGGGTGAGCG-3′) (Louws *et al.*, 1994), 0.2 mM each dNTP, 2 U Taq polymerase and 5 µl DNA extracted from xanthomonad strains. The reaction conditions are the same as for BOX PCR. Visualisation of PCR products is as for BOX PCR.

[128]   Fingerprints (band patterns) can be compared and analysed for similarity by eye, but patterns can also be transformed into peak patterns and strains compared using a computer software program such as

Bionumerics (Applied Maths NV, Belgium). Identification should be based on similarity to patterns of control (reference) strains (see section 4).

[129] **4.5.2 Genomic DNA fingerprinting**

[130] Genomic DNA fingerprinting can be used to characterize Xcc strains from different geographical locations (Hartung & Civerolo, 1987).

[131] **Extraction of DNA** (Berman *et al.*, 1981)

[132] Two ten millilitre liquid Luria Bertani (LB) cultures of the test bacteria and of positive controls of Xcc in 50 ml flasks are grown with gentle rotary shaking at 27 °C for 18 h. Genomic DNA is prepared as follows. The pooled 20 ml culture is centrifuged at 10 000 g for 10 min and the pellet is resuspended in 10 ml PBS (20 mmol/litre KH_2PO_4 buffer, pH 6.9, containing 150 mmol/litre NaCl). After a second centrifugation, the pellet is resuspended in 5 ml of 50 mmol/litre Tris, pH 8.0, containing 50 mmol/litre EDTA. Eggwhite lysozyme is added to a final concentration of 1 mg/ml and the tubes are incubated at 0 °C for 30 min. Then 1 ml of a freshly prepared lysing solution (0.5% SDS; 50 mmol/litre Tris-HCl, pH 7.5; 400 mmol/litre EDTA; 1 mg/ml pronase) is added to each tube, and the tubes are incubated at 50 °C until the suspension clears. The lysate is extracted with an equal volume of Tris buffer-saturated phenol (pH 7.8). After centrifugation (9 000 g for 10 min), the aqueous supernatant is transferred to a clean tube and sodium acetate is added to 0.3 mmol/litre. After addition of two volumes of ethanol and mixing by inversion, the nucleic acids are removed by spooling onto a glass pipette. They are dissolved in 3 ml Tris-EDTA (TE) buffer (10 mmol/litre Tris-HCl, pH 8.0; 1 mmol/litre EDTA) containing Ribonuclease (RNase) A (50 µg/ml). After 30 min at 37 °C, the solution is extracted with an equal volume of chloroform and the DNA is spooled out of the solution by a second ethanol precipitation. The DNA is dissolved in a minimal volume of TE buffer and stored at 4 °C until use. The concentration of DNA in the sample can be estimated spectrophotometrically.

[133] **DNA digestion**

[134] DNA extracts (3–5 µg) are digested with the restriction endonuclease *Eco*RI. Reaction volumes vary between 35 and 55 µl, buffer conditions are those recommended by the supplier, and incubation is at 37 °C for 4 h. Samples are loaded on a 1.5 mm thick, 14 cm long vertical 5% polyacrylamide gel, and fragments are separated by electrophoresis at 14 mA constant current for 14 h in Tris-borate-EDTA (TBE) buffer (89 mmol/litre Tris; 89 mmol/litre boric acid; 2 mmol/litre EDTA). During electrophoresis, the voltage increases from 50 V to 90 V. Gels are stained with ethidium bromide (2 µg/ml) for 60 min, then photographed on a transilluminator using both an orange and a yellow filter. Genomic fingerprints of the test and reference extracts are compared using the photograph or using the negative and the aid of a photographic enlarger.

[135] **5. Records**

[136] Records and evidence should be retained as described in section 2.5 of ISPM 27:2006.

[137] In instances where other contracting parties may be affected by the results of the diagnosis, retention of the original sample (labelled for traceability) culture(s) of the pest, preserved or mounted specimens, or test materials (e.g. photograph of gels, ELISA results printout, PCR amplicons) for at least for one year is recommended, especially in cases of non-compliance (ISPM 13:2001, *Guidelines for the notification of non-compliance and emergency action*) and where pests are found for the first time in a country or an area.

[138] **6. Contact Points for Further Information**

[139] General Direction of Agricultural Services, Biological Laboratories Department, Av. Millán 4703, CP 12900, Montevideo, Uruguay (Enrique F. Verdier, e-mail: emvermar@adinet.com.uy; tel.: +598 23043992).

[140] Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (María M. López, e-mail: mlopez@ivia.es; tel.: +34 963424000; fax: +34 963424001).

[141]   Instituto Nacional de Investigación Agraria y Tecnologia Alimentaria, INIA, Ctra de La Coruña km 6, Madrid, Spain (Jaime Cubero, e-mail: cubero@inia.es; tel.: +34 913473900; fax: +34 913572293).

[142]   **7. Acknowledgements**

[143]   The first draft of this protocol was written by E.F. Verdier, General Direction of Agricultural Services, Biological Laboratories Department, Uruguay (see section 6 for details), and revised by R. Lanfranchi, Plant Pests and Disease Laboratory, National Service of Agrifood Health and Quality, SENASA, Av. Ing. Huergo 1001 CP 1107, Buenos Aires, Argentina (Rita Lanfranchi, e-mail: ritalanfranchi@hotmail.com, tel.: +5411 43621177 int. 118); Ed Civerolo, USDA, United States; and M.M. López, IVIA, Spain (see section 6 for details). In addition, J. Cubero, INIA, Spain (see section 6 for details) was significantly involved in the development of this protocol.

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[184]   **9. Figures**

[185]   **Figure 1.** Typical citrus canker symptoms on leaves, stems and fruit of grapefruit

[186]

[187]   **Figure 2.** Twig symptoms of citrus canker: early lesions on grapefruit

[188]

[189]   **Figure 3.** Fruit symptoms of citrus canker on sweet orange (left) and grapefruit (right)

[190]

[191]   **Figure 4.** Leaf symptoms of citrus canker on lemon exacerbated by citrus leaf miner wounding

[192]

[193]   **Footnote 1.** The use of products of the brand QuantiMix in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[194]   **Footnote 2.** The use of products of the brand ABI in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[195]   **Footnote 3.** The use of products of the brand Agdia, Inc. in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.