Food and Agriculture Organization of the United Nations

International Plant Protection Convention
Protecting the world's plant resources from pests

ISPM 27
ANNEX 6

ENG

DP 6: *Xanthomonas citri* subsp. *citri*

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 6: *Xanthomonas citri* subsp. *citri*

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1. Pest Information

Xanthomonas citri subsp. *citri* is the major causal agent of citrus bacterial canker. It causes 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, United States (CABI, 2006; EPPO, 2006). Atypical strains of *X. citri* subsp. *citri* 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). Strain A* affects *Citrus aurantiifolia* (Mexican lime) under natural conditions in Asia. Strain A^w causes canker in *Citrus aurantiifolia* (Mexican lime) and *Citrus macrophylla* (Alemow) in Florida, United States under natural conditions (Cubero and Graham, 2002, 2004). Both of these strains have been reported to cause atypical lesions in other citrus species experimentally (Escalon *et al.,* 2013).

Citrus bacterial canker typically occurs on seedlings and on young and adult trees of susceptible hosts in which there is a flush of actively growing shoots and leaves from late summer through to autumn in most citrus growing areas. Canker lesions are formed on the leaves, shoots, twigs and fruits of susceptible hosts. Wounds caused by wind, thorns, insects, and physical or mechanical damage facilitate infection of mature tissues. Attacks of *Phyllocnistis citrella*, the citrus leaf miner, can increase the susceptibility of leaves to citrus canker (Hall *et al.*, 2010).

X. citri subsp. *citri* 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).

2. Taxonomic Information

Name:	*Xanthomonas citri* subsp. *citri* (Gabriel *et al.* 1989) Schaad *et al.* 2007
Synonyms:	*Xanthomonas smithii* subsp. *citri* Gabriel *et al.*, 1989, Schaad et *al.*, 2007
	Xanthomonas axonopodis pv. *citri* (Hasse) Vauterin *et al.*, 1995
	Xanthomonas citri (ex Hasse, 1915) Gabriel *et al.*, 1989
	Xanthomonas campestris pv. *aurantifolii* Gabriel *et al.*, 1989
	Xanthomonas campestris pv. *citri* (Hasse) Dye, 1978
	Xanthomonas citri f.sp. *aurantifoliae* Namekata and Oliveira, 1972
	Pseudomonas citri Hasse, 1915
Taxonomic position:	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae
Common names:	citrus canker, citrus bacterial canker, asiatic canker

Note: *X. citri* subsp. *citri* has been recently reclassified from *X. axonopodis* pv. *citri* (*X. campestris* pv. *citri* group A strains). 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 other group strains of *X. campestris* pv. *citri* have been reclassified as *Xanthomonas fuscans* subsp. *aurantifolii* (groups B, C and D) and *Xanthomonas alfalfae* subsp. *citrumelonis* (group E) (Schaad *et al*., 2006).

3. Detection

3.1 Detection in symptomatic plants

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

3.1.1 Symptoms

The disease characteristically causes scabs or crater-like lesions on the rind of 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).

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.

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 less susceptible 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.

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 or chlorotic halo margin.

Confusion may occur between symptoms on branches, leaves and fruit of citrus canker and scab or leaf spot-like symptoms caused by other bacteria or fungi that infect citrus or by physiological disorders. Other bacteria that can cause citrus canker-like symptoms are *X. alfalfae* subsp. *citrumelonis* and *X. fuscans* subsp. *aurantifolii*. Both of these bacteria have a limited host range, cause less aggressive symptoms and rarely produce lesions on fruit (Schaad *et al.,* 2005, 2006). Citrus scab caused by the fungus *Elsinoë fawcettii* has been reported to have symptoms similar to citrus canker, especially on host varieties that exhibit resistance to citrus scab (Taylor *et al.*, 2002), 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.

3.1.2 Isolation

Freshly prepared sample extracts are essential for successful isolation of *X. citri* subsp. *citri* from symptomatic plant material. Plant material should be analysed as soon as possible after collection; it may be stored at 4–8 °C until processing. When symptoms are very advanced or when environmental conditions are not favourable, the number of *X. citri* subsp. *citri* 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 not to confuse *X. citri* subsp. *citri* colonies with *Pantoea agglomerans*, which is also commonly isolated from canker lesions. and produces morphologically similar colonies on standard bacteriological media. *P. agglomerans* is generally faster growing and the colonies are a brighter yellow than the pale yellow/lemon colonies of *X. citri* subsp. *citri*.

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

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 beforehand with 1% NaClO for 1 min, rinsed three times with sterile distilled water, and pulverized. 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 7.0) 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 as a fungicide 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 not be easily cultured; therefore, longer incubations may be required or bioassays can be used to recover the bacteria from the samples, as described in section 3.1.6.2. Integration of kasugamycin and cephalexin in the medium (semi-selective KC or KCB medium) inhibits several saprophytic bacteria and facilitates isolation of the pathogen (Graham *et al.,* 1989; Pruvost *et al.,* 2005).

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. The use of names of chemicals (e.g. brand names) implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1.3 Serological detection: Indirect immunofluorescence

For serological detection (IF and enzyme-linked immunosorbent assay (ELISA)), appropriate controls are essential to ensure that test results are reliable. A positive and negative control should be included in each test. Positive controls can consist of a reference *X. citri* subsp. *citri* strain resuspended in healthy host plant extract (for detection in plant material) or in phosphate-buffered saline (PBS) (for identification of bacterial cultures). Negative controls should consist of healthy host plant extract (for detection in plant material) or a suspension of a non-target bacterial species (for identification of bacterial cultures).

For serological detection of bacterial cells, a loopful of fresh culture is collected from the plate and resuspended in 1 ml 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 (cfu)/ml (EPPO, 2009).

For serological detection in 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. 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) or in 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. Both solutions are filter-sterilized using a sterile 0.22 µm membrane.

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 or sample, and also for positive and negative controls as are used for ELISA. Commercially available antiserum or monoclonal antibodies are diluted with PBS (pH 7.2) and 25 µl of 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 of the appropriate anti-species gamma globulin-fluorescein isothiocyanate conjugate (FITC) at the appropriate dilution is pipetted into each window. The slides are incubated in the dark at 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.

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 show fluorescence, the sample windows are examined for fluorescent bacterial cells with the size and form of *X. citri* subsp. *citri*. This method permits detection of approximately 10^3 cfu./ml.

3.1.4 Molecular detection

3.1.4.1 Controls for molecular testing

For the test result obtained to be considered reliable, 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) are the minimum controls that should be used. These and other controls that should be considered for each series of nucleic acid extractions from your test samples as described below.

Positive nucleic acid control. Pre-prepared (stored) nucleic acid, whole genome DNA or a synthetic control (e.g. a cloned PCR product) may be used as a control to monitor the efficiency of PCR amplification.

Internal controls. For conventional and real-time PCR, a plant housekeeping gene (HKG) such as COX (Weller *et al.*, 2000), 16S ribosomal (r)DNA (Weisberg *et al.,* 1991) or GADPH (Mafra *et al.,* 2012) should be incorporated into the PCR protocol as a control to eliminate the possibility of false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

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.

Positive extraction control. This control is used to ensure that nucleic acid from the target is of sufficient quality for PCR amplification. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target at the concentration considered the detection limit of the protocol.

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 with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence, which, again, can be compared to PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises of nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended when large numbers of positive samples are tested.

3.1.4.2 DNA extraction from infected citrus tissue

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). DNA has also been successfully extracted from citrus tissue using commercial DNA extraction kits (e.g. Promega Wizard Genomic DNA Purification Kit) (Coletta-Filho *et al.,* 2006).

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 at 10 000 *g* for 20 min. 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% 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 is transferred to a new tube and mixed with 450 µl isopropanol. The suspension is mixed gently and left for 1 h at room temperature. 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.

3.1.4.3 Conventional PCR

Several primer pairs are available for diagnosis of *X. citri* subsp. *citri*. Hartung *et al.* (1993) primers 2 and 3 target a *Bam*HI restriction fragment length polymorphic DNA fragment specific to *X. citri* subsp. *citri* 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 *X. citri* subsp. *citri*, *X. fuscans* subsp. *aurantifolii* and the atypical *X. citri* subsp. *citri* strains A^* and A^w detected in Florida (Cubero and Graham, 2002). The primers are universal, but they have lower sensitivity (10^4 cfu/ml in plant material) than the Hartung *et al.* (1993) primers. However, the Hartung primers do not detect the *X. citri* subsp. *citri* strain A^w and all A^* strains or *X. fuscans* subsp. *aurantifolii*. In situations where the presence of atypical *X. citri* subsp. *citri* strains A^* and A^w is suspected – for example, where citrus canker symptoms are observed on the hosts *C. aurantiifolia* (Mexican lime) and *C. macrophylla* (Alemow) – both primer sets should be used.

PCR protocol of Hartung *et al.* (1993)

The primers are:

2 (Reverse): 5′-CAC GGG TGC AAA AAA TCT-3′

3 (Forward): 5′-TGG TGT CGT CGC TTG TAT-3′.

The PCR mixture is prepared in a sterile tube 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 each primer 2 and 3, 0.2 mM each deoxynucleotide triphosphate (dNTP) 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 ºC for 2 min followed by 35 cycles of 95 ºC for 60 s, 58 ºC for 70 s and 72 ºC for 75 s, and a final elongation step of 72 ºC for 10 min. The amplicon size is 222 bp.

PCR protocol of Cubero and Graham (2002)

The primers are:

J-pth1 (Forward): 5′-CTT CAA CTC AAA CGCC GGA C-3′

J-pth2 (Reverse): 5′-CAT CGC GCT GTT CGG GAG-3′.

The PCR mixture is prepared in a sterile tube and consists of 1× Taq buffer, 3 mM $MgCl_2$, 1 µM each primer *J-pth1* and *J-pth2*, 0.2 mM each dNTP 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 ºC for 5 min followed by 40 cycles of 93 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 45 s, and a final elongation step of 72 ºC for 10 min. The amplicon size is 198 bp.

Nested PCR, immunocapture and colorimetric detection of nested PCR products for direct and sensitive detection of *X. citri* subsp. *citri* 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).

3.1.4.4 Real-time PCR

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 use.

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 *X. citri* subsp. *citri* strains (Cubero and Graham, 2005). These strains include *X. citri* subsp. *citri*, *X. fuscans* subsp. *aurantifolii* and the atypical *X. citri* subsp. *citri* strains A^* and A^w detected in Florida.

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 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 protocol for real-time PCR has been developed using an ABI PRISM 7000 Sequence Detection System. Other equipment has provided similar results (María Lopez, pers. comm., 2013). Amplification conditions for 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. A complete real-time PCR kit based on this protocol and including master mix and enzyme is available from Plant Print Diagnostics (http://www.plantprint.net).

The real-time PCR provides similar specificity to the *pth* gene primers used in the conventional PCR method (Cubero and Graham, 2002, 2005) and enables reliable detection of approximately 10 cfu of *X. citri* subsp. *citri* 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 of detection of *X. citri* subsp. *citri* in fruit lesions was reported to be 10 cfu/ml.

3.1.5 Interpretation of results from conventional and real-time PCR

Conventional PCR

The pathogen-specific PCR will be considered valid only if the below criteria are met:

- the positive control produces the correct size amplicon for the bacterium
- no amplicons of the correct size for the bacterium are produced in the negative extraction control and the negative amplification control.

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 will produce an approximately 1.6 kilobase (kb) band (amplicon size will depend on which 16S rDNA primers are used (Weisberg *et al.*, 1991)). 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.

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

Real-time PCR

The real-time PCR will be considered valid only if the below criteria are met:

- the positive control produces an amplification curve with the pathogen-specific primers
- no amplification curve is seen (i.e. cycle threshold (Ct) value is 40) with the negative extraction control and the negative amplification control.

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 DNAhas degraded.

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.

3.1.6 Detection by bioassays

3.1.6.1 Inoculation test in leaf discs

In this test, citrus leaf tissue susceptible to *X. citri* subsp. *citri* is inoculated with diseased sample extracts and incubated under appropriate conditions for bacterial multiplication and development of incipient pustules of the disease.

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 leaves from *Citrus paradisi* var. Duncan (grapefruit) or other susceptible hosts, for example, *Citrus aurantifolia* (Mexican lime) or *Poncirus trifoliata* (trifoliate orange), are surface-disinfected for 1 min with 1% NAClO. The leaves should be fully expanded but not mature and hard. 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 95% ethanol), are placed adaxial surface down on the water agar in each well. Fifty microlitres of macerated citrus canker lesions (four replicate wells for each plant sample) are added.

An *X. citri* subsp. *citri* suspension of 10^5 cfu/ml is used as a positive control and sterile saline as a negative control (four replicates each). Plates are sealed (e.g. Parafilm), achieving a relative humidity of almost 100%, and incubated at 28 ºC for 12 days under constant light, with progress checked regularly. 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 *X. citri* subsp. *citri* 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 *X. citri* subsp. *citri* 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 cfu/ml) diagnostic method (Verdier *et al.*, 2008).

3.1.6.2 Detached leaf enrichment

X. citri subsp. *citri* can also be selectively enriched in wounded detached leaves of *C. paradisi* var. Duncan (grapefruit) or other highly susceptible hosts, for example, *C. aurantifolia* (Mexican lime) or *P. trifoliata* (trifoliate orange). 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 to the wounds. Positive and negative controls as for the leaf disc bioassay are used. After 4–12 days at 25 ºC in a lighted incubator, pustule development is evaluated and *X. citri* subsp. *citri* can be isolated from either the pustules or the symptomless wounded leaf tissue as described above (EPPO, 1998).

3.2 Detection in asymptomatic plants

Detection of *X. citri* subsp. *citri* in asymptomatic plants can be achieved by isolation and enrichment on semi-selective media (see below), serological techniques (IF (section 3.1.3)) and molecular testing (section 3.1.4).

Isolation of *X. citri* subsp. *citri* 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.

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.

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 (section 3.1.2).

4. Identification

Identification of presumptive *X. citri* subsp. *citri* colonies should be verified by several techniques because other species of *Xanthomonas*, such as *X. fuscans* subsp. *aurantifolii* and *X. alfalfae* subsp. *citrumelonis*, can be isolated from citrus. Techniques in addition to observing morphological characteristics on nutrient media, include serological testing, molecular testing, bioassay of leaf discs or detached leaves, and pathogenicity testing.

The minimum requirements for identification of a pure culture are a positive result from each of the following three techniques: (1) PCR using two sets of primers (section 4.1); (2) a serological technique (IF, double antibody sandwich (DAS)-ELISA or indirect ELISA sections 4.2, and 4.2.1 and 4.2.2)using specific monoclonal antibodies ; and (3) pathogenicity testing by inoculation of citrus hosts to fulfil the requirements of Koch's postulates (sections 4.3 and 3.1.6). Additional tests (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.

The following collections, among others, can provide *X. citri* subsp. *citri* reference strains (the *X. citri* subsp. *citri* isolates recommended for use as positive controls are given):

- NCPPB 3234 from National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom
- CFPB 2911 from Collection Française de Bactéries Phytopathogènes, INRA Station Phytobactériologie, Angers, France (this is a *X. citri* subsp. *citri* A* strain)
- ICMP 24 from International Collection of Microorganisms from Plants, Landcare Research (Manaaki Whenua) New Zealand Ltd, Auckland, New Zealand
- ATTC 49118 from American Type Culture Collection, Manassas, VA, United States

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

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

4.1 PCR methods

It is recommended that in addition to the PCR protocol described in section 3.1.4.3, the identification of pure cultures of suspect strains is confirmed by using two different sets of primers. One set should be the *J-pth1*/*J-pth2* or *J-Rxg*/*J-Rxc2* primers (Cubero and Graham, 2002) and the other set the Xac01/Xac02 (Coletto-Filho *et al.,* 2005) or XACF/XACR primers (Park *et al.*, 2006) (Table 1). This is because of the findings that most published primer pairs lack specificity (Delcourt *et al.*, 2013). Identification can be further confirmed by sequencing the resulting PCR amplicons and comparing their sequences with those of *X. citri* subsp. *citri* strains deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

PCR protocol of Cubero and Graham (2002) developed PCR primers for the internal transcribed spacer (ITS) regions of 16S and 23S rDNAs specific to *X. citri* subsp. *citri*. Variation in the ITS sequences allowed the design of specific primers for *X. citri* subsp. *citri* and these primers detect the atypical strains A* and A^w (Cubero and Graham, 2002). The primers are:

J-Rxg: 5′-GCGTTGAGGCTGAGACATG-3′

J-RXc2: 5′-CAAGTTGCCTCGGAGCTATC-3′.

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 3.1.4.3.

PCR protocol of Coletta-Filho *et al.* (2006) developed primers based on the *rpf* gene cluster. The primers are:

Xac01: 5′-CGCCATCCCCACCACCACCACGAC-3′

Xac02: 5′-AACCGCTCAATGCCATCCACTTCA-3′.

PCR is carried out in 25 µl reaction mixtures containing 1× Taq buffer, 2.0 mM $MgCl_2$, 0.36 µM each primer, 0.25 mM each dNTP and 1 U Taq DNA polymerase. The PCR amplification conditions are an initial denaturation step of 94 ºC for 3 min followed by 36 cycles of 94 ºC for 45 s, 60 ºC for 45 s and 72 ºC for 45 s, and a final elongation step of 72 ºC for 5 min. The amplicon size is 582 bp.

PCR protocol of Park *et al.* (2006) developed primers based on the *hrpW* gene sequences. The primers are:

XACF: 5′- CGTCGCAATACGATTGGAAC-3′

XACR: 5′- CGGAGGCATTGTCGAAGGAA-3′.

PCR is carried out in 25 µl reaction mixtures containing 1× Taq buffer, 1.5 mM $MgCl_2$, 0.10 µM each primer, 0.25 mM each dNTP, 0.01% gelatin and 2 U Taq DNA polymerase. The PCR amplification conditions are an initial denaturation step of 94 ºC for 5 min followed by 30 cycles of 94 ºC for 15 s, 60 ºC for 30 s and 72 ºC for 30 s, and a final elongation step of 72 ºC for 7 min. The amplicon size is 561 bp.

Table 1. Summary of PCR methods described in this diagnostic protocol.

Specificity data are taken from Delcourt *et al.* (2013). * Non-specific detection refers to the percentage of pathogenic xanthomonads and saprophytes that tested positive. ** Did not test positive with saprophytic strains.

Primer pair	Reference	Amplicon size (bp)	*X. citri* subsp. *citri* strain detection	Non-specific detection (%)*	Limits of detection in plant material
2/3	Hartung *et al.* (1993)	224	Does not detect A^w and all A* strains	17	10^2 cfu/ml
J-pth1/J-pth2	Cubero and Graham (2002)	198	All strains	51	10^4 cfu/ml
J-Rxg/J-Rxc2	Cubero and Graham (2002)	179	All strains	30	10^4 cfu/ml
Xac01/Xac02	Coletto-Filho *et al.* (2005)	582	All strains	16	10^4 cfu/ml
XACF/XACR	Park *et al.* (2006)	561	All strains	6**	Not reported

4.2 Serological detection

It is recommended that in addition to the IF protocol described in section 3.1.3, different antibodies should be used for identification of pure cultures. DAS- ELISA or Indirect ELISA can also be used as alternative serological tests for the identification of pure cultures.

4.2.1 DAS-ELISA

For the DAS-ELISA, microtitre plates are coated with 100 µl/well carbonate coating buffer (Na_2CO_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-*X. citri* subsp. *citri* immunoglobulins (IgG) and incubated overnight at 4 ºC. After washing the plates three times with PBS-Tween (NaCl, 8 g; KH_2PO_4, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 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 *X. citri* subsp. *citri*) is added (200 µl/well). The plates are incubated for 2 h at 37 ºC. After washing, anti-*X. citri* subsp. *citri* 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 cfu/ml (Civerolo and Fan, 1982). This method is not recommended for direct detection in plant tissue.

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 *X. citri* subsp. *citri* 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 *X. axonopodis* pv. *phaseoli*, *X. campestris* pv. *zinnea*, *X. alfalfae subsp. citrumelonis* and *Xanthomonas hortorum* pv. *pelargonii*; however, these pathovars are unlikely to be present on citrus.

4.2.2 Indirect ELISA

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 *X. citri* subsp. *citri* are available commercially (e.g. from Agdia, Inc.). In theory, all *X. citri* subsp. *citri* 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).

Pure culture suspensions are centrifuged at approximately 10 000 *g* for 2 min and the supernatant is discarded. One ml 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 cfu/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 bacterium 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.

4.3 Pathogenicity testing

X. citri subsp. *citri* should be identified by pathogenicity on a panel of indicator hosts such as *C. paradisi* var. Duncan (grapefruit), *Citrus sinensis* (Valencia sweet orange) or *C. aurantiifolia* (Mexican lime) for confirmation of the diagnosis.

Leaf assays by infiltration with a syringe with or without needle on susceptible cultivars of *Citrus* hosts allow demonstration of pathogenicity of bacterial colonies. Immature leaves that are 50–70% to fully expanded are preferred due to their higher level of susceptibility. 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 *X. citri* subsp. *citri* 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 cfu/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 separate from test plants.

4.4 Description and biochemical characteristics

X. citri subsp. *citri* 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 *X. citri* subsp. *citri* are listed in Table 2.

Table 2. Key biochemical characteristics of *Xanthomonas citri* subsp. *citri*

Test	Result
Catalase	+
Oxidase	– or weak
Nitrate reduction	–
Hydrolysis of:	
starch	+
casein	+
Tween 80	+
aesculin	+
Gelatin liquefaction	+
Pectate gel liquefaction	+
Utilization of asparagine	–
Growth requires:	
methionine	+
cysteine	+
0.02% triphenyl tetrazolium chloride (TTC) (w/v)	–

4.5 Molecular identification

Features of citrus-attacking xanthomonads including *X. citri* subsp. *citri* 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 (Hartung *et al.,* 1987; Lazo *et al.*, 1987), multilocus sequence analysis (Young *et al.*, 2008) and rep-PCR (Cubero and Graham, 2002, 2004).

4.5.1 Multilocus sequence analysis

A multilocus sequence analysis (MLSA) approach has been used for the specific identification of *X. citri* subsp. *citri*. (Almeida *et al.,* 2010; Bui Thi Ngoc *et al.,* 2010; Young *et al.,* 2008). Housekeeping genes are amplified using primers and PCR conditions as described by Almeida *et al.* (2010), Bui Thi Ngoc *et al.* (2010) and Young *et al.,* (2008). MLSA consists of sequencing multiple loci (typically four to eight housekeeping genes) and comparing these sequences with reference sequences of *Xanthomonas* species deposited in nucleotide databases; for example, the Plant Associated Microbes Database (PAMDB) (http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl) (Almeida *et al.*, 2010) and the MLVAbank for microbe genotyping (https://bioinfo-prod.mpl.ird.fr/MLVA_bank/Genotyping/).

4.5.2 Rep-PCR fingerprinting

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 and Graham, 2002).

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 3.1.4.2 can also be used.

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 DNA 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 for 30 s, 48 °C for 30 s and 72 °C for 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) run for 2 h at 110 V and stained with ethidium bromide.

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 DNA polymerase and 5 µl DNA extracted from xanthomonad strains. The reaction conditions are the same as for BOX PCR. Visualization of PCR products is as for BOX PCR.

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). Identification should be based on similarity to patterns of control (reference) strains (section 4).

Schemes for detection and identification of *Xanthomonas citri* subsp. *citri* on symptomatic and asymptomatic plant material are shown in figures 5 and 6, respectively.

5. Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

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 (*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.

6. Contact Points for Further Information

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).

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A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will forward it to the Technical Panel on Diagnostic Protocols (TPDP).

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addition, Mr J. Cubero, INIA, Spain (see section 6 for details) was significantly involved in the development of this protocol.

8. References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Typical citrus canker symptoms on leaves, stems and fruit of grapefruit (*Citrus paradisi*).

Figure 2. Twig symptoms of citrus canker: early lesions on grapefruit (*Citrus paradisi*).

Figure 3. Fruit symptoms of citrus canker on sweet orange (*Citrus sinensis*) (left) and grapefruit (*Citrus paradisi*) (centre and right).

Figure 4. Leaf symptoms of citrus canker on lemon (*Citrus limon*) exacerbated by citrus leaf miner wounds.

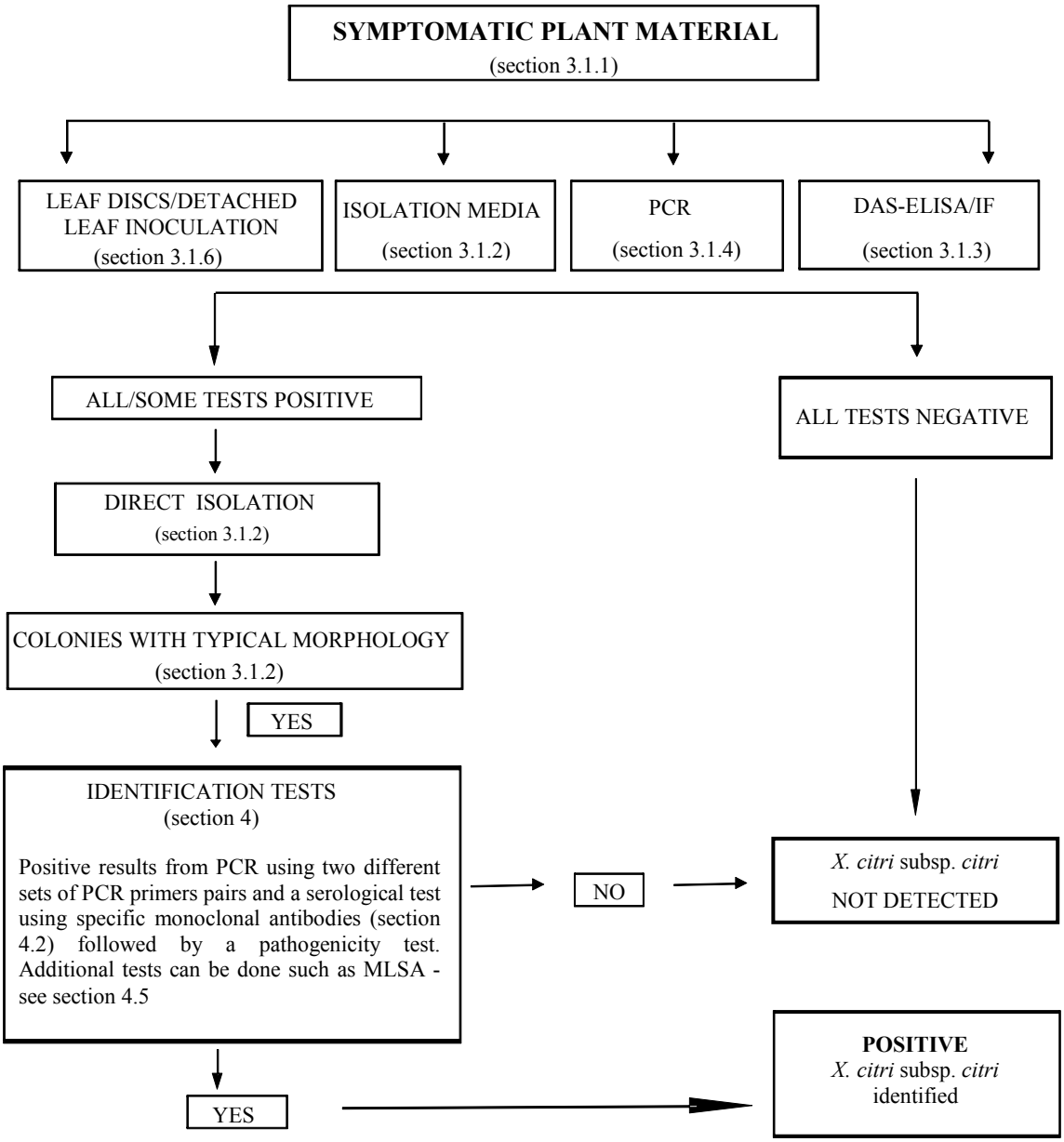

Figure 5. Scheme for detection and identification of *Xanthomonas citri* subsp. *citri* on symptomatic plant material.

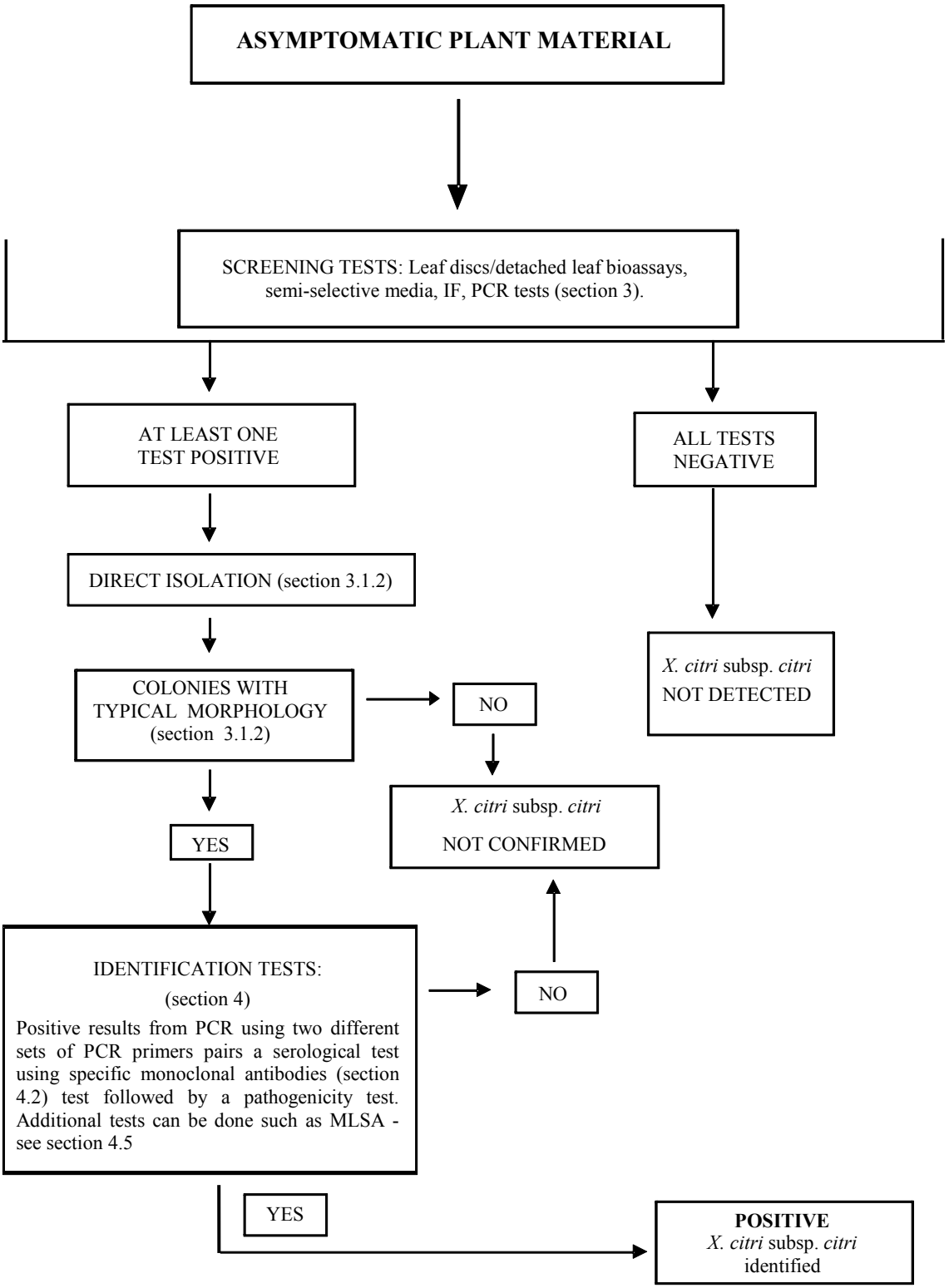

Figure 6. Scheme for detection and identification of *Xanthomonas citri* subsp. *citri* on asymptomatic plant material.

Publication history

This is not an official part of the standard

2004-11 SC added subject *Xanthomonas axonopodis* pv. *citri* (2004-011) to the work programme.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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