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

Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/24

Organization Européenne et Méditerranéenne pour la Protection des Plantes
1, rue Le Nôtre, 75016 Paris, France

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 Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

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 diagnostic protocols:

- 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).

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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.

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 Diagnostic Protocols for Regulated Pests 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

Nineteen 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

Several 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 ‘intercomparison’ 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 PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostic protocols for regulated pests[1]
Protocoles de diagnostic pour les organismes réglementés

Xylella fastidiosa

Specific scope

This standard describes a diagnostic protocol for *Xylella fastidiosa*.

Specific approval and amendment

Approved in 2003-09.

Introduction

Symptoms caused by *Xylella fastidiosa* were first observed in 1892 in the grape-growing region of southern California (US) and the syndrome was called 'Pierce's disease'. Subsequently, similar diseases were reported on many fruit-tree and ornamental species, such as phony peach, alfalfa dwarf, periwinkle wilt, citrus variegated chlorosis, leaf scald of plum and maple, leaf scorch of pear, almond, elm, mulberry, sycamore, oak, oleander and coffee, especially in north and south America (Hopkins, 1989). In Europe, the disease has been dubiously reported once on grapevine in the Kosovo region (Serbia). On most host plants, the pathogen induces leaf wilting, twig dieback and decline. However, in phony peach disease (Web Fig. 6), a reduction in plant growth, with shorter internodes, is seen. *X. fastidiosa* proliferates in the xylem vessels. Natural transmission occurs via insects feeding suctorially on xylem sap. Transmission efficiency varies widely among vector species. The bacterium overwinters in the xylem of the host plant as well as in weeds. From the latter, it is transmitted to the host plant in spring. This protocol gives methods only for strains from grapevine and citrus.

Identity

Name: *Xylella fastidiosa* Wells *et al*. (1987)
Taxonomic position: Bacteria, Gracilicutes, Gram-negative aerobic rods, Category I, Group 4, Subgroup 4 A (Holt, 1994)
Bayer computer code: XYLEFA
Phytosanitary categorization: EPPO A1 list no. 137 & 166; EU Annex designation I/A1 – as *Xylella fastidiosa* on grapevine, peach phony rickettsia on peach and variegated chlorosis on citrus

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

Detection

Disease symptoms

Inspection of crops suspected to be infected by *X. fastidiosa* is fundamental for detecting early symptoms of infection. The ELISA test and PCR are then utilized for rapid and reliable identification of the pathogen. There is a seasonal fluctuation in the detection of infected specimens. In citrus, recovery of the pathogen from roots and stems is best at two periods: midsummer (June–August) and midwinter (December–February). In grapevine, the best period for detection is late summer and early autumn.

On grapevine (Web Figs 1–3), the main symptom of primary infection is leaf scorching. An early sign of the disease is the sudden drying of a part of the leaf lamina which turns brown and is very often surrounded by a yellowish or a reddish halo. Subsequently, the wilting spreads and the whole leaf shrivels and drops; remnants of the leaf remain attached to the petiole. The wilting of the twig usually starts from the tip. Infected stems mature irregularly showing patches of green tissue. Infected plants sprout later than healthy ones. Heavily infected plants may die in one or two years. Symptoms can rarely be detected on 1-year-old plants. Symptoms on the twigs can be confused with those due to phytopathogenic fungi such as *Eutypa lata* and *Phomopsis viticola*.

On citrus, the disease is locally named 'amarelinho' in Brazil and 'pecosita' in Argentina. Symptoms are more obvious on 3–6-year-old trees and mainly on sweet orange cultivars. Affected trees show leaves with chlorotic yellow spots, recalling zinc deficiency; the lower surface shows slightly raised brownish necrotic spots (Web Figs 4 and 5). Fruits are much smaller than normal and very firm. Flower and fruit set occur at the same time on healthy and affected trees but fruits remain small and ripen earlier. The growth rate of affected trees is greatly reduced and twigs and branches may wilt. The plants do not usually die, nor do the roots show any apparent symptoms.

On peach (Web Fig. 6), young shoots are stunted and bear greener, denser foliage than healthy trees. Lateral branches grow horizontally or droop, so that the tree seems uniform, compact and rounded. Leaves and flowers appear early, and leaves remain on the tree longer than on healthy trees. Affected trees yield increasingly fewer and smaller fruits until, after 3–5 years, they become economically worthless.

Identification

Isolation

Xylella fastidiosa is difficult to isolate and grow in axenic culture. It does not grow on most common bacterial media.

Grapevine
To isolate *X. fastidiosa* directly on bacterial culture media, the procedure described for the preparation of plant extract for ELISA can be followed (see DAS-ELISA test). Isolation can be performed from leaf veins, petiole, small twigs or roots. In any case, it is critical to surface-sterilize the sample properly and to dilute the plant extract in sample buffer. The xylem sap obtained either from the crushing of leaf vein, petiole or small twig or from extraction with vacuum infiltration of small twigs and roots can be streaked on to CS20, PD2, PD3 or B.CYE media (Appendix I). Alternatively, the twigs can be sliced tangentially with a sterile scalpel and the slices can be directly placed on the agar medium in Petri dishes. The plates should be incubated at 28 °C for 8–10 days, in plastic bags to prevent desiccation.

Citrus
Symptomatic leaves are surface-sterilized with 10% bleach for 5 min, followed by two rinses in sterile distilled water. Midribs and petioles are aseptically excised and placed onto sterile Petri dishes containing 1–2 mL of PBS. Sections of 2–3 mm are obtained with a sterile scalpel. The sections are ground and the sap is streaked on to PW or SPW medium. The plates are incubated at 28 °C for 21 days. For isolation from roots and stem, after surface disinfection as above, segments of 4–12 mm in diameter and 2–3 cm long are vacuum-infiltrated with succinate-citrate-phosphate buffer (1.0 g disodium succinate, 1.0 g trisodium citrate, 1.5 g K_2HPO_4, 1.0 g K_2HPO_4, distilled water to 1 L, pH 7.0) as described for grapevine. Then, the vacuum extract (3–4 mL per sample) is centrifuged at 4.500 *g* for 15 min and resuspended in 0.8 mL of buffer. One drop (5 µL) is subsequently placed onto PW, SPW, CVC1 or CVC2 media (Appendix I). The plates are incubated at 27–30 °C, kept in plastic bags to prevent desiccation. They are observed for colony development at weekly intervals for a month with a binocular microscope.

Colony morphology and microscopical observation

Grapevine
After 10 days of incubation at 28 °C on PD2, PD3, CS20 or B.CYE media, *X. fastidiosa* yields colonies 0.5–2.0 mm in diameter, circular, with entire margins and convex elevation. Sometimes colonies are produced that are also circular with undulate margins with an umbonate or flat elevation.

Citrus
On SPW medium, isolated colonies are visible within 7 days after streaking. After 21 days of incubation at 27 °C, their diameter is 0.35 mm. On PW medium, colonies appear 10–14 days after isolation. On CVC1 and CVC2 media, colonies develop 25–30 days after isolation. Under dark field microscopy, the bacterium has a rod-shaped appearance with the following dimensions: 0.2–0.35 µm × 1–4 µm. Under the electron microscope, *X. fastidiosa* shows a characteristic rippled wall.

Pathogenicity test

Inoculation techniques should deliver the inoculum directly into the xylem vessels.

Grapevine
Pathogenicity tests use young grapevine plants grown in pots and colonies of the bacteria grown for 8–10 days on PD2, PD3, CS20 or B.CYE at 28–30 °C. Before inoculation, plants are removed from pots and roots are washed with water to remove soil particles. The inoculum is bacterial suspension of 10^7–10^8 CFU mL^{-1} in PBS. Test plants are cut off 15 cm above the collar and the roots are trimmed and immediately immersed in bacterial suspension. The stem is fitted to a Tygon tube connected to a vacuum and negative pressure is applied for 90 min. Control plants are treated in the same way except that PBS is used instead of bacterial suspension. The plants are then repotted and observed for symptom development. Alternatively, only one root per plant is trimmed and connected with a Tygon tube to a 10-mL pipette containing 10 mL of the bacterial suspension. The plants are repotted with the pipette reservoir connected to the root. The inoculum should be taken up in 3–4 days. Symptoms usually appear 60–80 days after inoculation. Small necrotic spots initially appear along the major veins, then enlarge and coalesce. Scorch symptoms then become apparent along the edge of the leaf, which can also wither. The bacterium should be re-isolated as for the primary isolation.

Citrus
For inoculation, a suspension of 10^8–10^9 CFU mL^{-1} in PBS is prepared from colonies grown on PW or SPW at 28 °C. Inoculation can be performed by the following three techniques: (1) 20 µL of suspension is placed on the surface of the citrus stem, and the stem is then pierced through the inoculum droplets with a syringe (multiple inoculation sites on the same stem raise the possibility of successful inoculation); (2) a flap of citrus branch is raised by cutting tangentially upward with a razor blade, and 10–30 µL of suspension is placed under the flap; (3) the flap of tissue is placed in a microcentrifuge tube containing 500 µL of bacterial suspension for 2 h, and the wound is then wrapped with grafting tape. These inoculations can be repeated after 2 months to increase the possibility of

reproducing the disease. Symptoms appear 10 months after inoculation, in the form of chlorotic spots on leaves. The bacterium should be re-isolated as for the primary isolation.

DAS-ELISA test

Kits for the serological detection of *Xylella fastidiosa* are supplied by AGDIA Inc., Elkhart (US).

Grapevine

A critical step for successfully performing the ELISA test is to obtain xylem sap in which the pathogen survives. To recover the bacterium from leaves, 1.0 g of petiole or leaf vein is first surface-sterilized in 0.5% NaOCl for 10 min, then rinsed in four changes of sterile distilled water and dried in a laminar flow cabinet. It is then ground in a sterile mortar containing 5 mL of sample buffer (SB) (disodium succinate 1.0 g; trisodium citrate 1.0 g; K_2HPO_4 1.5 g; KH_2PO_4 1.0 g; distilled water to 1 L; 0.02 M sodium ascorbate; 5% acid-washed insoluble polyvinylpyrrolidone; pH 7.0) or the grapevine sample extraction buffer provided by Agdia (tris-(hydroxymethyl)-aminomethane 60.5 g; NaCl 8.0 g; polyvinylpyrrolidone (PVP), MW 24–40 20.0 g; polyethylene glycol 10.0 g; NaN_3 0.2 g; Tween-20 0.5 g; distilled water to 1 L, pH 8.2). Twigs or roots have to be cut into 1–2-cm sections, stripped of bark, surface-sterilized for 5 min in 0.5% NaOCl with 3% of ethanol, then rinsed in four changes of sterile distilled water and crushed in a sterile mortar containing 5 mL of SB. Alternatively, twigs or roots may be cut into 5–6-cm lengths, stripped of bark and surface-sterilized as described above. Both ends are then connected to sterilized vacuum tube (e.g. Tygon), one piece being connected through a glass tube to a vacuum flask containing a sterile microcentrifuge tube to collect the liquid, and the other being filled with 15–20 mL of SB and sealed with parafilm or a sterile plastic tip. A negative vacuum pressure is applied to the flask to allow the buffer to pass through the xylem tissue and to be collected in the microcentrifuge tube. The collected liquid is then centrifuged for 3 min at 12.000 *g* and the pellet resuspended in 0.6 mL of SB.

Flat-bottom microtitre plates are coated with 0.2 mL of gamma globulin in coating buffer (Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; $NaNO_3$ 0.2 g, distilled water to 1 L; pH 9.6) in each well. The plates are incubated at 4 °C overnight, then washed with PBS-Tween (NaCl 8.0 g; KH_2PO_4 0.2 g; $Na_2HPO_4 \cdot 12H_2O$ 2.9 g; KCl 0.2 g; $NaNO_3$ 0.2 g; distilled water to 1 L; pH 7.4). 0.2 mL of plant extract in SB is added to each well. After incubating overnight at 4 °C, the plates are washed with PBS-Tween, and 0.2 mL of alkaline phosphatase conjugated antibody in conjugated buffer (polyvinylpyrrolidone 20 g; Ovo albumin 2 g; PBS-Tween 1 L) is added to each well. After incubation at 37 °C for 4 h, and washing with PBS-Tween, 0.2 mL of substrate solution (*p*-nitrophenyl phosphate: 1 mg/mL) in substrate buffer (diethanolamine 98 mL; NaN_3 0.2 g; distilled water to 1 L) is added to each well. The plates are incubated in the dark, at room temperature for 30-60 min. The reaction is terminated by adding 50 µL of 3M NaOH per well. Plates are read at 405–410 nm in a microplate auto reader. A suspension of 10^5 CFU mL^{-1} of *X. fastidiosa* type strain in SB is used as positive control, and SB alone as negative control. A mean absorbance greater than the mean of the negative control wells plus four times the standard deviation indicates a positive reaction.

Citrus

Leaves are taken from symptomatic trees. The leaf midribs and the petioles are aseptically excised, then surface-sterilized for 5 min with 10% NaOCl and rinsed twice with sterile distilled water. Midribs and petiole are homogenized or ground in sterile mortars, with 1 g of midribs or petiole in 3 mL of Phosphate-buffered Saline (PBS, 0.01 M KPO_4, 0.15 M NaCl, 0.02% NaN_3) plus 2% polyvinylpyrrolidone. 0.2 mL of plant extract is added to each well of the ELISA microplate. The method continues as for grapevine, above.

Polymerase Chain Reaction (PCR)

Grapevine

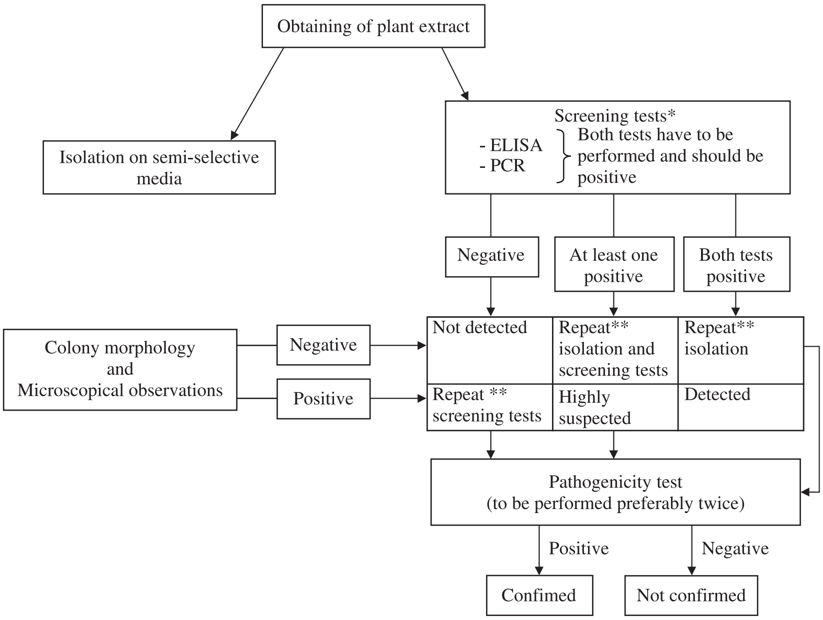
A sample of 1.0 g of leaf veins of the plant is ground to powder in liquid nitrogen and transferred directly into 10 mL of 65 °C preheated extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 500 mM NaCl, 1.25% sodium-dodecylsulphate, pH 8.0) containing 0.1 g of polyvinylpyrrolidone. After mixing, the sample is incubated at 65 °C in a water bath for 20 min. Then, 4 mL of 5 M potassium acetate is added and placed in ice for at least 20 min. Subsequently, the mixture is centrifuged at 12.000 *g* for 10 min at 4 °C. To the supernatant, 1 mL of 5% hexadecyltrimethylammonium bromide (CTAB), and 10 mL of chloroform, are added. The mixture is then centrifuged at 12.000 *g* for 10 min at 4 °C and 2/3 volume of isopropanol is added to precipitate DNA (Berisha *et al*., 1998).

Primers RST31 and RST33, which generate a PCR product of 733 base pairs, are used for detection of *X. fastidiosa* (Minsavage *et al*., 1994).

RST31: 5′-GCGTTAATTTTCGAAGTGATTCGATTGC-3′

RST33: 5′-CACCATTCGTATCCCGGTG-3′

Individual PCR samples (50 µL) contain 1X amplification buffer, 100 µM each of dNTP, 50 µM of each primer, 1.25 U of *Taq* DNA polymerase, 4 µL of plant sample extract (see ELISA test for extraction protocol) or 100 ng of purified *X. fastidiosa* genomic DNA in 4 µL of TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) for positive control. Each sample is covered with 50 µL of mineral oil before running the PCR reactions. Sterile 500-µL microcentrifuge tubes are used. PCR is carried out with an initial denaturation at 95 °C for 1 min followed by 40 cycles of denaturation step at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and DNA extension at 72 °C for 45 s. A final cycle consists of the denaturation and annealing step followed by an extension cycle at 72 °C for 5 min before cooling at 4 °C. After amplification, the products (15 µL) are analysed by electrophoresis in 1% agarose gel in TAE buffer. The gel is run at 5 V cm^{-1} for 1.5 h and then stained with ethidium bromide (10 mg mL^{-1}) for 20–30 min. PCR products can be photographed. A PCR product of 733 bp indicates the presence of an *X. fastidiosa* DNA fragment in the sample.



Both isolation and screening tests have to be performed.
The decision table indicates how to proceed according to the results obtained.
* for Citrus, positive results of presumptive tests may also be available (dot immunoblotting assay and membrane entrapment immunofloresence). These are sufficient to enter the decision table, but not as repeated screening tests.*
* if repeated tests are negative, *X. fastidiosa* is not detected. If they are positive, it is advisable to treat the result as 'highly suspected' and proceed to pathogenicity tests.

Fig. 7 Decision scheme for the detection and identification of *Xylella fastidiosa* in plant samples. It can be adopted either for visibly infected or for apparently healthy specimens.

Citrus

The technique for obtaining plant extract used for the DAS-ELISA test can also be followed for PCR. Plant extract from twigs is obtained by vacuum extraction as described for grapevine.

Primers CVC-1 and 272-2-int, which generate a PCR product of 500 base pairs, are used for the detection of *X. fastidiosa* in this case. DNA fragments are used (Pooler & Hartung, 1995):

CVC-1: 5′-AGATGAAAACAATCATGCAAA-3′

272-1-int: 5′-GCCGCTTCGGAGAGCATTCCT-3′.

Primers 272-1-int and 272-2-int that generate a PCR product of 600 bp may also be used for citrus as well as for grapevine.

Individual PCR samples (25 µL) containing: PCR buffer (20 mM NaCl, 50 mM Tris pH 9.0 1% Triton X-100, 0.1% gelatin, 3 mM $MgCl_2$), 200 µL dNTP, 0.4 µM primer, 1.0 unit of *Taq* DNA polymerase, 1–5 µL of plant sample extract or 5×10^3 CFU mL^{-1} of *X. fastidiosa* (CVC strain) cells as positive control. PCR is carried out with an initial lysis of bacteria at 94 °C for 4 min, followed by an amplification profile of 94 °C for 1 min, primer annealing at 62 °C for 1 min, a DNA extension at 72 °C for 1 min for 30 cycles, followed by a final extension at 72 °C for 10 min. PCR products are analysed by agarose gel electrophoresis and visualized with ethidium bromide.

Membrane entrapment immunofluorescence of plant extracts (MEIF)

This technique, developed for *Xanthomonas axonopodis* pv. *citri* (Brlansky *et al*., 1990), proved useful also for the preliminary detection of *X. fastidiosa* in citrus (Hartung *et al*., 1994). It is not conclusive for the completion of the diagnostic process. The plant extract is prepared as described for the ELISA test. Subsequently, it is centrifuged for 5 min at 1.000 *g* and the supernatant is transferred to a sterile syringe and passed to a multiple holder adapter containing a 5.0-µm membrane for trapping plant cellular debris plus a 0.2-µm polycarbonate membrane for trapping bacterial cells. After having passed the extract, the 0.2-µm membrane is removed and incubated for 1 h in *X. fastidiosa*-specific immunoglobulin in Tris-Bovine Serum Albumin-Gelatin Buffer (20 mM Tris, 0.9% NaCl, pH 8.2 plus 0.1% Bovine Serum Albumin and 1% gelatin). The membrane is then washed with PBS containing 2% polyvinylptlpyrrolidone (PVP-40) and incubated for 1 h in tetramethylrhodamine isothiocyanate (TRITC)-labeled goat antirabbit IgG. After a brief washing with PBS, the membrane is mounted in a epifluorescence microscope for observation. Cells of *X. fastidiosa* appear as fluorescent rods.

Dot immunoblotting assay (DIBA)

This technique (Lee *et al*., 1992) is useful for the preliminary detection of *X. fastidiosa* in citrus. It is not conclusive for the completion of the diagnostic process. The plant extract is prepared as described for the ELISA test. Subsequently, it is centrifuged for 5 min at 1.000 *g*. An aliquot of 2.0 µL of the supernatant is spotted onto a nitrocellulose membrane and allowed to dry. It is then blocked for 1 h in buffer (10.0 mM Tris, 0.15 M NaCl, 0.1% Tween 20, pH 8.0) (PBS-Tween) containing 1% Bovin Serum Albumin and 2.0% Triton X-100. After rinsing with PBS-Tween, the membrane is incubated overnight in IgG (1 µg/mL) specific for *X. fastidiosa* in conjugate buffer (PBS added with 2% PVP-40 and 0.5% Bovin Serum Albumin). The membrane is rinsed with PBS-Tween and, then, incubated for 2–4 h in goat antirabbit IgG with alkaline phosphatase in the conjugate buffer. The membrane is rinsed with PBS-Tween, then substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) BCIP-NBT are added. A purple-blue coloration of the membrane indicates the presence of the pathogen in the sample.

Reference material

Citrus strains are not maintained in ATCC. Individual researchers have their own strains. Pierce's disease ATCC 35877, 35879. Plum leaf scald ATCC 35871, 33490. Almond leaf scorch ATCC 35870. Mulberry leaf scorch ATCC 35868. Phony peach ATCC 33489. Elm leaf scorch ATCC 35873.

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 7, should have been followed. Disease symptoms, and morphological and pathogenic characteristics of the pathogen, should be in accordance with the descriptions. The detection techniques should yield a clear-cut positive result. A reference strain of the pathogen should be included as a positive control.

Report on the diagnosis

A report on the execution of the protocol should include:

- results obtained by the recommended procedures
- information and documentation on the origin of the infected material
- a description of the disease symptoms (with photographs if possible)
- a description of the morphological, biochemical and pathogenic characteristics of the bacterium
- a description of the isolation and detection techniques
- an indication of the magnitude of the infection
- comments as appropriate on the certainty or uncertainty of the identification.

Further information

Further information on this organism can be obtained from: M. Scortichini, Istituto Sperimentale per la Frutticoltura, 52 Via di Fioranello, I-00040 Ciampino aeroporto (Roma), Italy.

Acknowledgements

This protocol was originally drafted by: M. Scortichini, Istituto Sperimentale per la Frutticoltura, Rome (IT).

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Appendix I – Bacterial culture media

The following media are currently used for the primary isolation of *X. fastidiosa* from plant tissues. Ingredients should be dissolved in the order given. More than one single medium should be used for the primary isolation.

PD2 medium

This medium can be used for the isolation of *X. fastidiosa* from several host plants including grapevine (Davis *et al*., 1980): deionized distilled water 1.0 L; soy peptone 2.0 g; Bacto tryptone 4.0 g; disodium succinate 1.0 g; trisodium citrate 1.0 g; K_2HPO_4 1.5 g; KH_2PO_4 1.0 g; hemin chloride stock solution (0.1% in 0.05 N NaOH) 10.0 mL; Bacto agar 15.0 g; $MgSO_4{\cdot}7H_2O$ 1.0 g; bovine serum albumin fraction V (20% w/v) 10.0 mL pH 6.9. Autoclave at 121 °C for 15 min. Bovine serum albumin is filter-sterilized and added to the rest of the medium at 50 °C.

PD3 medium

This medium (Davis *et al*., 1980) is the same as PD2 but bovine serum albumin is replaced with soluble potato starch (2 g/L). All the ingredients can be directly sterilized in the autoclave.

CS20 medium

This medium (Chang & Walker, 1988) is for the isolation of *X. fastidiosa* from various host plants including grapevine): deionized distilled water 1100 mL; soy peptone 2.0 g; bacto tryptone 2.0 g; hemin chloride stock solution (0.1% in 0.05 N NaOH), 15.0 mL; $(NH_4)_2HPO_4$ 0.8 g; KH_2PO_4 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.4 g; phenol red stock solution (0.2%) 5.0 mL; L-glutamine 6.0 g; glucose 1.0 g; L-histidine-HCl 1.0 g; soluble potato starch 2.0 g; Bacto agar 12.0 g; pH 6.6. Autoclave at 121 °C for 15 min. To prepare 0.1% hemin chloride, dissolve 1.2 g of NaOH in 600 mL of distilled water and add 0.6 g of hemin chloride and dissolve. Keep in a dark container. To prepare 0.2% phenol red, dissolve 0.6 g of phenol red in 30 drops of 20% NaOH and bring the volume up to 300 mL with distilled water. Store in the refrigerator.

B.CYE medium

Deionized distilled water 1.0 L; yeast extract 10.0 g; activated charcoal 2.0 g; L-cysteine $HCl \cdot H_2O$ 0.4 g; ferric pyrophosphate (soluble) 0.25 g; ACES (N-2-acetamido-2-aminoethane sulfonic acid) 10.0 g; bacto agar 17.0 g. The L-cysteine HCl and ferric pyrophosphate are dissolved, filter-sterilized (0.2-µm filter), and added to the autoclaved basal media, pH 6.9 (Wells *et al*., 1981).

PW medium

This medium (Davis *et al*., 1983) is for isolation of *X. fastidiosa* from various host plants including citrus: deionized distilled water 1.0 L; soytone 4.0 g; bacto tryptone 1.0 g; hemin chloride stock solution (0.1% in 0.05 N of NaOH) 10.0 mL; $MgSO_4 \cdot 7H_2O$ 0.4 g; K_2HPO_4 1.2 g; phenol red stock solution (0.2%) 10.0 mL; L-glutamine 4.0 g; bovine serum albumin fraction V (20% w/v) 30.0 mL; bacto agar 12.0 g, pH 6.6. The bovine serum albumin is filter-sterilized and added to the rest of the medium at 50 °C.

SPW medium

This medium (Hartung *et al*., 1994) is for stimulating the growth of *X. fastidiosa* isolated from sweet orange. It is the same as PW supplemented with: malt extract 5.0 g; sucrose 10.0 g; myo-inositol 0.1 g; thiamine chloride 0.01 g; pyridoxine chloride 0.01 g; nicotinic acid 0.005 g; glycine 0.002 g.

CVC1 medium

This medium (Chang *et al*., 1993) was developed for the isolation of *X. fastidiosa* from citrus: deionized water 970 mL; bacto-peptone 4.0 g; tryptone 1.0 g; K_2HPO_4 1.2 g; KH_2PO_4 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.4 g; phenol red stock solution (0.2%) 10.0 mL; agar 12.0 g. After autoclaving, the following filter-sterilized compounds are added: glutamine stock solution (8%) 50 mL; bovine serum albumin stock solution Frac V (10%) 60 mL, pH 6.5.

CVC2 medium

This (Chang *et al*., 1993) is the same as CVC1 plus 10.0 mL of hemin chloride stock solution (0.1%) to be added before autoclaving.

Fig. 1 Pierce's disease: Marginal necrosis and leaf wilting caused by *Xylella fastidiosa* on grapevine leaves.

Fig. 2 Pierce's disease: Marginal necrosis surrounded by chlorotic halo induced by *X. fastidiosa* on grapevine leaf.

Fig. 3 Pierce's disease: "Green island" symptom on grapevine twig at the end of summer, caused by *X. fastidiosa.*

Fig. 4 Citrus variegated chlorosis (CVC): Typical spots caused by *X. fastidiosa* on sweet orange leaves.

Fig. 5 CVC: Twig die-back, reduction of production and dimension of sweet orange fruits induced by *X. fastidiosa.*

Fig. 6 Phony peach: Typical "phony peach" symptom on peach leaves caused by *X. fastidiosa*.