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EPPO STANDARD ON DIAGNOSTICS

PM 7/23 (3) *Xanthomonas phaseoli* pv. *dieffenbachiae*

Specific scope: This Standard describes a diagnostic protocol for *Xanthomonas phaseoli* pv. *dieffenbachiae*.[1] This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: First approved in 2003–09. Revised in 2009–09 and in 2023–08.

1 | INTRODUCTION

Anthurium bacterial blight caused by *Xanthomonas phaseoli* pv. *dieffenbachiae* (formerly *Xanthomonas axonopodis* pv. *dieffenbachiae*) is a major disease of anthurium and was reported for the first time in the United States in 1939 (McCulloch & Pirone, 1939) on *Dieffenbachia*, and then in 1952 in Brazil (Robbs, 1955) on *Anthurium* and in 1971 in Hawaii (US) also on *Anthurium* (Hayward, 1972).

Outbreaks or incursions of the pathogen in association with *Anthurium* were recurrently recorded in Europe since the 1990s, e.g. in the Netherlands (Sathyanarayana et al., 1998), Italy (Zoina et al., 2000), Turkey (Aysan & Sahin, 2003), Germany (Moltmann, 2005), Romania (Vlad et al., 2004) and Poland (Puławska et al., 2008). It is present in various countries of Central, South and North America, in Oceania, South Africa and Asia (Jouen et al., 2007; Robene-Soustrade et al., 2006). For an updated geographical distribution consult EPPO Global Database.

Anthurium is the major host of *Xanthomonas phaseoli* pv. *dieffenbachiae*. However, some other aroid species (e.g. in the genera *Aglaonema*, *Alocasia*, *Dieffenbachia*, *Philodendron* or *Syngonium*) (Chase et al., 1992; Constantin et al., 2017; Dickey & Zumoff, 1987) can also be infected by this pathogen. The control of *Anthurium* bacterial blight depends on sanitation and prophylactic measures and requires reliable and sensitive diagnostic tools for surveillance and certification programs.

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

2 | IDENTITY

Name: *Xanthomonas phaseoli* pv. *dieffenbachiae* (McCulloch & Pirone, 1939) Constantin et al., 2016.
Synonyms: *Xanthomonas dieffenbachiae* (McCulloch & Pirone, 1939) Dowson, 1943; *Xanthomonas campestris* pv. *dieffenbachiae* (McCulloch & Pirone, 1939) Dye et al., 1980; *Xanthomonas axonopodis* pv. *dieffenbachiae* (McCulloch & Pirone, 1939) Vauterin et al., 1995.
Taxonomic position: Kingdom: Bacteria, phylum: *Proteobacteria*; class: *Gammaproteobacteria*; order: *Lysobacterales* (previously known as *Xanthomonadales*); family: *Lysobacteraceae* (previously known as *Xanthomonadaceae*).

In 2016, strains belonging to the heterogeneous group named as *Xanthomonas axonopodis* pv. *dieffenbachiae* were reclassified into the species *Xanthomonas phaseoli*, *Xanthomonas citri* and *Xanthomonas euvesicatoria* based on different features, including multilocus sequence analysis, average nucleotide identity and homology in DNA–DNA hybridization analyses (Constantin et al., 2016). Strains responsible for *Anthurium* bacterial blight were reclassified as *Xanthomonas phaseoli* pv. *dieffenbachiae*. Strains isolated from *Syngonium* and causing severe leaf symptoms and systemic infection only on this host were reclassified as *X. phaseoli* pv. *syngonii*. Strains isolated from diverse aroid genera were reclassified in *X. euvesicatoria* (referred to as strains from *Philodendron*) and *X. citri* (as *X. citri* pv. *aracearum*) (Constantin et al., 2016). *X. citri* pv. *aracearum* strains are primarily pathogenic to their host of origin and slightly or not at all pathogenic to anthurium (Chase et al., 1992; Constantin et al., 2017; Lipp et al., 1992; Robene-Soustrade et al., 2006). The *X. euvesicatoria* strains isolated from *Philodendron* were considered as being non-pathogenic on araceae (hence were not assigned a pathovar extension).
EPPO Code: XANTPD.
Phytosanitary categorization: EPPO A2 list, no. 417.

[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.

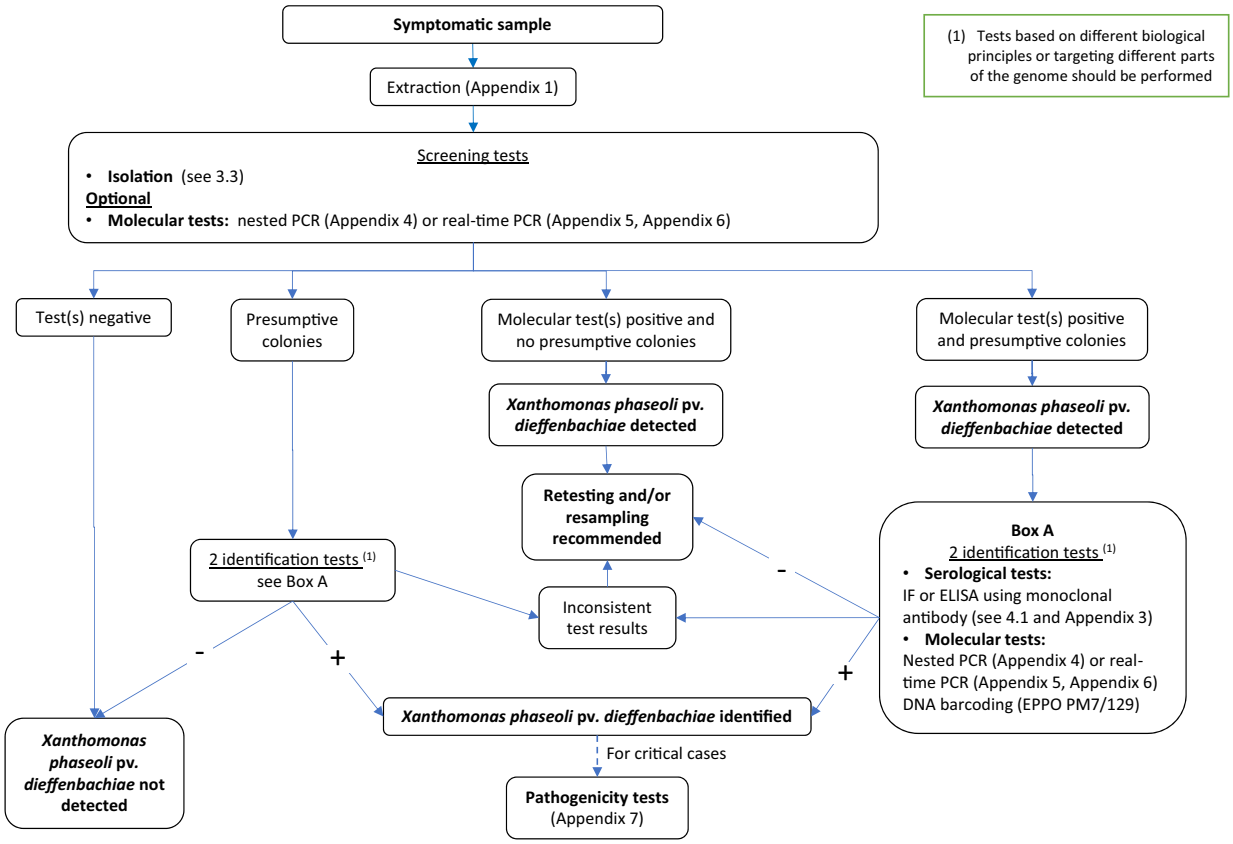

FIGURE 1 Flow diagram for the detection and identification of *Xanthomonas phaseoli* pv. *dieffenbachiae* in samples of symptomatic *Anthurium* or other host plants. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

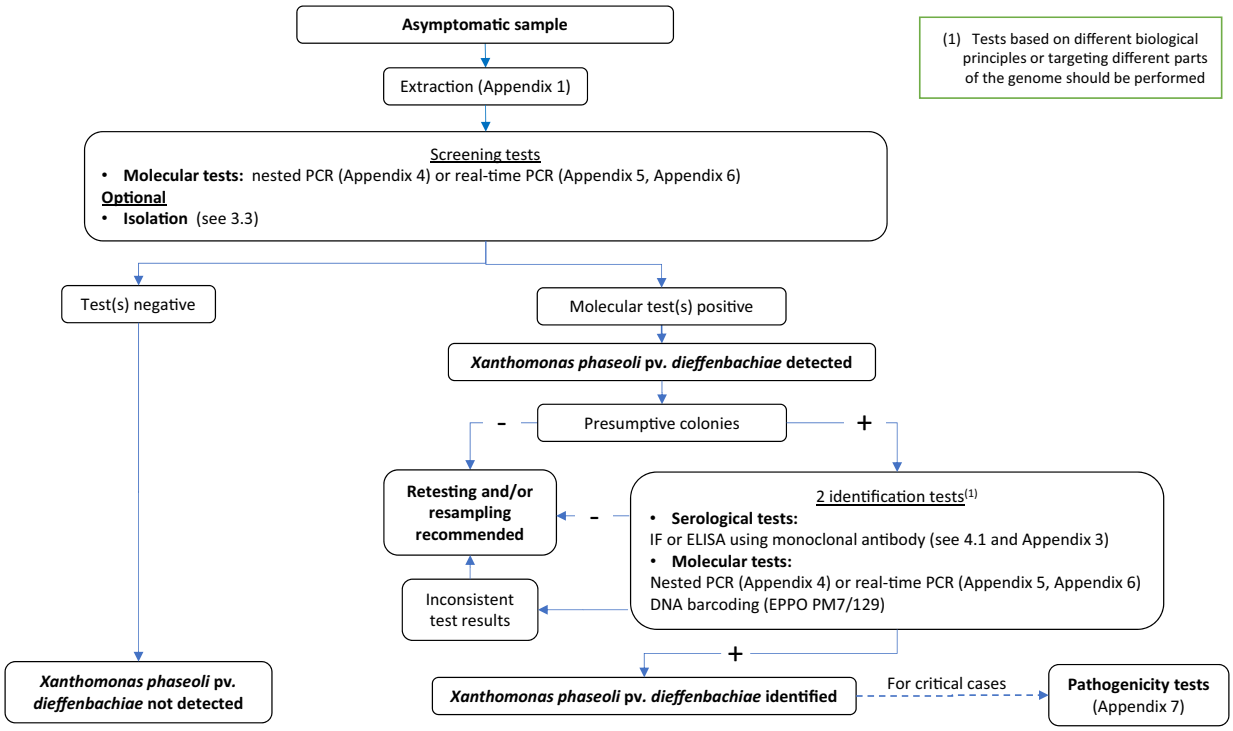

FIGURE 2 Flow diagram for the detection and identification of *Xanthomonas phaseoli* pv. *dieffenbachiae* in samples of asymptomatic *Anthurium* or other host plants. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

3 | DETECTION

3.1 | Disease symptoms

On *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 (Figure 3). Symptoms of systemic invasion by 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. Sometimes droplets of yellow bacterial slime occur on infected petioles. When petioles are cut, yellow-brown vascular bundles are visible. Eventually the entire plant can be 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. Symptoms of *X. phaseoli* pv. *dieffenbachiae* (especially the dry necrotic leaf spots) may also be confused with those of nutritional stress or injury. *X. phaseoli* pv. *dieffenbachiae* may occur in a latent form, and this can also occur in tissue culture (Fukui et al., 1996; Norman & Alvarez, 1994a).

3.1.1 | Confusion with other species

Symptoms caused by *X. phaseoli* pv. *dieffenbachiae* may be confused with those caused by *Acidovorax anthurii* (Gardan et al., 2000) (ex *Pseudomonas* sp., reference strain: CFBP3232, ICMP13404). These symptoms consist of 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. Infected plants may show yellowing of the entire leaf and black necrotic lesions progressing from leaf petioles into major veins and plants may eventually die. Nevertheless, no V-shaped water-soaked spots are formed, nor is there a large yellow halo surrounding necrotic spots, as in the case of *X. phaseoli* pv. *dieffenbachiae*. Symptoms of systemic infection may also easily be confused with those caused by *Ralstonia solanacearum* (Norman & Yuen, 1999).

3.2 | Test sample requirement and sample preparation

3.2.1 | Extraction

Extraction procedures for different plant material are presented in Appendix 1.

Extraction buffers may differ according to the test to be conducted subsequently. When different tests are to be used on a single extract, extraction should be done with sterile laboratory grade water or phosphate

FIGURE 3 Symptoms of *Xanthomonas phaseoli* pv. *dieffenbachiae* on *Anthurium* spp. Photo courtesy: CIRAD (FR).

buffer (PBS 0.01 M) and aliquots may need to be supplemented with a buffer according to the test to be done.

3.3 | Isolation

Isolation from infected tissue (stem or leaf) can easily be performed by plating 50 µL of plant extract onto a non-selective rich medium, such as YPGA or Wilbrink's medium.

The colony morphology of bacteria is observed after 2–3 days of incubation at 28°C. *X. phaseoli* pv. *dieffenbachiae* colonies on agar plates are circular, convex, mucoid and yellow (Figure 4). Comparison with a reference strain (positive control) on the same medium is recommended. On Wilbrink's, colonies appear bright, creamy yellow, circular, mucoid, smooth, slightly raised and glistening.

Additional plating on semi-selective media in parallel is advised, in order to facilitate diagnosis.

Different semi-selective media have been developed: NCTM4 medium (Laurent et al., 2009), CS medium (using cellobiose and starch as carbon sources) and modified ET medium (using esculin and trehalose as carbon sources) (Norman & Alvarez, 1989). Media are described in Appendix 2.

On NCTM4, colonies of *X. phaseoli* pv. *dieffenbachiae* appear at about 72 h and are circular, convex, mucoid, and yellow (Figure 5a).

On CS medium, a clear zone is formed around colonies hydrolysing starch. The non-hydrolysing strains form raised mucoid colonies on this medium (Figure 5b).

On ET medium, a dark diffusible pigment is formed around colonies hydrolysing esculin (Figure 5c).

As described previously, symptoms of systemic infection may easily be confused with those caused by *R. solanacearum*, but upon isolation of this bacterium it will produce fluid beige colonies on any general sucrose medium instead of the yellow colonies (Norman & Yuen, 1999).

Isolation from asymptomatic material is an optional detection test but is needed for identification purpose (see Section 4).

3.4 | Other screening tests

Immunofluorescence (IF), DAS-ELISA, nested-PCR and real-time PCRs can be used as screening tests on symptomatic material and nested-PCR and real-time PCRs can be used as screening tests on asymptomatic material. For symptomatic material, molecular and

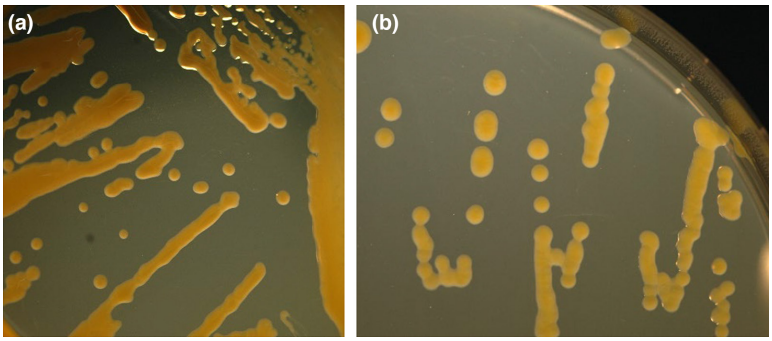

FIGURE 4 *Xanthomonas phaseoli* pv. pv. *dieffenbachiae* colonies on YPGA medium after 72 h at 28°C (a) LMG12734 (b) LB96. Photo courtesy: ANSES (FR).

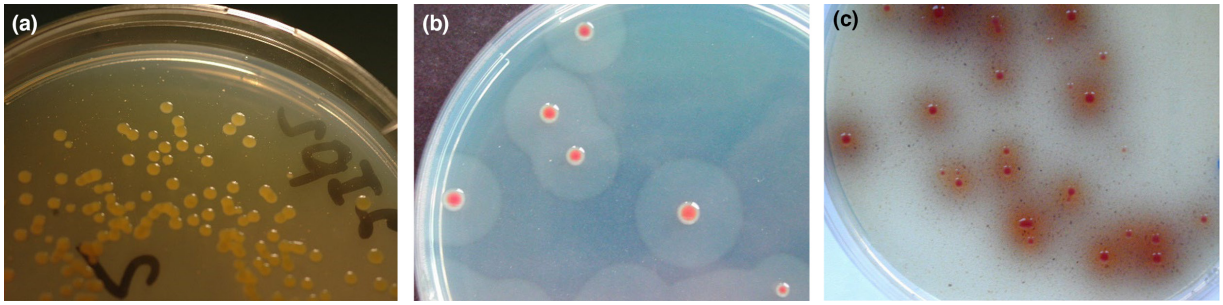

FIGURE 5 *Xanthomonas phaseoli* pv. *dieffenbachiae* on NCTM4 medium (a), CS medium (b) and ET medium (c). Photo courtesy: (a) ANSES (FR) and (b, c) Mr Van Vaerenbergh (ILVO, BE).

serological screening tests are optional, and may be used to support the results obtained using isolation.

For asymptomatic material, at least one molecular screening test should be used.

3.4.1 | Serological tests

Serological tests should only be used for symptomatic material.

Instructions for performing an IF test are provided in EPPO Standard PM 7/97 *Indirect immunofluorescence test for plant pathogenic bacteria* (EPPO, 2009) and those for performing ELISA are provided in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010). Performance characteristics are reported in Appendix 3.

Cross reactions with saprophytes leading to false positive responses can occur when performing serological tests, due to defaults in specificity of antibodies. Indirect–ELISA with monoclonal antibodies is advised for identification of pure cultures but not for plant extracts, due to low sensitivity for testing plant material. Note that excessive bruising of the material can lead to reduced staining in IF.

3.4.2 | Molecular tests

One nested-PCR test (Robene-Soustrade et al., 2006) and two real-time PCR tests (Jouen et al., 2019; van der Wolf et al., 2022) are recommended in this protocol:

- The nested-PCR test (Appendix 4) was validated in a test performance study (Chabirand et al., 2014). However, nested PCR protocols give an increased risk for false-positives due to contamination with amplicons produced in the first round of amplification.
- The two real-time PCR tests (Appendices 5 and 6) were only validated in an intralaboratory study but showed a better analytical sensitivity and specificity than nested -PCR.

A LAMP test suitable for on-site detection was developed by Niu et al. (2015). It was noted that its analytical sensitivity is lower than that of the nested-PCR and real-time PCR and therefore it should only be used for symptomatic material. There is no experience with this test within the EPPO region, so it is not recommended in this protocol.

4 | IDENTIFICATION

Identification of *X. phaseoli* pv. *dieffenbachiae* should be performed using at least two tests based on different biological principles or targeting different parts of the genome of the pathogen. Different tests are described below.

4.1 | Serological tests

4.1.1 | Immunofluorescence

For identification, 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) in phosphate buffer (PBS 0.01 M) as sample. The test is positive for a suspect culture if the size and form of the stained cells of the culture is equivalent to that of the positive control strain.

4.1.2 | ELISA

For identification, indirect-ELISA using specific monoclonal antibody Xad 47600 (AGDIA) should be performed as described in EPPO Standard PM 7/101 *ELISA tests for plant pathogenic bacteria* (EPPO, 2010) using a pure culture (approximately 10^7 cfu/mL) as sample.

DAS-ELISA with polyclonal antibodies should not be used for identification, due to possible non-specific reactions. Performance characteristics are reported in Appendix 3.

4.2 | Molecular tests

4.2.1 | PCR

Suspect cultures can be identified using the nested PCR and the real time PCR tests described in Appendices 4–6.

4.2.2 | Barcoding

Procedures are described in the EPPO Standard PM 7/129 on *DNA barcoding as an identification tool for plant pests* (EPPO, 2021). Multilocus sequence analysis based on partial 16S rRNA and gyrB gene sequences can be used to support the identification of *X. phaseoli* pv. *dieffenbachiae*. Sequences are available in EPPO-Q-bank https://qbank.eppo.int/bacteria/.

4.3 | Other tests

4.3.1 | Catabolic biochemical tests and other phenotypic properties

The *Xanthomonas* species *X. phaseoli* and *X. citri* can be differentiated based on phenotypic biochemical tests which are not detailed in this diagnostic protocol.

Xanthomonas phaseoli pv. *dieffenbachiae* strains are aerobic rods with one polar flagellum; producing yellow xanthomonadin pigment. Catabolic biochemical

characteristics that should be verified can be found in Chase et al. (1992) and Lelliott and Stead (1987).

4.3.2 | Pathogenicity tests

This test is used as a confirmation in the diagnosis of *X. phaseoli* pv. *dieffenbachiae* in critical cases.

Methods for inoculations are presented in Appendix 7.

5 | REFERENCE MATERIAL

Reference culture NCPPB 1833 (= PD 992 = LMG 695).

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:

- CIRAD, UMR PVBMT, Pôle de Protection des Plantes, Station de Ligne Paradis, 7 chemin de l'IRAT, 97410 St Pierre (FR, Reunion Island) isabelle.robene@cirad.fr.
- Fera Science Ltd., Sand Hutton, York (GB), andrew.aspin@fera.co.uk.
- French Agency for food, environmental and occupational health & safety (ANSES), Plant Health Laboratory, Unit for Tropical Pests and Diseases, Pôle de Protection des Plantes, 7 chemin de l'IRAT, 97410 St Pierre (FR, Reunion Island), aude.chabirand@anses.fr.
- Naktuinbouw, Sotaweg 22, 2371 GD Roelofarendsveen, P.O. Box 40, 2370 AA Roelofarendsvee (NL), e.meekes@naktuinbouw.nl.

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.

ACKNOWLEDGEMENTS

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APPENDIX 1 - EXTRACTION PROCEDURES

1. Extraction from symptomatic leaves or stems

Infected leaves or stems are quickly surface disinfected using 70% ethanol. Pieces of leaf or stem tissues taken at the margin of the spots or necrosis, or from systemic infected vascular tissue are removed with a disinfected scalpel blade and transferred to a small volume (e.g. 1–2 mL) of sterile distilled water or sterile phosphate buffered saline (PBS 0.01 M) (see Appendix 2). Tissue parts are cut aseptically into small pieces and then left 10–15 min for diffusion of bacteria.

Extraction can also be performed using a homogenizer grinder (e.g. Homex grinder from Bioreba) and extraction bags. This is particularly appropriate for rapid screening tests (DAS-ELISA, nested-PCR or real-time PCR).

Leaf and stem extracts should be analysed immediately and the remaining extracts should be kept refrigerated in sterile adequately labelled single use tubes for further use if necessary. For medium and long-term storage (more than 24 h), sterile glycerol is added to the remaining extract (final concentration 20–30% v/v) and it is kept at a temperature below −18°C.

2. Extraction from asymptomatic leaves or stems

Biological enrichment of the bacterium may be performed using a miniplate system, where large numbers of samples can be handled (Norman & Alvarez, 1994b). 150 µL of ET medium (see Appendix 2) is dispensed into each well of a microtitre plate. Leaf tissue samples are processed as described in Section 1 taking approximately 1 cm^2, and are soaked for 2–3 h in 1 mL of PBS (0.01 M) (see Appendix 2). Then 10 µL from each plant sample (or saline control) is added to individual wells. Miniplates are incubated at 29°C for 4 days and rapid screening tests (DAS-ELISA, IF, nested PCR or real-time PCR) are performed.

Extracts prepared in PBS 0.01 M are used immediately and if necessary, the remaining extracts are kept refrigerated in sterile disposable tubes for further use. For medium and long-term storage (more than 24 h), sterile glycerol (20–30% glycerol v/v) is added and then kept at a temperature below −18°C.

APPENDIX 2 - PREPARATION OF 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
Bactopeptone	7 g
Glucose	7 g
Microbiological grade agar	18 g
Distilled water	1.0 L
Adjust pH to 7.2 before autoclaving	

Cellobiose starch medium (CS)	
Cellobiose	5.0 g
$MgSO_4.7H_2O$	0.1 g
K_2HPO_4	0.4 g
KH_2PO_4	0.8 g
Microbiological grade agar	15.0 g
Distilled water	800 mL

Potato starch (10.0 g) should be added separately to 200 mL of demineralised water, brought to boil and added to the heated 800 mL, stirred on a hotplate, later 1.5 mL aqueous methyl green (1% w/v) is added.

The medium is autoclaved, and filter-sterilized solutions of the following antibiotics and other components are added:

Cyclohemixide	150.0 mg
Cephalexin	50.0 mg
Trimethoprim	30.0 mg
Pyridoxin HCl	1.0 mg
D-methionine	3.0 mg
Tetrazolium chloride	10.0 mg
Adjust pH to 6.8	

Modified esculin trehalose medium (ET) (Norman & Alvarez, 1989, 1994b)	
Esculin	1.0 g
Trehalose	0.5 g
$FeCI_3.6H_2O$	0.5 g
NaCl	5.0 g
$MgSO_4.7H_2O$	0.2 g
KH_2PO_4	1.0 g
Microbiological grade agar	15.0 g
Demineralised water	1.0 L
Adjust pH to 6.8 and autoclave immediately	

After autoclaving, filter-sterilized solutions of the following antibiotics and other components are added:

Cyclohemixide	200.0 mg
Cephalexin	50.0 mg
Trimethoprim	30.0 mg
Pyridoxin HCl	1.0 mg
D-methionine	3.0 mg
Tetrazolium chloride	10.0 mg
Final pH is 6.5	

NCTM4 medium (Laurent et al., 2009)	
Yeast extract	7.0 g
Bactopeptone	7.0 g
Glucose	7.0 g
Microbiological grade agar	18.0 g
Distilled water	1.0 L

The medium is autoclaved, and filter-sterilized solutions of the following antibiotics and other components are added:

Pivmecillinam	100.0 mg
Cephalexin	50.0 mg
Trimethoprim	10.0 mg
Neomycin	3.0 mg
Propiconazole (20 mg/L)	80 µL
Adjust pH to to 7.2	

Wilbrink's medium (Koike, 1965)	
Peptone special	5.0 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Sucrose	10.0 g
Microbiological grade agar	18.0 g
Distilled water	1 L
Adjust pH to 7.0 before autoclaving.	

2. Buffers

PBS buffer (0.01 M)	
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 if necessary before autoclaving	

PBS buffer (0.05 M)	
NaCl	8.2 g
Na_2HPO_4	2.52 g
NaH_2PO_4	2.5 g
Distilled water	1.0 L
pH is adjusted to 7.2 if necessary before autoclaving	

APPENDIX 3 - SEROLOGICAL TESTS

For extraction of the bacteria from plant samples or colonies, use a buffer recommended by the kit supplier.

The source of antibodies is critical. Kits for DAS ELISA using polyclonal antiserum are commercially available from different suppliers and may differ regarding their analytical sensitivity, analytical specificity and background noise. A kit for Indirect-ELISA using specific monoclonal antibodies Xad 47600 (Agdia) is commercially available. In general, it is recommended to follow the protocol provided by the supplier of the antiserum.

Performance characteristics reported below published by Chabirand et al. (2014) were obtained according to PM 7/98 and include data from an intra-laboratory comparative validation study and data from a test performance study involving 15 European laboratories.

Analytical sensitivity was evaluated using six independent 10-fold dilution series in the plant matrix (healthy anthurium leaves) for each of the strains LMG 695, NCPPB 3573, LB96 and LMG12720. Bacterial suspensions free of plant extract were also tested.

Inclusivity was evaluated on a set of 50 target strains of *X.* phaseoli pv. *dieffenbachiae*. Exclusivity was evaluated on a set of 53 non-target strains, including *X. citri* pv. *aracearum* and *X. euvesicatoria* 'Philodendron' strains pathogenic to some aroid genera but not anthurium (group A), strains belonging to different *Xanthomonas* pathovars or species not associated with *Araceae* (B), saprophytic bacteria isolated from anthurium (C) and strains pathogenic to anthurium belonging to other genera (D).

	Indirect ELISA (monoclonal antibodies, Agdia-biofords)	IF (PRI polyclonal antibodies, Wageningen, the Netherlands)	DAS-ELISA (PRI polyclonal antibodies)
Analytical sensitivity (DL100%)[a]	Bacterial suspensions: 5×10^3–5×10^4 cfu/mL Spiked plant extracts: >5×10^5 cfu/mL	Bacterial suspensions: 5×10^2–5×10^3 cfu/mL Spiked plant extracts: 5×10^4–5×10^5 cfu/mL	Bacterial suspensions: 5×10^3–5×10^4 cfu/mL Spiked plant extracts: 5×10^3–5×10^4 cfu/mL
Analytical specificity: inclusivity (%) (95% confidence intervals)[a]	100 ($CI_{95\%}$: 92.8–100)	100 ($CI_{95\%}$: 92.8–100)	100 ($CI_{95\%}$: 92.8–100)
Analytical specificity: Exclusivity (%) (95% confidence intervals)[a]	90.6 ($CI_{95\%}$: 79.7–95.9)	88.7 ($CI_{95\%}$: 77.4–94.7)	47.2 ($CI_{95\%}$: 34.4–60.3)
Repeatability (accordance) (%)[b]	–	82.9–83.9	89–91
Reproducibility (concordance) (%)[b]	–	69.6–75.5	74.3–78.5

$DL_{100\%}$: the smallest number of cells (as determined from agar plate counts of the corresponding suspensions) that can be detected 100% of the time.

[a] Intralaboratory study data.

[b] Interlaboratory comparison data.

The tests 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 4 - NESTED PCR TEST (ROBENE-SOUSTRADE ET AL., 2006)

The test below differs from the one described in the original publication (see 1.2).

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 following nested PCR test is suitable to detect and/or identify *Xanthomonas phaseoli* pv. *dieffenbachiae*. For the identification of *X. phaseoli* pv. *dieffenbachiae*, the first PCR round of the nested PCR followed by a digestion step with restriction enzyme HincII should be performed. This RFLP step allows *X. phaseoli* pv. *dieffenbachiae* strains to be distinguished from closely related strains belonging to *X. phaseoli* pv. *syngonii* that are not pathogenic to anthurium. For the detection of *X. phaseoli* pv. *dieffenbachiae* from plant material, the nested PCR is required because the second PCR round greatly increases sensitivity.

1.2 The test was developed by Robene-Soustrade et al. (2006) and further adapted and evaluated in a test performance study (Chabirand et al., 2014).

1.3 The target sequence is a bacterial gene encoding putative ABC transporter-type proteins in LPS cluster.

1.4 Oligonucleotides:

	Primer	Sequence	Amplicon size (bp)
First PCR round: Forward primer	PXadU	5′-AGG GCT CCC CAT GCC GGA AT-3′	1570
First PCR round: Reverse primer	PXadL	5′-ACG CAA TGC GCA GGG GAA AT-3′	
Nested PCR: Forward primer	NXadU	5′-AGC GCG GTA CAT TGT TGT TCG T-3′	785
Nested PCR: Reverse primer	NXadL	5′-GCG GAT CCT GAC TGA GCA AAG-3′	

1.5 Enzyme: Invitrogen Taq DNA polymerase, Eurogentec RedGoldStar enzyme and Promega G2 Hot Start Polymerase were used successfully.

1.6 The 9600, 9700 and Veriti thermal cyclers systems (Applied Biosystems) were used successfully.

2. Methods

2.1 *Nucleic acid extraction and purification*

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

2.1.1 *From plant material for detection*

DNA extraction should always be performed before amplification because it improves the analytical sensitivity of the test. Grind 0.25 g of symptomatic or asymptomatic anthurium leaves in 5 mL of 10 mM Tris buffer (pH 7.2) or in 5 mL of demineralised sterile water using a homogenizer grinder (e.g. Homex grinder from Bioreba). Centrifuge 2 mL for 10 min at 20 000 *g* and discard supernatant. The obtained homogenate can be stored at less than −18°C. Follow the manufacturer protocol provided with the Qiagen® Plant kit (Qiagen®, Hilden, DE) to extract DNA, starting with adding 400 µL buffer AP1 to the homogenate. Elute twice with 50 µL buffer AE.

2.1.2 *From bacterial suspension for identification*

Suspend a single colony of a fresh pure culture in 1 mL of PCR grade water. Boil for 1 min and immediately chill on ice for 1 min, and vortex vigorously.

Note that based on validation data gathered by ANSES (FR), similar results were obtained with and without thermal lysis.

2.2 *Polymerase chain reaction*

2.2.1 *First PCR round*

2.2.1.1. *Master mix*

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		17.95	
10× PCR buffer (Eurogentec)	10×	2.50	1×
$MgCl_2$ (Eurogentec)	25 mM	1.10	1.1 mM
dNTPs (NEB)	10 mM	0.25	0.1 mM of each of the dNTPs
PXadU	10 µM	0.50	0.2 µM
PXadL	10 µM	0.50	0.2 µM
RedGoldstarTaq polymerase (Eurogentec)	5 U/µL	0.20	1 U
Subtotal		23.00	
DNA		2.00	
Total		25.00	

2.2.1.2. PCR cycling parameters: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 70°C and 120 s at 72°C, a final step for 10 min at 72°C.

2.2.2 *Nested PCR*

To limit the risk of contamination, the nested PCR round is not recommended for identification (from bacterial suspension). Nevertheless, if this nested step is performed, amplicons from the first round should be 1/100 diluted in deionized water or all necessary precautions should be taken to avoid contaminations.

2.2.2.1. *Master mix*

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		18.95	
10× PCR buffer (Eurogentec)	10×	2.50	1×
$MgCl_2$ (Eurogentec)	25 mM	1.10	1.1 mM
dNTPs (NEB)	10 mM	0.25	0.1 mM of each of the dNTPs
NXadU	10 µM	0.50	0.2 µM
NXadL	10 µM	0.50	0.2 µM
RedGoldstarTaq polymerase (Eurogentec)	5 U/µL	0.20	1 U
Subtotal		24.00	
PCR product[a]		1.00	
Total		25.00	

[a] PCR tubes containing the first reaction amplicons must be opened with extreme care to avoid creation of aerosols which would cause contamination by amplification products.

2.2.2.2. PCR cycling parameters: 3 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 70°C and 30 s at 72°C, a final step for 5 min at 72°C.

2.3 *Restriction fragment length polymorphism (RFLP) reaction*

2.3.1 *Preparation of DNA Solution*

RFLP analysis are performed on DNA amplicons obtained after the first or the second nested-PCR step (DNA concentration around 20 ng/µL).

2.3.2 *RFLP Reaction*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	7.5	N.A.
Restriction enzyme buffer (NEB)	10×	1.5	1×
Hinc II	10 U/µL	1	10 U
Subtotal		10	
(purified) PCR product		5	
Total		15	

2.3.3 Incubation temperature 37°C for 3 h
2.3.4 Denaturation temperature 94°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. infected plant material or synthetic control prepared by adding pure culture of *X. phaseoli* pv. *dieffenbachiae* reference strain to a healthy *Anthurium* leaf homogenate).
- 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection. A pure culture extracted with the thermal lysis method, of the *X. phaseoli* pv. *dieffenbachiae* reference strain can be used as positive control (LMG695).

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 from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

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, PAC (and if relevant IPC) band(s) of the expected size (for PIC and PAC, see Table A1) is (are) visualized.

When these conditions are met:

- A test will be considered positive if band(s) of the expected size (see Table A1) is (are) visualized.
- A test will be considered negative, if no band(s) or (a) band(s) of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

TABLE A1 Expected band sizes.

Intended use of the test	Detection nested PCR followed by RFLP	Identification first round PCR followed by RFLP
Band size before digestion	758	1570
Bands size after digestion	460, 325	234, 711, 625

4. Performance characteristics available

Performance characteristics reported below published by Chabirand et al. (2014) were obtained according to

PM 7/98 and include data from an intra-laboratory comparative validation study and data from a test performance study involving 15 European laboratories. Further validation was performed for the nested PCR only (without the RFLP analysis) in an intra laboratory study by Jouen 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).

4.1 *Analytical sensitivity data*

Data from Chabirand et al. (2014) intralaboratory study:

Analytical sensitivity was evaluated using six independent 10-fold dilution series in the plant matrix (healthy anthurium leaves) for each of the strains LMG 695, NCPPB 3573, LB96 and LMG12720. Bacterial suspensions free of plant extract were also tested. $DL_{100\%}$: 5×10^{2}–5×10^{3} cfu/mL on bacterial suspension and 5×10^{3}–5×10^{4} cfu/mL on spiked plant extract.

4.2 *Analytical specificity data*

Data from Chabirand et al. (2014):

Inclusivity was evaluated on a set of 50 target strains of *X. phaseoli* pv. *dieffenbachiae*. Exclusivity was evaluated on a set of 53 non-target strains, including *X. citri* pv. *aracearum* and *X. euvesicatoria* 'Philodendron' strains pathogenic to some aroid genera but not anthurium (group A), strains belonging to different *Xanthomonas* pathovars or species not associated with Araceae (B), saprophytic bacteria isolated from anthurium (C) and strains pathogenic to anthurium belonging to other genera (D).

Inclusivity value (95% confidence interval): 100% (92.8–100%).

Exclusivity value (95% confidence interval): 100% (93.2–100%).

4.3 *Data on repeatability*

Data from Chabirand et al. (2014) test performance study: accordance: 93.9% ($CI_{95\%}$: 90.4–98.7%).

4.4 *Data on reproducibility*

Data from Chabirand et al. (2014) test performance study: concordance: 93.0% ($CI_{95\%}$: 91.2–95.5%).

APPENDIX 5 - DUPLEX REAL TIME PCR TEST (JOUEN 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 This test is suitable for the detection of *Xanthomonas phaseoli* pv. *dieffenbachiae* in plant material and for identification of *Xanthomonas phaseoli* pv. *dieffenbachiae* in pure bacterial colonies.

1.2 The test was developed and validated by van der Wolf et al. (2022).

1.3 The target sequences of the test is a portion of a gene encoding for a hypothetical protein and a gene encoding for a type I restriction endonuclease subunit S. An internal control is included. *Acidovorax cattleyae* is a bacterium added in a known quantity to check extraction and PCR efficiency. The primer set was originally developed by Syngenta and modified by Naktuinbouw (see Bonants et al., 2019).

1.4 Oligonucleotides:

1.4.1 Primers targeting *Xanthomonas phaseoli* pv. *dieffenbachiae*

	Primer/ probe name	Sequence	Amplicon size (including primer sequences)
Forward primer	Xad-ABC-F	5′-AAG TCA GGC GAG GCC AGT ATC-3′	63 bp
Reverse primer	Xad-ABC-R	5′-AGG CCG GGA AGG ATC GT-3′	
Probe	P-Xad-ABC	5′-6-FAM-TCG TTG ACC AAC ATC G-MGB-3′	
Forward primer	Anth-CHS-F	5′-GAC CAG AGC ACC TAC CCA GAC T-3′	61 bp
Reverse primer	Anth-CHS-R	5′-GCT CAA CCT GGT GCT CAC TGT-3′	
Probe	P-Anth-CHS	5′-VIC-CTA CTT CCG AAT CAC C-MGB-3′	

1.5 Enzyme: included in the TaqMan Universal PCR master mix (Applied Biosystem, Thermo Fisher Scientific).
1.6 Real-time PCR system: ABI PRISM 7000 SDS (Applied Biosytem, Thermo Fisher Scientific).

2. Methods

2.1 *Nucleic acid extraction and purification*

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

2.1.1 *From plant material for detection*

Grind 0.25 g of symptomatic or asymptomatic anthurium leaves in 5 mL of 10 mM Tris buffer (pH 7.2) (e.g. Homex grinder from Bioreba). Centrifuge 2 mL for 10 min at 20 000 *g* and discard supernatant. The obtained homogenate can be stored at <−18°C. Follow the manufacturer protocol provided with the Qiagen® DNeasy Plant mini kit (Qiagen®) to extract DNA, starting with adding 400 µL buffer AP1 to the homogenate. Elute twice with 50 µL buffer AE.

2.1.2 *From bacterial suspension for identification*

Suspend a single colony of a fresh pure culture in 1 mL of PCR grade water. Boil for 1 min and immediately chill on ice for 1 min, and vortex vigorously.

2.2 *Real-time polymerase chain reaction*

2.2.1 *Master Mix*

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		0.70	
TaqMan Universal master mix (Applied Biosystems)	2×	7.50	1×
Xad-ABC-F	10 µM	1.35	0.9 µM
Xad-ABC-R	10 µM	1.35	0.9 µM
P-Xad-ABC	12.5 µM	0.15	0.125 µM
Anth-CHS-F	10 µM	0.90	0.6 µM
Anth-CHS-R	10 µM	0.90	0.6 µM
P-Anth-CHS	5 µM	0.15	0.05 µM
Subtotal		13.00	
DNA		2.00	
Total		15.00	

Note that for small numbers of samples, the working concentration of the probes may be changes to 3.75 µM (P-Xad-ABC MGB) and 1.5 µM (P-Anth-CHS MGB) respectively so the volume of probe to be used per reaction is 0.5 µL. In that case no water should be used in the reaction.

2.2.2 PCR cycling conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 65°C.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

In addition to the external positive controls (PIC and PAC), an internal positive controls (IPC) is used to monitor each individual sample separately.

3.2 *Interpretation of results*

Verification of controls

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

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.
- The test should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Performance characteristics reported below were obtained according to PM 7/98 in the framework of an intra-laboratory validation study and were published by Jouen 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).

4.1 *Analytical sensitivity data*

Analytical sensitivity was evaluated using six independent 10-fold dilution series in the plant matrix for each of the strains LMG 695, NCPPB 3573, LB96 and JW127. At a concentration of 10^3 cfu/mL, 97.2% samples (70 out of 72) were tested positive. Two samples of JW127 strain tested negative with Ct values of 36.7 and 36.3 (higher than the 36.1 cut-off value). $LOD_{95\%}$ = 894 bacteria/mL ($CI_{95\%}$: 407.0–1965.2) corresponding to 18 bacteria per reaction.

4.2 *Analytical specificity data*

Inclusivity and exclusivity were evaluated on bacterial suspensions of 50 target and 92 non-target strains, respectively. The set of non-target strains included: *X. citri* pv. *aracearum* and *X. euvesicatoria* 'Philodendron'strains pathogenic to some aroid genera but not anthurium (group A), strains belonging to different *Xanthomonas* pathovars or species not associated with *Araceae* (B), saprophytic bacteria isolated from anthurium (C) and strains pathogenic to anthurium belonging to other genera (D).

Inclusivity value for bacterial suspension adjusted to 10^4 cfu/mL: 100% ($CI_{95\%}$: 91.1–100%).

Exclusivity value for bacterial suspension adjusted to 10^7 cfu/mL: 98.9% ($CI_{95\%}$: 93.2–99.9%). Cross reaction with *X. euvesicatoria* pv. *allii* CFBP 6380 was observed. Note: 10 other strains of *X. euvesicatoria* pv. *allii* did not cross react.

4.3 *Diagnostic sensitivity and specificity*

Diagnostic sensitivity (DSE) and specificity (DSP) was evaluated in 34 symptomatic anthurium samples originating from different locations in Reunion Island and 24 healthy anthurium plants.

DSE: 100% ($CI_{95\%}$: 86.2–100%).

DSP: 97.1% ($CI_{95\%}$: 85.5–99.5%).

4.4 *Data on selectivity*

The amplification of the internal control was assessed on 11 healthy commercial *Anthurium andraeanum* cultivars: Calore, Casino, Fire, Florida, Nunzia, Pistache, Presence, Simba, Spice, Tropical and Tropical Night. Amplification was observed on all the anthurium cultivars tested.

4.5 *Data on repeatability*

Accordance value for samples described in 4.1 and bacterial concentrations ranging from about 10^3 cfu/mL to 10^7 cfu/mL: 99.6%.

APPENDIX 6 - TRIPLEX REAL TIME PCR TEST (VAN DER WOLF ET AL., 2022)

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 test is suitable for the detection of *Xanthomonas phaseoli* pv. *dieffenbachiae* in plant material and for identification of *Xanthomonas phaseoli* pv. *dieffenbachiae* in pure bacterial colonies.

1.2 The test was developed and validated by Jouen et al. (2019).

1.3 The target sequence of the test is a portion of a gene encoding a putative ABC transporter-type protein (Wzt), a component of the o-antigen lipopolysaccharide (LPS) cluster, present in all *X. phaseoli* pv. *dieffenbachiae* strains pathogenic to anthurium. An internal control is used and targets an endogenous DNA sequence present in the plant sample, the *Anthurium andraeanum* chalcone synthase gene (*CHS*), encoding an enzyme involved in the flavonoid and anthocyanin biosynthesis pathway.

1.4 Oligonucleotides:

1.4.1 Primers targeting *Xanthomonas phaseoli* pv. *dieffenbachiae*

	Primer/ probe name	Sequence	Amplicon size (including primer sequences)
Forward primer	FwXpd866	5′-TAC CTG CCT CGC CTC TT-3′	94 bp
Reverse primer	RvXpd866	5′-GGA TCG TCG GTC TTG TGT TT-3′	
Probe	pXpd866	5′-FAM- CAA CAG CGT GAG AAA GAA ACT CGG CA3′[a]	
Forward primer	fwXpd4494	5′-GTA TAG ATG TAC TGA CGG CTC AC-3′	96 bp
Reverse primer	RvXpd4494	5′-CGC GAT CAT TCC CGA TAC TT-3′	
Probe	pXpd4494	5′-ATT0532-CGC TTG ATT GCA GTT CCA CTC AGG A-3′[a]	

[a] Probes were double quenched with ZEN/Iowa Black FQ. The use of single quenched probes is also possible: FAM and ATTO 532 (VIC replacement) with BHQ1.

1.4.2 Primers targeting *Acidovorax cattleyae*

	Primer/ probe name	Sequence	Amplicon size (including primer sequences)
Forward primer	Acat 2-F	5′-TGT AGC GAT CCT TCA CAA G-3	152 bp
Reverse primer	Acat 2-R	5′-TGT CGA TAG ATG CTC ACA AT-3′	
Probe	Acat 2-Pr	5′-Texas Red-CTT GCT CTG CTT CTC TAT CAC G-3′[a]	

[a] The probe was double quenched with ZEN/Iowa Black FQ. The use of single quenched probes is also possible: Texas Red with BHQ2.

1.5 Enzyme: PerfeCTa multiplex qPCR ToughMix 5x (Quantabio, Beverly, USA).

1.6 Real-time PCR system: Biorad CFX touch Real-Time PCR detection system (BioRad, Hercules, USA).

2. Methods

2.1 *Nucleic acid extraction and purification*

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

2.1.1 *From plant material for detection*

Crush plant material (e.g. 12 g) in a Bioreba bag with a synthetic intermediate layer (Bioreba, Kanton Reinach, Swiss) using a sample crusher (e.g. AAA lab equipment B.V. Roelofarendsveen). Subsequently, add 24 mL of 0.05 M PBS. From each dilution, use 1 mL of solution per replicates. Centrifuge the tubes for 10 min at 6000 g and discard the supernatant. Add 50 µL of a 1000-fold diluted suspension of *A. cattleyae* cells with an optical density at 600 nm of 0.8 (ca. 8×10^8 cells/mL). Perform DNA extraction with the AGOWA maxi kit (Nucleics, Woollahra, Australia), or mag maxi DNA extraction kit (LGC Genomics, Berlin, Germany) according to the manufacturer's instructions.

2.1.2 *From bacterial suspension for identification*

Collect bacteria from the agar surface in approximately 0.5 mL of water and store at −20°C until DNA extraction. Extract DNA using the Wizard Magnetic DNA purification System for Food (Promega, Leiden, The Netherlands). Alternatively, boil a suspension of 10^9–10^{10} cells/mL in 50 mM NaOH prior to testing.

2.2 *Real-time polymerase chain reaction*

2.2.1 *Master Mix*

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		9.75	
PerfeCTa multiplex qPCR ToughMix (Quantabio)	5×	5.0	1×
FwXpd866	10 µM	0.75	0.3 µM
RvXpd866	10 µM	0.75	0.3 µM
pXpd866	10 µM	0.25	0.1 µM
fwXpd4494	10 µM	0.75	0.3 µM
RvXpd4494	10 µM	0.75	0.3 µM
pXpd4494	10 µM	0.25	0.1 µM
Acat 2-F	10 µM	0.75	0.3 µM
Acat 2-R	10 µM	0.75	0.3 µM
Acat 2-Pr	10 µM	0.25	0.1 µM
Subtotal		20.00	
DNA		5.00	
Total		25.00	

2.2.2 PCR cycling conditions: 2 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C.

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 isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 spiked with the target organism). The concentration of the PIC should not be too high (e.g. set to obtain a C_t of 25–27).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of 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 PCR tests not performed on bacterial colonies, the PAC should preferably be near the limit of detection.

The gBlock containing the *A. cattleyae* internal positive control (IPC) amplicon sequence as described by Bonants et al. (2019) can be used to detect the *A. cattleyae* IPC. An early spike of *A. cattleyae*, as described in EPPO PM 7/127 (EPPO, 2016), is also possible.

3.2 *Interpretation of results*

Verification of controls

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

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve for the two sets of primers/probe.
- A test will be considered negative, if it does not produce an amplification curve for the two sets of primers/probe or if it produces a curve which is not exponential.
- The test should be repeated if any contradictory or unclear results are obtained (e.g. the two sets of primers/probe gave contradictory results).

4. Performance characteristics available

Performance characteristics reported below were published by van der Wolf et al. (2022) and were produced in the framework of an intra-laboratory validation study. 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 *Analytical sensitivity data*

The analytical sensitivity of the triplex TaqMan assay was determined using gBlocks, genomic DNA of the strain LMG 695 and supplementing an *Anthurium* leaf extract with a serial dilution of bacterial cells of strain IPO1104. The test was able to detect minimally 100 copies of a target sequence delivered as a gBlock, 100 fg of genomic DNA and 10^4 cells per mL in an *Anthurium* leaf extract.

4.2 *Analytical specificity data*

The analytical specificity of the two primer sets (Xpd866 and Xpd4494) in the triplex TaqMan was determined on the basis of an in silico analysis using the blastn function in NCBI database. The closest non-target sequences for Xpd866 and Xpd4494 were found in *Xanthomonas arboricola* and *Xanthomonas dyei*, respectively, but only with a low level of homology.

Inclusivity and exclusivity were further evaluated on genomic DNA of 22 target and 47 non-target strains, respectively. The set of non-target strains included *X. phaseoli* pv. *manihotis* (x6), *X. phaseoli* pv. *phaseoli* (x2), X. *axonopodis* pv. *poinsettiicola* (x3), *X. euvesicatoria* (x6), *X. axonopodis* pv. *begoniae* (x3), *X. citri* pv. *aracearum* (x5), *X. citri* pv. *fuscans* (x1), *X. sacchari* (x4), *Stenotrophomonas maltophilia* (x1), *X. hortorum* pv. *carotae* (x1), X. *hortorum* pv. *pelargonii* (x1), *X. fragariae*

(x2), *X. vesicatoria* (x2), *X. gardneri* (x2), *X. perforans* (x2), *X. dyei* (x1), *X. campestris* pv. *raphani* (x1), *X. campestris* pv. *campestris* (x3), *X. arboricola* (x1).

Inclusivity value: 100%.

Exclusivity value: 100%.

APPENDIX 7 - PATHOGENICITY TEST

Pathogenicity can be most easily determined by infiltrating a suspension, containing approximately 10^5–10^6 cfu/mL, prepared in Tris buffer solution (10 mM, pH 7.2) from a 24-h YPGA culture into the mesophyll of young *Anthurium* leaves. Inoculation of higher-titre suspensions is not recommended, as it can induce atypical reactions. Alternatively, inoculations using a suspension (10^6 cfu/mL) of a 24-h YPGA culture into stems of young *Anthurium* plants can also be performed. At least three test plants should be inoculated per suspected bacterial strain. The reference strain of *X. phaseoli* pv. *dieffenbachiae* (NCPPB 1833 = PD 992 = LMG 695) prepared as described previously should be used as positive control. Tris buffer solution (10 mM, pH 7.2) should be used as negative control.

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). Inoculation by atomizing is not recommended, except when facilities are adapted to the aerial containment of quarantine organisms.

Incubation should be for up to 4 weeks at 28°C under high (relative) humidity conditions (60–80%).

Symptoms should appear after 4 weeks in the inoculated plants and in the positive control but not in those inoculated with sterile distilled water. 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 re-isolated from plants (Section 3.3). Identity of re-isolated cultures can be checked by IF, ELISA or PCR.