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EPPO STANDARD ON DIAGNOSTICS

PM 7/44 (2) *Xanthomonas citri* pv. *citri* and *Xanthomonas citri* pv. *aurantifolii*

Specific scope: This Standard describes a diagnostic protocol for *Xanthomonas citri* pathovars responsible for citrus bacterial canker disease.
This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.[1]
Authors and contributors are given in the Acknowledgements section.
Specific approval and amendment: First approved in 2004–09. Revised in 2023–01.

1 | INTRODUCTION

Four distinct *Xanthomonas* pathovars that are members of two species, *X. citri* and *X. euvesicatoria*, are described as pathogens in rutaceous species, which are important fruit crops worldwide. Two taxa, *X. euvesicatoria* pv. *citrumelonis* and *X. citri* pv. *bilvae*, cause spot diseases of minor agricultural significance (Baker et al., 2014). The two other taxa cause bacterial canker on citrus. *Xanthomonas citri* pv. *citri* is the causal agent of Asiatic citrus canker whereas *X. citri* pv. *aurantifolii* is the causal agent of a disease referred to as South American citrus canker (or sometimes false canker). *X. citri* pv. *aurantifolii* and *X. citri* pv. *citri* induce morphologically indistinguishable symptoms (Graham et al., 2004). These two pathovars include strains differing in host range and agricultural significance, yielding the description of pathological variants referred to as pathotypes (i.e., groups of strains causing similar symptoms but differing in host range among rutaceous species; Rossetti, 1977; Rybak et al., 2009; Sun et al., 2004; Vernière et al., 1998). Diagnosing canker-causing bacterial strains at the pathotype level is important, as this information is crucial for guiding surveillance strategies following outbreak detection (e.g. defining the list of plant species to be investigated).

Xanthomonas citri pv. *aurantifolii* has solely been reported from four countries in South America (Argentina, Brazil, Paraguay and Uruguay) where two pathotypes have been reported (Rossetti, 1977). Pathotype B induces canker-like symptoms from natural infections primarily on lemon (*Citrus limon*) and Mexican lime (*C. aurantiifolia*) but also to a much lesser extent on sweet orange (*C. sinensis*), sour orange (*C. aurantium*), pummelo (*C. maxima*), citron (*C. medica*), rough lemon (*C. jambhiri*), or Rangpur lime (*C. limonia*). This pathotype was identified from all above-mentioned countries but not from Brazil. Pathotype C was originally reported as a group primarily infecting Mexican lime and to a much lesser extent Tahiti lime (*C. latifolia*), although a single report mentioned pathotype C strains pathogenic to Swingle citrumelo (*Poncirus trifoliata* × *C. paradisi*) but not Mexican lime (Behlau et al., 2020; Jaciani et al., 2009). This pathotype has only been reported in Brazil. While commonly observed on citrus in the decades following the initial description (ranging from 1933 to the 1960s; Rossetti, 1977), no significant outbreaks caused by *X. citri* pv. *aurantifolii* have been reported in recent years in these countries, suggesting that it has been outcompeted (Behlau et al., 2020) and consequently *X. citri* pv. *aurantifolii* is considered unlikely to be present in imported consignments.

Xanthomonas citri pv. *citri* is responsible for severe outbreaks in many areas of citrus production (EPPO, 2023a, 2023b). Its host range is strain-dependent and three pathotypes have been reported (Sun et al., 2004; Vernière et al., 1998). Pathotype A strains overall have the greatest impact on citrus industries. They are widely distributed and induce natural canker infections on a broad range of rutaceous species, including nearly all *Citrus* cultivars, hybrids as well as members of the following genera: *Casimiroa*, *Citropsis*, *Clausena*, *Eremocitrus*, *Fortunella*, *Naringi* (syn. *Hesperethusa*), *Microcitrus*, *Poncirus*, *Severinia*, *Swinglea* (syn. *Aegle*) and *Zanthoxylum* (Graham et al., 2004; Koizumi, 1978, 1981; Lee, 1918; Reddy, 1997; Stover et al., 2014). Pathotype A* strains are pathogenic to a restricted range of citrus species. Most outbreaks were reported on Mexican lime in Asia, the Arabian Peninsula and Eastern Africa although some strains can produce mild canker when inoculated to other Citrus species (Derso et al., 2009; Escalon et al., 2013; Pruvost et al., 2015; Vernière et al., 1998). In countries where the lime industry is economically important, pathotype A* can be of great concern, as at least some strains are highly aggressive (Bui Thi Ngoc et al., 2007). Pathotype A^{w} has been reported from the

[1]*Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.*

Indian subcontinent, the Arabian Peninsula, and the USA. Natural infections are restricted to Mexican lime and the related alemow (*C. macrophylla*).

Flow diagrams describing the diagnostic procedure for *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* are presented in Figures 1 and 2.

2 | IDENTITY

Name: *Xanthomonas citri* pv. *aurantifolii* (Schaad et al., 2006) Constantin et al., 2016

Synonyms: *X. fuscans* subsp. *aurantifolii* Schaad et al. (2006); *X. axonopodis* pv. *aurantifolii* (Gabriel et al., 1989)

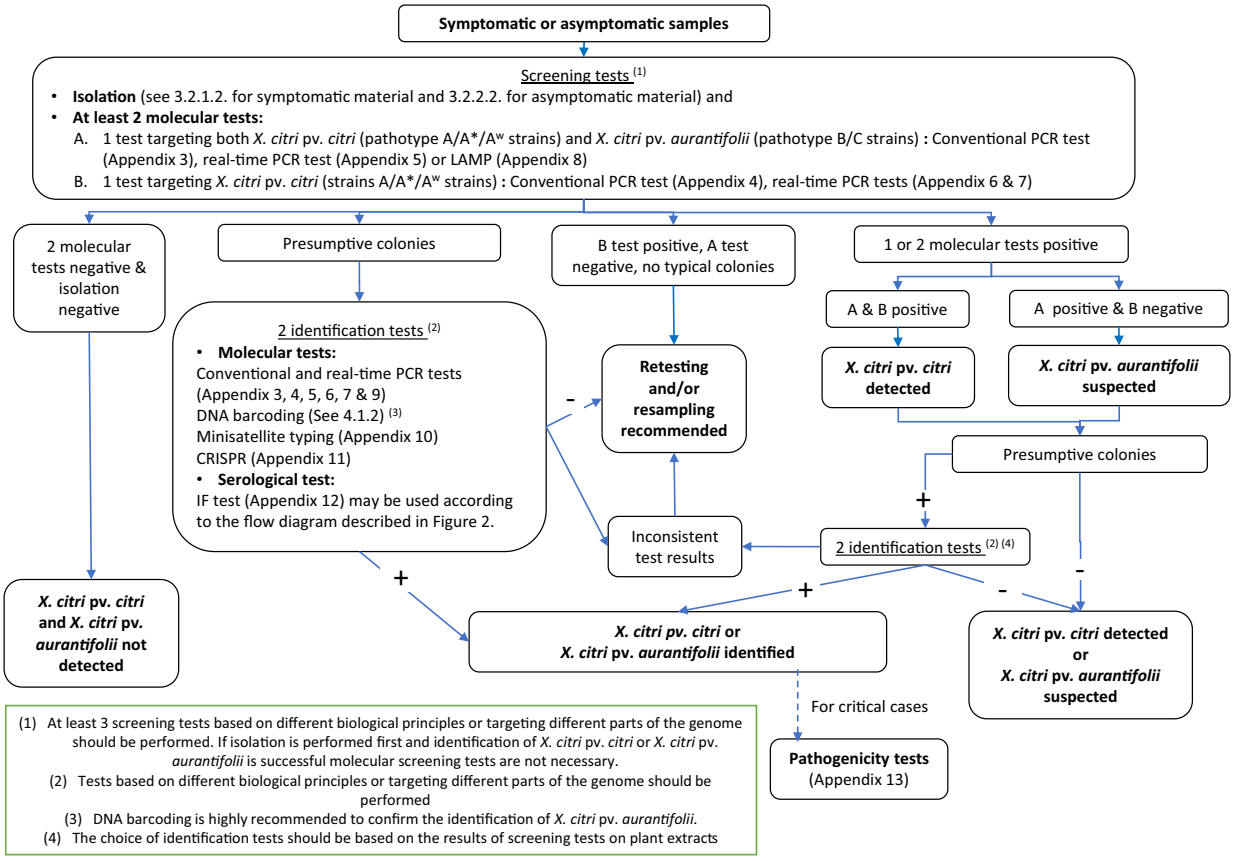

FIGURE 1 Flow diagram describing the diagnostic procedure for *Xanthomonas citri* pv. *citri* and *X. citri* pv. *aurantifolii*.

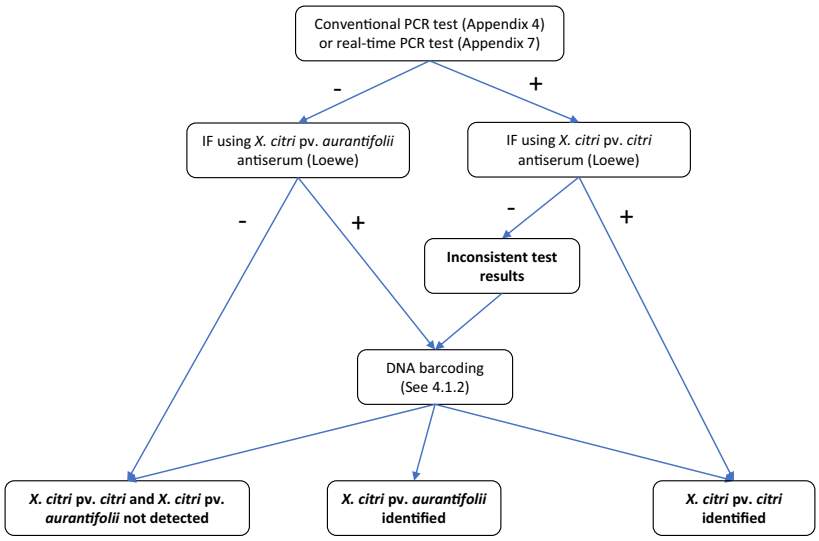

FIGURE 2 Flow diagram describing the identification procedure for *Xanthomonas citri* pv. *citri* and *X. citri* pv. *aurantifolii* using IF.

Vauterin et al., 1995; *X. campestris* pv. *citri* (Hasse, 1915) Dye, 1978.
Taxonomic position: Bacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae, Xanthomonas
EPPO Code: XANTAU
Phytosanitary categorization: EPPO A1 list no. 397; EU Annex IIA (as A1 Quarantine Pests)

Name: *Xanthomonas citri* pv. *citri* (Hasse, 1915) Constantin et al., 2016
Synonyms: *X. citri* subsp. *citri* (Hasse, 1915) Schaad et al., 2006; *X. axonopodis* pv. *citri* (Hasse, 1915) Vauterin et al., 1995; *X. campestris* pv. *citri* (Hasse, 1915) Dye, 1978; *X. citri* (Hasse, 1915) Dowson, 1939; *Pseudomonas citri* Hasse, 1915.
Taxonomic position: Bacteria, Gammaproteobacteria, Lysobacterales, Lysobacteraceae, Xanthomonas
EPPO Code: XANTCI
Phytosanitary categorization: EPPO A1 list no. 1; EU Annex IIA (as A1 Quarantine Pests)

3 | DETECTION

3.1 | Disease symptoms

Xanthomonas citri pv. *aurantifolii* and *X. citri* pv. *citri* can infect all aerial parts of their hosts. When the disease is severe, defoliation and early fruit drop can occur, but no tree death has been reported.

3.1.1 | Symptoms on leaves

On leaves, lesions first appear on the lower leaf surface as pin-point oily spots due to water-soaking of the tissue. Later the lesions become visible on both epidermal surfaces as slightly raised pustules or blister-like eruptions (Figure 3). As lesions develop, they increase in size, the epidermis ruptures and the lesions become erumpent, spongy or corky. The pustules then darken and thicken into light tan-brown corky lesions, which are rough to the touch. Eventually, their centre becomes crater-like. Diagnostic symptoms are tissue hyperplasia resulting in cankers sometimes with water-soaked margins and yellow halos surrounding the lesions. Lesions with an atypical morphology (flat or blister-like spots) can be sometimes observed, especially in the case of late fruit infections or lesions on some resistant cultivars.

3.1.2 | Symptoms on twigs

On twigs, the symptoms are similar: raised corky lesions initially surrounded by an oily or water-soaked margin. The lesions are generally irregularly shaped and may be sunken. Pustules may coalesce but chlorosis does not typically surround twig lesions. On removal of the corky layer, dark brown lesions are visible in the healthy green bark tissue. On highly susceptible citrus cultivars, diseased twigs can eventually show dieback symptoms.

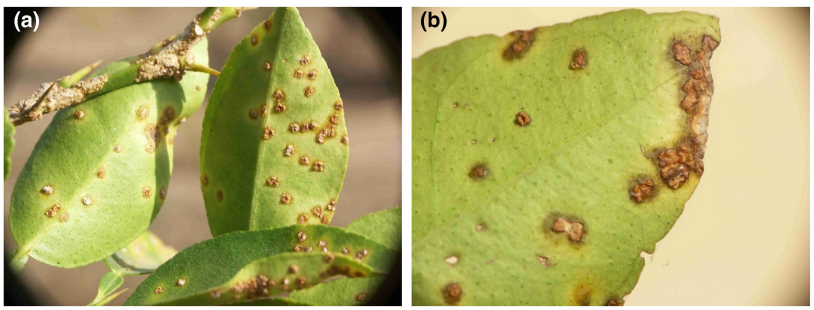

FIGURE 3 Lesions caused by *Xanthomonas citri* pv. *citri* on leaves of (a) *Citrus sinensis* and (b) *Citrus limon*. Photo courtesy: C. Vernière & O. Pruvost (CIRAD, FR).

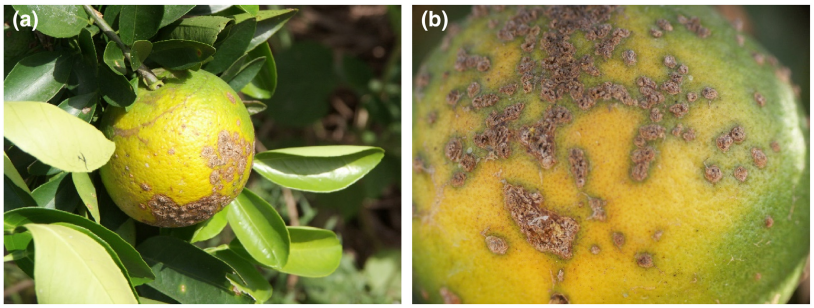

FIGURE 4 Lesions caused by *Xanthomonas citri* pv. *citri* on a (a) tangor fruit and (b) orange fruit. Photo courtesy: C. Vernière & O. Pruvost (CIRAD, FR).

3.1.3 | Symptoms on fruits

Lesions on fruits can appear when they are still small and green. They are similar to those on leaves, but tend to have more elevated margins and a sunken centre (Figure 4). These craters do not penetrate deep into the rind. Yellow chlorotic halos may or may not be present.

Harvestable infected fruit have a reduced value or can be unmarketable depending on the severity of infection.

3.1.4 | Possible confusion

Leaf and fruit symptoms of citrus bacterial canker may be confused with citrus scab (*Elsinoë fawcettii*; Figure 5), Phaeoramularia leaf and fruit spot disease (*Pseudocercospora angolensis*; Figure 6) and to a lesser extent other fungal diseases. Leaf symptoms, at early stages, and especially on young plants may be confused with citrus bacterial spot caused by *X. euvesicatoria* pv. *citrumelonis* (Figures 7 and 8).

FIGURE 5 *Elsinoë fawcettii* on *Citrus reticulata*. Photo courtesy: J. Mota (Direção de Serviços da Agricultura, PT).

FIGURE 6 *Pseudocercospora angolensis* on *Citrus paradisi*. Photo courtesy: J. Hubert (ANSES, FR).

3.2 | Screening tests

Isolation and at least two molecular tests, one targeting both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* and one targeting only *X. citri* pv. *citri* should be performed. However, if the isolation and identification of *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* are successful, performing two additional molecular tests on plant material is not required. No validated tests are currently available for the specific detection of *X. citri* pv. *aurantifolii*. Therefore, the detection of *X. citri* pv. *aurantifolii* is inferred based on the results of two molecular tests: one targeting both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* (giving a positive result) and one targeting specifically *X. citri* pv. *citri* (giving a negative result).

Serological tests are not recommended for the detection of *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* in plant material.

To conclude that *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* are not detected, two molecular tests and isolation should be negative.

FIGURE 7 Natural infection caused by *Xanthomonas euvesicatoria* pv. *citrumelonis* in *Citrus sinensis* 'Hamlin' Photo courtesy: J. Cubero (INIA, ES).

FIGURE 8 Inoculation with *Xanthomonas euvesicatoria* pv. *citrumelonis* in Swingle citrumelo Photo courtesy: J. Cubero (INIA, ES).

3.2.1 | Symptomatic material

3.2.1.1 | *Test sample requirement*

The plant material should be surface sterilized. Small pieces of the water-soaked tissue at the lesion margin are excised with a sterilized scalpel or razor blade, briefly cleaned with ethanol and allowed to dry. The tissue is chopped or diced in a drop of sterile distilled water or 0.01 M Tris buffer pH 7.2.

Young canker lesions should be preferred if available. A few canker lesions can be pooled to increase the probability of successful isolation.

3.2.1.2 | *Isolation*

Isolation from canker lesions is usually easy unless samples are in poor conditions or for some strains of *X. citri* pv. *aurantifolii*. However, in commercial fruit samples, the bacteria may be stressed e.g. due to different fruit treatments (washing, disinfection, chemical treatments, transport, and storage at low temperatures for variable periods of time) and may not be easily cultured (Golmohammadi et al., 2007); therefore, longer incubations may be required, or a bioassay (Appendix 13) may be performed (IPPC, 2016).

The chopped or diced tissue is soaked in distilled water for 10–15 min at room temperature and is streaked on the media.

- Generic media

Plating is done on generic media such as nutrient agar supplemented with 0.1% w/v d-glucose (Nutrient Glucose Agar – NGA), Yeast Peptone Glucose Agar (YPGA) or Wilbrink agar medium.

Pathotype B strains are known to be difficult to isolate (Canteros et al., 1985), and display better growth on modified Sucrose Peptone Agar (SPA).

- Semi selective media

The use of a semi-selective medium facilitates a successful isolation of xanthomonads. Semi-selective media are KCB or KC (media supplemented with kasugamycin, cephalexin and a fungicide) and are recommended for the isolation of *X. citri* pv. *citri* as reported by Gottwald and Graham (1992) and Pruvost et al. (2005). *X. citri* pv. *aurantifolii* does not form isolated colonies on semi-selective media (personal communication, O. Pruvost (CIRAD, FR)).

For *X. citri* pv. *citri* at least one generic and one semi-selective media is recommended to maximize the probability of detection. For *X. citri* pv. *aurantifolii*, SPA media should be used. Media are described in Appendix 1.

After 3–7 days of incubation at approximately 28°C, colonies of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* on agar plates are circular, convex, mucoid, shiny and creamy yellow[2] (Figure 9). On Wilbrink media, the colour ranges from cream to yellow for *X. citri* pv. *aurantifolii* and from dark cream/light yellow to bright yellow for *X. citri* pv. *citri* and some differences in sliminess have also been observed (Figure 10). Note that growth of *X. citri* pv. *aurantifolii* is delayed compared to *X. citri* pv. *citri* on all media but the growth of *X. citri* pv. *aurantifolii* is better on media supplemented with sucrose compared to that supplemented with glucose.

3.2.1.3 | *Molecular tests*

For a reliable detection of the citrus bacterial canker causing pathovars it is important to use molecular tests specific for *X. citri* pv. *citri* and/or *X. citri* pv. *aurantifolii*. Several sets of primers were designed for the detection of *X. citri* pv. *citri*. However, only a few tests have an appropriate analytical specificity (inclusivity or exclusivity).

Molecular tests for screening recommended below have been selected based on the validation data gathered from Delcourt et al. (2013), Robène et al. (2020) and from a Test Performance Study performed in the framework of the H2020 VALITEST project (Chabirand, 2021). DNA extraction is described in Appendix 2. No validated test is currently available for the specific detection of *X citri* pv. *aurantifolii*. Therefore, the detection of *X citri* pv. *aurantifolii* is inferred based on the results of two molecular tests: one targeting both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* (giving a positive result) and one targeting specifically *X. citri* pv. *citri* (giving a negative result).

Note that some tests are targeting a gene belonging to the *pthA* gene family which is essential for the bacteria to induce disease symptoms in citrus plants (Brunings & Gabriel, 2003). This gene is not present in non-canker-inducing bacteria isolated from citrus, such as *X. euvesicatoria* pv. *citrumelonis* and may be absent in *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* strains that lost virulence or have reduced virulence. Full virulence of those strains is restored with the introduction of the *pthA* gene (Ye et al., 2013).

- Conventional PCR
  - The conventional PCR test based on VM3 and VM4 primers targeting *pthA* (Delcourt et al., 2013; Mavrodieva et al., 2004) as described in Appendix 3 can be used for the detection of *X. citri* pv. *citri* (pathotypes A, A* and A^w strains) and *X. citri* pv. *aurantifolii* (pathotypes B and C strains).
  - The conventional PCR test based on primers XAC1051-F and XAC1051-R as described in Appendix 4 can be used for the detection of *X. citri*

[2]In a study conducted in La Réunion Island, strains of *Xanthomonas citri* pv. *citri* altered in xanthomonadin pigment production (and therefore cream-white to pale yellow) have been very occasionally observed (Vernière, 1992).

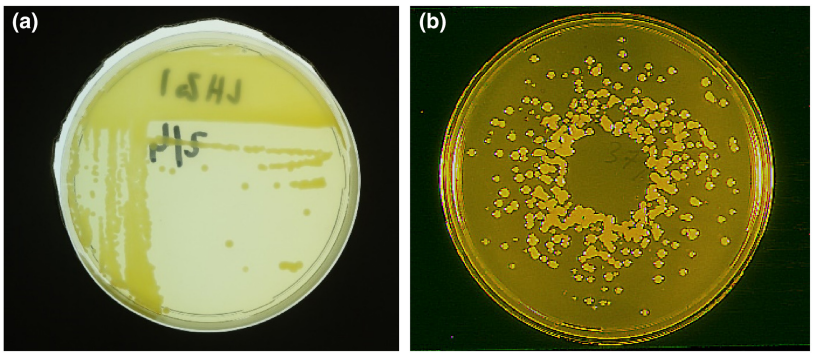

FIGURE 9 Typical colony morphology of *Xanthomonas citri* pv. *citri* on (a) YPGA medium and (b) KC medium after incubation at 28°C for 3 days. Photo courtesy: C. Vernière & O. Pruvost (CIRAD, FR).

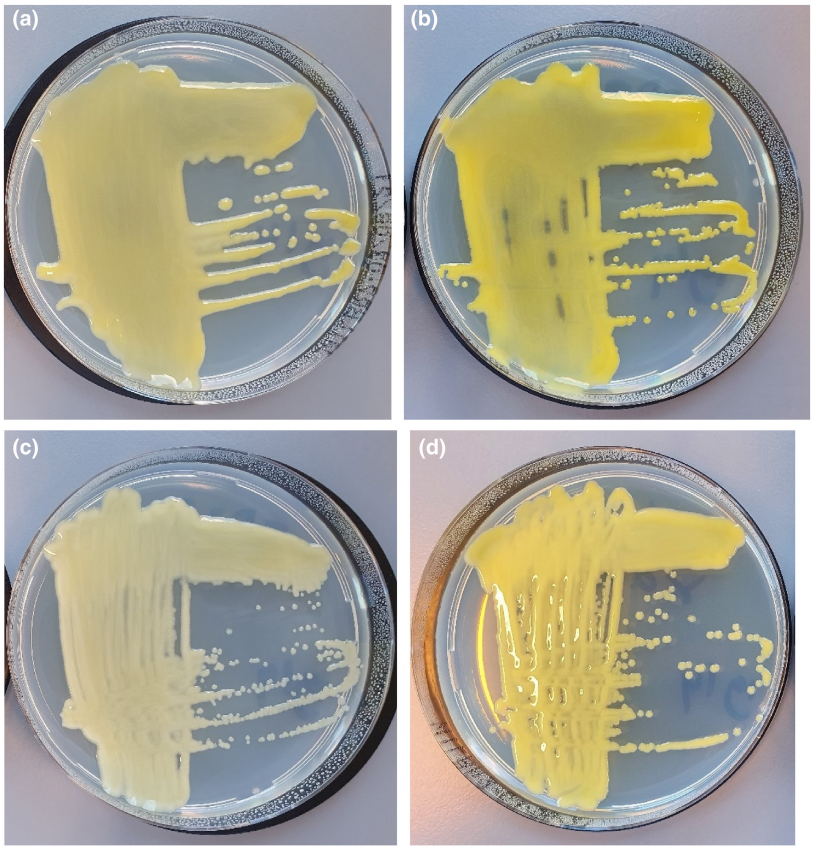

FIGURE 10 Typical colony morphology of *Xanthomonas citri* pv. *citri* (PD7215 (a) and NCPPB 409 (b)) and *X. citri* pv. *aurantifolii* (LMG 9182 (c) and LMG 9179 (d)) on Wilbrink media. Photo courtesy: R.J.M. Volkers & M. Bergsma-Vlami (NIVIP, NL).

pv. *citri* (pathotype A, A* and A^{W} strains; Robène et al., 2020).

- Real-time PCR
  - The real-time PCR test based on the primers VM3 and VM4 targeting *pthA*, using the fluorescent SYBR Green I detection dye (Mavrodieva et al., 2004) as described in Appendix 5 can be used for the detection of *X. citri* pv. *citri* (pathotype A, A* and A^{W} strains) and *X. citri* pv. *aurantifolii* (pathotype B and C strains).
  - The real-time TaqMan PCR test based on primers J-pth3 and J-pth4 targeting *pthA*, and the corresponding TaqMan probe (J-Taqpth2; Cubero & Graham, 2005) as described in Appendix 6 can be used for the detection of *X. citri* pv. *citri* strains (except for one pathotype A strain) but not for strains of *X citri* pv. *aurantifolii*.
  - The duplex TaqMan real-time PCR test XAC1051-2qPCR targeting the *X. citri* pv. *citri* XAC1051 gene (Robène et al., 2020) and the internal plant control (5.8S rDNA) as described in Appendix 7 can be

used for the detection of all *X. citri* pv. *citri* (evaluated on 98 strains).

- LAMP test

The loop-mediated isothermal amplification test (LAMP) based on the amplification of *pthA* gene as described in Appendix 8 can be used for the detection of pathotype A, A*, A^W, B and C strains of citrus bacterial canker from plant samples after a rapid extraction method using Chelex® (Rigano et al., 2010) or a simplified alkaline extraction protocol (Li et al., 2013).

3.2.2 | Asymptomatic plant material

3.2.2.1 | *Test sample requirement*

The test sample consists of 10 leaves or 1–10 fruits (depending on size of the fruits and feasibility). When sampling asymptomatic trees, leaves or fruits should be collected from all four quadrants of the trees.

Leaves or fruits should be covered with sterile 0.01 M Tris-Tween buffer (e.g. 20 mL per leaf, 50 mL per fruit) and washed at room temperature on a rotary shaker (200 rpm) for 20 min. Washings should then be centrifuged at 20 000 *g* for 5 min. The supernatant should be discarded, and the pellet resuspended in sterile distilled water or 0.01 M Tris buffer pH 7.2 at a volume corresponding to 1% of the original volume of Tris-Tween buffer.

3.2.2.2 | *Isolation*

Isolation can be performed directly by streaking the extracts prepared as described in 3.2.2.1 on the semi-selective media described in 3.2.1.2 (i.e. KCB or KC). However, as the *Xanthomonas* concentration is expected to be low in asymptomatic samples, enrichment may be attempted by performing a bioassay (see Appendix 13). If lesions develop the procedure described in Section 3.2.1 (symptomatic material) is followed.

3.2.2.3 | *Molecular tests*

The molecular tests as described in 3.2.1.3 can be performed on the extracts prepared as described in 3.2.2.1.

4 | IDENTIFICATION

Identification tests on pure bacterial cultures should be chosen based on the results of the detection tests on plant extracts (i.e., whether *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* is suspected). Pure cultures of presumptive *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* isolates should be identified with at least two tests, based on different biological principles or targeting different genetic loci. For critical cases (EPPO, 2017), a confirmative pathogenicity test is recommended. Recommended tests are listed in Section 4.1 and 4.2. Biochemical and physiological tests are not recommended because they are not specific to the targeted organisms.

Given the performance characteristics of the PCR based tests targeting *X. citri* pv. *aurantifolii* e.g. conventional and real-time PCR tests from Mavrodieva et al. (2004) and serological tests (cross reaction with *X. citri* pv. *citri* and other non-target bacteria), barcoding is highly recommended to confirm the identification of *X. citri* pv. *aurantifolii*.

4.1 | Molecular tests

4.1.1 | PCR based molecular tests

Conventional and real-time PCR tests can be used for identification at the pathovar level. The concomitant use of VM3/VM4 and XAC1051-F/ XAC1051-R primers (Mavrodieva et al., 2004; Robène et al., 2020) are recommended for conventional PCR (see Appendices 3 and 4). For real-time PCR, VM3/VM4 primers using the fluorescent SYBR Green I detection dye can be used (Mavrodieva et al., 2004; Robène et al., 2020; Appendix 5) together with one of the two following tests that are specific for *X. citri* pv. *citri*: J-pth3/J-pth4, and the corresponding TaqMan probe (J-Taqpth2; Cubero & Graham, 2005) or the XAC1051-2qPCR tests (Robène et al., 2020; Appendices 6 and 7).

One real-time TaqMan PCR test based on primers and probe targeting the *lrp* gene followed by allelic discrimination analysis can be used to differentiate the pathotypes of *X. citri* pv. *citri* (pathotype A vs. A* and A^W strains; Appendix 9; Cubero & Graham, 2005).

Additional conventional PCR tests for the identification of *X. citri* pv. *citri* (pathotype A, A* and A^W) and *X. citri* pv. *aurantifolii* (pathotypes B and C; based on Fonseca et al., 2019) were used in the framework of a proficiency test organized in 2021 by the EURL for bacteriology and gave satisfactory results when used as simplex PCRs. However, further validation data are needed before they can be included in a revision of the diagnostic protocol.

4.1.2 | DNA barcoding

DNA barcoding can be used for the identification of strains at the species/pathovar level but not at the pathotype level. The main advantage of DNA barcoding is that the obtained nucleotide sequences can be easily compared to reliable reference sequences. Protocols for routine barcoding of *Xanthomonas* spp. using 16S rDNA (to genus level), *gyrB* (to species level) and *avrBs2* (to subspecies level) are described in Appendix 2 of the EPPO Standard PM 7/129(2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021). Reference sequences for

those loci are available at https://qbank.eppo.int/bacteria/. General procedures for sequencing are described in Appendices 7 and 8 of the EPPO Standard PM 7/129 (EPPO, 2021).

4.1.3 | Minisatellite genotyping (MLVA)

MLVA has been increasingly proposed to genetically characterize *Xanthomonas* pathovars over the last decade. This method allows pathotype A, A* and A^w strains of *X. citri* pv. *citri* to be distinguished and to compare the identified strains with the global diversity of strains of *X. citri* pv. *citri* (Pruvost et al., 2014). Another advantage of this technique is that the obtained multilocus haplotypes can be compared to reference profiles online (http://www.biopred.net/mlva/). The detailed procedure is provided in Appendix 10.

4.1.4 | Clustered regularly interspaced short palindromic repeat genotyping (CRISPR)

This method allows pathotype A, A* and A^w strains of *X. citri* pv. *citri* to be distinguished and also to compare the identified strains with the global diversity of strains of *X. citri* pv. *citri* (Jeong et al., 2019). Spoligotyping (for spacer oligonucleotide typing), is based on the detection of unique spacers in the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) locus. All strains of *X. citri* pv. *citri* that have been analysed until now carry a CRISPR array that is built from a subset of 23 unique spacer sequences (Jeong et al., 2019). Spoligotypes of *X. citri* pv. *citri* are made available online (http://www.biopred.net/mlva/). Presence of CRISPR spacers can be elucidated by DNA sequencing of PCR amplicons or directly by oligonucleotide hybridization or using CRISPR/Cas-based detection technology (Li et al., 2019). To date, none of these technologies have been commercialized to type xanthomonads. The detailed procedure is provided in Appendix 11.

4.2 | Serological tests

Although not recommended by the drafting team during the preparation of the protocol because of specificity issues of the available antisera, the Panel on Diagnostics in Bacteriology suggested the addition of immunofluorescence (IF) in combination with, at least, one of the molecular tests based on Robène et al. (2020; Conventional or real-time PCR) and whenever needed DNA barcoding for the identification of pure cultures of presumptive *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* (see Figure 2) IF may be used providing that the following recommendations are met.

- The identification flow diagram described in Figure 2 should be followed.
- The antisera targeting *X. citri* pv *aurantifolii* or *X. citri* pv *citri* from Loewe should be used (Appendix 12). Other commercial kits using polyclonal or monoclonal antibodies against *X. citri* pv. *citri* and/or *X. citri* pv. *aurantifolii* are available. However, these tests are not able to identify all the pathotypes of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* or inclusivity is not precisely known.
- The test should be performed as described in EPPO Standard PM 7/97 Indirect immunofluorescence test for plant pathogenic bacteria (EPPO, 2009) using a pure culture (approximately 10^6 cfu mL^{-1}) in phosphate buffer (PBS 0.01 M) as the sample.

4.3 | Pathogenicity tests

For critical cases, pathogenicity can be confirmed either using a detached-leaf or attached-leaf assay (Appendix 13).

5 | REFERENCE MATERIAL

Strains of *X. citri* pv. *aurantifolii* (CFBP2904 = NCPPB3236 = LMG9179 pathotype B; CFBP2866 = NCPPB3233 = LMG9181) and *X. citri* pv. *citri* (CFBP2525 = NCPPB409 = LMG682) can be used as positive controls. It should be noted that several studies suggested that the pathotype strain of *X. citri* pv. *aurantifolii* lost its ability to produce symptoms during conservation or only produces mild symptoms on specific citrus species (Appendix 12). Other reference strains include LMG9322 (pathotype A), NCPPB3607 (pathotype A*), NCPPB3608 (pathotype A^w), CFBP2902 (pathotype B), CFBP2866 (pathotype C). The following collections can provide different reference strains:

(i) National Collection of Plant Pathogenic Bacteria (NCPPB), Fera, Sand Hutton, York (GB); https://www.fera.co.uk/ncppb;
(ii) International Center for Microbial Resources – French, Collection for Plant-associated Bacteria (CIRM-CFBP), IRHS – INRAE Beaucouzé (FR); https://cirm-cfbp.fr/page/Home.
(iii) Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig (DE); https://www.dsmz.de/l
(iv) Laboratorium voor Microbiologie Bacterial Collection (LMG), Universiteit Gent (BE); http://bccm.belspo.be/

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

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

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9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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APPENDIX 1 - MEDIA AND BUFFERS

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

1. Media

Yeast peptone glucose agar (YPGA)

Yeast extract	7 g
Peptone	7 g
Glucose	7 g
Agar	18 g
Distilled water	1.0 L
Adjust pH to 7.2.	

Nutrient glucose agar (NGA)

Nutrient broth without NaCl	8.0 g
Glucose	10 g
Agar	15.0 g
Distilled water	1.0 L
Adjust pH to 6.9.	

Wilbrink agar (Koike, 1965; Sands et al., 1986)

Peptone special	5.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
Sucrose	10.0 g
Agar technical no. 3	18.0 g
Distilled water to	1 L
Adjust pH to 7.0 (±0.2) with KOH/HCl before adding Agar	

Modified sucrose peptone agar (SPA; Canteros et al., 1985)

Sucrose	10.0 g
Peptone	5.0 g
K_2HPO_4	0.5 g
$MgSO_4$	0.3 g
Difco purified agar	15.0 g
Distilled water	1.0 L

Kasugamycin Cephalexin Bravo (KCB; Gottwald & Graham, 1992)

Nutrient broth without NaCl	8.0 g
Agar	15.0 g
Distilled water	1.0 L
Adjust pH to 6.9.	

After autoclaving and cooling down filter-sterilized solutions of antibiotics and other components are added.

Kasugamycin HCl	16.0 mg
Cephalexin	35.0 mg (add sodium hydroxide for dissolution)
Chlorothalonil	12.0 mg

If not used immediately the media can be kept refrigerated for a few weeks.

Kasugamycin Cephalexin (KC; Pruvost et al., 2005)

After autoclaving and cooling down the YPGA medium, filter-sterilized solutions of antibiotics and other components are added:

Kasugamycin HCl	20.0 mg
Cephalexin	40.0 mg (add sodium hydroxide for dissolution)
Propiconazole	20.0 mg
YPGA medium	1.0 L

2. Buffers

Tris-tween buffer	
Sigma 7-9®	1.21 g
Tween 20	250 µL
Distilled water	1.0 L
Adjust pH to 7.2 with 10N HCl.	

PBS buffer (1×)	
NaCl	8.0 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
KCl	0.2 g
Distilled water	1.0 L
Adjust pH to 7.4 of necessary. Sterilize by autoclaving	

APPENDIX 2 - SAMPLE PREPARATION AND DNA EXTRACTION FOR MOLECULAR TESTS

Different procedures can be used depending on the matrices and on the methods. LAMP tests are less sensitive than PCR to potential inhibitors contained in plant samples. Therefore, a simple DNA preparation method can be used before LAMP tests which allow them to be carried out directly on site. For example, Chelex® 100 resin can be added to the crushed samples (Mavrodieva et al., 2004; see 1.1). A fast and simple alkaline extraction previously reported for banana tissues (Li et al., 2013) can also be used with good results on citrus samples (I. Robène, pers. comm., see 1.2). PCR and LAMP tests can also be performed on DNA eluted from FTA® card (Whatman; see 1.3). The cards contain chemical that lyse cells, denature proteins, and protect DNA from degradation, whilst effectively inactivating the pathogen. Encouraging results have been obtained in interlaboratory tests on DNA samples eluted from FTA® card and tested with real-time quantitative PCR and CBC-LAMP tests (VALITEST project). Finally, PCR and LAMP tests can also be performed on DNA extracted using commercial kits (see 1.4) or other standard procedures (see 1.5 and 1.6).

1. DNA extraction from plant material

These methods can be used on symptomatic plant material, on the plant extract from symptomatic material (3.2.1.1).

For DNA extraction from the pellet obtained from the washing of the asymptomatic plant material (see 3.2.2.1), the use of commercial kits (see 1.4) is recommended

1.1. *Simplified extraction using Chelex® (Rigano et al., 2010)*

Lesions are placed in Eppendorf tubes containing a 100 µL $CaCO_3$–Silwet L-77–Chelex preparation: 5% Chelex 100 (Sigma Chemical) in Silwet L-77 in saturated $CaCO_3$ in sterile tap water 1:5000 (vol/vol). They are crushed with plastic disposable pestles and are shaken vigorously on a reciprocal shaker (250 cycles/min) for 1 h at room temperature and plant debris are pelleted by centrifugation for 5 min at 5000 rpm. The resulting supernatants are transferred to new tubes and 5 µL of each are used directly for LAMP tests (Appendix 8).

1.2. *Simplified alkaline extraction protocol (Li et al., 2013)*

A sample (10 mg) of symptomatic tissue is placed into 10 µL of freshly prepared 0.5 M NaOH and macerated with a plastic pestle. The tubes are then centrifuged at 12 000 *g* for 5 min, 5 µL of supernatant is removed and immediately diluted with 195 µL 100 mM Tris (pH = 8.0). Five microliters are used directly for LAMP tests (Appendix 8).

1.3. *Direct spot or on WhatmanTM FTA cards*

Direct spot or squash of symptomatic plant material on Whatman™ FTA cards yielded good results when evaluated in real-time PCR- and LAMP-based detection (Chabirand, 2021).

After spotting the plant sample on the FTA card, a disk of the sample area is punched out and placed in a PCR tube. A purification is performed using the Whatman™ FTA purification protocol reagent and following the manufacturer's instructions. After the drying step, 100 µL of molecular grade water is added into the tube containing the disk and is vortexed briefly. Sample extracts can be stored at less than −18°C. Two or 5 µL of this extract is used for real-time PCR tests and LAMP, respectively (Appendices 6–8, not evaluated for the test described in Appendix 5).

1.4. *DNA extraction using commercial kits*

The following general procedure should be applied for tissue preparation.

0.1 g of symptomatic plant material (one to three lesions) is ground in 10 mL of 0.01 M Tris buffer pH 7.2 (Sigma 7–9 Sigma-Aldrich) supplemented with 2% polyvinylpyrrolidone (PVP) average mol wt 40 000 (Sigma-Aldrich), using a homogenizer grinder (e.g. Homex grinder from Bioreba). Two millilitre is centrifuged for 10 min at 20 000 *g* and the supernatant is discarded. For DNA extraction from the pellet obtained from the washing of the asymptomatic plant material (see 3.2.2.1), the pellet can be used directly or if resuspended, 2 mL of the resuspended pellet is centrifuged in the same conditions as described above and the supernatant is discarded.

The pellet can then be stored at less than −18°C.

DNA has been successfully extracted from citrus tissue using commercial DNA extraction kits (e.g. Promega Wizard Genomic DNA Purification Kit (Coletta-Filho et al., 2006, Rigano et al., 2010); Qiagen DNeasy Plant Mini kit, (Robène et al., 2020)) used according to the manufacturer's instructions or with slightly adapted protocols (e.g. QuickPick™ Plant DNA kit (Bionobile) with two washing steps instead of three (NIVIP, NL)).

For the DNeasy Plant Mini kit (Qiagen), elute twice in the final step with 50 µL AE buffer. DNA thus extracted can be stored at less than −18°C.

Performance characteristics

An internal test performance study comparing four DNA extraction methods has been performed by CREA (IT) in the framework of the EURL in Bacteriology: The extraction methods compared were CTAB; DNeasy Plant Mini kit, Qiagen; DNeasy Mericon Food kit, Qiagen; QuickPick™ Plant DNA kit, Bionobile. The comparison was made using spiked samples (from 10^7 to 10^1) of different plant matrices (lemon leaves and fruits, orange fruit). All extraction methods were able to extract DNA from spiked samples of different plant/matrices and allowed a reliable detection of *Xanthomonas citri* pv. *citri* for samples spiked from 10^7 to 10^3 cfu mL^{-1} (100% diagnostic sensitivity, diagnostic specificity and accuracy). A background (late C_t values) was observed by real-time PCR (Cubero and Graham (2005)) when healthy plant matrices were tested, that was higher for samples extracted by CTAB. At lower bacterial concentrations (~10^2 cfu mL^{-1}) QuickPick™ Plant DNA kit (Bionobile) and DNeasy Plant Mini kit (Qiagen) performed better. In particular, analytical sensitivity of the Real-Time PCR of Cubero and Graham (2005) was better with QuickPick and DNeasy Plant Mini kit, intermediate for CTAB and less good for DNeasy Mericon Food kit. The validation report is available in the section on validation of the EPPO Database on Diagnostic Expertise.

1.5. *CTAB (hexadecyltrimethylammonium bromide) DNA extraction protocol (Hartung et al., 1993)*

Plant tissue (500 mg) is homogenized in 150 µL of extraction buffer [50 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 1% CTAB, 0.7 M NaCl and 0.1% 2-mercaptoethanol] and incubated at 60°C for 1 h. The mixture is extracted twice with chloroform-isoamyl alcohol (24:1), and the supernatant is precipitated with isopropanol and washed with 70% ethanol. The precipitate is dissolved in 150 µL of TE (10 mM Tris, 1 mM EDTA) buffer (pH 8.0) prior to the test.

Note that a slightly modified protocol was used for validation by CREA (see Section 1.4).

1.6. *Isopropanol DNA extraction method (Llop et al., 1999)*

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 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 5000 *g* for 5 min, after which 450 µL of the supernatant is discarded, and the pellet is dried. The pellet is resuspended in 100 µL water.

2. DNA extraction from pure cultures

Suspend a single colony of a fresh pure culture in 1 mL of 0.01 M Tris buffer pH 7.2 (Sigma 7–9 Sigma-Aldrich). If needed for quarantine purpose, inactivate the bacteria by boiling the suspension for 1 min and immediately chilling on ice for 1 min, and vortexing vigorously. In all cases, samples can be stored at less than −18°C.

The PCR tests can also be performed on DNA extracted from the pure culture of the bacterium. In this case, the DNA concentration should be adjusted to approximately 1 ng/µL.

APPENDIX 3 - CONVENTIONAL PCR ADAPTED FROM MAVRODIEVA ET AL. (2004)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for the detection of *X. citri* pv. *citri* (pathotypes A, A*, A^w) and *X. citri* pv. *aurantifolii* (pathotypes B, C) without distinction of the two pathovars. This conventional PCR is also suitable to identify the pathovars when used in combination with the conventional PCR according to Robène et al. (2020; Appendix 4).

1.2. The test is using primers developed by Mavrodieva et al. (2004) for a SYBR green real-time PCR test. The test was adapted to a conventional PCR test by Delcourt et al. (2013).

1.3. The target sequences are located in the *pthA* pathogenicity gene.

1.4. Oligonucleotides and amplicon size:

Primer	Sequence (5′→3′)	Amplicon size
VM3	GCA TTT GAT GAC GCC ATG AC	151 bp
VM4	TCC CTG ATG CCT GGA GGA TA	

1.5. The test has been successfully performed on a VeritiTM Thermal Cycler (Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.1. In planta *detection*

For the validation data reported in Section 4, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2, Section 1.

2.1.2. *Identification of pure bacterial cultures*

See Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. *Master Mix*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	14.3	N.A.
Buffer (Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	1	1.0 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Primer VM3	5 µM	1	0.2 µM
Primer VM4	5 µM	1	0.2 µM
GoTaq G2 Hot start polymerase (Promega)	5 U/µL	0.2	1 U
Subtotal		23	
DNA extracts		2	
Total		25	

2.2.2. *PCR cycling conditions:* 95°C for 5 min (initial denaturation), 40 cycles of 95°C for 45 s, 58°C for 45 s, 72°C for 45 s and a final extension step of 72°C for 10 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC: a band of the expected size (151 bp) is visualized.

When these conditions are met:

- The PCR test will be considered positive if a band of the expected size (151 bp) is visualized.
- The PCR test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained from Robène et al. (2020) and from the VALITEST project (Chabirand, 2021). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to Robène et al. (2020)*

Analytical sensitivity for both *Xanthomonas citri* pv. *citri* and *Xanthomonas citri* pv. *aurantifolii* ranged from 3×10^4 cfu mL^{-1} to 3×10^6 cfu mL^{-1} depending on the citrus matrices and strains tested.

Analytical specificity was tested on 78 strains of *X. citri* pv. *citri*, five strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, two strains of *X. euvesicatoria* pv. *citrumelonis*, seven *X. citri* pathovars not pathogenic to citrus, 11 other *Xanthomonas* species and 15 saprophytic *Xanthomonas* isolated from citrus.

Inclusivity: 100% of the *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* strains

Exclusivity: cross reactions were observed in eight non-target isolates out of 37. No cross reaction was observed with saprophytic strains isolated from citrus and with other citrus bacterial pathogens.

4.2. *Performance characteristics according to NIVIP (NL)*

Analytical specificity was tested on four isolates of *X. citri* pv. *aurantifolii*. One strain (LMG 9179, NCPPB 3236 and CFBP 2901) gave negative results because it lacks the target *pthA* gene (confirmed by Illumina sequencing).

4.3. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the test performance study (TPS) organized in the framework of the VALITEST project on *X. citri* pv. *citri* (2020) and in accordance with PM 7/98.

The panel of samples consisted of 24 DNA extracts (obtained from plant extracts spiked (or not) with bacterial suspensions) including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains: pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^w strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of conventional PCR Mavrodieva et al. (2004), were obtained from 16 participating laboratories.

Considering the performance for the detection of both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*, the results are given below.

Analytical sensitivity: estimated LOD 95% (i.e. the concentration at which a detection probability of 95% is expected) of 2 600 cfu mL^{-1}

Accuracy: 88%

Diagnostic sensitivity: 96%

Diagnostic specificity: 48%

Average repeatability: 93%

Average reproducibility: 83%

In this case, the samples including *X. citri* pv. *aurantifolii* were processed as target samples for the data analysis which is more relevant regarding the scope of this test.

Note that the low diagnostic specificity obtained for this test is mostly explained by the composition of the panel of samples: out of the four non-target samples used in the TPS, two (*X. citri* pv. *bilvae* in lime) are known to cross react with the Mavrodieva conventional PCR.

The performance characteristics for the detection *X. citri* pv. *citri* only (case where the samples including *X. citri* pv. *aurantifolii* were processed as non-target samples) are given below.

Analytical sensitivity: estimated LOD 95% (i.e. the concentration at which a detection probability of 95% is expected) of 2600 cfu mL^{-1}

Accuracy: 71%

Diagnostic sensitivity: 76%

Diagnostic specificity: 47%
Average repeatability: 93%
Average reproducibility: 83%

APPENDIX 4 - CONVENTIONAL PCR ACCORDING TO ROBÈNE ET AL. (2020)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This conventional PCR is suitable for the detection and/or identification of *X. citri* pv. *citri* (pathotypes A, A*, A^w) without distinction of the pathotypes.
1.2. The test is based on the publication of Robène et al. (2020).
1.3. The target sequences are located in the *XAC*1051 gene that encodes for a putative transmembrane protein.
1.4. Oligonucleotides and amplicon size:

Primer	Sequence (5′→3′)	Amplicon size
XAC1051-F	AAA TTC TTG TCG ATC TGC TGG CT	499 bp
XAC1051-R	GCC GCC GCA TAA TTC TTC TCA C	

1.5. The test has been successfully performed on a VeritiTM Thermal Cycler (Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.1. In planta *detection*

For the validation data reported in Section 4, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2, Section 1.

2.1.2. *Identification of pure bacterial cultures*

See Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	10.25	N.A.
Buffer (Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	2	2.0 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Primer XAC1051-F	5 µM	2.5	0.5 µM
Primer XAC1051-R	5 µM	2.5	0.5 µM
GoTaq G2 Hot start polymerase (Promega)	5 U/µL	0.25	1.25 U
Subtotal		23	
DNA extracts		2	
Total		25	

2.2.2. PCR cycling conditions: 95°C for 2 min (initial denaturation), 35 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 1 min and a final extension step of 72°C for 5 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.

- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC: a band of the expected size (499 bp) is visualized.

When these conditions are met:

- The PCR test will be considered positive if a band of the expected size (499 bp) is visualized.
- The PCR test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained from Robène et al. (2020) and from the VALITEST project (Chabirand, 2021). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to Robène et al. (2020)*

Analytical sensitivity (estimated LOD 95%) was 5 234 cfu mL^{-1} (95% CI 3656–7482).

Analytical specificity was tested on 78 strains of *X. citri* pv. *citri*, five strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, two strains of *X. euvesicatoria* pv. *citrumelonis*, seven *X. citri* pathovars not pathogenic to citrus, 11 other *Xanthomonas* species and 15 saprophytic *Xanthomonas* isolated from citrus.

Inclusivity: 100% of the *X. citri* pv. *citri*

Exclusivity: No cross reactions were observed with any other strains.

4.2. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the TPS organized in the framework of Valitest project on *X. citri* pv. *citri* (2020) and in accordance with PM 7/98.

The panel of samples consisted of 24 DNA extracts [obtained from plant extracts spiked (or not) with bacterial suspensions] including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains: pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^w strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of conventional PCR Robène et al. (2020), were obtained from 16 participating laboratories.

Analytical sensitivity: estimated LOD 95% of 3800 cfu mL^{-1}

Accuracy: 88%

Diagnostic sensitivity: 82%

Diagnostic specificity: 99%

Average repeatability: 98%

Average reproducibility: 92%

APPENDIX 5 - REAL-TIME PCR BASED ON MAVRODIEVA ET AL. (2004)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This real-time PCR is suitable for the detection of *X. citri* pv. *citri* strains (pathotypes A, A*, A^w) and *X. citri* pv. *aurantifolii* strains (pathotypes B, C) without distinction of the two pathovars.

1.2. The test is based on the publication of Mavrodieva et al. (2004).

1.3. The target sequences are located in the *pthA* pathogenicity gene.

1.4. Oligonucleotides and amplicon size:

Primer	Sequence (5′→3′)	Amplicon size
VM3	GCA TTT GAT GAC GCC ATG AC	151 bp
VM4	TCC CTG ATG CCT GGA GGA TA	

1.5. The test has been successfully performed on the following real-time PCR systems: StepOnePlus (Applied Biosystems), Light Cycler LC 480 (Roche Life Science) and Quantstudio5 (QS5; Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.1. In planta *detection*

For the validation data reported in Section 4, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2, Section 1.

2.1.2. *Identification of pure bacterial cultures*

See Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	4	N.A.
GoTaq® qPCR Master Mix (Promega)	2×	10	1×
Primer VM3	5 µM	2	0.5 µM
Primer VM4	5 µM	2	0.5 µM
Subtotal		18	
DNA extracts		2	
Total		20	

2.2.2. PCR cycling conditions: 95°C for 2 min (initial denaturation), 40 cycles (with measurement of fluorescence) of 95°C for 5 s, 57°C for 1 s, 72°C for 12 s.

For the melting curve analysis: 95°C for 5 s, 40°C for 90 s, 97°C with no holding time but a ramping at 0.1°C/s. (with measurement of fluorescence – continuous acquisition).

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include

nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC amplification curves should be exponential and the melting curve analysis should reveal a melting temperature (Tm) peak of approximately 90°C.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and if the melting curve analysis reveals a Tm peak of approximately 90°C.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential or if the Tm peak is different from the expected one.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained from Robène et al. (2020) and from the VALITEST project (Chabirand, 2021). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to Robène et al. (2020)*

Analytical sensitivity ranged from 3×10^2 to 3×10^5 cfu mL^{-1} depending on the citrus matrices and strains tested.

Analytical specificity was tested on 78 strains of *X. citri* pv. *citri*, five strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, two strains of *X. euvesicatoria* pv. *citrumelonis*, seven *X. citri* pathovars not pathogenic to citrus, 11 other *Xanthomonas* species and 15 saprophytic *Xanthomonas* isolated from citrus.

Inclusivity: 100% of the *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* strains

Exclusivity: Cross reactions were observed in eight non-target isolates out of 42 including *X. citri* pv. *bilvae*, a spot-causing pathogen of rutaceous species, *X. citri* pv. *bauhiniae*, *X. citri* pv. *cajani*, *X. citri* pv. *glycines*, *X. citri* pv. *malvacearum*, *X. axonopodis* pv. *phyllanthi*. No cross reaction was observed with saprophytic strains isolated from citrus and with other citrus bacterial pathogens.

4.2. *Performance characteristics according to NIVIP (NL)*

Analytical specificity was tested on 4 isolates of *X. citri* pv. *aurantifolii*. One strain (LMG 9179, NCPPB 3236 and CFBP 2901) gave negative results because it lacks the target *pthA* gene (confirmed by Illumina sequencing).

4.3. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the TPS organized in the framework of the VALITEST project on *X. citri* pv. *citri* (2020) and in accordance with PM 7/98.

The panel of samples consisted of 24 DNA extracts (obtained from plant extracts spiked (or not) with bacterial suspensions) including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains:

pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^W strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of real-time PCR Mavrodieva et al. (2004), were obtained from 17 participating laboratories.

The performance characteristics for the detection of both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*, are given below.

Analytical sensitivity: estimated LOD 95% of 110 cfu mL^{-1}

Accuracy: 78%

Diagnostic sensitivity: 84%

Diagnostic specificity: 47%

Average repeatability: 92%

Average reproducibility: 87%

In this case, the samples including *X. citri* pv. *aurantifolii* were processed as target samples for the data analysis which is more relevant regarding the scope of this test.

Note that the low diagnostic specificity obtained for this test is mostly explained by the composition of the panel of sample: out of the four non-target samples used in the TPS, two (*X. citri* pv. *bilvae* in lime) are known to cross react with the Mavrodieva real-time PCR.

If we consider the performance for the detection of only *X. citri* pv. *citri* (case where the samples including *X. citri* pv. *aurantifolii* were processed as non-target samples), the results are given below.

Analytical sensitivity: estimated LOD 95% of 110 cfu mL^{-1}

Accuracy: 61%

Diagnostic sensitivity: 80%

Diagnostic specificity: 24%

Average repeatability: 92%

Average reproducibility: 87%

APPENDIX 6 - REAL-TIME TAQMAN PCR BASED ON CUBERO AND GRAHAM (2005)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This real-time PCR is suitable for the detection of *Xanthomonas citri* pv. *citri* (pathotypes A, A*, A^W) without distinction of the pathotypes.

1.2. The test is based on the publication of Cubero and Graham, (2005).

1.3. The target sequences are located in the *pthA* pathogenicity gene.

1.4. Oligonucleotides and amplicon size:

Primer/probe	Sequence (5′→3′)
J-pth3	ACC GTC CCC TAC TTC AAC TCA A
J-pth4	CGC ACC TCG AAC GAT TGC
Probe J-Taqpth2	FAM - ATG CGC CCA GCC CAA CGC – TAMRA

1.5. The test has been successfully performed on the following real-time PCR systems: StepOnePlus (Applied Biosystems), Light Cycler LC 480 (Roche Life Science) and Quantstudio5 (QS5; Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.2. In planta *detection*

For the validation data reported in Section 4, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2, Section 1.

2.1.3. *Identification of pure bacterial cultures*

See Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	5	N.A.
GoTaq® probe qPCR Master Mix (Promega)	2×	12.5	1×
Primer J-pth3	10 µM	1	0.4 µM
Primer J-pth4	10 µM	1	0.4 µM
TaqMan probe J-Taqpth2	10 µM	0.5	0.2 µM
Subtotal		20	
DNA extracts		5	
Total		25	

2.2.2. PCR cycling conditions: 95°C for 10 min (initial denaturation), 45 cycles (with measurement of fluorescence) of 95°C for 15 s, 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC amplification curves should be exponential.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained from Robène et al. (2020) and from the VALITEST project (Chabirand, 2021). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to Robène et al. (2020)*

Analytical sensitivity was 3×10^3 cfu mL^{-1} for most of the citrus matrices and strains tested.

Analytical specificity was tested on 78 strains of *X. citri* pv. *citri*, five strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, two strains of *X. euvesicatoria* pv. *citrumelonis*, seven *X. citri* pathovars not pathogenic to citrus, 11 other *Xanthomonas* species and 15 saprophytic *Xanthomonas* isolated from citrus.

Inclusivity: All strains of *X. citri* pv. *citri* except one (NCPPB 211; but none of the *X. citri* pv *aurantifolii* strains).

Exclusivity: Cross reactions were observed in three non-target isolates (NCPPB 1759, LMG 760, LMG 844) out of 42 including *X. citri* pv. *bilvae*, a spot-causing pathogen of rutaceous species. No cross reaction was observed with saprophytic strains isolated from citrus and with other citrus bacterial pathogens.

4.2. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the TPS organized in the framework of VALITEST project

on *X. citri* pv. *citri* (2020) and in accordance with PM 7/98.

The panel of samples consisted of 24 DNA extracts (obtained from plant extracts spiked (or not) with bacterial suspensions) including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains: pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^w strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of real-time PCR Cubero and Graham, (2005), were obtained from 17 participating laboratories.

Analytical sensitivity: estimated LOD 95% of 110 cfu mL^{-1}

Accuracy: 85%

Diagnostic sensitivity: 98%

Diagnostic specificity: 59%

Average repeatability: 92%

Average reproducibility: 87%

APPENDIX 7 - DUPLEX REAL-TIME TAQMAN PCR ACCORDING TO ROBÈNE ET AL. (2020)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This real-time PCR is suitable for the detection of *X. citri* pv. *citri* (pathotypes A, A*, A^w) without distinction of the pathotypes. The real time PCR test includes an internal positive control sequence that is an endogenous plant DNA sequence present in the sample (5.8S rDNA). This internal control is co-extracted and co-amplified with the target DNA during the whole process.

1.2. The test is based on the publication of Robène et al., 2020.

1.3. The target sequences are located in the XAC1051 gene that encodes for a putative transmembrane protein.

1.4. Oligonucleotides:

Primer/probe	Sequence (5′→3′)
qPCR-XAC1051-F	AGA GGC GCA CTA TGG CTT TC
qPCR-XAC1051-R	CAA CCC AGG ACC TGC AAG AA
Probe P-Xcci-1051	FAM - CGG TGA GAA GCT GTA C - MGB
Citrus5.8S-F	GCG AAA TGC GAT ACT TGG TGT GA
Citrus5.8S-R	CGT GCC CTC GGC CTA ATG
Probe P-citrus5.8S	VIC - ATC CCG TGA ACC ATC G - MGB

1.5. The test has been successfully performed on the following real-time PCR systems: StepOnePlus (Applied Biosystems), Light Cycler LC 480 (Roche Life Science) and Quantstudio5 (QS5; Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.1. In planta *detection*

For the validation data reported in Section 4, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2, Section 1.

2.1.2. *Identification of pure bacterial cultures*

See Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	0.9125	N.A.
GoTaq® probe qPCR Master Mix (Promega)	2×	7.5	1×
Primer qPCR-XAC1051-F	24 µM	0.375	0.60 µM
Primer qPCR-XAC1051-R	24 µM	0.375	0.60 µM
Taqman Probe P-Xcci-1051	10 µM	0.6375	0.425 µM
Primer Citrus5.8S-F	12 µM	0.0625	0.05 µM

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Primer Citrus5.8S-R	12 µM	0.0625	0.05 µM
Taqman Probe P-citrus5.8S	10 µM	0.0750	0.05 µM
Subtotal		10	
DNA extracts		5	
Total		15	

2.2.2. PCR cycling conditions: 95°C for 2 min (initial denaturation), 45 cycles (with measurement of fluorescence) of 95°C for 15 s, 60°C for 1 min.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

The test includes an internal positive control (IPC), consisting of an endogenous plant DNA sequence present in the sample (5.8S rDNA). This internal control allows to check for flaws in DNA extraction or the presence of PCR inhibitors.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC (as well as IPC) amplification curves should be exponential.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential for the DNA target, and if the IPC produces a positive result.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained from Robène et al. (2020) and from the VALITEST project (Chabirand, 2021). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to Robène et al. (2020)*

Analytical sensitivity (estimated LOD 95%) was 754 cfu mL^{-1}.

Analytical specificity was tested on 98 strains of *X. citri* pv. *citri*, nine strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, nine strains of *X. euvesicatoria* pv. *citrumelonis*, 24 *X. citri* pathovars not pathogenic to citrus, 33 other *Xanthomonas* species and 24 saprophytic *Xanthomonas* isolated from citrus.

Inclusivity: 100% of the strains of *X. citri* pv. *citri* (but none of the *X. citri* pv *aurantifolii* strains).

Exclusivity: Cross reactions were observed in three non-target isolates (*X. citri* pv. *cajani*) out of 101 tested. No cross reaction was observed with saprophytic strains isolated from citrus and with other citrus bacterial pathogens.

4.2. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the TPS organized in the framework of VALITEST project on *X. citri* pv. *citri* (2020) and in accordance with PM 7/98.

The panel of samples consisted of 24 DNA extracts (obtained from plant extracts spiked (or not) with bacterial suspensions) including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains: pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^W strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of real-time PCR Robène et al. (2020), were obtained from 14 participating laboratories.

Analytical sensitivity: estimated LOD 95% of 380 cfu mL^{-1}

Accuracy: 91%

Diagnostic sensitivity: 95%

Diagnostic specificity: 85%

Average repeatability: 96%

Average reproducibility: 87%

APPENDIX 8 - LAMP TEST BASED ON RIGANO ET AL. (2010)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4.1. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This LAMP test (CBC-LAMP test) is suitable for the detection of *X. citri* pv. *citri* strains (pathotypes A, A*, A^W strains) and *X. citri* pv. *aurantifolii* strains (pathotypes B, C strains) without distinction of the pathovars.

1.2. The test is based on the publication of Rigano et al. (2010).

1.3. The target sequences are located in the *pthA* pathogenicity gene.

1.4. Sequences of primers used for CBC-LAMP test

	Primer name	Sequence (5′–3′)
Forward outer primer	XCC-F3	GGTGGATCTACGCACGC
Reverse outer primer	XCC-B3	GCTGCGATCATGTC CTGAT
Forward inner primer	XCC-FIP	GGTGCTGCGCCACT GTCGAAGCTACAGCC AGCAGCAACA
Reverse inner primer	XCC-BIP	GCACTGGTCGGCCA TGGGTA GCGACGGT CCCTAACG
Internal loop primer	XCC-LF	AACCTTCGGTTTGATCT TCTCC
Internal loop primer	XCC-LB	TTACACACGCGCAC ATCGT

1.5. The test has been successfully performed using a thermal dry block with a 0.5-mL PCR tube holder (end point reaction, Rigano et al., 2010). This end-point CBC –specific LAMP test has been successfully tested with the OptiGene Master mix ISO-DR004, using the Real-time LAMP instrument Genie II, (OptiGene) according to the reaction conditions recommended by the supplier (see below; VALITEST).

2. Methods

2.1. Nucleic Acid Extraction and Purification

For the validation data reported in Section 4.1, DNA from bacterial suspensions was extracted using the Wizard® Genomic DNA purification Kit (Promega), according to the manufacturer's instructions. A DNA purification method using Chelex® 100 resin (Biorad) was also tested on cultured bacteria and infected tissue as described in Mavrodieva et al. (2004; see Appendix 2 Section 1.1).

For the validation data reported in Section 4.2, DNA was extracted using the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, and obtained from infected tissue.

For alternative procedures, see Appendix 2.

DNA should preferably be stored at approximately −20°C.

2.2. LAMP reaction

2.2.1. Master Mix for end-point LAMP reaction (Rigano et al., 2010)

Reagent	Volume/quantity per reaction	Final concentration
XCC-FIP*	40 pmol	1.2 µM
XCC-BIP*	40 pmol	1.2 µM
XCC-F3*	5 pmol	0.8 µM
XCC-B3*	5 pmol	0.8 µM
XCC-LF*	20 pmol	0.4 µM

Reagent	Volume/quantity per reaction	Final concentration
XCC-LB*	20 pmol	0.4 µM
Bst DNA polymerase	8 U	
dNTP mix		1.4 mM
Reaction Buffer (pH 8.8)		Tris HCl, 20 mM
		KCl, 10 mM
		$(NH_4)_2SO_4$, 10 mM
		$MgSO_4$ 4.5 mM
		Triton X-100, 0.1%
		Betaine, 1.6 M
Molecular grade water	up to 20 µL	
DNA extract	5 µL	
Total	25 µL	

* A 10× primer mix can be prepared as described below and 2.5 µL of this primer mix added to the end-point LAMP mix.

2.2.2. Master Mix for real-time LAMP (VALITEST)

Prepare first a 10× primer mix as detailed below.

	Working concentration (µM)	Volume (µL) for 10× mix	Final concentration (µM)
XCC-FIP	100	8	8
XCC-BIP	100	8	8
XCC-LF	100	4	4
XCC-LB	100	4	4
XCC-F3	100	2	2
XCC-B3	100	2	2
Molecular grade water		72	
Total		100	

Then prepare the LAMP mix as detailed below.

Reagent	Volume per reaction (µL)
Primer mix (10×)	2.5
Molecular grade water	2.5
Mix OptiGene (ISO-DR004)	15
Subtotal	20
DNA extracts	5
Total	25

2.2.3. LAMP reaction conditions

For end-point reaction (Rigano et al., 2010), using a thermal dry block with a 0.5-mL PCR tube holder, incubate at 65°C for 30 min.

For the real-time LAMP using the Lamp instrument Genie II (OptiGene) or a classical real-time PCR thermocycler (e.g. Quantstudio (QS5)), use the following LAMP program: an isothermal amplification step at 65°C for 30 min and an annealing step from 80 to 98°C with a ramp rate of 0.05°C/s.

2.3. Analysis of LAMP products

2.3.1. End-point detection methods evaluated by Rigano et al. (2010)

Different methods were evaluated by Rigano et al. (2010) for the detection of LAMP products but are not detailed in this protocol. Indeed, electrophoresis of LAMP products or the addition of a DNA intercalating fluorescent dye such as SYBR Green at the end of the reaction are not recommended to avoid environmental contamination. However, note that adding SYBR Green to the inside of the lid prior to the reaction and mixing at the end of the reaction as described by Zhang et al. (2013) is an alternative. Lateral flow device strips in detection chambers were also used to detect LAMP amplicons with high sensitivity, however this method requires the use of oligonucleotides that are dual labelled with biotin and fluorescein and the lateral flow device used by Rigano et al. (2010) is no longer available (an alternative may be available http://en.bioustar.com/intro/15.html but has not been evaluated).

2.3.2. Real-time LAMP reaction (VALITEST)

The real-time LAMP evaluated during the VALITEST project is based on the use of a double strand specific dye included in the Master mix and the monitoring of the fluorescence during the reaction in a portable LAMP instrument (Genie II, OptiGene).

3. **Essential Procedural Information**

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For LAMPs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

3.2. Interpretation of results for real-time LAMP reaction

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC amplification curves should be exponential (presence of an annealing curve specific for the target with an annealing temperature (Ta) approximately 92°C).

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve (presence of an annealing curve specific for the target with a Ta approximately 92°C).
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential (no annealing curve specific for the target).
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data were obtained in the framework of the VALITEST project with the procedure described in Section 2 (Chabirand, 2021). Further validation data were reported by Rigano et al. (2010) using a different procedure and are reported in this appendix.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. *Performance characteristics according to VALITEST*

Data mentioned in this section were obtained from the TPS organized in the framework of VALITEST project on *X. citri* pv. *citri* (2020) and in accordance with PM7/98.

The panel of samples consisted of 24 DNA extracts (obtained from plant extracts spiked (or not) with bacterial suspensions) including:

- two non-target samples (diversity of *Citrus* plants (orange 'Washington Navel' (*Citrus sinensis*) and Mexican lime (*Citrus aurantiifolia*)))
- two non-target samples (*X. citri* pv. *bilvae* in lime (*Citrus aurantiifolia*))
- four target samples (*X. citri* pv. *aurantifolii* (pathotype B or C strain) in lime (*Citrus aurantiifolia*))
- and 16 target samples including heavily contaminated samples (diversity of *X. citri* pv. *citri* strains: pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*), pathotype A* and pathotype A^w strains in lime (*Citrus aurantiifolia*)) and target samples obtained from a serial dilution with repetitions (only pathotype A strain in orange 'Washington Navel' (*Citrus sinensis*)).

Data produced for the evaluation of the real-time LAMP PCR Rigano et al. (2010), were obtained from only seven participating laboratories.

The performance characteristics for the detection of both *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*, are given below.

Analytical sensitivity: estimated LOD 95% of 240 cfu mL^{-1}

Accuracy: 82%

Diagnostic sensitivity: 88%

Diagnostic specificity: 50%

Average repeatability: 93%

Average reproducibility: 83%

In this case, the samples including *X. citri* pv. *aurantifolii* were processed as target samples for the data analysis which is more relevant regarding the scope of this test.

If we consider the performance for the detection of only *X. citri* pv. *citri* (case where the samples including *X. citri* pv. *aurantifolii* were processed as non-target samples), the results are given below.

Analytical sensitivity: estimated LOD 95% of 240 cfu mL^{-1}

Accuracy: 79%

Diagnostic sensitivity: 96%

Diagnostic specificity: 46%

Average repeatability: 93%

Average reproducibility: 83%

4.2. *Performance characteristics according to Rigano et al. (2010)*

Analytical sensitivity was determined using the *X. citri* pv. *citri* pathotype A strain 306. The detection limit (per reaction) was 10 fg of pure DNA, 5 cfu of *X. citri* pv. *citri* cultured cells, and 18 cfu from artificially infected leaves with the strain 306.

Analytical specificity was tested on DNA extracted from 44 strains of *X. citri* pv. *citri* (pathotypes A and A*), three strains of *X. citri* pv. *aurantifolii* (pathotypes B and C), one strain of *X. euvesicatoria* pv. *citrumelonis* and nine other phytopathogenic bacteria (including two *Xanthomonas* species).

Inclusivity: 100% of the strains of *X. citri* pv. *citri* and *X. citri* pv *aurantifolii* strains.

Exclusivity: No cross reactions were observed.

APPENDIX 9 - REAL-TIME TAQMAN PCR AND ALLELIC DISCRIMINATION ACCORDING TO CUBERO AND GRAHAM (2005)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This real-time PCR is suitable to be used to distinguish *X. citri* pv. *citri* pathotypes A from A* and A^W.

1.2. The test is based on the publication of Cubero and Graham, (2005).

1.3. The target sequences are located in the leucine-responsive regulatory protein (lrp).

1.4. Oligonucleotides:

TaqMan MGB probes labelled with FAM (J-Alrpallelic1) and with the fluorescent dye VIC (J-Awlrpallelic1) were designed to hybridize with canker pathotype A strains and restricted host range pathotype A* and pathotype A^W strains, respectively.

Primer/probe	Sequence (5′→3′)
J-ADlrpU1	GCG TCC TAC CGC AAG TTG C
J-AD1lrpU2	CCT TGA CCT CTT CCA TCA CGA T
J-Alrpallelic1	FAM- AGC TCT TGG ATT CAC -MGBNFQ
J-Awlrpallelic1	VIC- AGC TCT TGG ACT CAC -MGBNFQ

1.5. The test has been successfully performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

DNA should preferably be stored at approximately −20°C.

2.1.1. In planta *detection*

The protocol described in Appendix 2, Section 1.6 was used to generate the validation data in Section 4. For alternative procedures, see Appendix 2, Section 1.

2.1.2. *Identification of pure bacterial cultures*

DNA was extracted from bacterial cells with a single phenol-chloroform-isoamyl-alcohol step, precipitated in ethanol, resuspended in ultrapure water, and stored at –20°C. For alternative procedures, see Appendix 2, Section 2.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	4.5	N.A.
PCR universal master mix (Applied Biosystems)	2×	12.5	1×
Primer J-ADlrpU1	10 µM	1	0.4 µM
Primer J-AD1lrpU2	10 µM	1	0.4 µM
Taqman Probe J-Alrpallelic1	10 µM	0.5	0.2 µM
Taqman Probe J-Awlrpallelic1	10 µM	0.5	0.2 µM
Subtotal		20	
DNA extracts		5	
Total		25	

2.2.2. A pre-read run at 60°C to determine the baseline fluorescence associated with primers and probes is run before amplification.

2.2.3. PCR cycling conditions: 95°C for 10 min (initial denaturation), 40 cycles (with measurement of fluorescence) of 95°C for 15 s, 60°C for 1 min.

2.2.4. A post- read run at 60°C that automatically subtracts the baseline fluorescence determined during the pre-read run is performed to assign alleles using the amplified data. The change in fluorescence from FAM and VIC dyes is measured and a x-y scatter plot used to identify the specific genotype based on their location in the graph.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification controls (PACs) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). PACs should include allele standards corresponding to *X. citri* pv. *citri* pathovars A and A^w or A*. It may include other *Xanthomonas* species (e.g. *X. euvesicatoria* pv. *citrumelonis*).

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. These can include specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PACs amplification curves should be exponential.

When these conditions are met:

- After the post read run the PCR software determines the contribution of FAM and VIC dyes and plots the results of the allelic discrimination run on a scatter plot of allele with FAM vs. allele with VIC. The clustering of the point samples according to the standards indicates their pathotypes.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Analytical specificity (according to Cubero & Graham, 2005) was tested on 14 strains of *X. citri* pv. *citri* (eight pathotype A strains, three pathotype A* strains and three pathotype A^w strains) and four strains of *X. euvesicatoria* pv. *citrumelonis*.

All the strains were included in the corresponding group according to strains characteristics, i.e. pathotype A strains grouped together, pathotype A* and A^w strains formed a second group and *X. euvesicatoria* pv. *citrumelonis* strains a third one.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

APPENDIX 10 - MINISATELLITE GENOTYPING ACCORDING TO PRUVOST ET AL. (2014)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. Minisatellite genotyping is suitable for the identification of *X. citri* pv. *citri* pathotypes (pathotypes A, A*, Aw). The nucleic acid is obtained from cell pellets of pure cultures as described below (Section 2.1).
1.2. The test is based on the publication of Pruvost et al. (2014).
1.3. The target sequences are minisatellites.
1.4. Sequences of primers used for MLVA-31 and MLVA-12 test are listed in Table A1.
1.5. For laboratories equipped with a genetic analyser, it is recommended to use the complete genotyping scheme (MLVA-31) in a multiplex PCR format. Other laboratories should use MLVA-12 in a simplex PCR format resolved by high resolution capillary electrophoresis. The test has been successfully performed on GeneAmp™ PCR system 9700 and HID Veriti™ 96-well thermal cyclers (PCR amplification), as well as ABI PRISM genetic analyzers (fragment resolution; Applied Biosystems).

2. Methods

2.1. Nucleic Acid Extraction and Purification

PCRs can be conducted from purified gDNA (see EPPO Standard PM 7/129 (1) Appendix 2 for details) or

boiled bacterial suspensions. For the latter option, a 1 µL loopful of bacterial culture derived from a single colony is vortexed in 0.4 mL of sterile distilled water or 0.01 M Tris buffer (pH 7.2), heated at 100°C for 2 min, placed on ice and once chilled, briefly centrifuged for pelleting debris. The supernatant is used as PCR target. Authenticated reference strains should be used as controls.

DNA should preferably be stored at approximately −20°C.

2.2. Polymerase chain reactions

2.2.1. Master Mix

For the MLVA-31 scheme, nine reaction mixes (corresponding to the PCR pools mentioned in Table A1) should be used (multiplex reactions). For the MLVA-12 scheme, 12 reaction mixes should be prepared (simplex reactions).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	Up to 14	N.A.
Type-it Multiplex PCR Master mix (QIAGEN)	2×	7.5	1×
Q-solution (Qiagen)	5×	1.5	0.5×
10X Minisatellite primer mix	10×	1.5	0.2–0.8 µM of each primer
Subtotal		14	
DNA extracts		1	
Total		15	

2.2.2. PCR cycling conditions: 5 min at 95°C for polymerase activation, followed by 25 cycles at 94°C for 30 s, annealing at temperatures ranging from 64 to 70°C (see Table A1) for 90 s, and 72°C for 90 s with a final extension step at 60°C for 30 min. Amplicons should be appropriately diluted prior to capillary electrophoresis in order to avoid signal saturation (a test run is recommended for determining the dilution ratio).

2.2.3. Fragment resolution

Resolution of multiplex PCRs is achieved by capillary electrophoresis (GS500LIZ (alternatively GS600LIZ) or GS1200LIZ size standard depending on PCR pools) using for example a 3130, 3730 or 3500 ABI PRISM Genotyper or equivalent. Simplex PCRs should be resolved using a high-resolution capillary electrophoresis apparatus (such as Agilent fragment analyser) or equivalent.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include boiled bacterial suspensions, nucleic acid extracted from the target organism or a synthetic control (e.g. cloned PCR product). A reference strain of *X. citri* pv. *citri* should be used.

3.2. Interpretation of results

Verification of the controls

- NAC should give no amplification.
- PAC should produce an amplicon at all loci and yield an allelic profile consistent with data available at http://www.biopred.net/mlva/.

When these conditions are met:

- A test will be considered positive if accurate sizing of amplicons at all loci has been achieved from bacterial cultures that have proved positive by diagnostic PCR targeting *X. citri* pv. *citri*.
- A test will be considered doubtful if any partial, contradictory or unclear results are obtained from bacterial cultures that have proved positive by diagnostic PCR targeting *X. citri* pv. *citri*.

4. Performance characteristics available

According to published studies (Leduc et al., 2015; Pruvost et al., 2014, 2015; Richard et al., 2017), amplicons were obtained for all targeted loci (822 strains tested), with the sole exception of a few pathotype A* strains from Iran for which no successful amplification was obtained for locus Xcc3324 (Pruvost et al., 2015).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

TABLE A1 Minisatellite and primer description, amplification conditions and amplicon sizes for the markers used for MLVA-31 and MLVA-12.

Name[a]	Tandem repeat length (bp)	Primers	Annealing temperature (°C)	Primer concentration (μM)	PCR pool	Range of repeat numbers	Range of amplicon sizes[b]
Xcc0292	25	5′ VIC-AGACATCTGCGCAAACGTCC 3′ 5′ CAGCACGGCAGGCGAGCATT 3′	64.0	0.2	6	3–5	143–193
Xcc0514	205	5′ NED-GGCGGAGTTGGCTGGCTAA 3′ 5′ GCGGCGTTGTTTCTGGCATC 3′	68.0	0.2	4	2–3	479–684
Xcc0677	133	5′ NED-ACACCATGGGCGCAGTCAAC 3′ 5′ TGCCGCAGGGAATGGACCGA 3′	70.0	0.6	5	2–4	363–629
Xcc0724	12	5′ NED-CAGCGAGATCGACCAATTGCC 3′ 5′ ATTCTATTGGTCGTGGAACCCC 3′	66.0	0.2	2	3–5	262–286
Xcc0912	173	5′ 6-FAM-ACGACAGAACCCGGCTTATC 3′ 5′ CAGGCGGTGGAAGGGAGT 3′	68.0	0.6	4	1–3	222–568
Xcc1014	11	5′ VIC-ATTGCTGCAGTTCCGTCCT 3′ 5′ TCGACCTCTTGCGGTTTCCAG 3′	64.0	0.2	9	4–5	162–173
Xcc1317	165	5′ VIC-TCGGCGATTATGCGTTCTGG 3′ 5′ TTGCGGCTGGCTGTCGTTTG 3′	70.0	0.4	5	2–4	527–857
Xcc1662	100	5′ NED-CCTGATTTCGCTTCGTGGTT 3′ 5′ GGCGCTCGTACCATGAG 3′	66.0	0.8	7	6–10	747–1147
Xcc1806	140	5′ 6-FAM- GAGGCGGCGATGTGGATCA 3′ 5′ CGCGCACCAGACACGGGAGA 3′	70.0	0.2	5	3–6	641–1061
Xcc1894	33	5′ VIC-AGGTTTGAGCAGCGGCCACA 3′ 5′ AAGCACGGGCGCGGTTAT 3′	64.0	0.6	1	2–4	189–255
Xcc2059	117	5′ PET-TGGAGTTGCGGCAGTCTTGA 3′ 5′ CGGTGGAGCGGTGGGTTA 3′	68.0	0.6	4	1–6	290–875
Xcc2072	18	5′ VIC-ACGGCCAACGCATTTCATCTCA 3′ 5′ CCAGCCCACCATCCAGGTCA 3′	66.0	0.2	2	1–7	366–474
Xcc2229	81	5′ NED-CTGCGGTGATCAGGTCCACT 3′ 5′ CCTCCAACGCGATTGC 3′	64.0	0.8	9	5–7	444–606
Xcc2741	49	5′ 6-FAM-CCGGCAAGGAAACTCTGGAT 3′ 5′ GGTGGCGACGCTGGAC 3′	68.0	0.6	3	2–4	292–390
Xcc2922	186	5′ 6-FAM-CGCTGAGTCAGGCAGTCGTT 3′ 5′ GCGTATTGCGGGCGTGTAGG 3′	66.0	0.4	7	3–5	545–917
Xcc3088	27	5′ PET-CTAAGCCTCCGCGCACCAG 3′ 5′ CGCTTGTTGCCGAAAACCGAA 3′	64.0	0.2	6	1–2	326–353
Xcc3324	26	5′ VIC-TGATCGAAGCACCGAGCAGT 3′ 5′ GCAACCGGGCAGACCGTTGT 3′	66.0	0.2	8	2–3	289–315
Xcc3510	24	5′ NED-ACCGCTCTACCGAATACGTCA 3′ 5′ ATCGGCATTGTCCATCAACGTC 3′	66.0	0.2	8	1–3	383–431

(Continues)

TABLE A1 (Continued)

Name[a]	Tandem repeat length (bp)	Primers	Annealing temperature (°C)	Primer concentration (μM)	PCR pool	Range of repeat numbers	Range of amplicon sizes[b]
Xcc3522	10	5′ NED-CCCAGCCACCGAACAGATCCG 3′ 5′ AAATCCCTATCGCGCCCAGGT 3′	64.0	0.2	1	2–5	273–303
Xcc3816	18	5′ PET-TGGACTGGCTCATGCGTCAG 3′ 5′ ACGAAGGGCTGGGAAT 3′	64.0	0.6	1	2–9	155–281
Xcc3993	12	5′ 6-FAM-CGGCGTGGCTGTTCGGTTCC 3′ 5′ AAGACATGGCGAATGCGTCA 3′	64.0	0.2	6	4–8	269–317
Xcc4071	22	5′ 6-FAM-ATTCTCAGTGTCTTAGGGGCCAT 3′ 5′ CGCCGTCCTTCATCACATCCAG 3′	66.0	0.2	8	2–3	212–234
Xcc4279	33	5′ PET-ATCGGTTCGGCGGCGGTGAT 3′ 5′ AGAAGGGCAGGCGGGCACTC 3′	64.0	0.2	9	2–3	311–344
Xcc4322	27	5′ NED-CAAGCACCGGCAGCAAGCGTA 3′ 5′ CGCTGGCCGAGCACTTCCTT 3′	68.0	0.4	3	2–4	408–462
Xcc4325	32	5′ 6-FAM-GCCTTGGCGGAACAGACTCA 3′ 5′ TGCCCGTATACGATATGGAT 3′	64.0	0.4	1	1–2	332–364
Xcc4372	158	5′ PET-CATGCTGGCGCTGACCTCGTT 3′ 5′ ATTCCCATCTCCCGCCACACC 3′	70.0	0.2	5	1–3	439–755
Xcc4424	15	5′ PET-CCGAGTTCGCCGACACTGCT 3′ 5′ AGTTTCTTCCACCGCTTCGTCCT 3′	66.0	0.2	2	2–4	386–416
Xcc4748	16	5′ PET-GAAGCCCTCAACGCGGTCAA 3′ 5′ CCTCCAACGCGCAATACCGA 3′	68.0	0.4	3	3–13	206–366
Xcc4799	217	5′ VIC-GACAACGCCATCAGCAGCAG 3′ 5′ CGCCGGTCGTCTCTAAC 3′	68.0	0.6	4	2–4	549–983
Xcc4927	27	5′ PET-CCCGAGCCAAACCGAATCAC 3′ 5′ GCAGCCGACCCGCGCATCCA 3′	66.0	0.2	8	3–5	268–322
Xcc4946	30	5′ 6-FAM-CCACAGGCACGCAAGGCCAC 3′ 5′ ACCCATGCCGATCAGGAACTGGA 3′	66.0	0.2	2	2–3	312–342

[a]Bold loci are proposed for routine analyses (MLVA-12) using unlabelled primers in a simplex PCR format.

[b]From the IAPAR 306 genome.

APPENDIX 11 - CRISPR TYPING ACCORDING TO JEONG ET AL. (2019)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. The CRISPR array is suitable to identify *X. citri* pv. *citri* pathotypes (pathotypes A, A*, A^{w}) and also to compare the identified strains with the global diversity of strains of *X. citri* pv. *citri*. The nucleic acid is obtained from cell pellets of pure cultures as described below (Section 2.1).
1.2. The test is based on the publication of Jeong et al. (2019).
1.3. The target sequences are located at the *X. citri* pv. *citri* CRISPR/Cas locus.
1.4. Sequences of primers used are listed in Table A2.
1.5. The test has been successfully performed with a 2720 thermal cycler version 2.08 (Applied Biosystems)

2. Methods

2.1. Nucleic Acid Extraction and Purification

Use purified genomic DNA (gDNA) obtained as described in EPPO Standard PM 7/129 (2) Appendix 2 (EPPO, 2021b).

2.2. Polymerase chain reactions and sequencing

As a first step, the full CRISPR array (S23-S01) is amplified by PCR and sequenced with the primers used for DNA amplification. In cases the PCR amplicon cannot be completely sequenced, they are re-sequenced using internal primers corresponding to the spacers Xcc_21, Xcc_19, Xcc_18 and Xcc_02, depending on the missing CRISPR regions.

TABLE A2 Sequences of primers used in CRISPR array.

			Primer used for	
CRISPR (sub)array	**Primer name**	**Primer sequence (5′–3′ orientation)**	**PCR**	**Sequencing**
S23-S01	Leader_fw	TCACGGGGTCCGCATGAC	X	X
	Terminator_rev	CTCGTCAGCGTCCGGCTG	X	X
	S#21_fw	TCGGGTTTCGGGATGTGC		X
	S#19_fw	CGAGCGCATCGATGACGG		X
	S#18_rev	CGTCGCTGTCGGTGGCAC		X
	S#02_rev	CCGGGACGGCGACGAAC		X
S23-S02	Leader_fw	TCACGGGGTCCGCATGAC	X	X
	S#02_rev	CCGGGACGGCGACGAAC	X	X
S23-S18	Leader_fw	TCACGGGGTCCGCATGAC	X	X
	S#18_rev	CGTCGCTGTCGGTGGCAC	X	X
S21-S01	S#21_fw	TCGGGTTTCGGGATGTGC	X	X
	Terminator_rev	CTCGTCAGCGTCCGGCTG	X	X
S21-S02	S#21_fw	TCGGGTTTCGGGATGTGC	X	X
	S#02_rev	CCGGGACGGCGACGAAC	X	X
S21-S18	S#21_fw	TCGGGTTTCGGGATGTGC	X	X
	S#18_rev	CGTCGCTGTCGGTGGCAC	X	X
S19-S01	S#19_fw	CGAGCGCATCGATGACGG	X	X
	Terminator_rev	CTCGTCAGCGTCCGGCTG	X	X
S19-S02	S#19_fw	CGAGCGCATCGATGACGG	X	X
	S#02_rev	CCGGGACGGCGACGAAC	X	X
S23-IS	Leader_fw	TCACGGGGTCCGCATGAC	X	X
	IS-1_rev	GTCGCCGAACAGGTTCACC	X	X
IS-S01	IS-2_fw	GCCGACCTGATGATGCA	X	X
	Terminator_rev	CTCGTCAGCGTCCGGCTG	X	X
IS-S18	IS-2_fw	GCCGACCTGATGATGCA	X	X
	S#18_rev	CGTCGCTGTCGGTGGCAC	X	X

Annealing temperatures used in the original study were 55°C but could be adapted if different PCR reagents are used.

If the PCR amplicon size is below 2500 bp but the amount or quality of the DNA does not allow sequencing, shorter subarrays are amplified (preferentially S23-S18, S21-02 and S19-S01, complemented with S23-S02, S21-S01, S21-S18 and/or S19-S02, if necessary) and sequenced with the primers used for DNA amplification.

If the PCR amplicon size for S23-S01 is larger than 2500 bp or if DNA amplification fails due to the presumed presence of an IS element in the CRISPR array, PCR reactions with one primer annealing to the IS element are recommended (S23-IS, IS-S01, IS-S18) and PCR products are sequenced with primers used for DNA amplification.

2.2.1. Master Mix

Reagent	Final concentration or quantity
Molecular grade water	To make up to 25 µL
Tris-HCl (pH 8.5)	10 mM
KCl	50 mM
$MgCl_2$	1.5 mM
Gelatine	0.01%
dNTP	0.2 mM each
CRISPR primers	0.4 µM
GoTaq® DNA polymerase (Promega, France).	0.25 U
DNA extracts	1 µL
Total	25 µL

2.2.2. PCR cycling conditions: All PCR protocols include an initial denaturation step of 1 min at 95°C, 30 cycles of a denaturation step of 2 min at 94°C, an annealing step of 30 s at 55°C, an elongation step of 2 min at 72°C and a final extension step of 2 min at 72°C.

2.2.3. Sequencing: General procedures for sequencing are described in Appendices 7 and 8 of the EPPO Standard PM 7/129 Appendix 8 (EPPO, 2021b).

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following external controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism or a synthetic control (e.g. cloned PCR product). A reference strain of *X. citri* pv. *citri* should be used.

3.2. Interpretation of results

Verification of the controls

- NAC should give no amplification.
- PAC should produce an amplicon and double-strand sequences consistent with data available at http://www.biopred.net/mlva/.

When these conditions are met:

- A test will be considered positive if sequence data allowing a complete spoligotype is obtained from DNA purified from bacterial cultures that have proved positive by diagnostic PCR targeting *X. citri* pv. *citri*.
- A test will be considered doubtful if any partial, contradictory or unclear results are obtained from bacterial cultures that have proved positive by diagnostic PCR targeting *X. citri* pv. *citri*.

4. Performance characteristics available

Complete spoligotypes were obtained from all *X. citri* pv. *citri* strain assayed ($n = 56$; Jeong et al., 2019).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

APPENDIX 12 - IMMUNOFLUORESCENCE

For general instructions on how to perform the immunofluorescence (IF) test see EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009). Only specific features are presented below.

Performance characteristics available (data from NIVIP (NL)).

Analytical specificity was evaluated on 38 strains of *X. citri* pv. *citri*, five strains of *X. citri* pv. *aurantifolii*, two strains of *X. citri* pv. *bilvae*, seven strains of *X. euvesicatoria* pv. *citrumelonis*, five saprophytic *Xanthomonas* sp. isolated from citrus and four epiphytic bacteria on *Citrus*.

	Loewe antiserum *X. citri* pv *aurantifolii* Cat. No 07303	**Loewe antiserum *X. citri* pv *citri* Cat. No 07302**
Inclusivity	100% of *X. citri* pv. *aurantifolii* strains	82% of *X. citri* pv. *citri* strains
Exclusivity	Cross reaction with 15 *X. citri* pv. *citri* strains Cross reaction with 10 non-target bacteria including strains of *X. citri* pv. *bilvae*, *X. euvesicatoria* pv. *citrumelonis*, and saprophytic *Xanthomonas* sp. isolated from citrus	Cross reaction with one *X. citri* pv. *aurantifolii* strain Cross reaction with five non-target bacteria including strains of *X. citri* pv. *bilvae*, *X. euvesicatoria* pv. *citrumelonis*, and saprophytic *Xanthomonas* sp. isolated from citrus

APPENDIX 13 - PATHOGENICITY TESTS AND BIOASSAY

For both detached or attached-leaf assays, negative (e.g., sterile water or buffer) and positive controls (e.g., a reference strain of *X. citri* pv. *citri* from an international culture collection) should be used. A panel of indicator host species such as grapefruit (*C* × *paradisi*), sweet orange (*C. sinensis*), *x Citrofortunella microcarpa*, Ortanique tangor ('*Citrus* × *tangerina*' × *Citrus sinensis*) and/or Mexican lime (*C. aurantiifolia*) should be used for assessing pathotype status. Young grafted plants (6–10 months) or seedlings should be preferred because the expression of symptoms is better in young leaves. Both *X. citri* pv. *aurantifolii* and *X. citri* pv. *citri* produce typical canker-like lesions on Mexican lime, which is regarded as the universal susceptible host species. However, *X. citri* pv. *aurantifolii* generally causes less pronounced symptoms than *X. citri* pv. *citri*. The susceptibility of other citrus species is pathovar and pathotype-dependent. Lesions develop on Mexican lime for *X. citri* pv. *citri* pathotype A* and A^w strains and on all citrus species for pathotype A strains. Pathotype A^w display the unique feature of inducing a hypersensitivity-like, necrotic reaction on grapefruit and sweet orange (Rybak et al., 2009) typically visible after 2–3 days from the inoculation sites where a thick suspension was infiltrated. Note that mild canker reactions can be caused on some citrus species by a few pathotype A* strains and that Ortanique tangor was identified as the species most clearly discriminating between pathotype A and A* strains (Escalon et al., 2013). However, no significant canker outbreak caused by pathotype A* has been reported in the field from species other than lime.

1. Attached leaf assay

Adult leaves from the youngest vegetative flush should be used. The attached leaf assay consists of infiltration (using a needle-free syringe) of bacterial suspensions containing approximately 5×10^8 cfu mL^{-1} ($OD_{600} \approx 0.3$ determined by spectrophotometry) and the same suspensions a hundred-fold diluted in 0.01 M Tris buffer (pH 7.2) over areas of approx. 1–2 cm^2 in the abaxial side of nearly expanded or recently fully expanded citrus leaves. Plants should be incubated at 28–30°C and canker-like lesions typically develop after 5–8 days. Note: if the starting bacterial concentration is more diluted (e.g. 10^6 cfu mL^{-1}), plants should be incubated for a longer period (e.g. 14 days).

2. Detached-leaf assay

Bacterial suspension (Pathogenicity test)

The detached-leaf assay involves the use of young leaves (ideally 70%–80% expanded immature leaves), submitted to a surface-sterilization in 1% sodium hypochlorite for 1 min, which are rinsed twice with sterile water and placed aseptically on 1% water agar plates optionally supplemented with a suitable fungicide (e.g., chlorothalonil, propiconazole) with the abaxial surface of the leaf up. Wounds (6–10 per leaf depending on its size) are created using a sterile needle and 10 μL of bacterial suspension containing approximately 10^6 cfu mL^{-1} is placed on each wound. More wounds per inoculation sites (e.g. 5) can be performed to enhance the chance of penetration in the same droplet. Leaves are incubated

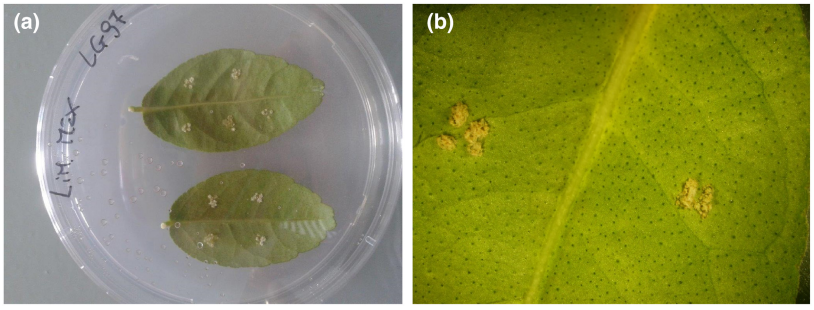

FIGURE A1 Inoculation of *Citrus aurantifolii* leaves with A. LG97 and B. LG115 strains of *X. citri* pv. *citri* (Photo courtesy: V. Catara (UNICT, IT)).

in an illuminated growth chamber at 28°C (12/12 h day/night) and observed for the development of tissue hyperplasia after 7–21 days. Authenticated reference strains should be used as controls (Vernière et al., 1998). Symptoms observed in detached-leaf assays following this protocol are shown in Figure A1.

Plant extract (Bioassay)

The same procedure as for bacteria colonies can be implemented using the plant extracts. Washings (see Section 3.2.2.1) can be concentrated by high-speed centrifugation (10 000 *g* for 2 min) and the pellets resuspended in a minimal volume of sterile 0.01 M Tris buffer pH 7.2.