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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/048 (3)

Diagnostics
Diagnostic

PM 7/048 (3) *Plenodomus tracheiphilus* (formerly *Phoma tracheiphila*)

Specific scope

This Standard describes a diagnostic protocol for *Plenodomus tracheiphilus* (formerly *Phoma tracheiphila)*.[1]

Specific approval and amendment

First approved in 2004–09.
Revision approved in 2007–09 and 2015–04.

1. Introduction

Plenodomus tracheiphilus is a mitosporic fungus causing a destructive vascular disease of citrus named 'mal secco'. The name of the disease was taken from the Italian words 'male' = disease and 'secco' = dry. The disease first appeared on the island of Chios in Greece in 1889, but the causal organism was not determined until 1929.

The principal host species is lemon (*Citrus limon*), but the fungus has also been reported on many other citrus species, including those in the genera *Citrus, Fortunella, Poncirus* and *Severina;* and on their interspecific and intergenic hybrids (EPPO/CABI, 1997 – Migheli *et al.*, 2009). Mal secco is present in the citrus-producing countries in the Mediterranean and Black Sea areas with the exception of Spain, Portugal and Morocco (CMI map no. 155, 2004).

The procedure for detection and identification of *P. tracheiphilus* on *Citrus* is summarized in the flow diagram in Fig. 1.

2. Identity

Name: *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley, comb. nov.,
Synonyms: *Phoma tracheiphila* (Petri) Kantschaveli & Gikashvili, *Bakerophoma tracheiphila* (Petri) Ciferri, *Deuterophoma tracheiphila* Petri.
Hyphomycetous anamorph: *Phialophora* sp.
Teleomorph: *Leptosphaeria* genus.
Taxonomic position: *Fungi, Ascomycota*, *Dothideomycetes, Pleosporales, Leptosphaeriaceae.*
EPPO code: DEUTTR.

Phytosanitary categorization: EPPO A2 list N°287; EU Annex designation II/A2.

3. Detection

3.1 Symptoms

Symptoms appear in spring as leaf and shoot chlorosis followed by a dieback of twigs and branches (Fig. 2A). On the affected twigs, immersed, flask-shaped or globose pycnidia appear as black points within lead-grey or ash-grey areas (Fig. 3B). On fruits, browning of vascular bundles can be observed in the area of insertion of the peduncle. *Colletotrichum gloeosporioides*, the anamorphic state of *Glomerella cingulata*, is a secondary invader of withered twigs. Acervuli of *C. gloeosporioides* are often associated with the pycnidia of *P. tracheiphilus*, but they can be easily distinguished by visual inspection. The acervuli of *C. gloeosporioides* are easily visible and appear arranged in concentric rings (Fig. 3B,C), whereas the pycnidia of *P. tracheiphilus* are scattered and cannot easily be distinguished by the naked eye, as they are immersed in the cortical tissues of the twig, under the epidermis (Figs 3B and 4A). If pycnidia are present, they can be mounted in distilled water or lactic acid and observed under the microscope. Withered twig pieces may be incubated in a humid chamber for 12–24 h to stimulate the development of pycnidia. After incubation, spore tendrils (cirrhi) protruding from pycnidia can easily be observed under the stereomicroscope.

Growth of sprouts from the base of the affected branches, and of suckers from the rootstock, is a very common response of the host to the disease. Gradually, the pathogen affects the entire tree, which eventually dies. When cutting into the twigs or after peeling off the bark of the branches and the trunk of the infected trees, characteristic salmon-pink or orange-reddish discoloration of the wood can be

[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.

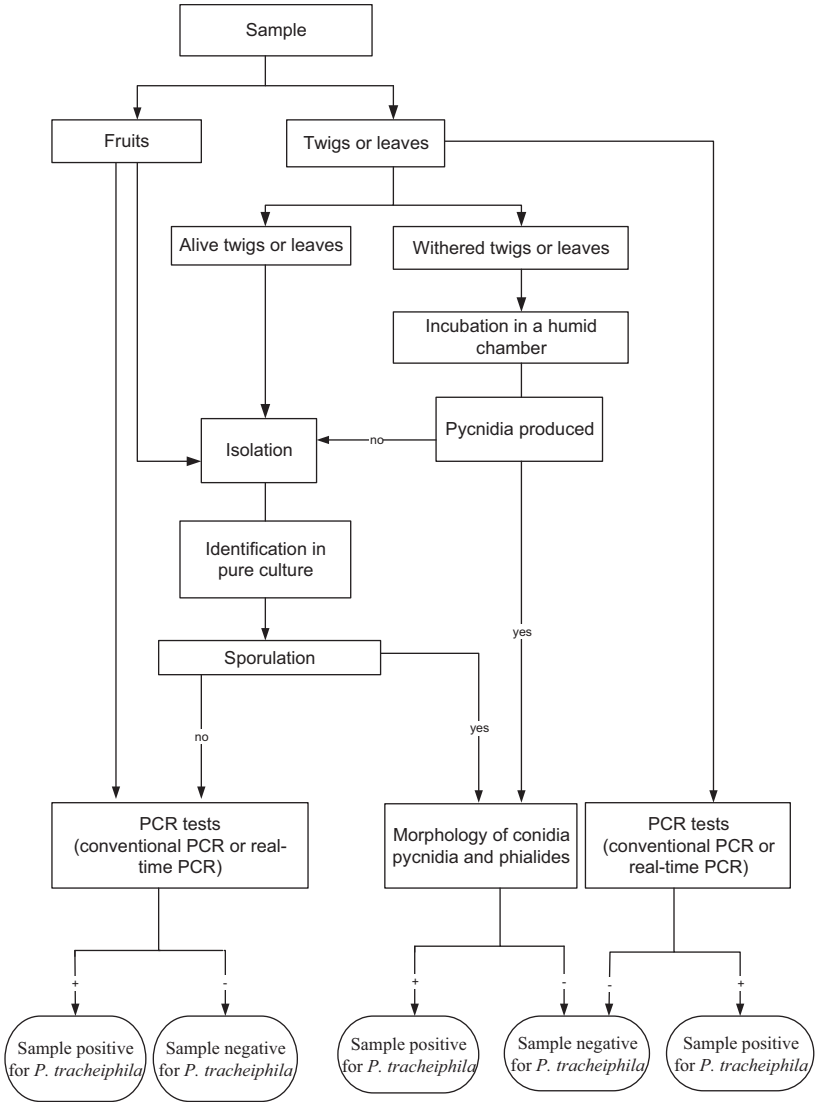

Fig. 1 Flow-diagram for detection and identification of *Plenodomus tracheiphilus* on *Citrus* sp.

seen (Figs 2A, B and 3A); this internal symptom is associated with gum production within the xylem vessels. On leaves, the typical symptom is the discoloration of small veins; epinasty of young leaves can also be seen in infected branches (Fig. 5). In addition to the more common form of mal secco, two different forms of the disease can be distinguished: 'mal fulminante', a rapid fatal form of the disease apparently due to root infection; and 'mal nero', a consequence of chronic infection of the tree leading to a browning of the heartwood.

The sampling procedure depends on the material to be investigated (fruits, leaves, living twigs or withered twigs). Twigs are chosen by visual inspection, and the edge of tissue with signs of discoloration of the xylem is sampled. Following isolation, an axenic culture of the fungus is obtained on common agar media and the fungus can be identified by microscopic observation of the phialoconidia.

In the field, samples (twigs and leaves) can be taken at any time of the year. If nursery plants are grafted on a susceptible rootstock, such as sour orange, the rootstock should also be inspected and tested. Samples should be processed within a few days after being collected. To avoid desiccation, they should be kept in plastic bags wrapped in a damp towel or paper and stored at 8–10°C.

3.2 Isolation

The fungus can be isolated by placing pieces of infected tissues taken from discoloured wood from living twigs or from symptomatic leaves on Potato Dextrose Agar (PDA), Carrot Agar (CA) or Malt Extract Agar (MEA) + chloramphenicol (Appendix 1). These are suitable media for both isolation and culture (Fig. 2C). Czapek-Dox agar may also be used as a culture medium (Fig. 2D). Petri dishes are

Fig. 2 (A) Defoliation and dieback on a lemon twig infected by mal secco. The bark of the twig has been peeled off to show the typical salmon-orange discoloration of the xylem. (B) Salmon-orange stain of the wood on the trunk of a lemon tree infected by mal secco. The bark has been peeled off to show the discoloration of the wood. (C) Isolation of *Plenodomus tracheiphilus* from an infected twig with the typical salmon-orange discoloration. Mycelium of a red-pigmented (chromogeneous) variant of the fungus developing from wood pieces plated on Potato-Dextrose Agar. (D) Colony morphology of three *P. tracheiphilus* isolates on two media: Czapek-Dox (top lane) and Potato-Dextrose Agar (bottom lane).

incubated in the dark at 22–24°C for 6–8 days. On agar media, the isolates soon lose their ability to produce pycnidia and differentiate only phialoconidia. After successive subculturing some isolates also lose the ability to produce phialoconidia. Isolations can also be performed from leaf veins: the leaf is cut into small pieces (2–3 mm), surface sterilized with 0.5–1% NaOCl for 40 s, rinsed in distilled water and then plated.

A slight modification of the conventional isolation method is to cut twig sections (2–5 mm thick), surface-sterilize with 0.5–1% NaOCl or 50% ethanol for 40 s to 2–5 min depending on the thickness and diameter of the sections, rinse in sterile distilled water and plate on PDA. Petri dishes are incubated in the dark at 23 ± 2°C for 6–12 days. Another suggested isolation method is to cut twig sections (5–10 mm thick), immerse them in sterile distilled water for 30–60 min, dry them on a sterile filter paper, then quickly flame and plate on agar medium.

Isolation on agar media is easy but time-consuming. It is still the most widely-used method for routine diagnosis for plants showing symptoms.

3.3 Growth characteristics in culture

Optimal temperature for growth is 23 ± 2°C on PDA and the growth rate is 3.8–6.0 mm per day at this temperature. The mycelium is at first hyaline and after a few days becomes brown or pinkish-red (Fig. 2C). After 10–12 days,

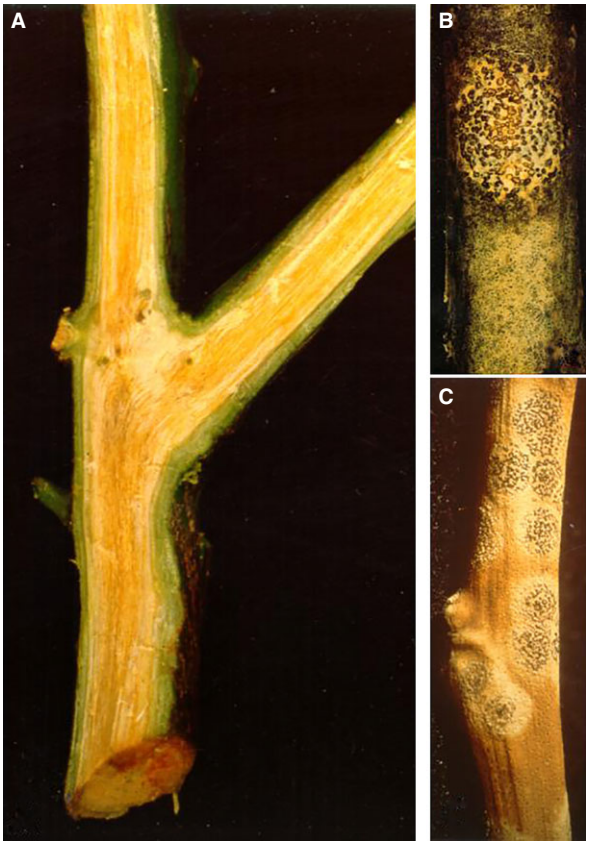

Fig. 3 (A) Longitudinal section of a lemon twig infected by *Plenodomus tracheiphilus* showing the typical salmon orange stain of the xylem (courtesy: S. Grasso). (B) Comparison between acervuli of *C. gloeosporioides* (top) and pycnidia of *P. tracheiphilus* (bottom) on a 2–3 year old dried lemon twig (courtesy: S. Grasso). (C) Dried lemon twig with acervuli of *C. gloeosporioides*.

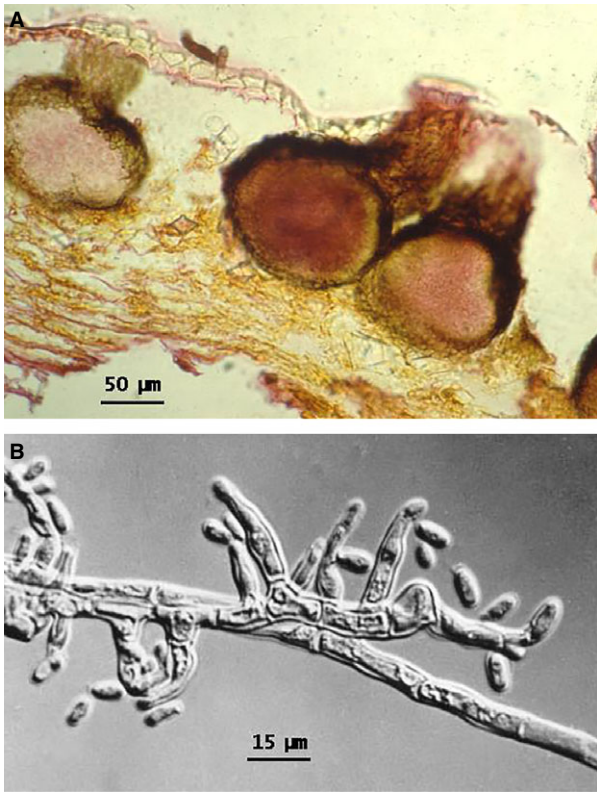

Fig. 4 (A) Hand-made tangential section of a withered lemon twig showing *Plenodomus tracheiphilus* pycnidia immersed in the cortex. Note the necks of pycnidia emerging through the epidermis (courtesy: S. Grasso). (B) Phialides and phialoconidia of *P. tracheiphilus* (differential interference contrast).

phialoconidia are produced and should be mounted in distilled water and observed under the microscope. In culture, confusion is possible with *Epicoccum* spp., which develop a brown-red to orange, relatively dense aerial mycelium and the agar medium gradually becomes orange-yellow to brownish in colour.

In the absence of sporulation, molecular methods should be used (see below).

3.4 Molecular methods

A conventional PCR test (Balmas *et al.*, 2005) and a real-time PCR test (Demontis *et al.*, 2008), developed for detection *in planta,* are described in Appendices 2 and 3, respectively. Diagnosis can be considered positive when a positive PCR result is obtained with DNA extracted directly from symptomatic twigs, leaves and fruits. The real-time PCR test was also evaluated for direct detection in soil. However, inhibition as well as reduction of sensitivity have been observed (Demontis *et al.*, 2008).

4. Identification

When sporulation occurs, identification is possible based on cultural and morphological characters. In the absence of sporulation, identification should be based on cultural characters and a molecular method.

4.1 Morphology

Mature black ostiolate pycnidia (60–165 × 45–150 µm diameter) with a neck (Fig. 4A); within the pycnidial cavity, minute unicellular, mononucleate and sometimes binucleate hyaline conidia (0.5–1.5 × 2–4 µm), are produced by conidiogenous cells (phialides). Sometimes conidia are extruded through ostioles in whitish cirrhi. Larger conidia (1.5–3 × 3–8 µm), usually named phialoconidia, are produced by phialides (12–30 × 3–6 µm) borne on free hyphae grown on exposed wood surfaces (Fig. 4B), wounded plant tissues, and within the xylem elements; they are hyaline, unicellular, uninucleate (sometimes binucleate or trinucleate), straight or curved with rounded apices. Ovoid subpyriform blastoconidia (15–17 × 7–9 µm) are

Fig. 5 (A) Discoloration of small veins on a sour-orange leaf infected by *Plenodomus tracheiphilus*. (B) Vein chlorosis on a lemon leaf infected by *P. tracheiphilus*. (C) Withering of young twigs of sour-orange infected by *P. tracheiphilus* (top); chlorosis and epinasty of young sour-orange leaves infected by *P. tracheiphilus* (bottom).

produced inside the xylem vessel of the host and in culture on liquid media. See Punithalingam & Holliday (1973).

Chromogenous and non-chromogenous variants have been distinguished in culture and isolates differing in virulence have been found to occur in nature (Magnano di San Lio *et al.*, 1992). The teleomorph is in the genus *Leptosphaeria* (Balmas *et al.*, 2005; Gruyter *et al.*, 2012) and the species of the teleomorph has never been described.

4.2 Molecular methods

The conventional PCR (Balmas *et al.*, 2005; described in Appendix 2) can be performed on plant material and cultures and this test is a standard diagnostic method, which may be performed both for testing young trees in nurseries for the sanitary certification of propagative material of lemon and for quarantine purposes. The real-time PCR (Demontis *et al.*, 2008; described in Appendix 3) can be performed on plant material and cultures and has allowed identification in asymptomatic plant material.

5. Reference material

Three strains, ATCC (1991) 26007 (Mandarin twigs, S. Grasso, Italy), 38206 (*Citrus jambhiri*; I. Barash, Z. Solel, Israel), and 48139 (*Citrus limon*; G. Perrotta, Italy) are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852- 1776 (USA). Strain Fc 40 has been deposited in the culture collection of the Dipartimento dei Sistemi Agroalimentari e Ambientali (DiGeSA), Plant Pathology Section, University of Catania, Via Santa Sofia, 100, 95123 Catania (Italy) (this strain was used for the isolation of Pt60 toxin; Fogliano *et al.*, 1998). A collection of isolates is available at the DiGeSA, (Italy).

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria 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: A. Ippolito, Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, University of Bari, via Amendola 165/A, 70126 Bari, Italy; Q. Migheli, Dipartimento di Agraria, University of Sassari, Via Enrico De Nicola 9, 07100 Sassari, Italy; A. Graniti, University of Bari, via Amendola 165/A, 70126 Bari (Italy).

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 addi-

tional 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.

11. Acknowledgements

This protocol was originally drafted by Ms SO Cacciola, University of Catania (IT) and Mr G Magnano di San Lio, Mediterranean University of Reggio Calabria (IT). It has been revised by Ms Cacciola.

12. References

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Appendix 1 – Culture Media for isolation and identification of *Plenodomus tracheiphilus*

All media are sterilized by autoclaving at 121°C for 15 min.

Potato Dextrose Agar (PDA)	
PDA (potato extract 0.4%, dextrose 2%, microbiological grade agar 1.5%)	39.0 g
Distilled water	1 L
pH = 5.6 ± 0.2	
Malt Extract Agar (MEA)	
Malt extract	20.0 g
Agar	20.0 g
Distilled water to	1 L
Chloramphenicol 1 μg mL^{-1}	
Carrot Piece Agar (CPA)	
(Werres *et al.*, 2001)	
Agar	22.0 g
Carrot pieces (grown without fungicide treatment)	50.0 g
Distilled water to	1 L
Czapek-Dox Agar	
$NaNO_3$	3.0 g
KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4$	0.01 g
Sucrose	30.0 g
Agar	15.0 g
Distilled water to	1 L

Appendix 2 – PCR (Balmas *et al.*, 2005)

1. General information

1.1 This protocol was developed by Balmas *et al.* (2005).

1.2 Nucleic acid source is infected *Citrus* tissue or cultures.

1.3 The target regions are two polymorphic regions of the internal transcribed spacer region (ITS)

1.4 Amplicon size is 378 bp.

1.5 Pair of *P. tracheiphilus*-specific primers based on the consensus sequence (555 residues) obtained from the alignment of ITS1-5.8S-ITS2 sequences of *P. tracheiphilus* isolates. Sequences of the primers are:
Forward primer: Pt-FOR2: 5′-GGATGGGCGC CAGCCTTC-3′
Reverse primer: Pt-REV2: 5′-GCACAAGGGC AGTGGACAAA-3′.

1.6 0.8 μL of crude recombinant Taq DNA polymerase prepared according to Desai & Pfaffle (1995)

1.7 200 μM each of dATP, dCTP, dGTP, and dTTP (Promega) and 1.0 μM of each primer
1.8 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 50 mMKCl.
1.9 PCR grade water is used for all reactions

2. Methods

2.1 Extraction and purification of nucleic acid

2.1.1 DNA extraction from *Citrus* tissues or cultures is performed by following the protocol of Balmas *et al.* (2005): approximately 500 mg of wood or leaf tissue is ground in a mortar with liquid nitrogen; then 450 μL of extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2.0 mM EDTA pH 8.0, 400 μg mL^{-1} proteinase K, 2% sodium dodecyl sulfate) are added to 100 mg of ground tissue and mixed well. Samples are incubated for 1 h at 65°C. After adding 300 μL of 6.0 M NaCl, samples are briefly vortexed and centrifuged at maximum speed for 30 min, the supernatant is extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and precipitated with one volume of isopropanol. The pellet is then rinsed with 100% ethanol and resuspended in TE pH 8.0. Nucleic acid extraction method details, buffer composition and pH, concentration of all constituents are listed below.

Alternatively commercial kits can be used. In this case instructions provided by the manufacturing should be followed.

2.1.2 Purified DNAs were stored at 4°C in TE buffer, pH 7.5 (Maniatis *et al.*, 1982).

2.2 Conventional PCR

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	11.8	N.A.
PCR buffer: Tris-HCl, pH 9.0	100 mM	2.5	10 mM
$MgCl_2$	25 mM	1.5	1.5 mM
KCl	500 mM	2.5	50 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Pt-FOR2	10 μM	2.5	1.0 μM
Pt-REV2	10 μM	2.5	1.0 μM
Recombinant Taq DNA polymerase (Promega)	5 U μL^{-1}	0.8	1 U
Subtotal		24	
Genomic DNA extract		1	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 lm filtered) and nuclease-free.

Commercial kits for PCR reactions can be also used and in this case instructions provided by the manufacturer should be followed.

2.2.1 Thermocycler conditions

Initial denaturation at 94°C for 5 min; 30 cycles of 30 sec denaturation at 94°C, 60 sec annealing at 65°C, 90 sec extension at 72°C; and 5 min final extension at 72°C.

2.2.2. Electrophoresis

PCR products are separated on 1.5% agarose gel in 1 × TAE running buffer according to standard methods (Maniatis *et al.*, 1982), and amplimers are observed over a UV light source.

3. Essential procedural information

3.1 Controls

In order to obtain a reliable result of the test, the following controls should be included in the isolation and amplification of the target organism.

- 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 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 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 in planta PCRs, the PAC should preferably be near to the limit of detection;

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA) amplification

of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons;
- PIC, PAC (and if relevant IC) should produce amplicons of the 378 bp.

When these conditions are met

- A test will be considered positive if amplicons of 378 bp are produced;
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Performance criteria are as presented in Balmas *et al.* (2005), using the DNA extraction method and the mastermix described in the specific section.

4.1 Analytical sensitivity data

The primer pair Pt-FOR2 + Pt-REV2 allowed detection of the specific fragment in 10 pg of total genomic DNA.

4.2 Analytical specificity data

The primer pair Pt-FOR2 + Pt-REV2 amplified a 378-bp DNA fragment from 36 *P. tracheiphilus* isolates tested that were collected from *Citrus* orchards at different locations in different years. No fragments were amplified from representatives of other *Phoma* species or from any other fungus associated with *Citrus* spp. (for details on the species see Balmas *et al.*, 2005).

4.3 Diagnostic sensitivity

100%: The test for detecting *P. tracheiphila* on lemon tissues was consistently in agreement with the isolation of the pathogen on PDA.

The specific amplicon was obtained by PCR test from all samples infected with *P. tracheiphila* and showing symptoms of "mal secco" disease; samples collected from infected symptomless plants gave rise to a faint, although reproducible, amplification signal.

4.4 Data on repeatability

Repeatability was evaluated in two laboratories and was evaluated as 100%.

4.5 Data on reproducibility

Reproducibility was evaluated in two laboratories and was evaluated as 100%.

Appendix 3 – Real-time PCR test based on SYBR® Green I or TaqMan® technologies (Demontis *et al.*, 2008)

1. General information

1.1 This protocol was developed by Demontis *et al.* (2008).
1.2 Nucleic acid source is infected Citrus tissue, conidia and pure culture.
1.3 The target region is the internal transcribed spacer (ITS) region of the nuclear rRNA.
1.4 Sequences of the primers and probe:
Phomafor: 5′-GCT GCG TCT GTC TCT TCT GA-3′
Phomarev: 5′-GTG TCC TAC AGG CAG GCAA-3′
Phomaprobe: 5′-FAM CCA CCA AGG AAA CAA AGG GTG CG BHQ-3′.
1.5 200 nM of Phomafor, 200 nM of Phomarev, 100 nM of Phomaprobe.
1.6 2XiQ SYBR® Green Supermix or iQSupermix (Biorad).
1.7 PCR grade water is used for all reactions.
1.8 PCR is monitored on an iCycleriQ Real-Time Detection System.

2. Methods

2.1 Nucleic acid extraction and purification

DNA is obtained by grinding plant tissues and cultures in liquid nitrogen, taking an aliquot of 50 mg from the homogenate and then extracting the DNA by following a standard method (Balmas *et al.*, 2005) slightly modified as follows: 200 µL of extraction buffer (50 mM Tris–HCl, pH 8.0; 2% SDS (sodium dodecyl sulphate), 0.75 M NaCl; 10 mM EDTA and 100 µg ml−1 proteinase K) are added to each sample and mixed well. Samples are incubated for 1 h at 65°C. Samples are extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and precipi-

tated by adding one volume of cold isopropanol. The pellet is then washed once with 100% ethanol and twice with 70% ethanol, re-suspended in 50 µL of TE (10mMTris–HCl, pH 8.0; 1 mM EDTA) and stored at −20°C. Alternatively, commercial kits can be used.

2.2 Real-time PCR

SYBR® Green I Real-time PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	10.5	N.A.
2X iQ SYBR® Green Supermix (Biorad)	2X	12.5	1X
Phomafor	10 µM	0.5	0.2 µM
Phomarev	10 µM	0.5	0.2 µM
Subtotal		24	
Genomic DNA extract		1	
Total		25	

TaqMan® Reall-time PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	N.A.	10.25	N.A.
2X iQSupermix (Biorad)	2X	12.5	1X
Phomafor	10 µM	0.5	0.2 µM
Phomarev	10 µM	0.5	0.2 µM
Phomaprobe	10 µM	0.25	0.1 µM
Subtotal		24	
Genomic DNA extract		1	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 lm filtered) and nuclease-free.

Commercial kits for PCR reactions can be also used and in this case instructions provided by the manufacturer should be followed.

2.2.1 Thermocycler conditions

Initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR is monitored on an iCycleriQ Real-Time Detection System.

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, 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 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 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 *in planta* PCRs, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA) amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC 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;

- For SYBR® Green based real-time PCR tests: the TM value should be as expected;
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

The validation data was obtained using the SYBR green chemistry; both the TaqMan® and SYBR® Green I tests proved sensitive and specific, and the results obtained with the two chemistries were consistent and equivalent, however the SYBR green chemistry was considered cheaper and easier to use.

4.1 Analytical sensitivity data

The test reliably detected 10 copies of the cloned target sequence.

For DNA extracted from conidia, the minimum amount of DNA that could be quantified with the SYBR® Green based test was 15 pg corresponding to less than one fungal spore per reaction.

4.2 Analytical specificity data

For the SYBR® Green based test specificity was evaluated by mixing 54 pg μL^{-1}genomic DNA from non-target species (for details on the species see Demontis *et al.*, 2008) with a serial dilution of *P. tracheiphilus*. No cross reaction was noted, but sensitivity was reduced.

4.3. Data on repeatability

Repeatability was evaluated in two laboratories and was evaluated as 100%.

4.4. Data on reproducibility

Reproducibility was evaluated in two laboratories and was evaluated as 100%.