Diagnostics
Diagnostic

Xanthomonas axonopodis pv. *dieffenbachiae*

Specific scope

This standard describes a diagnostic protocol for *Xanthomonas axonopodis* pv. *dieffenbachiae*[1].

Specific approval and amendment

First approved in 2003–09.
Revised in 2009–09.

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) (McCulloch & Pirone, 1939) and described 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* (*An. amnicola*, *An. andraeanum*, *An. crystallinum*, *An. scherzerianum*), *Caladium* (*C. bicolor*, *C. lindenii*), *Colocasia esculenta*, *D. maculata*, *Epipremnum aureum*, *Philodendron* (*P. bipinnatifidum*, *P. scandens*, *P. scandens* subsp. *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 1952 in Brazil (Robbs, 1955) and then in 1971 in Hawaii (US) on Kauai island (Hayward, 1972). Since then bacterial blight has been a major problem in Hawaii, and in Guadeloupe and Martinique.

Outbreaks or incursions of the pathogen were recurrently recorded in Europe in association with Anthurium during the last decade, e.g. in 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).

There are at least two groups of strains affecting *Araceae*: (1) strains highly virulent to *Anthurium*, and with a broad host range. These strains are the causal agent of Anthurium bacterial blight (Chase *et al.*, 1992; Lipp *et al.*, 1992; Robene-Soustrade *et al.*, 2006); (2) strains originating from other Araceae genera that are primarily pathogenic to their host of origin and weakly or not pathogenic to Anthurium (Chase *et al.*, 1988; Lipp *et al.*, 1992; Robene-Soustrade *et al.*, 2006). There are no reports of outbreaks on Anthurium caused by this heterogeneous group of strains. This group of strains includes the ones designated as pv. *syngonii* which are serologically closely related to strains causing Anthurium bacterial blight (Chase *et al.*, 1988; Dickey & Zumoff, 1987; Lipp *et al.*, 1992). 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 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 within pathovars. 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). The description below is mainly for strains from *Anthurium*.

The diagnostic procedure for the identification of *X. axonopodis* pv. *dieffenbachiae* is presented in Fig. 1.

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; Dickey & Zumoff, 1987; Lipp *et al.*, 1992).

[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.

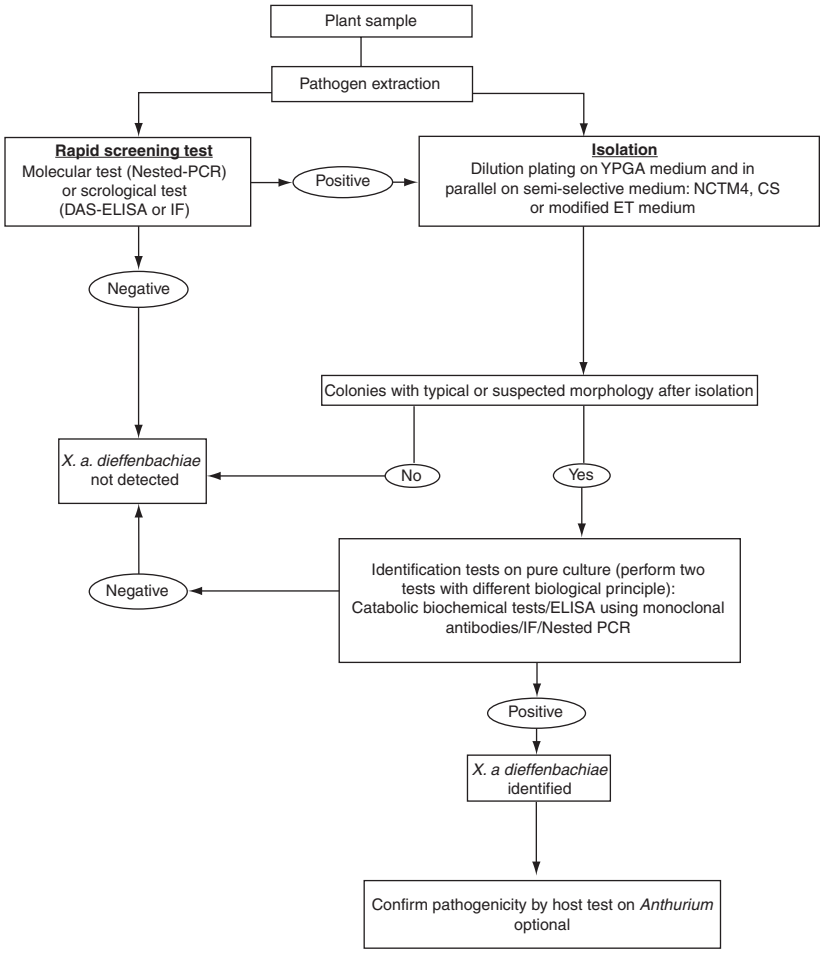

Fig. 1 Flow diagram for the detection and identification of *Xanthomonas axonopodis* pv. *dieffenbachiae* in samples of symptomatic or asymptomatic *Anthurium* or other host plants.

The phylogeny based on partial sequencing of the Gyrase B gene (Parkinson *et al.*, 2009) places the *X. axonopodis* pv *dieffenbachiae* pathotype strain (NCPPB 1833) in a clade, with *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *manihotis*, *X. campestris* pv. *syngonii*, *X. campestris* pv. *begoniae* and *X. campestris* pv. *passiflorae*.

EPPO code: XANTDF.

Phytosanitary categorization: EPPO A2 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 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. axonopodis* pv. *dieffenbachiae* (especially the dry necrotic leaf spots) may also be confused with those of nutritional stress or injury. *X. axonopodis* pv. *dieffenbachiae* may occur in a latent form, also in tissue culture (Fukui *et al.*, 1996; Norman & Alvarez, 1994a).

Symptoms caused by *X. axonopodis* pv. *dieffenbachiae* may be confused with those caused by *Acidovorax anthurii* (Gardan

et al., 2000) (ex *Pseudomonas* sp., reference strain: CFBP3232, ICMP13404). 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 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. axonopodis* pv. *dieffenbachiae*. Symptoms of systemic infection may also easily be confused with those caused by *Ralstonia solanacearum* (Norman & Yuen, 1999).

Extraction

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

Extraction buffers may be different 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 buffer (PBS 0.01 M) and aliquots should be supplemented with appropriate concentrated buffers (to a 1x final concentration in plant sample, avoiding a too high dilution) according to the test to be done.

Screening tests

Direct isolation, immunofluorescence, DAS-ELISA and nested-PCR can be used as screening tests. The Immunofluorescence test is described in PM 7/97, ELISA and PCR are described in Appendices 3 and 4. Using nested PCR protocols gives an increased risk for false-positives due to contamination with amplicons produced in the first round of amplification.

Cross reactions with saprophytes leading to false positive responses could 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 extract, due to low sensitivity for testing plant material.

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.

The colony morphology of bacteria is observed after 2–3 days of incubation at 28°C. *X. axonopodis* pv. *dieffenbachiae* colonies on agar plates are circular, convex, mucoid and yellow. Comparison with a reference strain on the same medium is recommended.

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. axonopodis* pv. *dieffenbachiae* appear at about 72 h and are circular, convex, mucoid, and yellow.

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

On ET medium, a dark diffusible pigment is formed around colonies hydrolysing esculin.

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 instead of the yellow colonies (Norman & Yuen, 1999).

Identification

Identification of *X. axonopodis* pv *dieffenbachiae* should be performed using at least two tests based on different biological properties of the pathogen. Different tests are described below. Figure 1 summarizes the testing scheme for *X. axonopodis* pv. *dieffenbachiae* in symptomatic and asymptomatic material.

Catabolic biochemical tests, other phenotypic properties and their taxonomic relations.

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

Catabolic biochemical characteristics that should be verified for *X. axonopodis* pv *dieffenbachiae* strains are given in Table 1.

Pectolytic activity is variable: strains from *Colocasia*, *Dieffenbachia* and *Philodendron* are highly pectolytic, strains from *Xanthosoma* and many from *Syngonium* are non-pectolytic (Chase *et al.*, 1992).

Syngonium strains defined as *Xanthomonas axonopodis* 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). Two biochemical variants of the bacterium were found by studying 75 strains with the Biolog GN Microplate Identification System in Taiwan (Hseu & Lin, 1998). 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.

Serological tests

Immunofluorescence

For identification, the test should be performed as described in PM 7/97 using a pure culture (approximately 10^6 CFU mL^{-1}) 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.

Table 1 Catabolic biochemical characteristics for *Xanthomonas axonopodis* pv *dieffenbachiae* (Chase *et al.*, 1992; Lelliott & Stead, 1987)

Characteristics	Results
Gram's reaction	−
Hydrolysis of starch	v (+ biotype B, − biotype A)
Aerobic metabolism of glucose	+
Anaerobic metabolism of glucose	−
Catalase test	+
Gelatine test	+
Casein hydrolysis	+
Tributyrin hydrolysis	+
H_2S production	+
Nitrate reduction	−
Oxidase reaction	−
Urease test	−
Indol production	−
Fluorescent pigment production (on Kings Medium B)	−
Hypersensitivity reaction on tobacco	+
Acid production from:	
Arabinose	+
Cellobiose	+
Fructose	+
D(+) galactose	+
Glycerol	+
Lactose	+
Maltose	+
D-mannose	+
Melibiose	+
Raffinose	+
D-ribose	+
Sucrose	+
Trehalose	+
Xylose	+
Glutamate	+
Propionate	+
Succinate	+
Dulcitol	−
Erythritol	−
Inositol	−
Inulin	−
Mannitol	−
Rhamnose	−
Salicin	−
Sorbitol	−

−, negative; +, positive; v, variable.

ELISA

For identification, indirect-ELISA using specific monoclonal antibody Xcd 108 (AGDIA) should be performed as described in Appendix 3 using a pure culture (approximately 10^7 CFU mL^{-1}) in specific ELISA extraction buffer (Carbonate Coating Buffer) as sample. The test result is positive for an identification of a suspect culture if its optical density (O.D.) is in the range of those obtained with positive controls.

DAS-ELISA with polyclonal antibodies should not be used for identification, due to possible unspecific reactions.

Molecular tests

PCR

A suspension containing approximately 10^8 CFU mL^{-1} in molecular grade sterile water is prepared from a 24 h growing culture on YPGA. No DNA extraction is required when pure cultures are used but cell lysis by boiling as described in Appendix 4 should be performed. Appropriate PCR[2] procedures are applied to amplify specific amplicons of *X. axonopodis* pv. *dieffenbachiae* as described in Appendix 4 (Robene-Soustrade *et al.*, 2006).

In this test the first PCR round of the nested PCR is performed followed by a digestion step with restriction enzyme HincII.

Sequencing

Single gene sequencing (e.g. partial sequencing of the Gyrase B and other genes), once fully validated, is likely to offer rapid and accurate identification of *Xanthomonas* species and pathovars, including *X. axonopodis* pv. *dieffenbachiae* (Parkinson *et al.*, 2009; Young *et al.*, 2008).

Pathogenicity tests

This test is used as a confirmation in the diagnosis of *X. axonopodis* pv. *dieffenbachiae* (when necessary). Inoculation by atomizing is not recommended, except when facilities are adapted to the aerial containment of quarantine organisms.

Methods for inoculations are presented in Appendix 5.

Reference material

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

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (EPPO, 2006).

Further information

Further information on this organism can be obtained from:

- Plant Protection Service, National Reference Laboratory, PO Box 9102, 6700 HC, Wageningen (NL), fax 0031 317421701
- Food and Environment Research Agency, Sand Hutton, York (GB)
- French National Laboratory for Plant Health (LNPV), Pôle de Protection des Plantes, Station de Ligne Paradis, 7 chemin de

[2]The Anthurium Bacterial Blight pathogen primers and their use in diagnostic assays are the subject of French (FR2848222) and Dutch (NL1024929CC2) patents application by the CIRAD. No express or implied rights for their use are provided under any patent applications, trade secrets or other proprietary rights via this publication. Those desiring to use or license the patent rights are asked to contact the CIRAD to request permission to do so at the following address: Office of Technology Transfer and Development, TA 40/PS1 Boulevard de la Lironde 34398 Montpellier Cedex 5, FR (or e-mail: valo-bios@cirad.fr).

l'IRAT, 97410 St Pierre (FR, Reunion Island), e-mail:lnpv.sdqpv.dgal@agriculture.gouv.fr
- CIRAD, UMR PVBMT, Pôle de Protection des Plantes, Station de Ligne Paradis, 7 chemin de l'IRAT, 97410 St Pierre (FR, Reunion Island) isabelle.soustrade@cirad.fr

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Appendix 1 – Extraction procedures

1. Extraction from symptomatic plant material (leaves, stems)

Infected leaves or stems are quickly surface disinfected using 70% ethanol. Pieces of leaves or stems 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 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 transferred to sterile distilled water or PBS. The preparation is 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 (IF, DAS-ELISA or Nested-PCR).

Leaves and stems extracts should be analyzed 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 double distilled 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 by enhancement 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) are dispensed in 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. 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 or nested 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), double distilled glycerol (20–30% glycerol v/v) is added and then kept at a temperature below −18°C.

Appendix 2 – Preparation of media and buffers

Autoclavage (sterilization): products processed by 1 bar overpressure at 121°C for 15 min can be considered as sterile.

1-Media

Yeast peptone glucose agar (YPGA)

Yeast extract	7 g
Bactopeptone	7 g
Glucose	7 g
Agar	18 g
Distilled water	1.0 L

pH is adjusted to 7.2. Sterilized by autoclaving

Cellobiose starch medium (CS)

Cellobiose	5.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
K_2HPO_4	0.4 g
KH_2PO_4	0.8 g
Agar	15.0 g
Potato starch	10 g
Aqueous methyl green (1%)	1.5 mL
Distilled water	1.0 L

Potato starch 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 aqueous methyl green is added.

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

Cycloheximide	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

The pH is adjusted to 6.8

Modified esculin trehalose medium (ET) (Norman & Alvarez, 1989, 1994b)

Esculin	1.0 g
Trehalose	0.5 g
$FeCl_3 \cdot 6H_2O$	0.5 g
NaCl	5.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KH_2PO_4	1.0 g
Agar	15.0 g
Demineralised water	1.0 L

The pH is adjusted to 6.8 and the medium autoclaved immediately

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

Cycloheximide	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
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)	80µL

The pH is adjusted to 7.2

2-Buffers for Indirect-ELISA (AGDIA)

Carbonate Coating buffer (1X) pH = 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1.0 L

pH is adjusted to 9.6 with HCl. Sterilize by autoclaving

PBS buffer (1x)	
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
pH is adjusted to 7.4 if necessary. Sterilize by autoclaving	
PBS-L buffer (1x)	
Non fat dry milk	50.0 g
PBS 1X	1.0 L
PBS-TL buffer (1x)	
Non fat dry milk	25.0 g
Tween 20	0.5 mL
PBS 1X	1.0 L
Substrate buffer (for alkaline phosphatase)	
Diethanolamine	97.0 mL
Distilled water	1.0 L
pH is adjusted to 9.8 with concentrate HCl solution (HCl solution volume is deduced from distilled water volume). Sterilize by autoclaving	
Washing buffer (PBS-T)	
Tween 20	0.5 mL
PBS 1X	1.0 L

3-Buffers for DAS-ELISA (e.g. PRI)	
Carbonate buffer pH = 9.6	
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1.0 L
pH is adjusted to 9.6 with HCl (HCl solution volume is deduced from distilled water volume). Sterilize by autoclaving	
Extraction buffer (SEB)	
Tween 20	1.0 mL
PVP-25	20.0 g
(ovalbumine, grade IV	2.0 g)
PBS (0.01M)	1.0 L
Phosphate-buffered saline (0.01M) (PBS 0.01M)	
NaCl	8.0 g
KH_2PO_4	1.0 g
Na_2HPO_4, 12 H_2O	14.5 g
NaH_2PO_4, 2 H_2O	0.4 g
Distilled water	1.0 L
pH is adjusted to 7.4 if necessary. Sterilize by autoclaving	
Substrate buffer (for alkaline phosphatase)	
Diethanolamine	97.0 mL
Distilled water	1.0 L
pH is adjusted to 9.8 with concentrate HCl solution (HCl solution volume is deduced from distilled water volume). Sterilize by autoclaving	
Washing buffer (PBS-T)	
Tween 20	1.0 mL
PBS (0.01 M)	1.0 L

Appendix 3 – ELISA

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

Kits for DAS ELISA using polyclonal antiserum are commercially available from different suppliers. A kit for Indirect-ELISA using specific monoclonal antibodies Xcd108 is commercially available. Perform the test as described by the supplier or follow instructions below in the absence of instructions from the supplier.

1. DAS-ELISA

(1) Dilute the coating antibody 1000 times in carbonate buffer (see Appendix 2);
(2) Dispense 200 µL of the antibody solution into the wells of the ELISA plate;
(3) Cover the plate with a lid and place the plate in a humid box (wet tissue on the bottom of the box). Close the box and incubate the box over-night in the refrigerator (5°C) or 3 h at 37°C;
(4) Wash the plate three times 5 min with the washing buffer PBS-T (see Appendix 2);
(5) Add 200 µL per well of the plant extract or bacterial suspension (plant extract and bacterial suspension are to be realized into extraction buffer SEB, see Appendix 2);

Fill at least one well with the positive control and 3 wells with the negative control;

(6) Cover the plate with a lid and place the plate in a humid box. Close the box and incubate the box over-night in the refrigerator;
(7) Wash the plate four times 5 min with the washing buffer PBS-T;
(8) Dilute the alkaline phosphatase conjugate 1000 times in SEB (see Appendix 2) and add 200 µL of the conjugate solution in each well;
(9) Cover the plate with a lid and place the plate in a humid box. Close the box and incubate the box over-night in the refrigerator or 3 h at 37°C;
(10) Wash the plate as above;
(11) Prepare a 1 mg/mL alkaline phosphatase substrate (para nitrophenyl-phosphate) in substrate buffer (see Appendix 2);
(12) Add 200 mL to each well and incubate the plate at room temperature in a dark place;
(13) Read the optical density (O.D.) for each well with a microplate ELISA reader set to 405 nm, after different incubation times.

Results may be interpreted after more than 60 min of incubation as long as negative wells remain virtually clear (see supplier recommendations).

Samples are negative when their O.D. are less than twice the one obtained with the negative control.

Samples are positive when their O.D. are at least three times the one obtained with the negative control. Samples are considered as doubtful when their O.D. are between twice and three times the one obtained with the negative control. In this last case, it is recommended to repeat the test or to perform a second technique to confirm the result.

2. Indirect-ELISA

(1) Prepare the samples: suspend a single colony of a fresh pure culture in carbonate coating buffer (see Appendix 2). It is recommended to adjust the solutions optical

density at 600 nm to 0.1 and to perform a 10^{-1} dilution which is equivalent to about 10^6 CFU mL^{-1};
(2) Dispense 100 µL of prepared sample into the wells of the ELISA plate. Fill at least one well with the positive control and 3 wells with the negative control.
(3) Place the plate in an oven at 37°C overnight to dry. Be sure wells are completely dry before continuing;
(4) Add 200 µL of blocking solution PBS-L (see Appendix 2) to each well, and incubate the plate in a humid box for 30 min at room temperature;
(5) Wash the plate three times 5 min with the washing buffer PBS-T (see Appendix 2);
(6) Dilute the coating antibody 200 times in PBS-TL (see Appendix 2);
(7) Dispense 100 µL of prepared detection antibody per well;
(8) Set the plate inside the humid box and incubate for 1 h at room temperature;
(9) Wash the plate eight times with PBS-T;
(10) Dilute the alkaline phosphatase conjugate 200 times in PBS-TL and dispense 100 µL of the prepared enzyme conjugate per well;
(11) Set the plate inside the humid box and incubate for 1 h at room temperature;
(12) Wash the plate eight times with PBS-T;
(13) Prepare a 1 mg/mL alkaline phosphatase substrate (para nitrophenyl-phosphate) in substrate buffer (see Appendix 2);
(14) Add 200 mL to each well and incubate the plate at room temperature in a dark place;
(15) Read the optical density (O.D.) for each well with a microplate ELISA reader set to 405 nm, after different incubation times.

Results may be interpreted after more than 60 min of incubation as long as negative wells remain virtually clear (see supplier recommendations).

Samples are negative when their O.D. are less than twice the one obtained with the negative control.

Samples are positive when their O.D. are at least three times the one obtained with the negative control. Samples are considered as doubtful when their O.D. are between twice and three times the one obtained with the negative control. In this last case, it is recommended to repeat the test or to perform a second technique to confirm the result.

Appendix 4 – Nested PCR

1. General information

The following PCR protocol was previously published (Robene-Soustrade *et al.*, 2006). The nested PCR protocol is suitable to detect and/or identify *Xanthomonas axonopodis* pv. *dieffenbachiae* strains pathogenic to anthurium. A large collection (n = 97) of unrelated phytopathogenic bacteria or saprophytic bacteria from the Anthurium phyllosphere were tested negative with this PCR assay. The detection threshold obtained with pure cultures or plant extracts was approximately 10^3 CFU mL^{-1}. Using nested PCR protocols gives an increased risk for false-positives due to contamination with amplicons produced in the first round of amplification.

Identification of *X. axonopodis* pv. *dieffenbachiae* strains pathogenic to anthurium is achieved with the first PCR round of the nested PCR followed by a digestion step with restriction enzyme HincII. This RFLP step allowed to distinguish between *X. axonopodis* pv. *dieffenbachiae* strains pathogenic to anthurium and closely related strains belonging to *X. campestris* pv. *syngonii* and not pathogenic to anthurium. The nucleic acid source can be either pure bacterial cultures or plant extracts, prepared as described below (section 2.1). The target sequence revealed sequence similarity with bacterial genes encoding putative ABC transporter-type proteins in LPS cluster. The first PCR round produces an amplicon of 1570 bp, using primers PXadU (5′-AGGGCTCCCCATGCCGGAAT-3′) and PXadL (5′-ACGCAATGCGCAGGGGAAAT-3′).

For the detection of *X. axonopodis* pv. *dieffenbachiae* from plant material, the nested PCR protocol is required because the second PCR round greatly increases sensitivity. The primers used in the second round are NXadU (5′-AGCGCGGTACATTGTTGTTCGT-3′) and NXadL (5′-GCGGATCCTGACTGAGCAAAG-3′), producing an amplicon of 785 bp. Among various Taq polymerases tested especially Invitrogen Taq DNA polymerase and Eurogentec RedGoldStar enzyme were successful. The same amount of enzyme is necessary for both enzymes, 1 U for a 25 µL mix. A mix containing the four nucleotides, 10 mM each, is used (OZYME, New England Biolabs, GB). The 10x concentrated buffer and 25 mM MgCl2, supplied with the Eurogentec Taq DNA polymerase are used. The 9600 and 9700 thermal cyclers systems (Applied Biosystems, Foster City, CA, US) were successfully used. All reactions were performed with HPLC grade water.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. In planta detection

DNA extraction [e.g. with the DNeasy® Plant kit (Qiagen®, Hilden, DE)] should always be performed before amplification because sensitivity of the PCR reaction is then greatly improved. For detection purpose follow the nested PCR protocol. 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 protocol provided with the Qiagen® Plant kit to extract DNA, starting with adding 400 µL buffer AP1 to the homogenate. Elute twice with 50 µL buffer AE. DNA thus extracted can be stored at less than −18°C.

2.1.2. 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. Sample can be stored at less than −18°C.

2.2. Polymerase chain reaction

2.2.1. First PCR round

Prepare the following master mix for each sample (final volume 25 µL):
2.5 µL PCR buffer 10x (Eurogentec),
1.1 mM $MgCl_2$ (Eurogentec),
100 µM of each dNTP (NEB),
0.2 µM of each primer (PxadU and PxadL),
1 U RedGoldstarTaq polymerase (Eurogentec)
PCR molecular grade water to reach a final volume of 23 µL.
2 µL of plant or bacterial pure culture extracts.
PCR cycling parameters: 94°C for 3 min (initial denaturation), 35 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 2 min and a final extension step of 72°C for 10 min. PCR amplification products are detected by electrophoresis in 1% agarose and are stained with ethidium bromide. If an amplicon of 1570 pb is observed on gel, a restriction fragment length polymorphism (RFLP) analysis is performed on this PCR product using Hinc II as described in 2.3.

2.2.2. Nested PCR

For the second round of PCR prepare the same master mix than described above except primers which are NXadU and NXadL for this round. 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. Pipette 1 µL from the first reaction mixture into this master mix. Use the following PCR cycling parameters: 94°C for 3 min (initial denaturation), 20 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 30 s and a final extension step of 72°C for 5 min. PCR amplification products are detected by electrophoresis in 1% agarose and are stained with ethidium bromide.

A RFLP analysis can be performed on the nested product in the conditions described in 2.3 to distinguish between *X. axonopodis* pv. *dieffenbachiae* strains and *X. campestris* pv. *syngonii strains.*

To limit contamination risks, 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.

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). These DNA solutions can be stored at less than −18°C.

2.3.2. RFLP Reaction

Total reaction volume 15 µL
PCR grade water 7.35 µL
5 µL DNA solution (20 ng/µL)
1.5 µL RFLP buffer
0.15 µL BSA
1 µL Hinc II (=10 units)
Incubation temperature 37°C for 3 h
Denaturation temperature 94°C for 10 min

3. Essential procedural information

3.1. Controls

Every time a PCR-based test is performed, the following controls should be included:

3.1.1. Identification

- A **negative control** containing no target nucleic acid
- A **positive control** containing nucleic acid that will be amplified by the target test.

A pure culture extracted with the thermal lysis method, of the *X. axonopodis* pv. *dieffenbachiae* reference strain can be used as positive control (LMG695).

3.1.2.Detection

- A **negative extraction control**. This consists of performing a nucleid acid extraction using a known 'blank' sample that does not include target nucleid acid (*e.g.* uninfected plant material or clean extraction buffer)
- A **positive extraction control.** This consists of performing a nucleid acid extraction using a known sample that includes target nucleid acid (e.g. infected plant material or synthetic control prepared by adding pure culture of *X. axonopodis* pv. *dieffenbachiae* reference strain to a healthy *Anthurium* leaf homogenate).

3.2.Interpretation of results

The PCR test (identification or detection) is *negative* if the expected amplicon is not observed or if the observed amplicons are not of the expected size, and if the amplicon is detected for positive control samples.

The PCR identification test is positive if an amplicon of the expected size is observed (1570 bp), and if there is no amplification for the negative control. Then, the RFLP analysis should be performed on the amplicon using Hinc II (3 fragments: 234 bp, 711 bp and 625 bp).

The PCR detection test is *positive* if an amplicon of the expected size is observed (785 bp) and if there is no amplification for the negative control sample. If necessary, RFLP analysis can be performed on nested PCR amplicons using Hinc II (2 fragments: 460 bp and 325 bp).

Tests should be repeated if any contradictory or unclear results are obtained.

Appendix 5 – Pathogenicity test

Pathogenicity can be most easily determined by infiltrating a suspension, containing approximately 10^5–10^6 CFU mL^{-1}, 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-titer suspensions is not recommended, as it can induce atypical reactions. Alternatively, inoculations using a suspension (10^6 CFU mL^{-1}) 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. axonopodis* 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).

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 (refer to appropriate chapter in this text). Identity of re-isolated cultures can be checked by IF, ELISA or PCR.