

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 7/23



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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 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).
- 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.

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
Protocoles de diagnostic pour les organismes réglementés

Xanthomonas axonopodis* pv. *dieffenbachiae

Specific scope

This standard describes a diagnostic protocol for *Xanthomonas axonopodis* pv. *dieffenbachiae*.

Introduction

Bacterial blight of aroids caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* was reported for the first time on *Dieffenbachia maculata* in glasshouses in New Jersey (US) by McCulloch & Pirone (1939) and described by them as *Bacterium dieffenbachiae*. Since then it has been reported from a broad range of ornamental and edible species of the family *Araceae*, including *Aglaonema* (*A. commutatum*, *A. crispum*), *Anthurium* (*A. amnicola*, *A. andraeanum*, *A. crystallinum*, *A. scherzerianum*), *Caladium* (*C. bicolor*, *C. lindenii*), *Colocasia esculenta*, *Dieffenbachia maculata*, *Epipremnum aureum*, *Philodendron* (*P. bipinnatifidum*, *P. scandens*, *P. scandens* ssp. *oxycardium*), *Rhaphidophora*, *Scindapsus pictus*, *Spathiphyllum* (*S. floribundum*, *S. wallisii*), *Syngonium podophyllum* and *Xanthosoma* (*X. caracu*, *X. sagittifolium*). The major host is *Anthurium*, reported for the first time in 1971 in Hawaii (US) on Kauai island. Since then bacterial blight has been a major problem in Hawaii, and in Guadeloupe and Martinique. Within the EPPO region, the pathogen has been isolated from *Anthurium* imported into the Netherlands and later from plants in glasshouse production from this country, and from Italy and Turkey. It is present in various countries of Central and South America, in some Pacific countries, in South Africa and in USA (California, Florida).

There are at least three groups of strains affecting *Araceae*: (1) strains from *Anthurium*, which are more virulent on *Anthurium* than other strains and have a broader host range (2) certain strains from *Syngonium*, serologically closely related to *Anthurium* strains, also virulent on *Anthurium*, with a narrow host range; (3) strains from other *Araceae*, including strains from *Syngonium* other than those mentioned above, which are weakly virulent on *Anthurium* and have a narrower host range. The disease on *Anthurium* appears most damaging and is described here in more detail. On *Anthurium*, two biotypes have been described on the basis of starch hydrolysis

Specific approval and amendment

Approved in 2003-09.

on cellobiose starch agar: biotype A (no hydrolysis) and B (hydrolysis). On the basis of reactivity to 8 different monoclonal antibodies, at least 12 serotypes have been determined. Most *Anthurium* strains are found in serogroups 1–6 and react with an additional monoclonal antibody Xcd108. Strains from other hosts are found mainly in serogroups 7–12 (Lipp *et al.*, 1992; Norman & Alvarez, 1996). For certain strains on *Syngonium*, the name pv. *syngonii* has been proposed (Chase *et al.*, 1992). The description below is mainly for strains from *Anthurium*.

Identity

Name: *Xanthomonas axonopodis* pv. *dieffenbachiae* (McCulloch & Pirone) Vauterin, Hoste, Kersters & Swings

Synonyms: *Xanthomonas campestris* pv. *dieffenbachiae* (McCulloch & Pirone) Dye. *Xanthomonas dieffenbachiae* (McCulloch & Pirone) Dowson

Taxonomic position: Bacteria, Gracilicutes, *Proteobacteria* β subdivision. Strains from *Syngonium* have been described as a separate pathovar, viz. pv. *syngonii* (Chase *et al.*, 1988)

Bayer computer code: XANTDF

Phytosanitary categorization: EPPO A1 list, no. 180

Detection

Disease symptoms

On *Aglaonema* and *Anthurium*, the disease has two stages (leaf infection and systemic infection), while other hosts only show leaf infections. The foliar symptoms are found on the leaves and spathe. They start close to the leaf margin on the underside of the leaf as small star-shaped water-soaked spots, eventually with some yellowing around the spots. Infection is usually through hydathodes and/or wounds and occasionally through

stomata. Under dry conditions the small, initial spots are dry and may appear dark brown. In later stages, the pathogen usually invades the vascular tissue and leaf spots coalesce, resulting in large, V-shaped to irregular brown necrotic areas with a bright yellow border. Symptoms of systemic invasion of the pathogen start with yellowing of the older leaves and petioles. Systemically infected leaves or flowers easily break off and may show dark brown streaks at their base, which gradually enlarge. Sometimes droplets of yellow bacterial slime occur on infected petioles. When petioles are cut, yellow-brown vascular bundles are visible. Eventually the entire plant is killed. Sometimes systemic infection also produces new water-soaked leaf spots, when bacteria invade the leaf parenchyma from the infected vascular bundles. These water-soaked spots are mainly found near the main veins. Especially the dry necrotic leaf spots may be confused with symptoms of other diseases, injury or nutritional deficiencies, making a laboratory diagnosis obligatory.

Detection of latent infections and isolation

X. a. dieffenbachiae may occur in a latent form, also in tissue culture (Fukui *et al.*, 1996; Norman & Alvarez, 1994a). Two selective media, using cellobiose and starch in one (CS medium) and esculin and trehalose in the other as carbon sources, were developed by Norman & Alvarez (1989). CS medium contains per 800 mL distilled water: cellobiose, 5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2HPO_4 , 0.4 g; KH_2PO_4 , 0.8 g; agar, 15 g. Potato starch (10 g) is added separately to 200 mL of distilled water and brought to boil and added to the heated 800 mL, stirred on a hotplate; later 1.5 mL of 1% aqueous methyl green is added. The medium is autoclaved and filter-sterilized solutions of the following antibiotics added: cycloheximide, 150 mg; cephalixin, 50 mg; trimethoprim (prepared in methanol) 30 mg; pyridoxin HCl, 1 mg; D-methionine 3 mg. Tetrazolium chloride is added at a final concentration of 0.001% (10 mg). On CS medium, a clear zone is formed around colonies hydrolysing starch. The non-hydrolysing strains form raised mucoid colonies on this medium. Modified ET medium (Norman & Alvarez, 1989, 1994b) contains, per L distilled water: esculin, 1 g; trehalose, 0.5 g; $FeCl_3 \cdot 6H_2O$; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4 , 1 g; agar, 15 g. The pH is adjusted to 6.8 and the medium autoclaved immediately. After autoclaving, filter-sterilized antibiotics and cycloheximide (200 mgL⁻¹), and other components as described for CT medium above, are added. Final pH is 6.5. On ET medium, a dark diffusible pigment is formed around colonies hydrolysing esculin. A miniplate system has been developed, where large numbers of samples can be handled. Tissue exudates are incubated in 150 µL of ET medium in a 96-well microtitre plate. Identity is confirmed by ELISA using specific monoclonal or polyclonal antibodies (Hseu & Lin, 2000; Norman & Alvarez, 1994b; Norman *et al.*, 1992). Classical immuno-fluorescence test (EU, 1997) using poly or monoclonal antisera can also be performed. Isolation from symptomatic material can easily be performed using YPGA non-selective medium.

Identification

Description and biochemical tests

Aerobic Gram-negative rods with one polar flagellum; yellow xanthomonadin pigment produced; starch hydrolysed (biotype B) or not (biotype A); positive for aerobic metabolism of glucose, gelatin, catalase, casein and tributyrin hydrolysis, H_2S production, hypersensitivity reaction on tobacco; acid produced from arabinose, cellobiose, fructose, D(+)-galactose, glycerol, lactose, maltose, D-mannose, melibiose, raffinose and D-ribose, sucrose, trehalose, xylose, glutamate, propionate, succinate; no acid produced from dulcitol, erythritol, inositol, inulin, mannitol, rhamnose, salicin and sorbitol; negative for anaerobic metabolism of glucose, nitrate reduction, oxidase reaction, urease, indol production and fluorescent pigment production (Lelliott & Stead, 1987). Pectolytic activity is variable: strains from *Colocasia*, *Dieffenbachia* and *Philodendron* are highly pectolytic, strains from *Xanthosoma* and many from *Syngonium* are non-pectolytic. *Syngonium* strains defined as pv. *syngonii* can be differentiated to a certain extent on the basis of host range, symptomatology, pigmentation and sensitivity to pH (Chase *et al.*, 1992). Hseu & Lin (1998) found two biochemical variants of the bacterium studying 75 strains with the Biolog GN Microplate Identification System in Taiwan. Group A utilized L-fucose, α -lactose, β -methyl D-glucoside, turanose, D-glucuronic acid, β -hydroxy butyric acid, γ -hydroxy butyric acid, α -keto valeric acid, quinic acid, L-asparagine, hydroxy-L-proline, L-Leucine, glycerol, D, L- α -glycerol phosphate and glucose-6-phosphate, whereas group B did not. Malonic acid and glucose-1-phosphate were not utilized by group A, whereas group B utilized these compounds.

A quick presumptive identification can be made by the combination of four (simple) tests: characteristic orange-reddish raised translucent colonies on TZC medium (with 0.001 instead of 0.005% TZC), growth on CS, brown halos on ET medium and a positive reaction with monoclonal antibody Xcd 108. This monoclonal antibody is now commercially available from Agdia company.

Pathogenicity test

Pathogenicity can be determined by inoculating a suspension (10⁶ cfu mL⁻¹) of a 48-h nutrient agar/YPGA culture into stems of young *Anthurium* plants. Another method of inoculation is by atomizing the bacterial suspension onto leaf surfaces and maintaining them at 100% relative humidity for 12–16 h (e.g. by putting inoculated plants in plastic bags). Incubation should be for up to 4 weeks at 28 °C under high (relative) humidity conditions (60–80%). Symptoms are water-soaked lesions near veins in the leaf blade, necrotic spots surrounded by a yellow halo, and plant collapse in later stages. Sometimes drops of yellow bacterial ooze are observed on infected tissue. The bacterium should be reisolated from plants by taking a stem or petiole section above the inoculation point or from the margin of symptomatic tissue and placing it in a small volume of sterile distilled water or 50 mM phosphate buffer, for plating

on YPGA and/or CS medium, followed by observation for typical colonies. Colonies can be used for confirmation by IF, ELISA.

A quick, diagnostic pathogenicity test for *Anthurium* is to inject healthy *Anthurium* leaves with thick suspensions (10^7 cfu mL⁻¹) in small (1 cm diameter) areas. Within 4–10 days, a virulent strain gives a definite water-soaked spot around the injected area.

Possible confusion with similar species

Symptoms caused by *X. a. dieffenbachiae* may be confused with those caused by *Pseudomonas* spp. These are small, angular, greasy spots on the lower leaf surface near veins and leaf margins, and on spathes. These lesions may develop into large, black necrotic spots, distorting the leaf. Necrotic spots are surrounded by water-soaked margins and bright chlorotic halos, or by violet halos on the spathe. Infection may progress into veins causing soft rot. Systemically infected plants may show yellowing of the entire lamina and black necrotic lesions progressing from leaf petioles into major veins. Plants may eventually die. No V-shaped water-soaked spots are formed, nor is there a large yellow halo surrounding necrotic spots, as in the case of *X. a. dieffenbachiae*. Symptoms of systemic infection may easily be confused with those caused by *Ralstonia solanacearum* biovar 1, race 1 (Norman & Yuen, 1999). Upon isolation, fluid beige colonies instead of the yellow colonies will be obtained. Symptoms of *X. a. dieffenbachiae* are also easily confused with those of nutritional stress or injury.

Reference material

Reference culture NCPPB 1833 (= PD 992).

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 1, should have been followed. When the organism is diagnosed for the first time, or in critical (import/export) cases, the following is required:

- disease symptoms, morphological and biochemical characteristics of the pathogen and its pathogenic properties should be in accordance with the descriptions in the protocol
- for isolation of the bacterium and descriptions of morphological, biochemical and pathogenic characteristics, the procedures and requirements of the protocol should have been followed
- in the case of latent infections, after an initial screening test, the pathogen should be isolated through semiselective plating and correctly identified, including a hypersensitivity test on tobacco and/or a pathogenicity test with the pure culture on anthurium or other host
- the original sample (with labels, if applicable), sample extract, IF-slide and pure culture should be kept under proper conditions

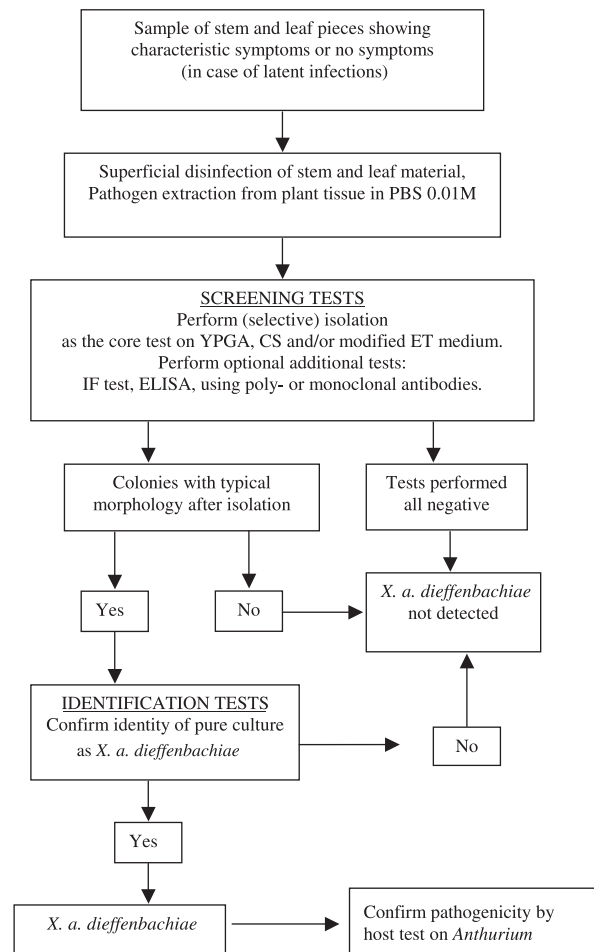


Fig. 1 Decision scheme for the detection and identification of *Xanthomonas axonopodis* pv. *dieffenbachiae* in samples of symptomatic or asymptomatic *Anthurium* or other host plants.

with correct registration and should be available in case of uncertainty

- 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.

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
- 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: Department Bacteriology, Plant Protection Service, PO Box 9102, 6700 HC, Wageningen, the Netherlands Fax 0031 317421701; E-mail: j.d.janse@pd.agro.nl; CSL Diagnostics, Central Science Laboratory, Sand Hutton, York, UK

Acknowledgements

This protocol was originally drafted by: J.D. Janse, Plant Protection Service, Wageningen (NL).

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