Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

# Normes OEPP
# EPPO Standards

## Diagnostics
## Diagnostic

PM 7/44

Organisation Européenne et Méditerranéenne pour la Protection des Plantes
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## Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

## Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations.

## Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

## Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

## Scope

EPPO Standards on Diagnostics are intended to be used by NPPOs in their capacity as bodies responsible for the application of phytosanitary measures. Standards on diagnostic protocols are concerned with the diagnosis of individual pests and describe different methods which can be used to detect and identify pests of phytosanitary concern for the EPPO region. General Standards on diagnostics are in preparation on: (1) the purpose of diagnostic protocols (which may differ according to the circumstances of their use); and (2) reporting and documentation of diagnoses.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all EPPO Standards on Diagnostics:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

## References

EPPO/CABI (1996) *Quarantine Pests for Europe*, 2nd edn. CAB International, Wallingford (GB).

EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

FAO (1997) *International Plant Protection Convention* (new revised text). FAO, Rome (IT).

IPPC (1993) *Principles of plant quarantine as related to international trade*. ISPM no. 1. IPPC Secretariat, FAO, Rome (IT).

IPPC (2002) *Glossary of phytosanitary terms*. ISPM no. 5. IPPC Secretariat, FAO, Rome (IT).

OEPP/EPPO (2003) EPPO Standards PM 1/2(12): EPPO A1 and A2 lists of quarantine pests. *EPPO Standards PM1 General phytosanitary measures*, 5–17. OEPP/EPPO, Paris (FR).

## Definitions

*Regulated pest*: a quarantine pest or regulated non-quarantine pest.
*Quarantine pest*: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

## Outline of requirements

EPPO Standards on Diagnostics provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

## Existing EPPO Standards in this series

Forty-one EPPO standards on diagnostic protocols have already been approved and published. Each standard is

numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69

PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77

PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253

PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259

PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265

PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275

PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279

PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292

PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298

PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310

PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256

PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264

PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270

PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280

PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288

PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

PM 7/20 (1) *Erwinia amylovora*. *Bulletin OEPP/EPPO Bulletin* **34**, 159–172

PM 7/21 (1) *Ralstonia solanacearum*. *Bulletin OEPP/EPPO Bulletin* **34**, 173–178

PM 7/22 (1) *Xanthomonas arboricola* pv. *corylina*. *Bulletin OEPP/EPPO Bulletin* **34**, 179–182

PM 7/23 (1) *Xanthomonas axonopodis* pv. *dieffenbachiae*. *Bulletin OEPP/EPPO Bulletin* **34**, 183–186

PM 7/24 (1) *Xylella fastidiosa*. *Bulletin OEPP/EPPO Bulletin* **34**, 187–192

PM 7/25 (1) *Glomerella acutata*. *Bulletin OEPP/EPPO Bulletin* **34**, 193–200

PM 7/26 (1) *Phytophthora cinnamomi*. *Bulletin OEPP/EPPO Bulletin* **34**, 201–208

PM 7/27 (1) *Puccinia horiana*. *Bulletin OEPP/EPPO Bulletin* **34**, 209–212

PM 7/28 (1) *Synchytrium endobioticum*. *Bulletin OEPP/EPPO Bulletin* **34**, 213–218

PM 7/29 (1) *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **34**, 219–228

PM 7/30 (1) *Beet necrotic yellow vein benyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 229–238

PM 7/31 (1) *Citrus tristeza closterovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 239–246

PM 7/32 (1) *Plum pox potyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 247–256

PM 7/33 (1) *Potato spindle tuber pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **34**, 257–270

PM 7/34 (1) *Tomato spotted wilt tospovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 271–280

PM 7/35 (1) *Bemisia tabaci*. *Bulletin OEPP/EPPO Bulletin* **34**, 281–288

PM 7/36 (1) *Diabrotica virgifera*. *Bulletin OEPP/EPPO Bulletin* **34**, 289–294

PM 7/37 (1) *Thaumetopoea pityocampa*. *Bulletin OEPP/EPPO Bulletin* **34**, 295–298

PM 7/38 (1) *Unaspis citri*. *Bulletin OEPP/EPPO Bulletin* **34**, 299–302

PM 7/39 (1) *Aphelenchoides besseyi*. *Bulletin OEPP/EPPO Bulletin* **34**, 303–308

PM 7/40 (1) *Globodera rostochiensis* and *Globodera pallida*. *Bulletin OEPP/EPPO Bulletin* **34**, 309–314

PM 7/41 (1) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *Bulletin OEPP/EPPO Bulletin* **34**, 315–320

Some of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'inter-comparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM 7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

**Diagnostics**[1]
**Diagnostic**

# *Xanthomonas axonopodis* pv. *citri*

## Introduction

*Xanthomonas axonopodis* pv. *citri* (formerly *Xanthomonas campestris* pv. *citri*) is the causal agent of citrus bacterial canker (EPPO/CABI, 1997; OEPP/EPPO, 1990). The pathogen causes necrotic lesions on leaves, stems and fruits. Severe infections can cause defoliation, badly blemished fruits, premature fruit drop, twig dieback and general tree decline. Known hosts are in the family *Rutaceae*, *Citrus* spp. being the hosts of major economic importance. Natural infections are known to occur on *Citrus* hybrids, and on *Poncirus trifoliata*, *Fortunella japonica*, *Fortunella margarita*, *Severinia buxifolia* and *Swinglea glutinosa*. The disease is present in Asia, Africa, North America, South America and Oceania (EPPO/CABI, 1997). Several pathotypes have been recognized within *X. a. citri*. Different types of bacterial canker occur and are induced by variants of the same causal agent, primarily distinguished by their geographical origin and host range in addition to certain genotypic characteristics. Pathotype A has the widest host range and a global distribution and causes Asiatic citrus canker (CBC-A). Pathotype B is restricted to lemon, although Mexican lime, sour orange and pummelo are also susceptible (CBC-B). Pathotype C is restricted to Mexican lime (*C. aurantifolia*) (CBC-C). These two canker types were described in South America and were gradually supplanted by CBC-A. Two groups of strains, with restricted host range strains, have recently been recently identified within pathotype A (Vernière *et al*., 1998; Sun *et al*., 2000), and designated as A* and A$^{w}$. These are closely related to type A strains (Cubero & Graham, 2002, 2004) but affect only Mexican lime and Alemow. The proposal has been made to restrict the name *X. a. citri* to pathotype A, and to use *Xanthomonas axonopodis* pv. *aurantifolii* for pathotypes B and C (Vauterin *et al*., 1995). Despite the fact that these distinct names have been extensively and commonly used, the proposal has not been validated by the Committee on Taxonomy of Plant Pathogenic Bacteria. So all the strains are still considered as *X. a. citri*. Another disease, citrus bacterial spot, is caused by a *Xanthomonas* whose strains are referred to as *Xanthomonas axonopodis* pv. *citrumelo* (Rossetti, 1977; Gottwald *et al*., 1991). Reported only in Florida (US), this was wrongly named in the past as pathotype E of *X. c. citri*. Similarly, a strain originally described as pathotype D of *X. c. citri* was at one time recorded in Mexico. The disease with which it was associated is now attributed to the fungus *Alternaria limicola*, and the status of the bacterial strains isolated is obscure. These so-called pathotypes D and E are not covered by this protocol.

## Identity

**Name:** *Xanthomonas axonopodis* pv. citri (Hasse) Vauterin *et al*. (1995)
**Synonyms:** *Pseudomonas citri* Hasse, *Xanthomonas citri* (Hasse) Dowson, *Xanthomonas citri* f.sp. *aurantifoliae* Namekata & Oliveira, *Xanthomonas campestris* pv. citri (Hasse) Dye 1978, *Xanthomonas campestris* pv. *aurantifolii* Gabriel *et al., Xanthomonas citri* (ex Hasse) nom. rev. Gabriel *et al., Xanthomonas axonopodis* pv. *aurantifolii* Vauterin *et al*.
**Taxonomic position:** *Bacteria*: *Gracilicutes*
**EPPO computer code:** XANTCI
**Phytosanitary categorization:** EPPO A1 list no. 1; EU Annex designation II/A1

## Detection

### Disease symptoms

*X. a. citri* generally occurs on the aerial part of its hosts. 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. 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 and may fall out leaving a hole. Diagnostic symptoms are tissue hyperplasia resulting in cankers with water-soaked margins and yellow halos surrounding the lesions.

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

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

twig lesions. On removal of the corky layer, dark brown lesions are visible in the healthy green bark tissue.

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

Symptoms of citrus bacterial canker may be confused with citrus scab caused by *Elsinoe fawcettii* on sweet orange fruits, on leaves, fruits and branches of lemon, and on leaves of Rangpur lime. There are some similarities also with anthracnose (*Glomerella cingulata*) on fruits and melanose (*Diaporthe citri*) on leaves.

### Screening tests

To confirm the presence of *X. a. citri*, it is necessary to isolate the bacterium from lesions and to perform a pathogenicity test on citrus. However, in view of the difficulty of isolating *X. a. citri*, especially from asymptomatic plants or parts of plants, a PCR screening test with specific primers is recommended as the only reliable method for rapid analysis of suspect samples. Immunofluorescence can also be used but no commercial antibodies have been evaluated. Monoclonal antibodies are available for ELISA, but are mostly advised for identification of pure cultures, due to low sensitivity.

*DNA extraction from infected citrus tissue*

A DNA extraction protocol should be used before amplification from plant material. The original DNA extraction by Hartung *et al*. (1993) was performed with a CTAB protocol, but there are also methods that do not require phenol (Llop *et al*., 1999). Lesions or other suspicious infected plant materials are cut into small pieces, covered with PBS buffer and shaken in a rotary shaker for 20 min at room temperature. The supernatant is filtered and centrifuged for 20 min at 10 000 *g*. The pellet is resuspended in 1 mL of PBS. 500 µL is saved for further analysis or for direct isolation on agar plates. 500 µL of the sample is centrifuged at 10 000 *g* for 10 min. The pellet is resuspended in 500 µL of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP) vortex and left for 1 h at room temperature with continuous shaking. The suspension is centrifuged at 5000 *g* for 5 min, 450 µL of the supernatant is transferred and 450 µL isopropanol is added. The suspension is mixed gently and left at room temperature for 1 h. Precipitation can be improved by the use of Pellet Paint Coprecipitant (Novagen, Darmstadt, DE) (Cubero *et al*., 2001). The suspension is centrifuged at 13 000 *g* for 10 min, the supernatant is discarded and the pellet is dried. The pellet is resuspended in 100 µL water. 5 µL of sample is used in a 50 µL PCR reaction.

*Primers used in PCR*

Several sets of primers are available for diagnosis of *X. a. citri*. Based on Hartung *et al*. (1993), primers 2 (5′-CAC GGG TGC AAA AAA TCT-3′) and 3 (5′-TGG TGT CGT CGC TTG TAT-3′) allow the amplification of a 222 bp DNA fragment only in A strains and are the most frequently used in assays on plant material. Primers J-pth1 (5′-CTTCAACTCAAAC-GCCGGAC-3′) and J-pth2 (5′-CATCGCGCTGTTCGGGAG-3′) based on the nuclear localization signal in virulence gene *pthA* allow the amplification of a 197 bp fragment in A, B and C strains (Cubero & Graham, 2002) but they showed lower sensitivity in plant material. Primers J-Rxg (5′-GCGTTGAGGCTGAGACATG-3′) and J-RXc2 (5′-CAAGTTGCCTCGGAGCTATC-3′), based on the internally transcriber spacer (ITS) between the 16S ansd 23S genes, have also been used for universal identification on pure culture of A strains (Cubero & Graham, 2002).

For amplification with primers 2 and 3, the PCR reaction mix is prepared in a sterile vial (per 50 µL reaction): PCR buffer (50 mM Tris HCl pH 9, 20 mM NaCl, 1% Triton ×100, 0.1% gelatin, 3 mM $MgCl_2$), 1 µM each primer, 200 µM deoxynucleoside triphosphates, and 1.25 units of *Taq* DNA polymerase. The components are mixed and 45 µL of the mix is transferred into sterile PCR vials. The vials are kept with the PCR reaction mix on ice. 5 µL of the extracted DNA, water control and positive control are added in the specified order to the vials with the PCR reaction mix. The vials are placed in the heating block of the thermal cycler and the following programme is run: 35 cycles of 70 s at 95°C (denaturation), 70 s at 58°C (annealing of primers), 60 s at 72°C (extension of copy). The PCR product is analysed and the vials are stored at 4°C for use on the same day, or at –20°C for longer. The PCR fragment of 222 bp should be detected in positive samples in 2% agarose gel after electrophoresis and staining with ethidium bromide. The water control should be negative in each case. If positive, the test should be repeated. The gel is photographed if a permanent record is required.

Primer pair 2/3 is the most commonly used. Pair 4/7 [4-5′-TGT CGT CGT TTG TAT GGC-3′; 7-5′-GGG TGC GAC CGT TCA GGA-3′] has proved useful for identification of A strains and shows variable results for B and C strains (Vernière *et al*., 1998). Nested PCR, immunocapture and colorimetric detection of nested PCR products for direct and sensitive detection of *X. a. citri* in plants have also been developed (Hartung *et al*., 1996).

For identification or detection in plant material of *X. a. citri* based on the *pth* gene, PCR reactions are performed in volumes of 25 µL containing 1× Taq buffer, 3 mM $MgCl_2$, 0.1 µM concentration of primers J-pth1 and J-pth2, 0.2 mM each deoxynucleoside triphosphate, 1 U of Taq polymerase and 5 µL of extracted DNA. Amplification reaction conditions consist of 93°C for 30 s, 58°C for 30 s, and 72°C for 45 s for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min. For identification of A strains based on ribosomal primers, PCR reactions are carried out in volumes of 25 µL containing 1× Taq buffer, 1.5 mM $MgCl_2$, 0.04 µM concentration of primers J-RXg and J-RXc2, 0.2 mM each deoxynucleoside thriphosphate, 1 U of Taq polymerase and 5 µL of extracted DNA with the same PCR profile as used the other primers. PCR products are visualized and stained as above.

Real time PCR has also been applied for quantitative PCR and for the rapid, on site identification of this bacterium in plant material, but it has not been compared in routine detection with standard or nested PCR.

### Isolation

Isolation in culture is possible from fruit, leaf or stem lesions. Small pieces of the water-soaked tissue at the lesion margin are excised with a sterilized scalpel or razor blade. The tissue is chopped or diced in a drop of sterile distilled water. The resulting suspension is streaked on standard culture media as nutrient agar supplemented with 0.1% w/v D-glucose (NGA) or YPGA (0.5% peptone, 0.5% yeast extract, 1% glucose, 1.5% agar). Some B and C strains are reportedly difficult to isolate, and may be cultured initially on 1% sucrose, 0.5% peptone, 0.05% $K_2HPO_4$, 0.03% $MgSO_4$ and purified agar (Canteros *et al*., 1985). After initial culturing, strains adapt to other nutrient media. Semi-selective isolation can be achieved on KCB medium, containing NGA supplemented with kasugamycin (16 µg mL$^{-1}$), cephalexin (16 µg mL$^{-1}$) and Bravo or Daconil (12 µg a.i. mL$^{-1}$). For samples isolated from the environment, a lower concentration of glucose (0.01%) should be used.

Colonies on agar plates are circular, convex, mucoid, shiny and yellow; they cannot be distinguished by morphological characteristics from those of *Xanthomonas campestris* pv. *campestris* strains or from those of *X. a. citrumelo*.

## Identification

### General characteristics of Xac

General characteristics of the bacterial strains can be used after isolation to support the identification at species level. Methods to perform physiological and biochemical tests are available in laboratory manuals (Lelliott & Stead, 1987; Schaad, 1988; Schaad *et al*., 2001). *X. a. citri* is a rod shaped, Gram-negative aerobic bacterium with a single polar flagellum, producer of xanthomonadin, oxidase negative, catalase positive, urease negative, casein and aesculin hydrolysis positive; nitrates not reduced; acid is produced from arabinose, glucose, saccarose, mannose, galactose and trehalose. The A, B and C pathotypes, and *X. a. citrumelo*, are not easily distinguished by their biochemical and physiological characteristics.

### Pathogenicity tests

*X. a. citri* and its pathotypes should be identified by pathogenicity on a panel of indicator hosts such as Duncan grapefruit, Valencia sweet orange or Mexican lime, for confirmation of the diagnosis. Leaf assays on susceptible cultivars of *Citrus* hosts allow rapid and accurate evaluation of bacterial strains. Lesions develop 7–14 days after inoculation of intact leaves or detached leaves. With these assays, the flat lesion types of *X. a. citrumelo* can readily be distinguished from the eruptive callus-like reaction of *X. a. citri*. The bacteria are grown in liquid media or cells are scraped off from a freshly streaked agar plate and suspended in sterile distilled water for inoculation into citrus. Concentration is adjusted from $10^6$ to $10^8$ cfu mL$^{-1}$. Leaves can also be inoculated with aqueous lesion extracts. A negative control should always be included and a positive control where possible. Plants inoculated with the positive control strain should be kept apart from other test plants.

*Attached leaf assay*

With a syringe without the needle, citrus leaves are infiltrated with the bacterial suspension by gently pressing the blunt end against the abaxial leaf surface until about 2 cm$^2$ of the leaf is water-congested. Six different strains may be inoculated into the same leaf, three on each side of the mid-vein. The leaves are then observed for development of tissue hyperplasia. Symptoms can generally be observed after 7–14 days after incubation in a glasshouse at about 25°C, as lesions with a raised margin surrounding a slightly chlorotic region. The raised margin then becomes pronounced, roughened and corky, the central region of the lesion becomes necrotic and collapsed; the necrotic lesions may split and the leaves abscise after several weeks.

*Detached leaf assay*

Detached fully expanded but still immature leaflets of grapefruit, or other susceptible citrus, should be surface-sterilized in 70% ethanol for 1 min. They are rinsed twice with sterile water and placed aseptically on 1% water agar plates with the adaxial surface of the leaf exposed. A triangle is cut in each side of the mid-vein with a sterilized scalpel and 20 µL of bacterial suspension is placed on each wound. Leaves are incubated in a growth chamber at 28°C and observed for the development of tissue hyperplasia after 7–14 days.

*Inoculation of plants growing* in vitro

Use of *in vitro* plants for inoculation provides for greater security. Susceptible cultivars of grapefruit like 'Marsh', or of sweet orange like 'Parson Brown', can be used. The seedlings are grown under sterile conditions in Murashige and Skoog salt solutions by placing a drop of about $10^9$ cfu mL$^{-1}$ in wounded leaves. Incubation at 25–28°C for 7–14 days is required for symptom appearance (López & Navarro, 1981).

### Indirect ELISA

ELISA kits containing all the necessary components for the identification of *X. a. citri* are available commercially from Agdia (US). Positive control samples are also commercially available from the manufacturers. The method used is indirect ELISA by detection with monoclonal antibodies described by Alvarez *et al*. (1991). In theory, all strains can be identified, but it has been reported that some phenotypically distinct A strains isolated in South-west Asia do not react with the monoclonal antibodies. The sensitivity of the detection in symptomatic plant material is about$10^6$ cfu mL$^{-1}$.

For identification of pure cultures, suspensions are centrifuged at about 10 000 rev min$^{-1}$ for 2 min and the supernatant

is discarded. 1 mL 1× PBS buffer is added and the cells are resuspended by vortexing. The operation is repeated twice more. After the third wash, the cells are resuspended in coating buffer. Bacterial concentration is adjusted spectrophotometrically to $OD_{600}$ 0.01 (about $2.5 \times 10^7$ cfu $mL^{-1}$). 100 µL aliquots of the samples are loaded onto ELISA microtiter plates (two wells per sample). Positive control sample (from a reference culture or provided by manufacturer) and buffer control wells should be included. The plates are incubated overnight at 37°C until dry. 200 µL blocking solution is added to each well (5% non-fat dried milk in PBS buffer, 0.05 blocking component per mL buffer). The plates are incubated for 30 min at room temperature and washed twice with 1× PBST. 100 µL of prepared primary antibody is dispensed (prepare at the appropriate dilution in a solution of 2.5% of dried milk in PBST). Plates are incubated 1 h at room temperature, and washed five times with 1× PBST. 100 µL of prepared enzyme conjugate per well is dispensed (prepared at the appropriate dilution in a solution of 2.5% of dried milk in PBST). Plates are incubated for 1 h at room temperature. After washing the plates five times with 1× PBST, 100 µL per well of freshly prepared substrate solution containing 1 mg $mL^{-1}$ p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8 is dispensed. The plates are incubated for 30–60 min at room temperature. The O.D. is measured using a plate reader at 405 nm. Positive samples should be at least 2× O.D. of the negative control on each plate.

#### Molecular identification

Other techniques than those here described have been reported for identification and strain assignation, such as RFLP analysis, genomic fingerprinting, etc. The same sets of primers indicated for detection can be used for identification of suspect strains. Another approach for producing universal primers for canker-producing strains utilizes specific sequences in the intergenic spacer (ITS) regions of 16S and 23S ribosomal DNAs. Variation in the ITS sequences allows the design of specific primers for A strains, to identify the $A^w$ as an A strain, and readily to differentiate the $A^w$ strain from the B and C pathotypes, even though these strains have a very similar host range (Cubero & Graham, 2002).

BOX- and ERIC-PCR (Louws *et al*., 1994) can be used for strain identification and characterization under specific PCR conditions (Cubero & Graham, 2002). BOX PCR reactions are carried out in 25-µL volumes containing 1× Taq buffer, 6 mM $MgCl_2$, 2.4 µM concentration of primer BOX1R (5′-CTACGGCAAGGCGACGCTGCAG-3′), 0.2 mM each deoxynucleoside thriphosphate and 2 U of Taq polymerase with a profile of 94°C (30 s), 48°C (30 s), and 72°C (1 min) for 40 cycles plus an initial step of 94°C for 5 min and a final step of 72°C for 10 min and using 5 µL of DNA extracted from xanthomonad strains. DNA is extracted from bacterial supensions (absorbance at 600 nm from 0.2 to 0.5) following a single step of phenol-chloroform-isoamyl alcohol, precipitated in ethanol, and re-suspended in ultrapure water. DNA is stored at –20°C until further use. ERIC PCR reactions are carried out also in 25 volumes containing 1× Taq buffer, 3 mM $MgCl_2$, 1.2 µM concentration of primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Louws *et al*., 1994), 0.2 mM each deoxynucleoside triphosphate and 2 U of Taq polymerase with the same profile as for BOX-PCR reactions. PCR products are analysed by 3% agarose gel in 1X TAE buffer for 2 h at 110 V and stained with ethidium bromide.

Real time PCR has also been applied for quantitative PCR and for the rapid, on site identification of *X. a. citri* in plant material. Approaches using SYBR green dye and Taqman probes have been evaluated in conjunction with primers based on sequences from the pth and ribosomal genes (Cubero & Graham, 2002), as well as on a gene for the leucine-responsive regulatory protein (Irp) (Cubero & Graham, 2004).

#### Automated techniques

Fatty acid analysis for identification of pure cultures is available from MIDI (Newark, US) and from NCPPB (CSL,York, GB). Biolog GN is an automated method for identifying bacteria, based on the use of 95 substrates. It can be used for identification at the species level and is commercially available from Biolog (Hayward, US).

For a positive diagnosis, disease symptoms, morphological and biochemical characteristics of the pathogen and its pathogenic properties should be in accordance with the descriptions of the protocol and the pathotype should be identified. A flow diagram is presented in Fig. 1.

*Note on* X. a. citrumelo

*X. a. citrumelo* is considered in Florida (US) to be the cause of 'citrus bacterial spot', a disease observed in the rootstock Swingle citrumelo, causing flat necrotic lesions with water-soaked margins. Isolation from affected plants gives strains indistinguishable by cultural and physiological characteristics from *X. a. citri*. Accordingly, it is necessary to perform the pathogenicity and other tests to obtain a correct diagnosis. Monoclonal antibodies and PCR protocols used for *X. a. citri* do not recognize *X. a. citrumelo*. In addition, BOX- and ERIC-PCR profiles from *X. a. citrumelo* strains are quite different from those of *X. a. citri*. As opportunistic *Xanthomonas* strains have also been isolated from *Citrus* spp., it is always advisable to check strains for pathogenicity.

## Reference strains

NCPPB 409 (Type strain); CFBP 2525.
NCPPB = National Collection of Plant Pathogenic Bacteria, CSL York, UK.
CFBP = Collection Française de Bactéries Phytopathogènes, INRA, Angers, FR.

## Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

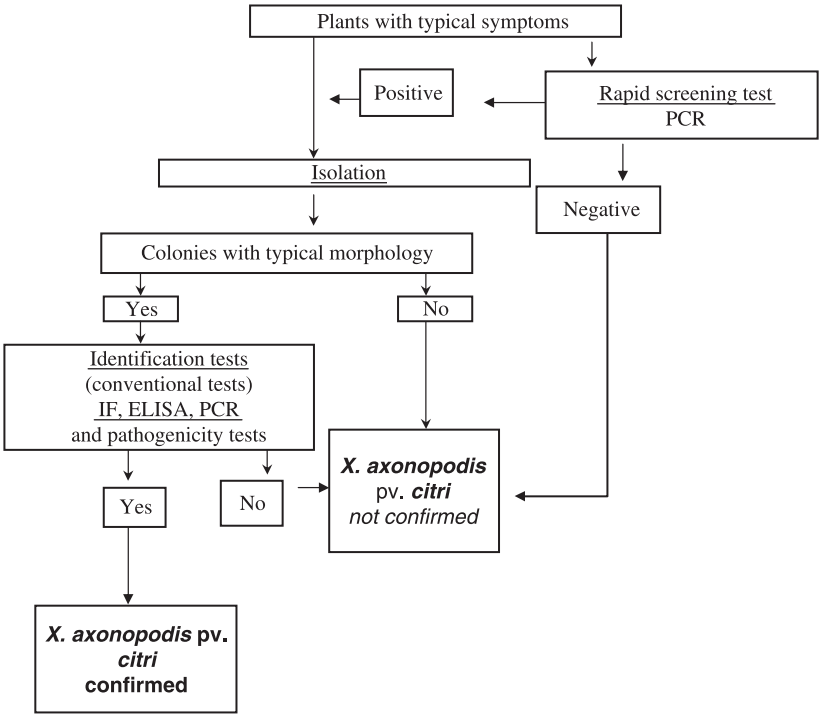


**Figure 1** Flow diagram for the diagnosis of *Xanthomonas axonopodis* pv. *citri* on symptomatic samples.

## Further information

Further information on this organism can be obtained from:

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## Acknowledgements

This protocol was originally drafted by V. Catara, DISTEF, University of Catania (IT) and revised by J. Cubero, INIA, Madrid (ES) and M.M. López, IVIA, Moncada, Valencia (ES).

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