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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/14 (2)

Diagnostics
Diagnostic

PM 7/14 (2)*: Ceratocystis platani*

Specific scope

This Standard describes a diagnostic protocol for *Ceratocystis platani*.[1]

Specific approval and amendment

First approved in 2002–09. Revised in 2014–06.

Introduction

Ceratocystis platani causes a destructive tracheomycosis in *Platanus* spp. In the EPPO region, *Platanus* × *acerifolia* (Ait.) Willd., suffers severe damage. Infected trees usually die within 3–7 years (EPPO/CABI, 1997). The disease is native to the USA, and in Europe its presence is confirmed in Italy, France, Switzerland and Greece. An outbreak was detected in Spain in 2010 and has been eradicated. The fungus is transmitted by contaminated pruning tools and terracing machinery which causes wounding to the roots. It may also be transmitted by root contact (anastomosis), and infected dead plant tissue in soil which may be infectious for up to 5 years. Sawdust and wood chips from diseased trees are highly infective. Apart from phytosanitary measures which prevent the spread of the disease to new areas, no control methods are available.

Ceratocystis platani belongs to the *C. fimbriata* sensu latu (s.l.) species complex, which includes several host-specialized cryptic species and not completely characterized lineages in the process of speciation or awaiting species description. All these groups are morphologically similar or (nearly) indistinguishable. Phylogenetic analyses of DNA sequences revealed three geographic clades within the *C. fimbriata* complex which are located respectively in North America, Latin America and Asia.

The list of described cryptic species within *C. fimbriata* s.l. and their hosts: *C. albifundus* M.J. Wingf., De Beer & Morris (from *Acacia mearnsii*), *C. pirilliformis* Barnes & M.J. Wingf. (from *Eucalyptus* spp.), *C. polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew (from *Syzygium aromaticum*), *C. fimbriata* sensu stricto (s.s.) Ellis & Halsted (from *Ipomea batata*), *C. cacaofunesta* Eng. &Harr. (from *Theobroma cacao*), *C. variospora* (Davids.) C. Moreau (from *Quercus* spp.), *C. populicola* J. A. Johnson and Harrington (from *Populus* spp.), *C. caryae* J.A. Johnson and Harrington (from *Carya* spp., *Ulmus* spp., *Ostrya virginiana*), *C. smalleyi* J.A. Johnson and Harrington (from *Carya* spp.), *C. manginecans* M. van Wyk, A Al Adawi & M.J. Wingf. (from *Mangifera indica*), *C. atrox* M. van Wyk & M.J. Wingf. (from *Eucalyptus grandis*), *C. tsitsikammensis* Kamgan & Jol. Roux (from *Rapanea melanophloeos*), *C. neglecta* M. van Wyk, Jol. Roux & C. Rodas (from *Eucalyptus* spp.), *C. fimbriatomima* M. van Wyk & M.J. Wingf (from *Eucalyptus* sp.). Phylogenetic analyses of DNA sequences clearly showed that among the above cited cryptic species, *C. fimbriata* s.s., *C. cacaofunesta*, *C. manginecans*, *C. fimbriatomima* and *C. neglecta* are the closest to *C. platani* (Baker *et al.*, 2005; Van Wyk *et al.*, 2007b, 2009; Rodas *et al.*, 2008).

Host specialization is considered a major factor driving speciation within the genus and appears to be a major factor which helps to define the groups/populations as cryptic species within *C. fimbriata* s.l. (Baker *et al.*, 2003; Johnson *et al.*, 2005; Baker-Engelbrecht & Harrington, 2005). In inoculation trials *C. platani* showed a strong host specialization and, as a host, *Platanus* was shown to be strongly selective when challenged with *C. fimbriata* isolates obtained from a variety of host species (Baker *et al.,* 2003; Baker-Engelbrecht & Harrington, 2005). To date, *C. platani* is the only *Ceratocystis* species that has been isolated from and which is able to cause a disease in the genus *Platanus*. Thus, it is unlikely (although it cannot be excluded) that *Platanus* will become infected by *C. fimbriata* cryptic species other than *C. platani*.

[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.

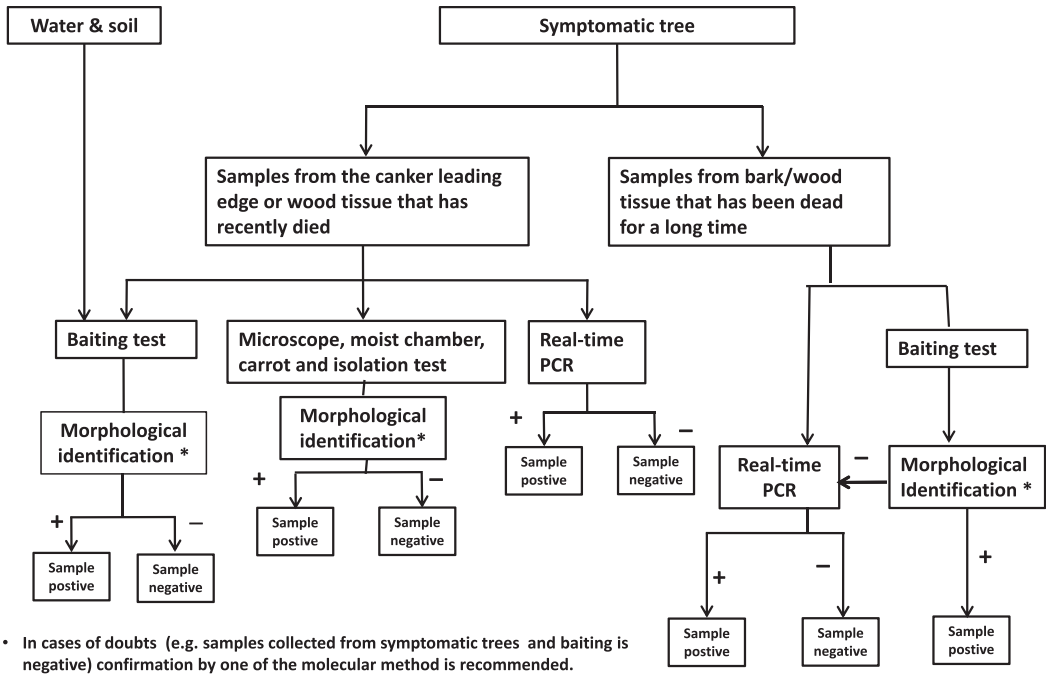

Fig. 1 Flow diagram for the detection and identification of *Ceratocystis platani.*

A flow diagram describing the diagnostic procedure for *C. platani* is presented in Fig. 1.

Identity

Name: *Ceratocystis platani* (J. M. Walter) Engelbr. & T. C. Harr.
Synonyms: *Endoconidiophora fimbriata* (Ellis & Halsted) Davidson f. sp. *platani* Walter, *Ceratocystis fimbriata* sp *platani*
Taxonomic position: Fungus; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreomycetidae; Microascales; Ceratocystidaceae
EPPO code: CERAFP
Phytosanitary categorization: EPPO A2 list no. 136; EC Annex designation II/A2

Detection

Symptoms

Platanus species are the only hosts of *C. platani*. The pathogen can be present in growing plants, wood, wood chips, and sawdust. Plants may be affected singly or in groups. Externally, the whole canopy or a single branch can begin to decline showing sparse chlorotic foliage. Infection and progress of the pathogen in the roots and the stem cause a greyish to brown discoloration of the wood and a reddish to bluish staining of the bark. These are the signs of necrosis and loss of function of the vascular system, as well as of the parenchymatous tissue, cambium and bark (Fig. 2). In general, the edges of the lesions, where necrosis advances, show no callus reaction. The pathogen is able to enter the vessel elements and progresses vertically. It also invades the xylem parenchyma and proceeds radially. In the latter case *C. platani* can reach the bark (Fig. 2B) causing the appearance of typical reddish/bluish spots in the external surface. After death of the tree or of sections of the stem, the bark becomes hazel brown and detaches from the vascular cylinder.

Detection from recently infected plant material or plant material that has recently died

Light microscopy: *C. platani* can be detected in samples collected, preferably, on the necrotic edge of an advancing canker by means of an increment borer. Thin hand-made sections are then directly prepared from cores, and the presence of the fungus can be ascertained by light microscope observation of dark diagnostic aleurioconidia inside xylem vessels (200–400×) (Fig. 3). Wood sections should be prepared longitudinally (vessel-oriented) to maximise the chance of detecting aleurioconidia. Aleurioconidia are present only in the necrotic tissue, not in the healthy-looking tissue. Aleurioconidia observed in the plant tissue may have the same shape and colour as those observed in culture. However, the plant tissue-inhabiting aleurioconidia can also be deformed to different degrees, depending on their location inside the vessels and their age. Specifically, in tissue that died some time ago and in dehydrated wood, aleurioconidia are rarely observable and they frequently appear as a deflated ball in which one half incorporates the other. Detection of aleurioconidia, both in the canonical and non-canonical form, is sufficient for a positive detection. If rainy or humid weather

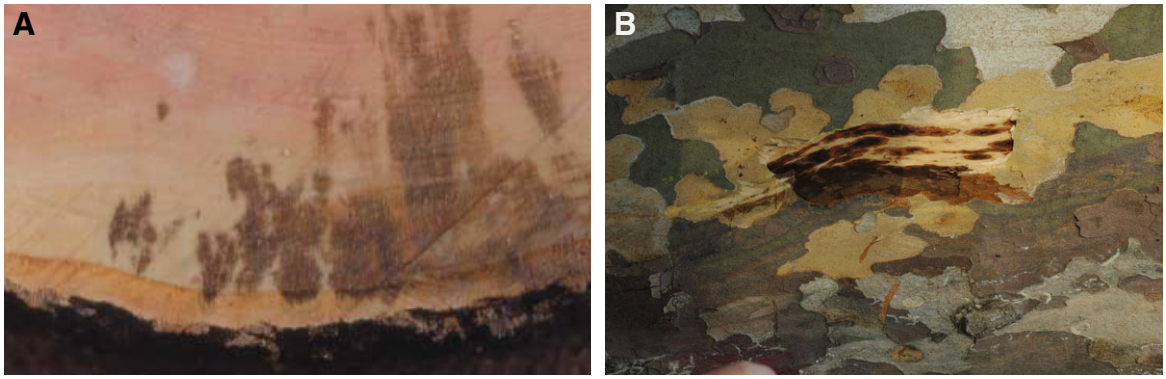

Fig. 2 A) Typical discolored areas in a transversal section of a trunk of *Platanus* infected by *Ceratocystis platani*, B) Symptoms on bark.

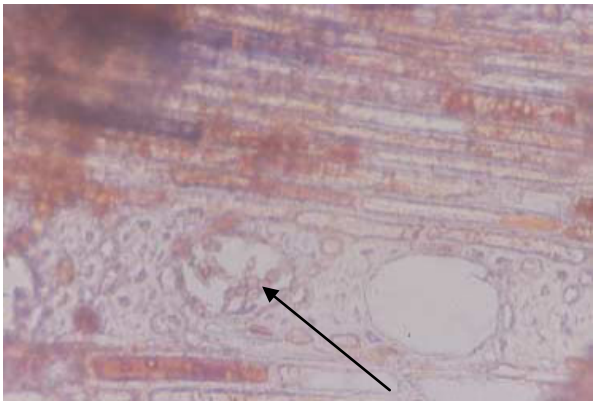

Fig. 3 Presence of aleurioconidia of *Ceratocystis platani* inside xylem vessels.

conditions persist, *C. platani* perithecia can also be present. The presence of perithecia with typical ascospores is sufficient for a positive diagnostic.

In freshly collected samples, in the absence of perithecia or aleurioconidia, the following methods can be used for the detection of *C. platani*:

- Moist chamber test: cores collected on the edge of lesions by means of an increment borer are cut into discs (2–3 mm thick) and incubated in a moist chamber at 20–25°C. *Ceratocystis platani*, if present, can produce endoconidia and perithecia after 3–8 days (Vigouroux, 1979);
- Isolation on media: the pathogen can be isolated from pieces of necrotic wood collected from the leading edge of a lesion on a freshly diseased plant. These are chopped into small pieces, rapidly flamed and placed on potato dextrose agar (PDA), malt agar (MA) or malt yeast extract agar MYEA. All media are supplemented with streptomycin (see Appendix 1). Plates should be incubated at 20–25°C for 3–8 days. Colony growth characteristics are presented below.
- Carrot test: small fragments of wood samples are inoculated by inserting through the cork into the parenchymatous cortex of entire carrots (15 fragments per carrot). Two carrots are inoculated per sample. Carrots are placed into polyethylene bags and incubated in the dark at 20–25°C. *Ceratocystis platani* is detected when perithecia emerge from the inoculation points and ascospores, if present, are observed by light microscopy. The whitish/greyish mycelium typical of *C. platani* has also a diagnostic value if endoconidia are observed by light microscopy (Pilotti *et al.*, 2009, 2012).
- Baiting test: to detect *C. platani* in wood samples, soil or water a trap technique has been adapted from Grosclaude *et al.* (1988) and improved by ANSES (FR). This test is presented in Appendix 2.
- Real-time PCR (see Appendix 3): this test is particularly valuable due to its high analytical and diagnostic sensitivity and specificity. Confirmation using real-time PCR is recommended in case of negative results with microscope examination, moist chamber, isolation and carrot tests, as these tests are reported to give false negative results. It is also recommended that a negative result of a baiting test on samples collected from symptomatic trees is confirmed using real-time PCR. Note that real-time PCR methods have not been validated on water and soil.

When detection is based on the observation of perithecia (freshly collected samples, moist chamber, carrot test) typical *C. platani* ascospores should be observed (at the top of the perithecium neck or inside the perithecium).

Detection from material which died a long time ago, soil or water

From tissue that has been dead for a long time (collected either from a dead tree or from a dead part of a living tree), successful isolation on agar media is highly unlikely, as many saprophytic fungi colonize dead wood and will outcompete *C. platani* on nutritive media. The moist chamber and carrot test also have a very limited success when applied to such samples (see validation data comparing these tests with real-time PCR in Appendix 3). Therefore it is recommended to use one of the following tests for such samples:

- Baiting test Grosclaude *et al.* (1988) (see in Appendix 2).

- Real-time PCR (see Appendix 3) for plant material only. This real-time PCR has been demonstrated to be able to detect *C. platani* in artificially infected plants up to 28 months after their death. No information is available for longer periods. When a baiting test gives a negative result, confirmation using real-time PCR is recommended.

Growth characteristics in culture

Growth rate is 0.3–0.5 cm day^{-1} on average on PDA at approximately 24°C. The mycelium is at first hyaline. The colonies are thicker on PDA than on MA. Later, the colonies can produce three types of conidia and, very often, perithecia. With age, the colonies become from greyish to brownish-green on the underside because of the presence of aleuroconidia, and are grey-whitish on the surface due to the presence of hyaline endoconidia, with dark spots due to perithecia. Each isolate takes a different time for the production of conidia and perithecia. Some isolates do not produce perithecia, others loose this capacity after a period of conservation. Some isolates yield colonies on PDA in which perithecia-producing sectors coexist with asexual sectors.

Identification

Morphology

The morphological description is based on Panconesi (1999) and Baker-Engelbrecht & Harrington (2005) as well as the previous version of this Diagnostic Protocol.

Ceratocystis platani is characterised by ascospore-producing perithecia, while the anamorph, *Thielaviopsis*, is recognised by endoconidia-producing endoconidiophores.

Perithecia are dark-brown, superficial or partly immersed in the substrate, with a globose base 120–330 µm wide (Fig. 4a). The base is ornamented with hyphal filaments (they can also be the endoconidiophores). Perithecia have a long, dark brown to black, straight neck, 400–1000 µm long, provided with divergent ostiolar hyphae, 20–102 µm long. Asci are evanescent. The ascospores are expelled from the ostiole and accumulate in a yellowish creamy matrix at the tip of the neck. They are characteristically shaped like bowler hats (4.0–6.5 µm long – 3.0–4.5 µm wide, 3.0–4.5 µm tall) (see Fig. 4b). Some strains produce no perithecia and others produce only aborted ones which are sterile.

The anamorph produces three types of conidia:

- Cylindrical endoconidia: unicellular, hyaline to light brown, smooth, with truncate ends, straight, biguttulate, borne in chains, of variable length (8–43 µm long, 3.0–6.0 µm wide);
- Doliform or barrel-shaped endoconidia (less frequent): hyaline to light brown, borne in short chains (6–17 µm long, 3.5–9 µm);
- Globose to pyriform thick-walled aleurioconidia becoming dark brownish with time, produced in chains (10–20 × 6–12 µm). These can be very numerous (but also absent) in freshly infected wood and are useful for direct diagnosis.

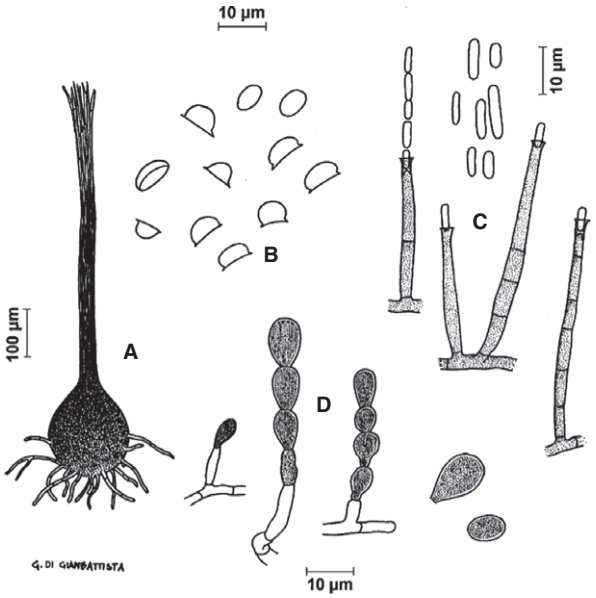

Fig. 4 *Ceratocystis platani*. (a) perithecium; (b) ascospores; (c) cylindrical endoconidia; (d) aleurioconidia. Original drawings by G. Di Giambattista (Plant Pathology Research Center – CRA-PAV, Via C.G. Bertero 22, I-00156 Rome, Italy).

Other species have similar anamorphs (e.g. *Ceratocystis paradoxa*, *Ceratocystis moniliformis* species complex), but none of these have been reported on *Platanus*. The morphological diagnostic characteristics are:

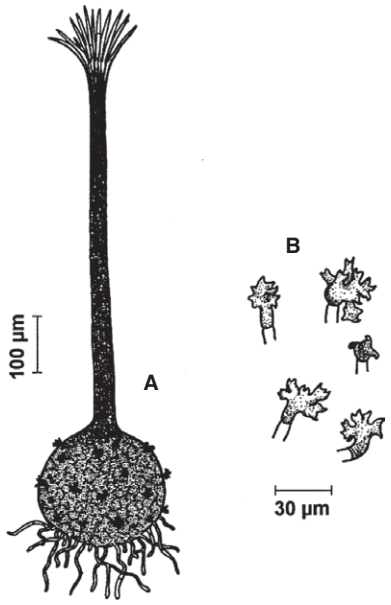

Fig. 5 *Ceratocystis moniliformis*. (a) perithecium; (b) perithecial appendages (schematic). Redrawn from Luc (1952).

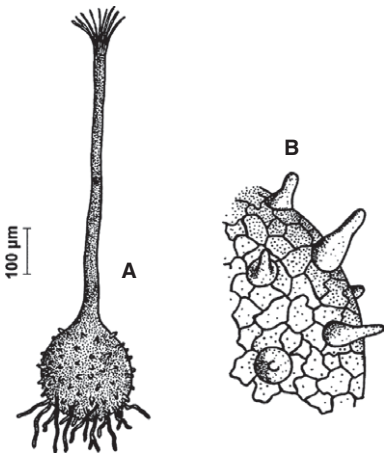

Fig. 6 *Ceratocystis paradoxa*. (a) perithecium; (b) perithecial appendages. Redrawn from Dade (1928).

- *C. platani* produces perithecia without appendages (Fig. 4), unlike the similar species (*C. paradoxa* Fig. 5 and *C. moniliformis* Fig. 6);
- *C. moniliformis* s.l. species complex does not produce aleurioconidia (Harrington pers. comm. 2014). In culture *C. moniliformis* species complex grows faster, has a coarser mycelium, and a light brown colour, which is different from the dark colour of *C. platani;*
- *C. paradoxa* produces very abundant dark conidia that confer a black powdery appearance to the colonies. Growth is rapid (more than 1 cm day^{-1} on PDA at 24°C).

Despite morphological similarity, some characteristics help *C. platani* to be distinguished from other *C. fimbriata* cryptic species and are presented in Table 1.

Molecular tests

In case of doubts regarding morphological identification a real-time PCR (see Appendix 3), a PCR-RFLP test (see Appendix 4) or sequencing can be performed. An EPPO Standard PM 7/XX on *DNA barcoding as an identification tool for plant pests* is in preparation and Internal transcribed spacers (ITS) and Translation elongation factor 1-alpha (EF1α also referred to as TEF) sequences for *C. platani* are currently available in Q-bank (http://www.q-bank.eu/Fungi/) for this pest. In Q-bank, multilocus analysis is recommended for this pest based on a combination of ITS and EF1α.

Reference material

ATCC (1984) no. 38160 (from *Platanus orientalis*), S. Accordi Mutto, Dipartimento territorio e sistemi agro-forestali, Patologia Vegetale– Università degli Studi di Padova, Campus di Agripolis – Viale dell'Università, 16 – 35020 – Legnaro Padova (IT).

no. 44186 (from *Platanus* × *acerifolia*), A. Vigouroux, INRA, Unité de Formation et de Recherche de Biologie et Pathologie Végétales, 34060 Montpellier (FR).

Table 1 Key features for distinguishing *Ceratocystis platani* from other *C. fimbriata* cryptic species

Morphological elements *C. fimbriata* cryptic species	Barrel-shaped endoconidia	Aleurioconidia	Perithecial base	Perithecial neck	Mycelium	References
C. platani	Present	Present produced in chain	Globose dark-brown to black	Absent a distinct collar at the base	From greyish to brownish-green	Baker-Engelbrecht & Harrington (2005)
C. fimbriata s.s.	Absent					Baker-Engelbrecht & Harrington (2005)
C. pirilliformis			Pyriform			Barnes *et al.* (2003)
C. albifundus			Yellowish brown			Wingfield *et al.* (1996a)
C. fimbriatomima	Absent					Van Wyk *et al.* (2009)
C. polychroma					Mostly submerged in medium with only sparse white aerial mycelium, whose colour changes according to growth temperature	Van Wyk *et al.* (2004)
C. atrox		Absent			Much darker than *C. Platani*	Van Wyk *et al.* (2007a)
C. variospora				Present a collar at the base		Johnson *et al.* (2005)
C. populicola				Present a collar at the base		Johnson *et al.* (2005)
C. smalleyi	Absent	Absent				Johnson *et al.* (2005)
C. tsitsikammensis	Not described	Produced singly				Kamgan *et al.* (2008)

Plant Pathology Research Center (Agricultural Research Council of Italy) CRA-PAV, 22 Via C.G. Bertero, 00156 Rome (IT) has a collection of about 20 Italian isolates.

Sources of reference material can be identified via the Fungi database of Q-bank http://www.q-bank.eu/Fungi/.

Reporting and documentation

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

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.).

Further information

Further information on this organism can be obtained from:

M. Pilotti – Plant Pathology Research Center (Agricultural Research Council of Italy) CRA-PAV 22 Via C.G. Bertero, 00156 Rome (IT).

S. Accordi Mutto, Dipartimento territorio e sistemi agro-forestali, Patologia Vegetale – Università degli Studi di Padova, Campus di Agripolis – Viale dell'Università, 16 – 35020 – Legnaro Padova (IT).

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.

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

This protocol was prepared by: M. Pilotti, A. Brunetti & V. Lumia, Plant Pathology Research Center (CRA-PAV), Agricultural Research Council, Via C.G. Bertero 22, 00157 Rome (IT).The previous version was by: T. Annesi, E. Motta & M. Pilotti, Plant Pathology Research Center (CRA-PAV), Agricultural Research Council, Via C.G. Bertero (IT).

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Appendix 1 – Media preparation

Media is sterilized by autoclaving at 121°C for 15 min unless stated otherwise by the supplier. Prepared plates should be stored at 2–8°C in the dark.

• **PDA (potato dextrose agar)**	
PDA (potato extract 0.4%, dextrose 2%, microbiological grade agar 1.5%)	39.0 g
Distilled water to make up to pH = 5.6 ± 0.2	1 L
If the medium is to be used for fungal isolation, after autoclaving and cooling at about 50°C, add a sterile water-diluted streptomycin aliquot, at a final concentration of 0.2 g L^{-1}	
• **MA (malt agar)**	
Malt extract	20.0 g (i.e. 2%)
Microbiological grade agar	15.0 g (i.e. 1.5%)
Distilled water to make up to pH = 5.4 ± 0.4	1 L
If the medium is to be used for fungal isolation, after autoclaving and cooling at about 50°C, add a sterile water-diluted streptomycin aliquot, at a final concentration of 0.2 g L^{-1}	
• **MYEA (malt yeast extract agar)**	
Malt extract	20.0 g (i.e. 2%)
Yeast extract	2.0 g (i.e. 0.2%)
Microbiological grade agar	15.0 g (i.e. 1.5%)
Distilled water to make up to	1 L
If the medium is to be used for fungal isolation, after autoclaving and cooling at about 50°C, add a sterile water-diluted streptomycin aliquot, at a concentration of 0.2 g L^{-1}	

Appendix 2 – Biological baiting test (adapted from Grosclaude *et al.*, 1988)

Nature of the sample

The sample may consist of one of the following
- Wood cores of approximately 5 cm in length (preferably at least two pieces)
- Small wood fragments or sawdust (for a volume preferably ≤200 mL)
- Soil sampled in the vicinity of the infested trees (for a volume preferably ≤200 mL)
- Water taken from a stream/river crossing an infested area (for a volume preferably ≤200 mL)

Samples should preferably be processed upon arrival at the laboratory. However, if needed the samples can be stored up to 5 days at approximately 5°C.

Description of the test

Containers of a minimum of 200 mL are used for the test (e.g. Erlenmeyer).

Preparation of trap twigs
Fresh twigs of *Platanus* of 5–15 mm diameter taken from a healthy tree are cut into pieces of the size of the container to be used for the test (these are subsequently referred to as 'trap twigs'). Trap twigs are stored at approximately 5°C. Extreme ends are protected with parafilm to limit drying. Before starting the test, the phloem should still be green. A test should be performed with trap twigs from the same lot. The bark should be stripped from the trap twigs before use.

Preparation of the sample
Wood samples to be tested should be cut into pieces small enough to be placed in the container. Half of the container should be filled with water (deionized water, distilled water or spring water).

For soil, water or sawdust, a volume of water (deionized water, distilled water or spring water) equivalent to the volume of the sample should be added.

An aeration system should be placed in the container (e.g. aquarium pump). The trap twig is placed in the container. The top half of the trap twig should not be covered by water. The container should be covered with parafilm in order to prevent any cross contamination (see Fig. 7).

Controls
A negative and a positive control should be prepared for each lot of samples to be tested.

The pieces of wood used for the negative and for the positive control should come from the same lot of trap twigs used in the test. The negative control should be prepared first and the container should be covered after all samples have been prepared.

The positive control should consist of a fragment of pure culture placed in water and should be prepared after the negative control and the samples.

Incubation
The sample, positive and negative control should be incubated at approximately 22°C at normal light or in a system with a 12 h white light photoperiod.

The aeration system should also ensure adequate water movement during the whole incubation period.

Samples should be incubated for up to 3 weeks and checked from day 5 at a frequency of 2–3 time per week for the presence of white mycelium or a black colouration on the part of the trap twig exposed to air.

Fig. 7 Experimental design for the biological baiting test (Grosclaude *et al.*, 1988).

After several days of incubation, the following structures can be observed if *C. platani* is present:

- White humid masses (resembling melting snow) when examined under the microscope conidiogenous cells and endoconidia characteristic of *C. platani* can be seen.
- Perithecia (not always produced)
- Aleurioconidia

For identification of the different structures see the relevant sections in the protocol.

Appendix 3 – Real-time PCR (Pilotti *et al.*, 2012)

Test version 1: intercalating dye (EvaGreen).
Test version 2: dual-labelled probe (Taqman).

1. General information

1.1 The scope of the test is the detection of *C. platani* in *Platanus* wood with two versions of a real-time PCR test Pilotti *et al.* (2012). The test can also be performed on pure cultures.

1.2 Internal Transcribed Spacer 1 region (ITS1) is the molecular target.

1.3 Amplicon location: from position 89 to 183 of the *C. platani* reference sequence DQ399853.

1.4 The expected size of the amplicon is of 95 bp (including primer sequences).

1.5 Forward primer: C.P.Sn.For.I 5′-CGTACCTATCTT GTAGTGAGATGAATGC-3′ (from position 89 to 116 of the *C. platani* reference sequence DQ399853); reverse primer: C.P.Sn.Rev.I 5′-GAGTTTACAGTG GCGAGACTATACTG-3′ (from position 158 to 183); the Taqman probe: C.P.TM.Pr. 5′-CGGTGCCCTTCA GAAGGGCCCTACCACC-3′ (from position 123 to 150). The probe was labeled with FAM (6- carboxy-fluorescein), at 5′ end, and contained Black Hole Quencher™ 1 (BHQ-1) at 3′ end.

1.6 The real-time PCR system is: CFX96 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA).

1.7 Real-time PCR results were analyzed in terms of threshold cycle value (C_T) by using the Bio-Rad CFX Manager™ Software 2.1. The default threshold setting was used throughout.

2. Methods

2.1 Nucleic Acid Extraction, Purification and Cloning

2.1.1 Pure fungal cultures, infected bark/wood tissue and a PCR target-containing plasmid can be tested with both real-time methods.

2.1.2 DNA extraction from pure cultures: mycelium (100 mg) is collected with a sterile pipette tip by scraping from an actively growing culture.
DNA extraction from wood, either healthy or *C. platani*-infected, use a 100 mg aliquot.
Grind fungal or plant aliquots to powder with liquid nitrogen using an autoclaved pestle and mortar and extract DNA using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. This kit was evaluated during the development of the test, however other commercial kits may also be suitable. Extraction can be performed soon after sample collection or after storage preferably at −80°C.

2.1.3 DNA can be stored at −20°C

2.2 Real-time PCR – EvaGreen test

2.2.1 Master Mix (See Table 2)

2.2.2 PCR conditions: initial denaturation at 96°C for 3 min; 40 cycles at 95°C for 10 s (denaturation) and 66°C for 20 s (annealing/extension); final extension at 72°C for 5 min. Fluorescence capture: Eva Green has a maximum absorbance at 500 nm and a maximum emission at 530 nm; the dedicated excitation led in CFX 96 is channel 1 (450–490 nm) and the photo-body detector is channel 1 (515–530 nm).

2.3 Real-time PCR – Dual-labelled probe (Taqman) test

2.3.1 Master Mix (See Table 3)

2.3.2 PCR conditions: initial denaturation is at 96°C for 3 min; 40 cycles at 95°C for 10 s (denaturation) and 66°C for 20 s (annealing/extension); final extension at 72°C for 5 min. Fluorescence capture: 6-FAM (6-carboxy-fluorescein) has a maximum absorbance at 495 nm and a maximum emission at 518 nm; the dedicated excitation led and photo-body detector in CFX 96 is the same as that reported for real-time PCR – Eva Green method (see above).

Table 2 Mastermix real-time PCR EvaGreen test

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	To make up to 20	N.A.
Supermix (dNTPs, Sso7d-fusion polymerase, $MgCl_2$, EvaGreen as the saturating dsDNA fluorescent dye, and stabilizers) (BIO-RAD)	2×	10	1×
Forward Primer (C.P.Sn.For.I)	10 μM	1	0.5 μM
Reverse Primer (C.P.Sn.Rev.I)	10 μM	1	0.5 μM
Subtotal		Variable	
Diluted DNA sample		Up to 8	Variable
Total		20	

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

Table 3 Mastermix real-time PCR Dual-labelled probe (Taqman) test

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	To make up to 20	N.A.
Supermix (dNTPs, Sso7d-fusion polymerase, $MgCl_2$, enhancers and stabilizers) (BIO-RAD)	2×	10	1×
Forward Primer (C.P.Sn.For.I)	10 μM	1	0.5 μM
Reverse Primer (C.P.Sn.Rev.I)	10 μM	1	0.5 μM
Probe 1 (C.P.TM.Pr.)	20 μM	0.3	0.3 μM
Subtotal		Variable	
Diluted DNA sample		Up to 7.7	Variable
Total		20	

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

3. Essential procedural information (valid for both methods)

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 of a sample collected from uninfected plane tree wood. Clean extraction buffer can also be used to monitor contamination;
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a pure culture of *C. platani* or a matrix sample that contains *C. platani* (e.g. host tissue which has been previously demonstrated to be *C. platani*-infected, either naturally or artificially);
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water and of the supermix solution 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 a pure culture of *C. platani*, DNA extracted from an infected host tissue, or a synthetic control (e.g. the cloned ITS region). 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 (IPC) can be used to monitor each individual sample separately. IPC can be amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls).

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. The necrotic nature of *C. platani*-infected samples pushed the authors to investigate possible inhibitory effects introduced by the nucleic acid extract. With EvaGreen and Taqman methods standard curves were generated which were based on dilution series of DNA extracted from *C. platani*-infected necrotized wood and of fungal gDNA alone or with additional plant gDNA. These experiments showed the repeatability of the assays, high/maximum values of the amplification efficiency (94–105%) and the linearity of the data (R^2 values = 0.99–1.00). Particularly amplification efficiency did not change substantially in the experiments based on the different templates. This showed that the test should not be influenced by

inhibitory effects (Pilotti *et al.*, 2012). The inclusion of a control represented by healthy wood spiked with nucleic acid from *C. platani* is not useful, as natural samples are usually represented by necrotic wood. Instead a host tissue which has been previously demonstrated to be *C. platani*-infected, either naturally or artificially, can be included as IC (=PIC).

Although the method does not seem to be influenced by inhibitory effects, the above described IPC can be used as IC.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IPC if applicable) 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 produces no amplification curve or if it produces a curve which is not exponential;
- For EvaGreen® based real-time PCR tests the melting temperature of the amplicon (TM) should be 81–82°C. Checking the melting curve is a manner to verify the specificity of real-time signal, in case of a positive detection, although specificity of the test has been widely verified (see 4.2 below);
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

With both methods – EvaGreen and Taqman-based – the detection limit tested on the ITS region-containing plasmid is 20 copies of the target amplicon. Detection limit tested on *C. platani* gDNA is 3 fg per PCR reaction with both methods [i.e. 0.15 fg μL^{-1} of the PCR reaction or 1.5 fg μL^{-1} of the diluted sample to be tested (2 μL)].

4.2 Analytical specificity data

Twenty six Italian isolates of *C. platani* were tested and positively detected with both EvaGreen and Taqman methods. Fifty four isolates of fungal species colonizer of *Platanus* wood, other than *Ceratocystis* spp., were tested and did not give any cross reaction. The *in silico* investigation on the ITS regions of *Ceratocystis* spp., showed that the primers and probe have a good potential to distinguish *C. platani* from all *Ceratocystis* species except for some isolates and species within the complex species *C. fimbriata* (for details see Pilotti *et al.*, 2012). However, in the context of the pathosystem *C. platani-Platanus*, this is of no significance as *C. platani* is the only *Ceratocystis* species which has been reported to infect *Platanus* spp., and the tests were developed to be directly applied on *Platanus* species.

4.3 Diagnostic sensitivity data

Diagnostic sensitivity was evaluated in two manners:

(i) Both real-time PCR tests were performed on 20 canker stain-affected trees. *Ceratocystis platani* was always positively detected from trees showing canker stain symptoms, except in one sample tested with EvaGreen. Microscope examination enabled a positive detection in 16 trees out of 20.

(ii) Both real-time PCR tests were performed on necrotic wood samples collected from 110 artificially-infected trees which were sampled at different times from the moment of the death up to 27 months after death (11 time-points, 10 trees tested for each time-point). *Ceratocystis platani* was detected in all samples of all time points except one sample, for which the Ct values were above the cut off value for both tests (see Pilotti *et al.*, 2012 for details of the experiment). Thus diagnostic sensitivity was 99.1%. Microscopic examination and a carrot test were performed on the same set of samples. They enabled a 100% positive detection up to 1 and 2 months after plant death, respectively. After these time points, microscope observation led to a positive detection declining from 90 to 50% in the time points from between 2 and 9 months, at which point the dryness, hardness and fragility of the wood then impeded the test from being performed. The carrot test led to a positive detection in 80% of the samples in the time points from 3 to 9 months, 50% at 12 months, and failed to give any detection in the subsequent time-points. From these results it was concluded that the diagnostic sensitivity of the molecular methods (99.1%) is higher than that of the traditional tests, particularly when detection is performed on wood which has been dead for a long time.

EvaGreen fluorescence signals were always confirmed as specific by querying the *C. platani* specific melting profiles.

4.4 Diagnostic specificity data

The application of both real-time tests on some trees affected by diseases other than canker stain, never yielded any fluorescence signals above the background threshold.

4.5 Data on repeatability

All the experiments for the development of the methods were repeated at least twice with similar results and each sample was tested in triplicate (inference of standard curves for the evaluation of amplification efficiency, linearity of the data, inhibitory effects and analytical sensitivity) or in duplicate (experiments of diagnostic sensitivity and analytical/diagnostic specificity) (for details see, Pilotti *et al.* >2012). In addition to the data from Pilotti *et al.* (2012), the same authors determined the detection limit in more detail by inferring standard curves using 5-fold serial dilutions and adopting an experimental design according to EPPO PM 7/98 (Pilotti, unpublished data). For each method the standard curve was repeated three times. Then

the selected detection limit was tested in eight replicates and the experiment was repeated three times for each method (repeatability experiments). All this work was performed in parallel by two operators. Thus a total of six standard curves to identify the detection limit for each method and additional 48 replicates at the detection limit to test the repeatability, for each method. Repeatability of the detection limit was fully confirmed as 3 fg DNA/PCR reaction were always detected with both methods and with a negligible variation among C_t values. Detection limit was in the range 35.4–35.9 for EvaGreen method and 34.3–35 for Taqman method (in the range: the lowest and the highest average values of the experiments).

4.6 Data on reproducibility

Reproducibility of the detection limit was tested as described above (par 4.5). The detection limit was highly reproducible with a negligible variation among average C_t values obtained by the two operators.

Appendix 4 – PCR-RFLP

1. General information

1.1 Identification of the genus *Ceratocystis* by polymerase chain reaction (PCR) based on fungal specific primers. Distinguishing *C. platani* from the other *Ceratocystis* spp. is then possible by performing restriction fragment length polymorphisms (RFLP) after PCR. This test is applied to fungal pure cultures only (i.e. not for diagnosis of *C. platani* on diseased plant material).

1.2 The test is adapted from Witthuhn *et al.* (1999) for the PCR-based RFLP identification method. See Witthuhn *et al.* (1998) for PCR conditions and Harrington & Wingfield (1995) for the preparation of the crude template.

1.3 Amplicon location: 3′ end of the small sub-unit (SSU) rRNA gene, the 5.8S rRNA gene, part of the large sub-unit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2.

1.4 Amplicon size: 1600 bp (Witthuhn *et al.*, 1999).

1.5 Oligonucleotides: Primer ITS1: 5′-TCCGTAGGT GAACCTGCGG-3′; Primer LR6: 5′-CGCCAGTT CTGCTTACC-3′.

1.6 Any thermal cycler is suitable.

1.7 Any gel imaging and documentation system is suitable to visualize amplicons after gel electrophoresis analysis.

2. Methods

2.1 Template preparation

2.1.1 PCR amplification is performed directly from fungal mycelium, as follows: scrape a pipette tip approximately 1 cm across the actively growing mycelium at the edge of the colony. Dip the tip of the pipette into the PCR tube containing the reaction mix, then stir vigorously with the tip. This is done in order to adequately suspend the fungal material attached to the tip in the reaction mixture. Controls should also be set up, including mycelium from a known culture of *C. platani* (positive control) and a PCR tube containing the reaction mixture without any template (negative control).

2.2 Conventional PCR

2.2.1 Master Mix (see Table 4)

2.2.2 PCR conditions: Initial denaturation is at 96°C for 3 min; cycling denaturation is at 92°C for 60 s; cycling annealing temperature is at 55°C for 30 s; cycling extension temperature is at 72°C for 60 s; 35 cycles; final extension temperature is at 72°C for 5 min.

2.3 Restriction Fragment Length Polymorphism (RFLP) Reaction

2.3.1 Procedure and restriction pattern

In order to distinguish *C. platani* from other *Ceratocystis* spp., digest amplification products separately with the restriction enzymes *Alu*I and *Dra*I according to the manufacturer's instructions. These enzymes have a working temperature of 37°C. Analyse the restriction pattern in a 2% agarose gel. Determine the size of the restriction products against a 50–2000 bp DNA molecular weight marker.

Digestion of the *C. platani* amplicon with *Alu*I produces a restriction pattern that is different from most *Ceratocystis* species including *C. fimbriata* isolates from *Populus* and *Prunus* spp. Consequently it is recommended to use this enzyme. Fragments obtained are of the following sizes: 150, 180, 220 + 720 bp. *C. albofundus* shows the same *Alu*I restriction pattern as *C. platani* however distinction can be made by digestion with *Dra*I, as the amplification product of *C. albofundus* is cut into three fragments (150, 320 + 1200 bp) and that of *C. platani* is left substantially uncut. Use of Hae III and Rsa I also enable a distinction with *C. coerulescens* species complex as in these species the enzymes produce a restriction pattern distinguishable from *C. platani* whose amplicon is left uncut.

For details on the restriction patterns of *Ceratocystis* species see Table 5 (modified from Witthuhn *et al.*, 1999).

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 of a clean extraction buffer;

Table 4 Mastermix conventional PCR

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	N.A.	To make up to 50	N.A.
PCR buffer (*any producer suitable*)	10×	5	1×
$MgCl_2$ (*any producer suitable*)	50 mM	6.25	6.25 mM
dNTPs (*any producer suitable*)	10 mM	1	0.2 mM each
Forward primer (ITS1)	10 μM	2.5	0.5 μM
Reverse primer (LR6)	10 μM	2.5	0.5 μM
Polymerase (*any producer suitable*)	5 U μL^{-1}	0.5	2.5U 50 μL^{-1}
Subtotal		50	
A minimum quantity of fungal material		Not specified	
Total		50	

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

- 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;
- 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 or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results

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

Conventional PCR tests

Verification of the controls

- NIC and NAC have not to produce any amplicons;
- PIC, PAC have to produce amplicons of the expected size.*When these conditions are met:*
- A test will be considered positive if amplicons of 1600 bp are produced and the expected sizes of the restriction fragments are obtained (see Table 5).
- A test will be considered negative, if it produces no band or a band of a different size after PCR or if a not expected restriction pattern is obtained;
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

No performance criteria are available.

Table 5 Restriction patterns of *Ceratocystis* species

Restriction enzymes *Ceratocystis* species	Alu I	Dra I	Dra I/Hae III Double digest	Rsa I
C. platani	720, 220, 180, 150	1600	1600	1600
C. fimbriata from *Populus* and *Prunus*	560, 220, 180, 150	uc	uc	uc
C. fagacearum	440, 400, 200, 180, 150	uc	uc	uc
C. moniliformis	400, 350, 260, 220, 150	uc	uc	uc
C. albofundus	720, 220, 180, 150	1200, 320, 150	uc	uc
C. adiposa	400, 340, 200, 180, 150	uc	uc	uc
C. paradoxa	400, 340, 200, 180, 150	uc	uc	uc
C. radicicola	400, 280, 200, 180, 150	uc	uc	uc
*C. pinicola**	400, 280, 200, 180, 150	uc	620, 550, 280	700, 600, 250
*C. coerulescens**	400, 280, 200, 180, 150	uc	620, 550, 280	700, 600, 250
*C. resinifera**	400, 280, 200, 180, 150	uc	620, 550, 280	700, 600, 250
*C. rufipenni**	400, 280, 200, 180, 150	uc	620, 550, 280	700, 600, 250
*C. virescens**	400, 280, 200, 180, 150	uc	620, 550, 280	600, 480, 250
*C. laricicola**	400, 280, 200, 180, 150	uc	550, 510, 360	700, 600, 250
*C. polonica**	400, 280, 200, 180, 150	uc	550, 510, 360	700, 600, 250

uc = uncut i.e. 1600.

*Species within *C. coerulescens* species complex (Witthuhn *et al.*, 1999).

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ADDENDUM

Addendum – PM 7/14(2) *Ceratocystis platani*

Since the last revision of the diagnostic protocol PM 7/14 *Ceratocystis platani* (EPPO, 2014), new validation data were produced for the real-time PCR test from Pilotti et al. (2012). Lumia et al. (2018) published the results of an in-house validation study of the test performed by CREA-DC and Brunetti et al. (2022) published the results of an interlaboratory comparison (test performance study) involving nine European laboratories.

A SYBR green version of the test was developed and was included in those validation studies.

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