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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/017(3)

Diagnostics

PM 7/017 (3) *Phyllosticta citricarpa* (formerly *Guignardia citricarpa*)

Specific scope

This Standard describes a diagnostic protocol for *Phyllosticta citricarpa*.[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

First approved in 2002–09. First revision 2009–09. Second revision 2020–07.

This revision was initially prepared taking into account the IPPC Diagnostic Protocol adopted in 2014 (Appendix 5 to ISPM 27, *Phyllosticta citricarpa* (McAlpine) van der Aa on fruit). However, the EPPO Diagnostic Standard includes other tests developed after the adoption of the IPPC diagnostic protocol and covers additional matrices than fruits. It also includes new species described since the adoption of the IPPC protocol.

1. Introduction

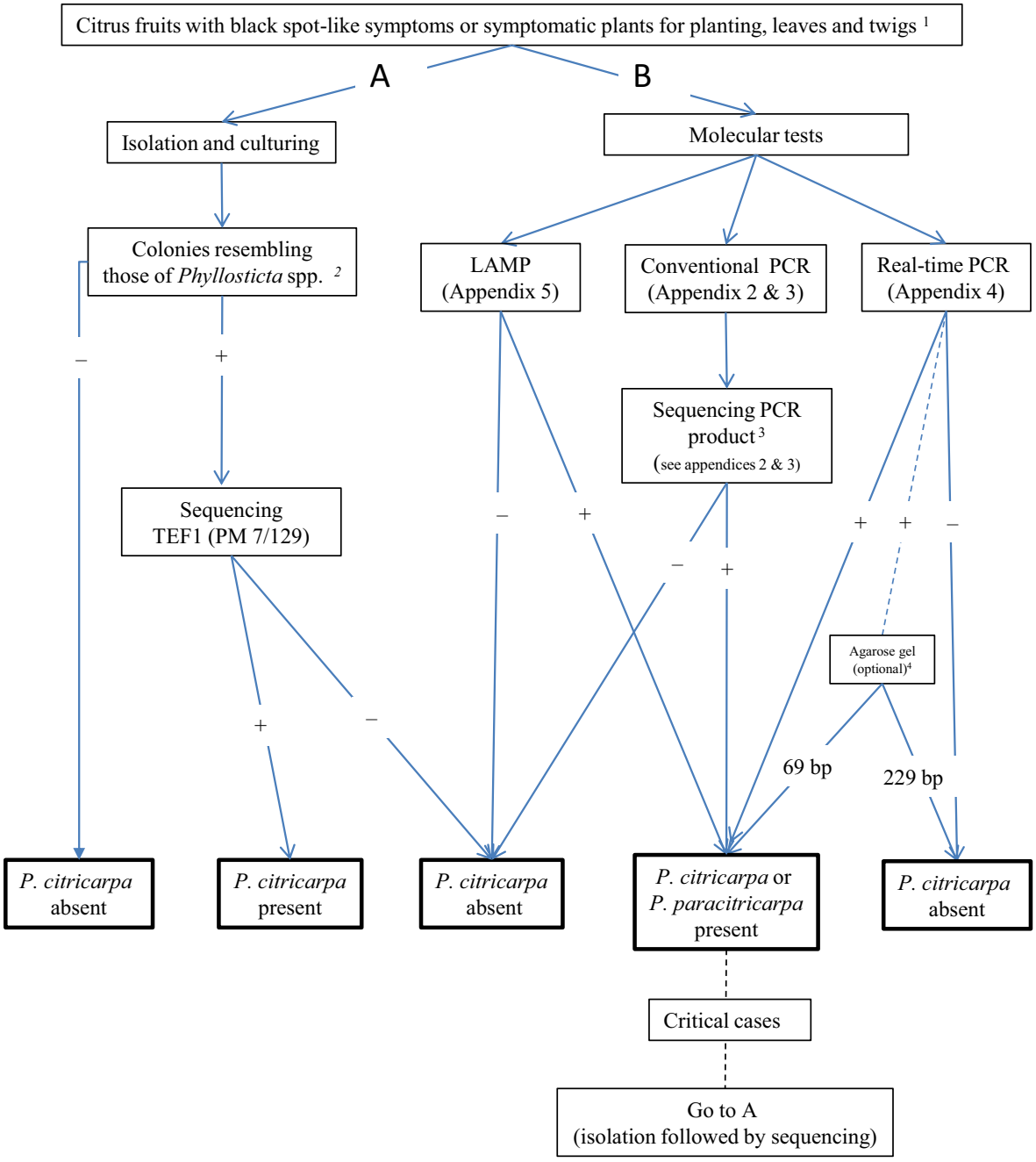
Phyllosticta citricarpa (McAlpine) Aa, the causal agent of "citrus black spot" disease, is a leaf-spotting and fruit-blemishing fungus affecting *Citrus*, *Poncirus* and *Fortunella* and their hybrids. Except for *C. aurantium* and its hybrids and *C. latifolia*, all commercially grown *Citrus* species are susceptible to the disease (Kotzé, 2000; Aguilar-Vildoso *et al*., 2002). *C. limon* is particularly susceptible and thus it is usually the first *Citrus* species to show symptoms of the disease once the pathogen is introduced into a new area (Kotzé, 2000).

Symptoms of citrus black spot were first reported in Australia in 1895 on *C. sinensis* (Benson, 1895). The disease is now present in some citrus-producing areas of Africa, Asia, Australia, and North and South America (CABI, 2011; NAPPO, 2010; Schubert *et al*., 2012). *P. citricarpa* has been reported in Italy, Malta and Portugal by Guarnaccia *et al*. (2017); however, neither symptoms nor the pathogen have been detected during official surveys in the areas where *P. citricarpa* has been reported by these authors. The organism has not been reported from Central America and in the Caribbean region it has only been reported in Cuba (CABI, 2011; EPPO/CABI, 1997; CABI/EPPO, 1998; Hidalgo & Pérez, 2010; NAPPO, 2010).

P. citricarpa has economic impact mainly because of the external blemishes it causes, which makes citrus fruit unsuitable for the fresh market (Spósito, 2003). Severe infections may cause premature fruit drop (Kotzé, 2000). Some losses due to fruit drop occur in years favourable for disease development and when fruit is held on the trees past peak maturity (CABI, 2011). In addition, latently infected (asymptomatic) fruit at harvest may still develop symptoms during transport or storage (Kotzé, 1996).

The epidemiology of citrus black spot is influenced by the availability of inoculum, the occurrence of environmental conditions favourable for infection (i.e. warm, wet and humid conditions), the growth cycle of the citrus tree, and the age of the fruit and leaves in relation to their susceptibility to infection (Kotzé, 1981, 2000). When two complementary mating types of *P. citricarpa* are present (Tran *et al*., 2017), sexual reproduction occurs in the form of pseudothecia with ascospores which are produced exclusively on leaf litter and represent the main source of inoculum. Pycnidia with conidia resulting from the asexual reproduction of *P. citricarpa* can also be important sources of inoculum (Kotzé, 1981; Spósito *et al*., 2008, 2011). In Florida, USA, where a clonal population of *P. citricarpa* with a single mating type is present, conidia are the only inoculum source driving citrus black spot epidemics (Wang *et al*., 2016).

[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.



[1] The molecular tests have been validated on fruits not on other plant material
[2] Morphological identification is difficult but may allow the distinction of *P. citricarpa* or *P. paracitricarpa* from other *Phyllosticta* species. However, sequencing is needed to distinguish *P. citricarpa* from *P. paracitricarpa*
[3] To exclude *P. citriasiana*
[4] Depending on internal validation data this step can be omitted

Fig. 1 Flow diagram for the detection and identification of *P. citricarpa*. [Colour figure can be viewed at wileyonlinelibrary.com]

Pseudothecia develop 40–180 days after leaf drop, depending on the frequency of wetting and drying, and prevailing temperatures (Kotzé, 1981). Citrus leaves drop all year round, but some seasonality is observed in certain countries, which affects the development of pseudothecia and ascospores. The optimum temperature for pseudothecial formation is 21–28°C; no pseudothecia are formed below 7°C or above 35°C (Lee and Huang, 1973). Ascospore release is closely influenced by the rainfall pattern (Kotzé, 1981) and can take place occasionally during irrigation or when there is heavy dew (Kiely, 1948; Kotzé, 2000). Windborne ascospores are forcibly released and are carried by air currents throughout the canopy and over long distances (Kiely, 1948). The critical period for infection starts at fruit set and lasts 4–7 months (Lanza *et al*., 2018). Depending on fruit age and inoculum concentration, hard spot symptoms (see Section 3.1.1) may appear from 113 to 360 days after inoculation (Frare *et al*., 2019). After infection, the fungus remains in a quiescent state until the fruit becomes fully grown or mature, with hard spot symptoms becoming apparent many months after infection has taken place (Kotzé, 2000). Leaves remain susceptible to infection from development up to 8–10 months of age (Truter *et al*., 2007).

Pycnidia with conidia are produced on fruit, leaves, twigs, fruit pedicels and in abundance on leaf litter (Kotzé, 2000). They may be splash-dispersed onto the canopy or washed off infected twigs or late-hanging fruit onto younger fruit and leaves that are still at the susceptible stage (Agostini *et al*., 2008; Spósito *et al*., 2008). *P. citricarpa* also has microconidia called spermatia[2] arising from the 'spermogonial' state (Kiely, 1949a), which usually appears on fallen leaves before pseudothecia develop. Spermatia, which function as male gametes, were shown *in vitro* to fertilize the receptive organs during sexual reproduction of *P. citricarpa* (Tran *et al*., 2017).

Symptom development on mature fruit is enhanced by rising temperatures, high light intensity, drought and poor tree vigour. Older trees usually have more citrus black spot than younger trees (Kotzé, 2000).

It should be noted that the endophytic non-pathogenic *Phyllosticta capitalensis* Henn. (formerly incorrectly referred to as *Guignardia mangiferae* A.J. Roy) (Glienke *et al*., 2011) is frequently detected in citrus fruits. The cultural, morphological and molecular characteristics that differentiate *P. capitalensis* from *P. citricarpa* have been described by Baayen *et al*. (2002). A non-pathogenic species closely related to this species, *P. paracapitalensis* (Guarnaccia *et al*., (2017), occurs in asymptomatic leaves of *Citrus* spp. in Italy and Spain. Furthermore, symptoms of *P. citricarpa* may be confused with those associated with *Phyllosticta citriasiana* Wulandari, Crous & Gruyter on pomelo (*C. maxima*) (Wulandari *et al*., 2009; Wang *et al*., 2012). Association of *P. citriasiana* with any other *Citrus* species has not been reported. The cultural, morphological and molecular characteristics that differentiate *P. citriasiana* from *P. citricarpa* have been described by Wulandari *et al*. (2009). Additional *Phyllosticta* species associated with *Citrus* spp. have been described:

- *P. citrichinaensis* has been isolated in Asia from mandarin and sweet orange fruits showing small sunken grey–brown spots with a dark-brown margin and from olive-green halos on pomelo leaves (Wang *et al*., 2012). However, pathogenicity tests were not conducted in this study.
- *P. citribraziliensis* is an endophyte in asymptomatic leaves of *Citrus* spp. in Brazil (Glienke *et al*., 2011).
- *P. paracitricarpa*, a species very closely related to *P. citricarpa*, was described by Guarnaccia *et al*. (2017) from leaf litter of *C. limon* in Greece and causing symptoms in mature Citrus sinensis (sweet oranges) by artificial inoculation.

Many new *Phyllosticta* species closely related to *P. citricarpa* have been described in the past 10 years (Glienke *et al*., 2011; Wang *et al*., 2012; Guarnaccia *et al*., 2017). These species are mostly defined on the basis of DNA sequence differences, and delineation on the basis of morphology is time-consuming and difficult (Guarnaccia *et al*., 2019).

This protocol allows the diagnosis of *P. citricarpa*, the causal agent of citrus black spot in fruits, plants for planting, leaves and twigs, and its distinction from *P. capitalensis* (previously referred to as 'non-pathogenic strains of *P. citricarpa*'), *P. citriasiana, P. citrichinaensis* and *P. paracitricarpa*. Distinction between *P. citricarpa* and *P. paracitricarpa* is currently only possible based on sequencing of phylogenetic markers, such as the translation elongation factor 1-alpha (TEF1) gene, after of isolation of the fungus in pure culture.

A flow diagram for the detection and identification of *P. citricarpa* is presented in Figure 1.

2. Identity

Name: *Phyllosticta citricarpa* (McAlpine) Aa
Other scientific names: *Guignardia citricarpa* Kiely, *Phoma citricarpa* McAlpine, *Phyllostictina citricarpa* (McAlpine) Petr., *Leptodothiorella* sp.
Taxonomic position: Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Botryosphaeriales, Phyllostictaceae
EPPO Code: GUIGCI
Phytosanitary categorization: EPPO A1 list no. 194, EU Annex IIA

3. Detection

3.1. Symptoms

3.1.1. Disease symptoms on fruits

Several symptoms (e.g. hard spot, freckle spot, false melanose, virulent spot) appear on fruit, depending on the temperature and on fruit maturity (Kotzé, 2000). The presence

[2]Described in the genus *Leptodothiorella* (Kiely, 1949a).

of *P. citricarpa* on fruit is unlikely to be accurately confirmed based on visual examination alone, as symptoms are variable in appearance and can easily be confused with those caused by other citrus pathogens or by mechanical, cold or insect damage (Snowdon, 1990; Kotzé, 2000; L. Diaz, pers. comm.). The following four symptoms are widely recognized as described by Kiely (1949a,1949b, 1960).

Hard spot. The most typical symptom of citrus black spot consists of shallow lesions, 3–10 mm in diameter, with a grey to tan centre and a dark-brown to black margin (Fig. 2A). At advanced stages of symptom development, the centre of the lesions becomes crater-like. Individual hard spot lesions may either remain small or coalesce to form larger lesions. A yellow halo, when the fruit is green, or a green halo, when the fruit is yellow or orange, may appear around these lesions. Quite often, pycnidia are produced in the centre of these spots (Fig. 2a) and can be detected by using a hand lens or a dissecting microscope. Hard spot usually appears when fruit starts maturing, even before colour change, and on the side of the fruit most exposed to sunlight (Kotzé, 1981, 2000). In many cases, citrus black spot can be easily identified by hard spot lesions with pycnidia.

Freckle spot. Grey, tan, reddish or colourless spots, 1–3 mm in diameter, slightly depressed at the centre and with no halo around them (Fig. 2B). The spots turn brown with age and are almost always devoid of pycnidia (Fig. 2b). Freckle spots mostly develop after the fruit has changed colour and may also appear as satellite spots around hard spot lesions (Bonants *et al.*, 2003) (Fig. 2C). Individual

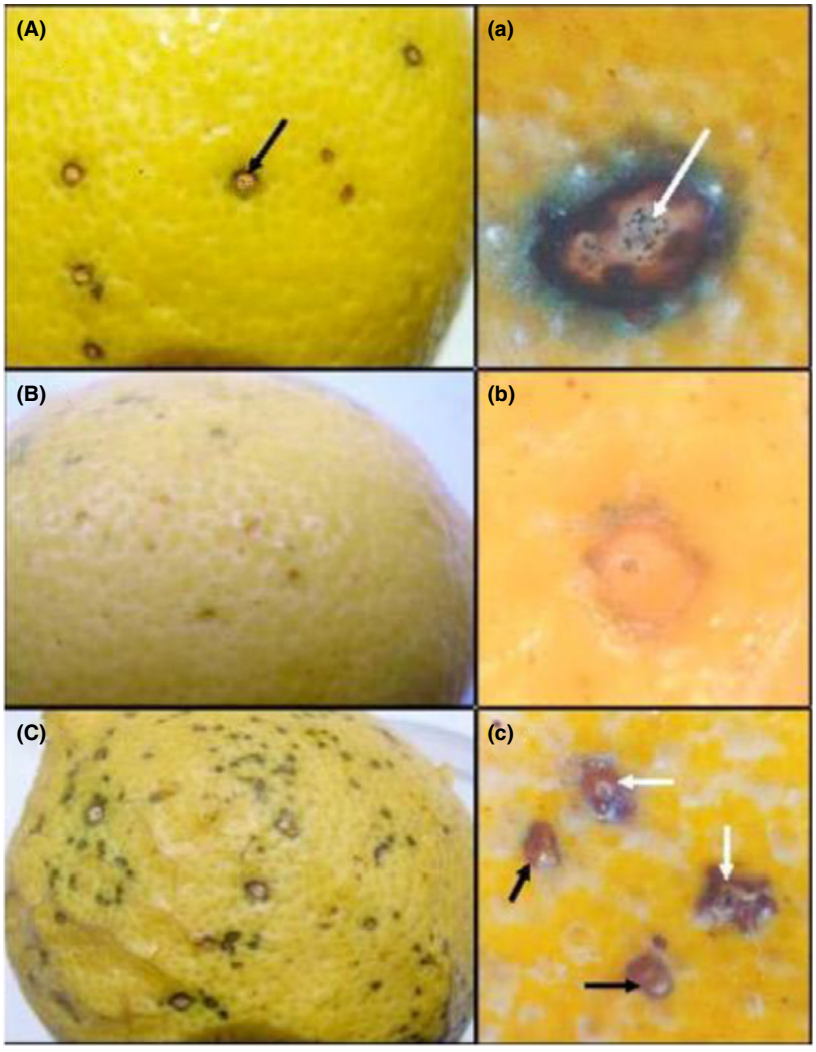


Fig. 2 Hard spot and freckle spot symptoms caused by *P. citricarpa* on sweet orange (*C. sinensis*) and lemon (*C. limon*) fruits: (A) and (a) hard spot lesions on sweet orange (black arrow) with the larger lesions containing pycnidia (white arrow); (B) freckle spot lesions on lemon; (b) freckle spot lesions on sweet orange (the lesions are slightly depressed in the centre and devoid of pycnidia); (C) hard and freckle spot lesions on lemon; (c) freckle spot lesions (black arrows) and intermediate stage between freckle and hard spot lesions with pycnidia (white arrows) on sweet orange. Photographs courtesy of E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil.

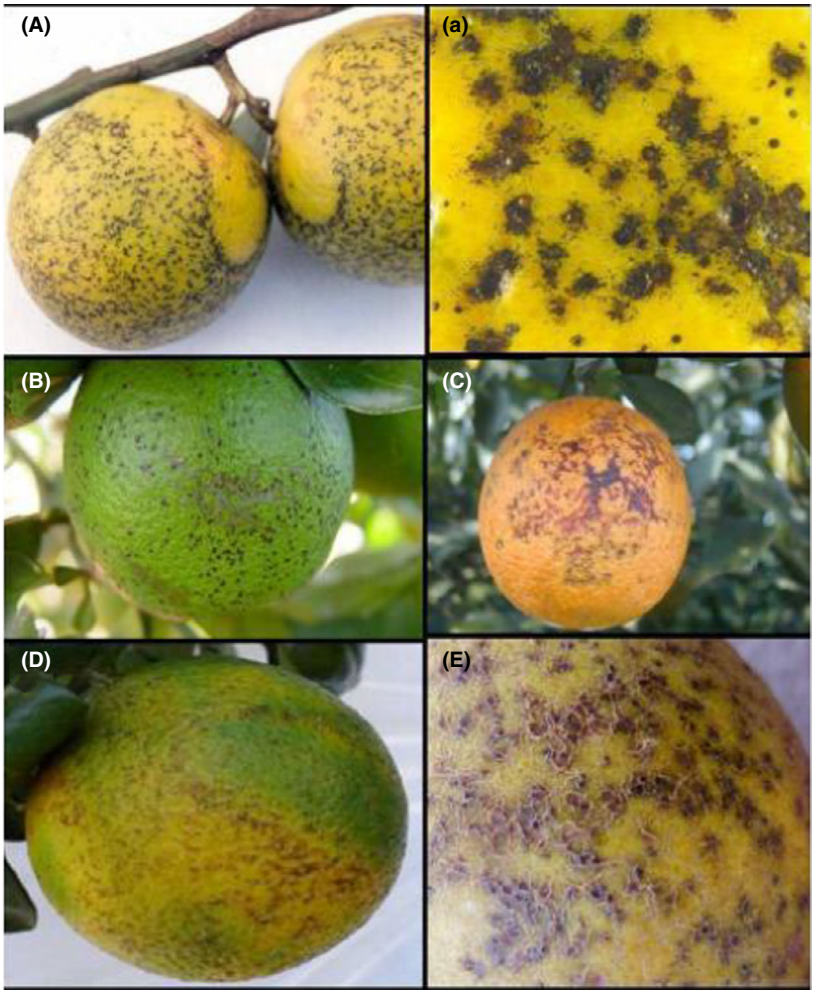


Fig. 3 False melanose, virulent spot, lacy spot and cracked spot symptoms caused by *P. citricarpa* on sweet orange (*C. sinensis*) and lemon (*C. limon*) fruits: (A) false melanose lesions on mature sweet orange; (a) false melanose lesions surrounded by dark specks on mature sweet orange; (B) false melanose lesions on a green sweet orange; (C) virulent spot lesions on sweet orange (the lesions are depressed and extend deeply into the albedo); (D) lacy spot symptoms on a green sweet orange; (E) cracked spot lesions on sweet orange (the lesions are slightly raised, cracked with irregular margins and devoid of pycnidia). Photographs courtesy of FUNDECITRUS (A, B, C, D, E) and E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (a).

freckle spots may coalesce to form larger lesions that turn into virulent spots (Fig. 2C), especially during fruit storage (Kotzé, 1981, 2000).

False melanose or *speckled blotch.* Usually appears on green fruit as small, raised, dark-brown to black lesions, often surrounded by dark specks (FUNDECITRUS, 2005) (Fig. 3A,3a,3B). The lesions are devoid of pycnidia and may coalesce as the season progresses (CABI, 2011). This symptom is observed in citrus-growing areas where *P. citricarpa* has been present for a long time and when infections occur in young fruit (FUNDECITRUS, 2005; Frare *et al*., 2019).

Virulent spot, spreading spot or *galloping spot.* Sunken irregular red to brown or colourless lesions that appear on heavily infected mature fruit towards the end of the season (Fig. 3C). Numerous pycnidia eventually develop in these lesions under conditions of high humidity (Kotzé, 2000). Virulent spots grow rapidly, covering two-thirds of the fruit surface within 4 to 5 days. Virulent spot and hard spot are the most damaging symptoms as they extend deep into the mesocarp (albedo), occasionally involving the entire thickness of the rind, causing premature fruit drop and serious post-harvest losses (Kotzé, 1981).

Three additional symptoms, as follows, have also been reported to occur on citrus fruit, though infrequently.

Lacy spot. Superficial yellow lesions with a dark-yellow to brown centre, a smooth texture and no defined margins (Aguilar-Vildoso *et al*., 2002) (Fig. 3D). This symptom appears on green fruit and may cover a big part of its surface (Goes, 2001). The lesions are devoid of pycnidia and frequently appear as brown netting on a yellow background.

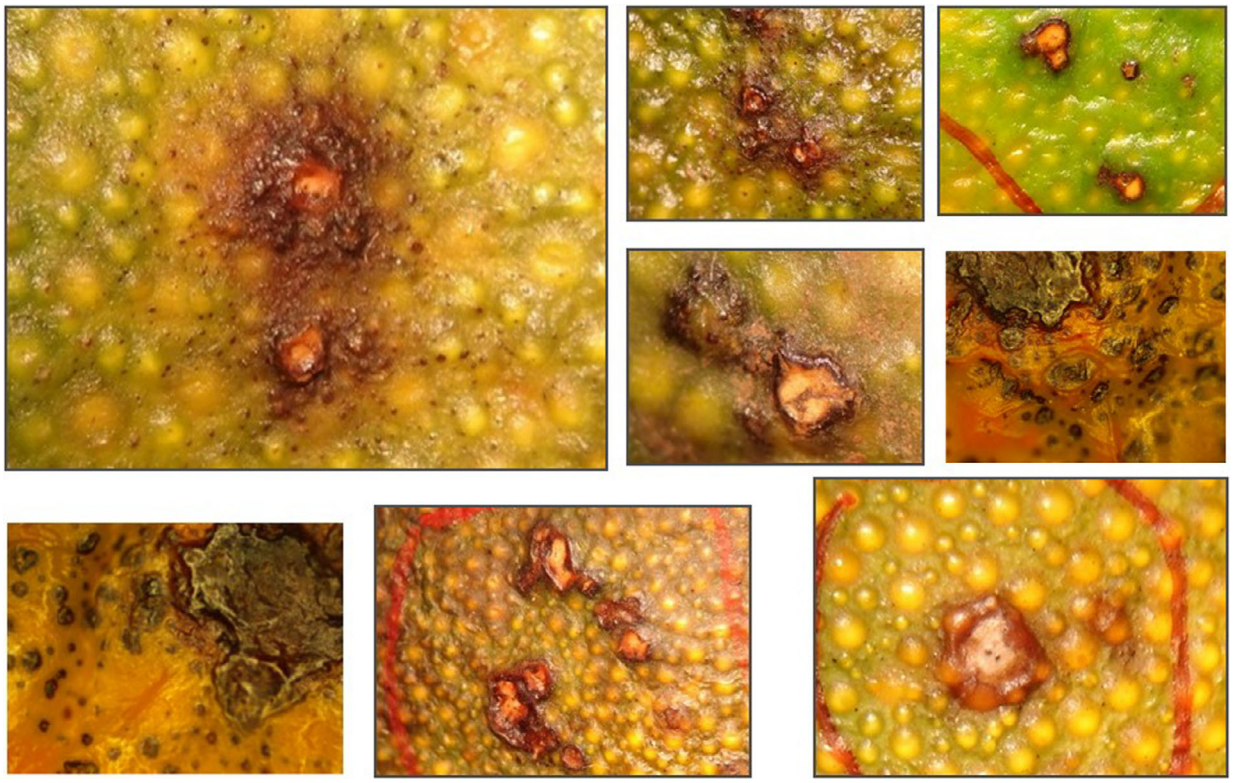


Fig. 4 Constellation symptoms on tangerine hybrid fruits. Photographs courtesy of Instituto Nacional de Tecnología Agropecuaria Argentina.

Fruits showing lacy spot usually appear to be aggregated in the tree canopy (M. Spósito, pers. comm.).

Cracked spot. Superficial slightly raised dark-brown to black lesions, variable in size, with a cracked surface and irregular margins (Goes *et al*., 2000) (Fig. 3E). The lesions are devoid of pycnidia and appear on fruit older than 6 months. This symptom has been associated with the presence of the citrus rust mite, *Phyllocoptruta oleivora* Ashmead (FUNDECITRUS, 2005; Spósito, 2003).

Constellation. Symptoms on tangerine hybrids, called constellation, have been reported in Argentina (see Fig. 4). This is not a typical symptom of the disease. Symptoms are formed of a stroma surrounded by small brown spots. In a few cases fruiting bodies develop in the centre of the stroma.

It should be noted that more than one of the symptoms described above, or intermediate stages between symptoms, may be observed on the same fruit (Fig. 2C,2c).

In some areas with high inoculum pressure, symptoms may also appear on small fruit, calyxes and peduncles. The symptoms on calyxes are red to dark-brown lesions similar to freckle spots. On small fruit and peduncles, symptoms appear as small black spots (Aguilar-Vildoso *et al*., 2002). Such symptoms on small fruit, calyxes and peduncles have been reported from Brazil only.

Fruit lesions caused by *P. citricarpa* can appear very similar to those found on fruits, from which *P. citriasiana* (symptoms on fruits are shown in Fig. 5ABC) and *P. citrichinaensis* were isolated, as well as fruits with

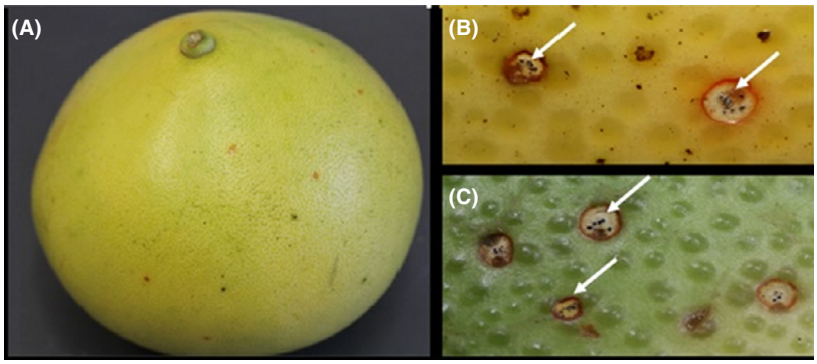


Fig. 5 Tan spot disease caused by *P. citriasiana* on pomelo (*C. maxima*). (A)–(C) Symptoms with lesions containing pycnidia (arrows).

Alternaria brown spot (*Alternaria alternata*), anthracnose (*Colletotrichum* spp.), melanose (*Diaporthe citri*), greasy spot (*Zasmidium citri-griseum*), Septoria spot (*Septoria* spp.), or mechanical and insect damage (Snowdon, 1990). In particular, Alternaria brown spot, anthracnose and Septoria spot may cause small, sharply black-rimmed, depressed lesions that are similar to tiny black spot lesions. By themselves, symptoms are not sufficiently distinctive. Other pictures of symptoms on fruits are available in the Global Database (https://gd.eppo.int/taxon/GUIGCI/photos).

3.1.2. Disease symptoms on leaves and twigs

P. citricarpa is usually present on leaves as a quiescent infection without visible symptoms (Sutton & Waterston, 1966). If symptoms do appear, they start as pinpoint spots visible on both leaf surfaces. The spots, which may increase in size up to 3 mm in diameter, are circular, with their centres becoming grey or light brown surrounded by a dark-brown to black margin and a yellow halo (Kotzé, 2000) (Fig. 6). Pycnidia may occasionally be present in the centre of the lesions on the adaxial leaf surface.

Fig. 6 Symptoms of citrus black spot caused by *P. citricarpa* on lemon (*C. limon*) leaves. Photographs courtesy of E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (A) and M. Truter, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa (B).

Lesions similar to those on leaves may also occur on small twigs, more commonly on *C. limon* than on other citrus species (M. Truter, pers. comm.). Symptoms are small (0.5–2 mm in diameter), round and slightly sunken lesions with a brown to black margin and a grey to light-brown centre (Fig. 7). Pycnidia may occasionally be present in the centre of the lesions.

3.2. Detection from fruits

Citrus fruits should be inspected for any symptoms typical of citrus black spot (see Section 3.1.1). If suspected symptoms are present in the form of spots or lesions, they are examined with a magnifying lens or a dissecting microscope for the presence of pycnidia. If pycnidia are present in hard spot lesions as described in Section 3.1.1, morphological characteristics of the pycnidia and conidia should be examined (see Section 4.1). However, as the pycnidia and conidia of *P. citricarpa* are very similar to those of *P. paracitricarpa* and *P. citriasiana*, the pathogen described on *C. maxima* (Wulandari *et al*., 2009), procedure A or B should be followed (see Fig. 1). *P. citriasiana* was isolated only from pomelos, and never from lemons, mandarins or sweet oranges (Wang *et al*., 2012). This is confirmed by data collected from nearly 250 citrus fruit consignments using two species specific real-time PCR tests validated for *P. citricarpa* and *P. citriasiana* by the National Reference Centre, Wageningen (NL) (unpublished data, van Raak, pers. comm., 2020).

It is noted that ethephon has been used in Brazil, Uruguay and South Africa to induce symptom expression in citrus fruits intended for export (Baldassari *et al*., 2007). However, there is insufficient experience with testing asymptomatic citrus fruits and with this treatment in European laboratories. Accordingly, the Panel on Diagnostics in Mycology considered that no appropriate recommendation for laboratory analysis can be made at this stage.

- Procedure A based on isolation followed by sequencing.

Procedure A is based on isolation from lesions on appropriate media (see Section 3.4.1), followed by morphological examination. When colonies resemble those of *Phyllosticta* spp TEF1 sequencing should be performed and will also allow the distinction between *P. citricarpa* and *P. paracitricarpa* (see Section 4).

Fig. 7 Symptoms of citrus black spot caused by *P. citricarpa* on lemon (*C. limon*) twigs.

Culturing and morphological examination requires 14 days, with an efficacy of 10–25% (Bonants *et al*., 2003; NRC Wageningen, unpublished data, van Leeuwen, pers. comm., 2019).

- Procedure B based on molecular tests.

Different molecular tests have been developed for direct testing on fruit lesions (Bonants *et al*., 2003; Meyer *et al*., 2006, 2012; van Gent-Pelzer *et al*., 2007; Peres *et al*., 2007; Stringari *et al*., 2009; Tomlinson *et al*., 2013). None of these tests, which target the ITS region, allow *P. citricarpa* to be distinguished from *P. paracitricarpa.*

The following tests are described in this protocol.

- Conventional PCR test (Bonants *et al*., 2003), Appendix 2.
- Conventional PCR test (Peres *et al*., 2007), Appendix 3.
- Real-time PCR (van Gent-Pelzer *et al*., 2007), Appendix 4.
- LAMP test (modified from Tomlinson *et al*., 2013), Appendix 5.

A recent real-time PCR test that can differentiate between *P. citricarpa* and *P. citriasiana* was developed by Schirmacher *et al*. (2019) on the basis of the test by van Gent-Pelzer *et al*. (2007).

The conventional PCR tests are based on the use of ITS sequence-based primers and detect *P. citricarpa*, *P. paracitricarpa* and *P. citriasiana.* Sequencing of the conventional PCR product will allow the distinction of *P. citricarpa* and *P. paracitricarpa* from *P. citriasiana.*

Real-time PCR and LAMP will allow the distinction of *P. citricarpa* and *P. paracitricarpa* from *P. citriasiana.*

Isolation followed by TEF1 sequencing should be performed for critical cases (EPPO, 2018) to allow the distinction between *P. citricarpa* and *P. paracitricarpa* to be made.

TEF1 sequencing is described in Appendix 3 of PM 7/129 (EPPO, 2016 under revision).

Real-time PCR tests are under development to allow a rapid distinction between P. citricarpa *and* P. paracitricarpa, *and will be included when they are validated.*

3.3. Detection from plant material other than fruits

Symptomatic detached leaves, twigs or planting material belonging to the genera *Citrus*, *Fortunella*, *Poncirus* and their hybrids can be examined following procedure A or B (see Section 3.2). Most EPPO countries prohibit the import of plants for planting of these genera from most origins, so in practice this mostly refers to material detained under quarantine.

Meyer *et al*. (2012) describe a 'wet-dry' technique to enrich fungal mycelial mass and stimulate fruiting body formation. The technique includes alternate daily wetting and drying of leaves. Leaves are rinsed in tap water to remove excess dirt, after which surfaces are disinfected with sodium hypochlorite (1.5% NaOCl) for 2 min, followed by thorough rinsing with sterile water. The following four steps are repeated for 4–10 consecutive days:

(1) Leaves are submerged in sterile tap water at 35°C for 30 min.
(2) Excess water is removed by draining the leaves on paper towels for 5 min.
(3) Leaves are placed in plastic bags (250 mm × 380 mm × 20 µm) and incubated at 42°C for 6 h.
(4) Leaves are air dried at room temperature (22–26°C) for 17.5 h under fluorescent light in open bags.

After 4 days, leaf material with noticeable mycelium colonization is tested with PCR tests (see Section 3.2).

3.4. Isolation

3.4.1. Isolation procedure

Fruits with lesions should be disinfected with a filter paper soaked in a 96% or 70% ethanol. Lesions are then excised with a cork borer or scalpel, or conidia are picked from pycnidia if present (van Raak, pers. comm., 2020). Subsequently, the lesions or conidia are placed aseptically on Petri dishes (9 cm in diameter) with cherry decoction agar (CHA), malt extract agar (MEA), malt extract agar chloramphenicol (MALTCHL), or potato dextrose agar (PDA) (see Appendix 1) or PDA supplemented with 50 µg/mL penicillin and 50 µg/mL streptomycin. If PDA is used and slow-growing, dark *P. citricarpa*-like cultures develop on it, which are subsequently transferred to CHA dishes for testing the growth rate of the colonies and oatmeal agar (OA, see Appendix 1) dishes to evaluate the yellow pigment production. At the same time, the cultures grown on PDA medium should be placed under near-ultraviolet (NUV) light at 22–26°C to induce formation of pycnidia.

3.4.2. Colony characteristics

Cultures are illustrated in Figures 8–11.

3.4.2.1. PDA. Colonies of *P. citricarpa* have irregular margins lined by a much wider translucent zone of colourless submerged mycelium (Figs 8 and 10A). The centre of the colony is dark with grey to glaucous aerial mycelium, often with numerous small tufts. The reverse of the colony is very dark in the centre and surrounded by areas of grey sepia and buff (Baayen *et al*., 2002). Stromata start to develop after 7–8 days, whereas mature pycnidia with conidia are generally produced within 10–14 days (Fig. 10B).

On PDA colonies of *P. paracitricarpa* are flat, rather regular and slow growing, initially white-grey mycelium, gradually becoming greenish to dark green, with white hyphae at the margin; reverse black (from Guarnaccia *et al*., 2017).

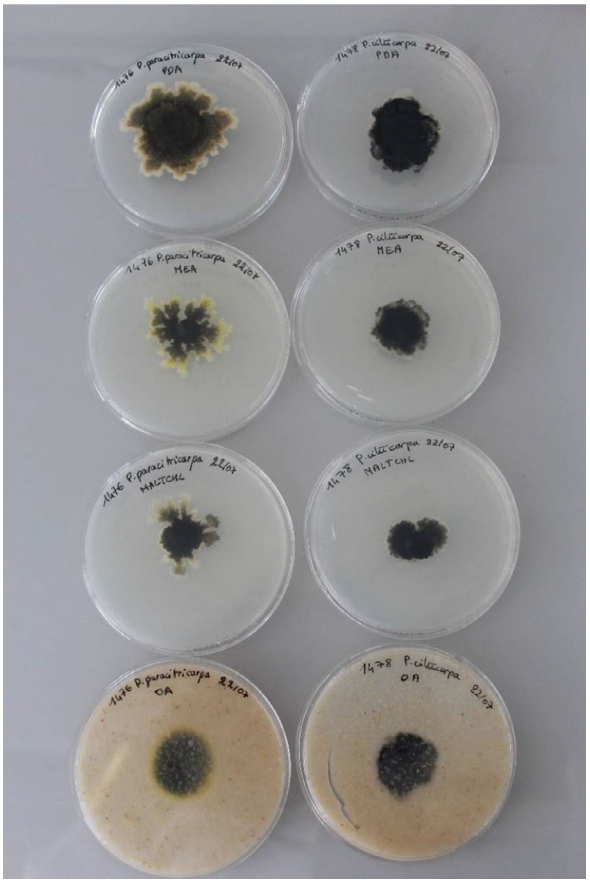


Fig. 8 Cultures of *P. paracitricarpa* (left) and *P. citricarpa* (right) on (from top to bottom) PDA, MEA, MALTCHL and OA (9 days at 26°C 12 h light). Photograph courtesy of J. Hubert, ANSES.

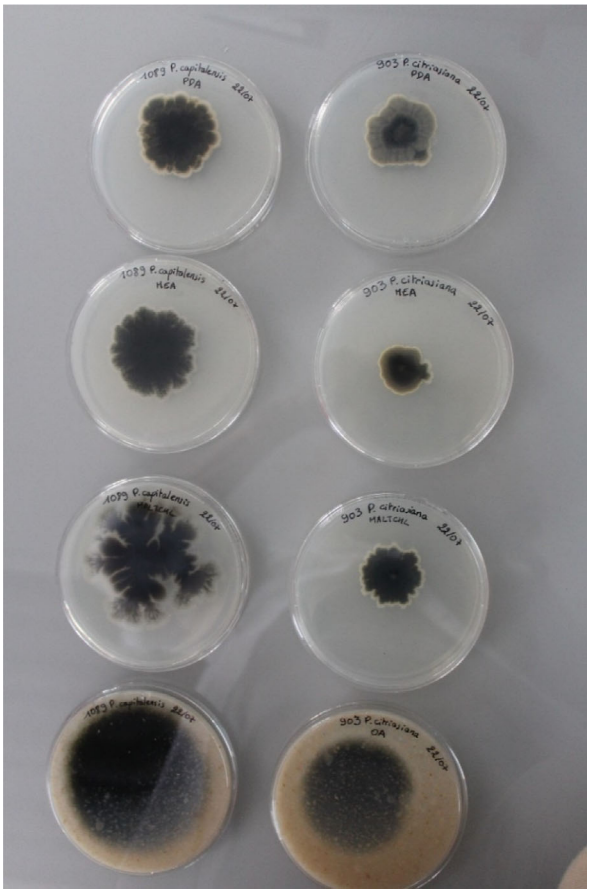


Fig. 9 Cultures of *P. capitalensis* (left) and *P. citriasiana* (right) on (from top to bottom) PDA, MEA, MALTCHL and OA (9 days at 26°C, 12 h light). Photograph courtesy of J. Hubert, ANSES.

3.4.2.2. CHA. P. citricarpa colonies grow slowly on CHA; they have an average diameter of 16–33 mm[3] after 7 days at 22°C in darkness (Baayen *et al*., 2002). The mycelium is submerged, dark, forming a plectenchymatous crust (see Fig. 11D). Stromata develop within 8 days as hard, black masses, with one to numerous cavities for the release of spores and spermatia in the upper region.

3.4.2.3. MEA or MALTCHL. These are additional media compared to Appendix 5 of ISPM 27 *Phyllosticta citricarpa* (McAlpine) Aa on fruit.

P. citricarpa

On MEA colonies are flat, with irregular edge; surface is initially olivaceous grey becoming black in the centre and sometimes yellow or cream at the margin.

Characteristics on MALTCHL are similar but growth is slower (Hubert, pers. comm. 2020). This is an additional medium compared to Appendix 5 of ISPM 27 *Phyllosticta citricarpa* (McAlpine) Aa on fruit.

P. paracitricarpa (from Guarnaccia *et al*., 2017)

On MEA colonies are flat, with irregular edge; surface is initially yellow becoming leaden grey in the centre, yellow at the margin, and leaden grey underneath.

Colonies are different from *P. citricarpa* colonies, which are olivaceous grey.

3.4.2.4. OA. P. citricarpa on OA after 14 days at 25°C in the dark, colonies are flat, spreading, olivaceous-grey, becoming pale olivaceous-grey towards the margin, with sparse to moderate aerial mycelium (Glienke *et al*., 2011). A distinct yellow pigment is often produced that diffuses into the medium around the colony (Fig. 11D, top row), although not all *P. citricarpa* isolates produce a yellow pigment (Baayen *et al*., 2002), as shown in Fig. 8. This yellow pigment is weakly produced on CHA and PDA.

Within 10–14 days, mature pycnidia should have been formed and a watery squash preparation of the pycnidium

[3]The IPPC protocol refers to 25–30 mm but fig 4 in Baayen *et al.* (2002) shows a variation from 16 to 33 mm.

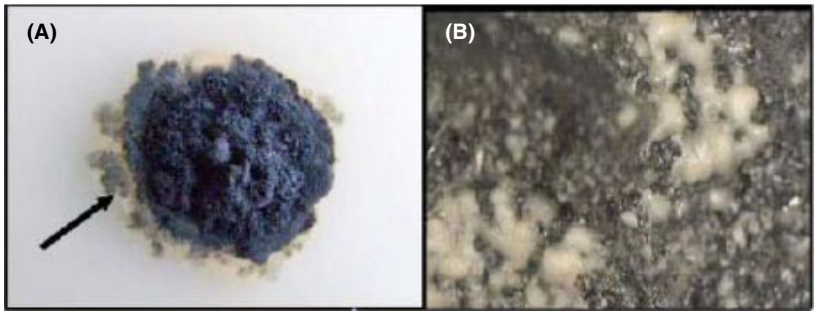


Fig. 10 Colony characteristics of *P. citricarpa*: (A) colony with irregular margin surrounded by a translucent zone of colourless submerged mycelium (arrow) after 30 days of growth on potato dextrose agar at 25°C and a 12 h photoperiod; (B) conidial slime oozing from mature pycnidia. Photographs courtesy of L.E. Diaz, Ministry of Husbandry, Agriculture and Fisheries, Montevideo, Uruguay.

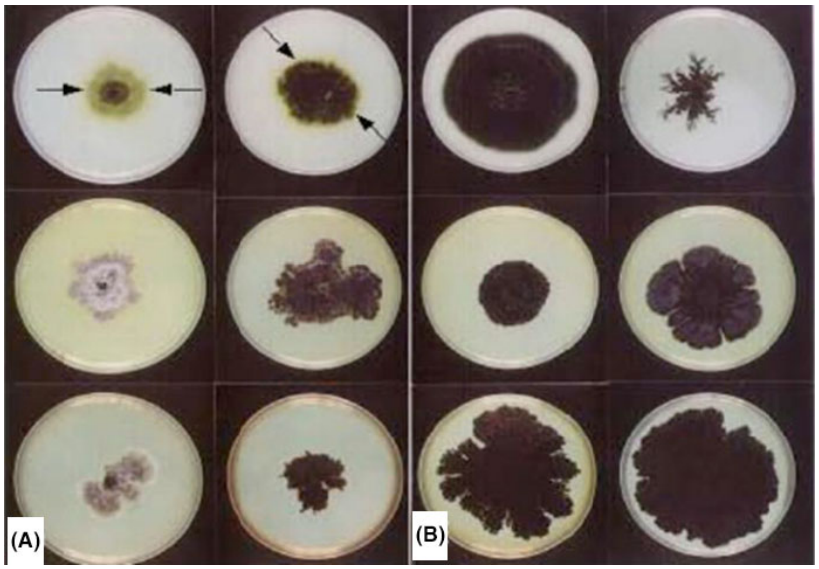


Fig. 11 Cultural characteristics of *P. citricarpa* and *P. capitalensis*: colonies of *P. citricarpa* (A) and *P. capitalensis* (B) after 7 days of growth on oatmeal agar (top row), malt extract agar (middle row) and cherry decoction agar (bottom row) [note that some, but not all strains of *P. citricarpa* form a yellow pigment around the colony on oatmeal agar (A, arrows) and the absence of this pigment in cultures of *P. capitalensis* grown on the same medium (B)]. Photographs courtesy of W. van Lienden, National Plant Protection Service, Wageningen, the Netherlands.

(or of the spores' slime oozing from it) can be examined under the microscope (See Section 4.1).

P. paracitricarpa on OA are flat, spreading, olivaceous grey, becoming pale dark grey towards the margin, with sparse to moderate aerial mycelium; surrounded by a diffuse yellow pigment in the agar medium (from Guarnaccia *et al*., 2017).

In general, cultures of *P. citricarpa* are very similar to those of *P. citriasiana* (Wulandari *et al*., 2009), *P. paracitricarpa* (Guarnaccia *et al*., 2017) and the endophytic, non-pathogenic *P. capitalensis* (Baayen *et al*., 2002; Glienke *et al*., 2011).

P. citricarpa colonies present the following cultural characteristics:

(1) Slow growth on CHA (although the ranges may overlap with those of *P. citriasiana* and *P. capitalensis*). For *P. paracitricarpa,* no similar growth rate data are available for CHA medium.
(2) Presence of yellow pigment on OA, although not all *P. citricarpa* isolates produce a yellow pigment (see above).

Detailed information on the distinctive characteristics of *P. citricarpa* and its related species is given in Table 1.

Colonies of *P. citriasiana* show darker shades of grey and black on OA, MEA and PDA than observed in *P. citricarpa* and *P. capitalensis*. An illustration of the colony reverse on OA, MEA, PDA and MALTCHL is given in Figs 8 and 9.

Pictures of colony growth of *P. capitalensis*, *P. citriasiana*, *P. citricarpa* and *P. paracitricarpa* on PDA and OA at different days are available as Supporting Information.

Table 1. Main cultural and morphological characteristics of *P. citricarpa*, *P. citriasiana*, *P. capitalensis* and *P. paracitricarpa* [data from Baayen *et al*., 2002; Wulandari *et al*., 2009 and ANSES (Hubert, pers. comm., 2019)]

Characteristic	*P. citricarpa*	*P. citriasiana*	*P. capitalensis*	*P. paracitricarpa*
Average colony diameter (mm)*	16–33[†]	18–20	>33	Unknown
Maximum growth temperature (°C)	30–36	30–33	30–36	30–36
Production of yellow pigment on oatmeal agar medium	Yes[§]	No	No	Yes[‡]

*On cherry decoction agar (CHA) medium after 7 days at 22°C in darkness.
[†]The IPPC protocol refers to 25–30 mm but fig 4 in Baayen *et al*. (2002) shows a variation from 16 to 33 mm.
[‡]Data from ANSES (Hubert, pers. comm., 2019).
[§]It should be noted that not all *P. citricarpa* isolates produce a yellow pigment.

4. Identification

Identification of *P. citricarpa* can be performed by sequencing. TEF1 sequencing (see PM 7/129 Appendix 3) will allow unambiguous identification of *P. citricarpa* and especially its distinction from *P. paracitricarpa*.

Identification based on morphology is not recommended because the distinction between *P. citricarpa* and *P. paracitricarpa* based on morphological examination is very difficult. However, morphological examination may allow the exclusion of non-target species and information on morphology is provided in Appendix 6.

5. Reference material

Reference cultures can be obtained from the Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT Utrecht, NL.

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis* (EPPO, 2019).

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 that this database is consulted 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:

GCM van Leeuwen, National Reference Centre (NRC), Plant Protection Service, PO Box 9102, 6700 HC Wageningen, NL (e-mail: g.c.m.vanleeuwen@nvwa.nl).

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

Supporting information

For readers looking at a paper or pdf copy of this Standard please consult the html version to see the supporting information.

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This protocol was originally drafted by R.P. Baayen, Plant Protection Service, Wageningen (NL), P.J.M. Bonants, Plant Research International, PO Box 26, 6700 AA Wageningen (NL), G.C. Carroll, 1210 University of Oregon, Eugene, Oregon (USA). It was revised by J.P. Meffert and L.F.F. Kox (NPPO-NL). The alignment with the IPPC Diagnostic Protocol was prepared by A. Chandelier and the EPPO Secretariat. The further revision of this Standard was prepared by an Expert Working Group composed of G.C.M. van Leeuwen (NPPO, NL), A. Barnes (Fera, GB), E. Diogo (INIAV, PT), C. Douala-Meli (JKI, DE), K. Heungens (ILVO, BE), J. Hubert and R. Ioos (ANSES, FR), J. Pages (IAM-UPV, ES), L. Riccioni (CREA, IT) and A. Vicent (IVIA, ES); it was reviewed by the Panel on Diagnostics in Mycology.

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Appendix 1 – Media

Cherry decoction agar (CHA) recipe 1

Simmer 1 kg of cherries (without stones and petioles) in 1 L of boiling tap water for 2 h, filter and bottle. Autoclave bottles for 30 min. Separately sterilize 0.8 L of water and 20 g of microbiological-grade agar at 121°C for 15 min, add 0.2 L of the above cherry extract and mix well. Adjust (if necessary) pH to 4.5 and resterilize for 5 min at 102°C.

Cherry decoction agar (CHA) recipe 2

Agar	16.7 g
Cherry juice	111 mL
Distilled water	1 L

Filter the 111 mL of cherry juice through muslin and adjust the pH to 4.4 with KOH. Dissolve the agar thoroughly first, then add cherry juice. Autoclave at 102°C for 5 min.

Malt extract agar (MEA)

Commercially available (e.g. Difco). or

Malt extract	20.0 g
Microbiological-grade agar	15.0 g
Distilled water to	1 L

Potato dextrose agar (PDA)

Commercially available (e.g. Difco).

Oatmeal agar (OA), Crous *et al*., 2009

Commercially available (e.g. Difco), or

Agar	20 g
Oatmeal flakes	30 g
Distilled water	1 L

Wrap 30 g of oatmeal flakes in cloth and hang in a pan of tap water. Bring to the boil and simmer for 2 h. Squeeze and filter through cloth. Sterilize 15 min at 121°C. Add 20 g of microbiological-grade agar to 1 L of the oatmeal extract and sterilize for 15 min at 121°C.

Malt extract agar chloramphenicol (MALTCHL)

Malt extract	12.0 g
Chloramphenicol solution*	2.0 mL
Microbiological-grade agar	15.0 g
Distilled water to	1 L

*Chloramphenicol solution: 10.0 g chloramphenicol in 100 mL of ethanol (absolute), stored at 5°C.

Note: Chloramphenicol can be added before autoclaving the medium, since it is heat and pressure stable.

Appendix 2 – Conventional PCR (Bonants *et al.*, 2003)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This protocol was developed by Bonants *et al.* (2003).
1.2. Nucleic acid source: mycelium or plant tissue (dissected fruit lesions).
1.3. The test is designed to rDNA internal transcribed spacer (ITS) sequences.
1.4. Amplicon size: 490 bp.
1.5. Oligonucleotides:

Forward primer	GcF3	5′-AAA AAG CCG CCC GAC CTA CCT-3′
Reverse primer	GcR7	5′-TGT CCG GCG GCC AG-3′

1.6. Buffer: 10X PCR buffer containing 15 mM $MgCl_2$ (Roche).
1.7. Taq DNA polymerase (Roche).
1.8. Amplification is performed in thin-walled PCR tubes in a Peltier-type thermocycler with heated lid, e.g. PTC 200 (MJ Research).

2. Methods

2.1. Nucleic acid extraction and purification
i. From Gent-Pelzer *et al.* (2007): DNA is extracted from plugs of mycelium (approximately 0.5 cm in diameter) or fruit. Lesions are dissected from the peel by removing as much of the surrounding pith and peel tissue as possible. Mycelium plugs or lesions are cut into small pieces and placed in a 1.5-mL microcentrifuge tube with a secure fitting flattop cap (e.g. Superlock tubes, BIOzymTC) containing a stainless-steel bead (3.97 mm in diameter) and 125 µL of extraction buffer (0.02 M PBS, 0.5% Tween 20, 2% polyvinylpolypyrrolidone, 0.2% bovine serum albumin). The tube is placed in a bead mill (e.g. Mixer Mil MM 300, Retsch) for 80 s at 1800 beats per min. The mixture is centrifuged for 5 s at maximum speed in a microcentrifuge (16 100 × g) and 75 µL of the resulting supernatant is used for DNA extraction.
ii. DNA can be extracted using commercially available DNA extraction kits, [e.g. Puregene kit (Gentra)], according to the manufacturer's instructions. The final volume of the DNA solution is 50 µL.
iii. The DNA is further purified using spin columns filled with polyvinylpolypyrrolidone (PVPP [9]). The columns are prepared by filling Axygen Multi-Spin columns (Dispolab, Asten, the Netherlands) with 0.5 cm PVPP, placing it on an empty reaction tube and washing twice with 250 µL of molecular-grade water by centrifuging the column for 5 min at 4000 × g. The DNA suspension is applied to a PVPP column and centrifuged for 5 min at 4000 × g. The flow-through fraction is used as input for the PCR.
iv. Either use purified DNA immediately or store overnight at 4°C or –20°C for longer periods.
2.2. Polymerase chain reaction.
2.2.1. Master Mix (concentration by 25 µL of single reaction)

Reagent	Working concentration†	Volume per reaction (µL)	Final concentration
Molecular-grade water*	NA	14.3	NA
10× reaction buffer (Roche)‡	10×	2.5	1×
dNTPs	600 µM	2.5	60 µM
Primer GcF3	60 µM	0.25	0.6 µM
Primer GcR7	60 µM	0.25	0.6 µM
Taq DNA polymerase (Roche)	5 U/µL	0.20	1 U
Subtotal		20.0	
DNA suspension		5.0	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.
†These figures are indicative. They can be modified provided that the final concentration in the PCR reaction is respected.
‡The buffer contains 15 mM $MgCl_2$. If a buffer without $MgCl_2$ is used, the final concentration of $MgCl_2$ should be 1.5 mM.

2.2.2. PCR conditions
The PCR reaction conditions include an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation 30 s at 94°C, annealing 30 s at 65°C and elongation for 1 min at 72°C. A final elongation is carried out at 72°C for 10 min.

3. Essential procedural information

3.1. Controls
For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid 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 of a sample of uninfected host tissue or clear extraction buffer or water.

[9] Incorrectly referred to as PVP in ISPM 27 Annex 5.

- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quality and quantity 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 PCR-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 (cloned PCR product). The PAC should preferably be near the limit of detection of the method.

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately for PCR inhibition by amplification of a duplicate sample spiked with the target nucleic acid. Alternatively, the internal control developed from heterologous *Lolium perenne* DNA can be obtained from P.J.M. Bonants, Plant Research International, Wageningen, NL. This control uses the same primers as those used for the amplification of the target (GcF3 and GcR7).

3.2. Interpretation of results

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 and PAC (and IPC if used*): should produce a band of the expected size 490 bp.

When these conditions are met

- A test will be considered positive if a band of 490 bp is visualized. Note that the test will amplify *P. citricarpa*, *P. citriasiana* or *P. paracitricarpa* DNA. *P. citricarpa* and *P. paracitricarpa* can be distinguished from *P. citriasiana* either by sequencing of the amplification product generated with primers GcR3/GcR7 and comparisons with accession numbers corresponding to both species (JF343584.1 for *P. citricarpa* and FJ538362.1 for *P. citriasiana*) or by performing a real-time PCR (Appendix 4) or LAMP test (Appendix 5).
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

*If the internal control developed from heterologous *Lolium perenne* DNA is used, the amplification size should be 230 bp.

4. Performance characteristics available

4.1. Analytical sensitivity

The analytical sensitivity (detection limit) indicated in Bonants *et al*. (2003) is 1 pg of DNA per reaction. Experience in CREA (IT) shows that analytical sensitivity is 20 pg of DNA per reaction (L. Riccioni, pers. comm., 2019).

4.2. Analytical specificity

Inclusivity: was evaluated with 37 isolates of *P. citricarpa* from different hosts and origins.

The exclusivity of the test was assessed by Bonants *et al*. (2003) using *P. citricarpa*, *P. capitalensis*, *P. citriasiana* and the citrus pathogens *Alternaria alternata*, *Colletotrichum acutatum*, *C. gloeosporioides*, *Diaporthe citri*, *Zasmidium citri-griseum* and *Penicillium digitatum*.

Further evaluation of exclusivity was performed subsequently with *P. citriasiana* and *P. paracitricarpa.* Both species gave a positive reaction.t

Appendix 3 – Conventional PCR according to Peres *et al.* (2007)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This protocol was developed by Peres *et al*. (2007).

1.2. Nucleic acid source: mycelium or plant tissue (dissected fruit lesions).

1.3. The target sequence is located in the rDNA internal transcribed spacer (ITS) sequences.

1.4. Amplicon size: 300 bp.

1.5. Oligonucleotides used:

Forward primer	GCN	5′-CTG AAA GGT GAT GGA AGG GAG G-3′
Reverse primer	GCMR	5′-CAT TAC TTA TCG CAT TTC GCT GC-3′

1.6. Amplification is performed in thin-walled PCR tubes in a Peltier-type thermocycler with heated lid, e.g. PTC 200 (MJ Research).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. DNA is extracted from mycelium or fruit lesions. Mycelium could be recovered from liquid or solid cultures.

2.1.2. Total DNA from mycelium is extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

Fruit lesions are dissected from the peel, removing as much of the surrounding pith and peel tissue as possible. For DNA extraction, alkaline lysis protocol (Klimyuk *et al*.,

1993) can be used, followed by purification using a dipstick method, which has proven to be the most effective (Peres *et al*., 2007).

However, other DNA extraction protocols may be used providing that they are proved to yield total DNA at least equivalent with at least similar quality and quantity.

2.1.3. Either use purified DNA immediately or store overnight at 4°C or −20°C for longer periods.

2.2. Conventional PCR

2.2.1. Master Mix

The conditions (below) are as recommended in Peres *et al*. (2007). Other Master Mixes (not indicated in this Diagnostic Protocol) have given similar results.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	NA	8.4	NA
Master Mix (Eppendorf)	2.5×	8	1×
Forward primer (GCN)	10 µM	0.8	0.4 µM
Reverse primer (GCMR)	10 µM	0.8	0.4 µM
Subtotal		18	
Template DNA		2	
Total		20	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.2.2. PCR conditions

The PCR reaction conditions include an initial denaturation at 94°C for 2 min, followed by 39 cycles of denaturation 30 s at 94°C, annealing 30 s at 64°C and elongation 1 min at 72°C. A final elongation is carried out at 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or if not available clean extraction buffer or water.
- 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 PCRs not performed on isolated organism, the PAC should preferably be near to the limit of detection (LOD) of the method.

As an 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. Positive internal controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA, ITS, etc.)
- 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) 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 the results

To assign results from the PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC (and IC if used) should produce a band of the expected size 300 bp.

When these conditions are met

- A test will be considered positive if amplicons of 300 bp are produced. Note that the test will amplify *P. citricarpa*, *P. citriasiana* or *P. paracitricarpa* DNA. *P. citricarpa* and *P. paracitricarpa* can be distinguished from *P. citriasiana*: either by sequencing of the amplification product generated with primers GCN/GCMR and comparisons with accession numbers corresponding to both species (JF343584.1 for *P. citricarpa* and FJ538362.1 for *P. citriasiana*) or by performing a real-time PCR (Appendix 4) or LAMP test (Appendix 5).
- A test will be considered negative if it produces no band or a band of different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1. Analytical sensitivity

The analytical sensitivity (detection limit) is 2 pg of DNA per reaction.

4.2. Analytical specificity

Inclusivity: evaluated with 30 isolates of *P. citricarpa* from Brazil.

Exclusivity: assessed using isolates of *P. capitalensis* and some common citrus pathogens, including *Alternaria alternata*, *Colletotrichum acutatum*, *C. gloeosporioides*, *Diaporthe citri*, *Mycosphaerella citri* and *Penicillium digitatum*.

Further evaluation of exclusivity was performed subsequently with *P. citriasiana* and *P. paracitricarpa.* Both species gave a positive reaction.

Appendix 4 – Identification of Phyllosticta citricarpa by real-time PCR (van Gent-Pelzer *et al.,* 2007)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. Protocol developed by van Gent-Pelzer *et al.* (2007).

1.2. Nucleic acid source: mycelium or dissected fruit lesions.

1.3. The test is designed to internal transcribed spacer (ITS) sequences.

1.4. Amplicon size: 69 bp.

1.5. Oligonucleotides:

Forward primer	GcF1	5′-GGT GAT GGA AGG GAG GCC T-3′
Reverse primer	GcR1	5′-GCA ACA TGG TAG ATA CAC AAG GGT-3′
Taqman probe	GcP1	5′- AAA AAG CCG CCC GAC CTA CCT TCA-3′ FAM label and TAMRA or Eclipse Dark quencher (Eurogentec)

1.6. 2× Premix Ex Taq (Takara) containing Taq polymerase, reaction buffer containing $MgCl_2$ and nucleotides (Applied Biosystems) is used for PCR.

1.7. ROX reference Dye (50× concentrated; Takara) is added to Premix Ex Taq if applicable.

1.8. Amplification is performed using a real-time PCR thermal cycler, e.g. 7700 Sequence Detector (Applied Biosystems).

A recent real-time PCR test that can differentiate between *P. citricarpa* and *P. citriasiana* was developed by Schirmacher *et al*. (2019) on the basis of the test by van Gent-Pelzer *et al*. (2007).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. DNA is extracted from plugs of mycelium (approximately 1 cm in diameter) or fruit lesions, as described in Appendix 2.

2.1.2. DNA can be extracted using commercially available DNA extraction kits [e.g. Puregene kit (Gentra) or QuickPick Plant DNA kit (Bionobile)], according to the manufacturer's instructions. The final volume of the DNA solution is 50 µL.

2.1.3. DNA purification using spin columns filled with polyvinylpolypyrrolidone (PVPP) is necessary for DNA isolated using the Puregene kit. The procedure is described in Appendix 2. For DNA isolated using the QuickPick kit no DNA purification is necessary.

2.1.4. Either use extracted DNA immediately or store overnight at 4°C or –20°C for longer periods.

2.2. Polymerase chain reaction

2.2.1. Master Mix (concentration per 30 µL single reaction).

Reagent	Working concentration[†]	Volume per reaction (µL)	Final concentration
Molecular-grade water*	NA	13.1	NA
2× Premix Ex Taq Master Mix (Takara)[‡]	2×	15.0	1×
Primer GcF1	50 µM	0.15	0.25 µM
Primer GcR1	50 µM	0.15	0.25 µM
Probe GcP1	5 µM	0.6	0.10 µM
Subtotal		29.0	
DNA		1.0	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

[†]These figures are indicative. They can be modified provided that the final concentration in the PCR reaction is respected.

[‡]0.6 µL of 50× ROX Reference Dye can be added if applicable; in that case, 12.5 µL of molecular-grade water is used

2.2.2. PCR conditions

The real-time PCR conditions include an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 15 s at 95°C and 1 min at 60°C, respectively. The Ct value for each reaction is determined using the software provided with the thermocycler.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following 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 extrac-

tion and subsequent amplification of a sample of uninfected host tissue or clear extraction buffer or water
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quality and quantity 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 PCR-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 organisms, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (cloned PCR product). The PAC should preferably be near the limit of detection of the method.

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately for PCR inhibition by amplification of a duplicate sample spiked with the target nucleic acid.

Alternatively, an internal amplification control (IAC; 12.5 fg), 75 nM of IAC forward primer FIAC (5′-TGG CCC TGT CCT TTT ACC AG-3′), 75 nM of IAC reverse primer RIAC (5′-TTT TCG TTG GGA TCT TTC GAA-3′), 50 nM of IAC MGB TaqMan probe (5′-ACA CAA TCT GCC-3′), VIC label and quencher dye Eclipse Dark Quencher can be added to the reaction mixes. The target DNA of IAC is a green fluorescent protein (GFP) construct in *Escherichia coli* containing plasmid DNA and genomic *E. coli* DNA (Klerks *et al*., 2004). It can be obtained from P.J.M. Bonants, Plant Research International, Wageningen, NL.

3.2. Interpretation of the results

To assign results from PCR-based test the following criteria should be followed:

Verification of the controls
- The PIC and PAC (see *Note 1*) 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 (see *Note 2*).
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note 1: If a PAC at the limit of detection (PAC-LOD) is used, plant samples whose DNA extract yield a Ct lower or equal to $Ct_{PAC\text{-}LOD}$ should be considered as infected by *P. citricarpa*, whereas samples whose DNA extract yields $Ct > Ct_{PAC\text{-}LOD}$ should be considered as negative only providing that the DNA extract was amplifiable and that there was no significant inhibitory effect. Doubtful or borderline results should be re-analyzed using the same or another technique (e.g. sequencing).

Note 2: It is stated in ISPM 27 (Annex 5) that there is no cross-reaction with *P. citriasiana*; however, two laboratories in the EPPO region have noted cross-reaction when performing this test on *Citrus maxima*. Migration of the product on an agarose gel allows the distinction of the two species. The size of the amplicon is 69 bp for *P. citricarpa* and 229 bp for *P. citriasiana*.

4. Performance characteristics available

4.1. Analytical sensitivity data

The analytical sensitivity (detection limit) is 10 fg DNA per reaction.

4.2. Analytical specificity data

Inclusivity: Not evaluated – only one isolate used in the study van Gent-Pelzer *et al*. (2007). However, this test is widely used in the region and has produced a number of positive results with other isolates.

The exclusivity of the test was assessed with *P. capitalensis*, *P. citriasiana*, *Guignardia bidwellii* and 14 other citrus pathogens (*Alternaria* spp., *Penicillium* spp., *Colletotrichum* spp. and *Phyllosticta artocarpina*). Cross-reaction with *P. citriasiana* has been noted in two laboratories in the EPPO region when the test was performed on *Citrus maxima.*

In addition, isolates of *P. citrimaxima* (one isolate), *P. citribraziliensis* (one isolate), and *P. citrichinaensis* (two isolates) were tested in the National Reference Centre in Wageningen, and no cross-reaction was observed (unpublished data). Testing by Anses (FR) revealed that DNA from the newly described species *P. paracitricarpa* (Guarnaccia *et al*., 2017) provided positive results.

4.3. Diagnostic sensitivity

Diagnostic sensitivity is 100%.

Appendix 5 – LAMP test (Tomlinson *et al.*, 2013, modified)

1. General Information

1.1. The LAMP test described in this section is performed to detect *Phyllosticta citricarpa* on fruit tissues.

1.2. The test was developed by Fera Science Ltd (Tomlinson *et al*., 2013) and additional validation was provided by CREA-PAV through a test performance study. It can be used on-site or for laboratory testing.

1.3. The test targets the internal transcribed spacer (ITS) region of the rDNA gene.

1.4. LAMP primers were designed by studying available *P. citricarpa* ITS sequences from NCBI GenBank, as well as sequences for related non-target species.

1.5. Oligonucleotides from Tomlinson *et al*. (2013).

Forward primer	GcF3	GGTTTTGACCCGGGCGG
Reverse primer	Gc B3	CGATGATTCACTGAATTCTGCAA
Forward primer	Gc FIP	AATAATCGCTGGAGTTTTGTATACTGGCGCCCMCAGYCTAGTCTC
Reverse primer	Gc BIP	CTGTGTAGTCCTGAGAATTCATTTAATGTTTCGCTGCGTTCTTCATCG
Forward primer	GcF-loop	CCAGGCGTCCTGGCCTA
Reverse primer	Gc B-loop	AATAAAACTTTCAACAACGGATCTC

1.6. The test can be performed using the Genie II instrument for real-time LAMP (OptiGene Ltd) or a qPCR platform.

2. Methods

2.1. No DNA extraction needed, the homogenized sample can be tested directly.

2.2. Process for sample preparation is performed using the extraction kit by OptiGene Ltd (Plant Material Lysis Kit) with some modifications:

2.2.1. Cut a piece of suspected tissue 2/3 × 2/3 mm from the surface of the fruit.

2.2.2. Place into a 1.5 mL microcentrifuge tube containing stainless-steel beads 3 mm diameter and add 100 µL of lysis buffer then cap securely.

2.2.3. Shake manually for 20–30 s so that the ball bearings slightly break up the tissue.

2.2.4. Transfer 10 µL of extract into a vial of dilution buffer, cap securely and mix.

2.2.5. 5 µL of the prepared plant material in the dilution buffer can be used directly as template in a LAMP test

2.3. LAMP

2.3.1. The reaction is carried out with the Isothermal Master mix ISO-004 and *P. citricarpa*-specific primer mix from Tomlinson *et al*. (2013).

2.3.2. Set up reactions as shown in the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Isothermal Master Mix ISO-004	NA	15	1×
GcFIP + GcBIP	25 µM each	2	2 µM
GcF3 + GcB3	5 µM each	1	0.2 µM
GcF-loop + GcB-loop	12.5 µM each	2	1 µM
Subtotal		20	
DNA template		5	
Total		25	

2.3.3. LAMP amplification conditions:

2.3.3.1. for *genie* users: pre-heat: none; 65°C for 20 min; melting curve analysis: 95–75°C, with ramp rate of 0.05°C per second.

2.3.3.2. For real-time PCR platform users: 20 cycles at 65°C for 1 min with acquisition of fluorescence (FAM channel) at the end of each cycle; melting curve analysis: 95–75°C, with ramp rate of 0.1°C per second; final hold: 4°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.
- 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. This can include nucleic acid extracted from the target organism *P. citricarpa* in culture or in infected material, or synthetic control [e.g. a cloned PCR product or commercially available control, e.g. cat. no CD-GCIT-050 (OptiGene)].

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. A positive internal control can be the 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) or amplification of a duplicate sample spiked with the target nucleic acid. In the case of detection of *P. citricarpa* in an infected host, the pathogen-specific test can be used in conjunction with a test to detect DNA from the host plant, such as the plant cytochrome oxidase (COX) test described by Tomlinson *et al*. (2010) or the LAMP plant control test kit available from OptiGene Ltd (cat. no. PK-COX-050W).

3.2. Interpretation of results

Verification of controls

- NAC (and if relevant NIC) should produce no fluorescence.
- The PAC (and if relevant PIC) amplification curve should be exponential. The T_m (melting temperature) should be

in the range 84–87°C in a GENIE instrument, depending on the starting material from which DNA is extracted (mycelium, conidia, fruit lesions).

When these conditions are met

- A test will be considered positive if it produces a positive reaction as defined for PAC (see above).
- A test will be considered negative if it produces no fluorescence. (Note that if an IPC is used, the results obtained will allow the identification of false negative *P. citricarpa* results caused by suboptimal processing, e.g. sample too small or too large.)
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test was validated on fruit tissues using a Genie II instrument for real-time LAMP (OptiGene Ltd) and a q-PCR platform. The primer mix was prepared as described by Tomlinson *et al*. (2013) using the plant material Lysis Kit for DNA extraction from tissue and the Isothermal Master mix ISO-004 (Optigen Ltd).

4.1. Analytical sensitivity data

During the validation performed by CREA-DC the analytical sensitivity from tissue was determined evaluating the minimum quantity of *P. citricarpa* conidia in healthy orange peel from which a detectable amount of target DNA can be detected. Serial dilutions of conidial suspensions were analysed and the analytical sensitivity was 500 conidia per reaction.

4.2. Analytical specificity

Inclusivity was evaluated on DNA of four different isolates of *P. citricarpa.* All isolates were detected.

The LAMP test is observed to produce false positive results in the presence of very high concentrations of *P. citriasiana* DNA (>50 ng per reaction). However, such high levels cannot be obtained by the extraction method from tissue described above.

The exclusivity was evaluated on DNA of isolates of *P. capitalensis* (two), *P. citriasiana, Phomopsis citri*, *Colletotrichum gloeosporioides*, *Alternaria* sp., *Mycosphaerella* sp. and DNA of sweet orange, clementine, grapefruit and lemon. No cross-reactions were observed in the experimental conditions described above.

Cross-reaction with *P. paracitricarpa* has not been evaluated but is expected to happen as the DNA target region of the test is identical for the two species.

Cross-reaction with *P. citrichinaensis* and *P. citribraziliensis* has not been evaluated but is expected not to happen as the DNA target region contained several mismatches within the primer binding sites (Tomlinson *et al*., 2013).

4.3. Data on repeatability and reproducibility

Test performance study with the participation of four laboratories organized by CREA-DC of Rome, Italy using the test to analyse sweet orange samples (fragments of sweet orange peel of about 2–3 mm^2) spiked with known levels of target organism (conidia), including positive and negative controls (eight replicates for each sample):

(i) a negative control (containing only *P. citricarpa*-free fruit samples)
(ii) a contamination level slightly above the relative limit of detection (1000–2000 conidia)
(iii) a contamination level equal to 10 times the relative limit of detection (10 000 conidia)
(iv) in addition, a 'specificity' (negative) control has been added to assess the specificity of the protocol: fruit samples artificially contaminated with *P. citriasiana* (40 000 conidia), a species phylogenetically or morphologically close to the target. Each sample is analysed in duplicate.

Results: 92% of repeatability and 91% of reproducibility.

Appendix 6 – Information on morphology

In addition to the colony features described in Section 3.4.2, discrimination of *P. citricarpa* or *P. paracitricarpa* from other *Phyllosticta* species may be possible by studying the following features when these are observable:

(a) the thickness of the mucoid sheath surrounding the conidia (Fig. 10A)
(b) the length of the conidial appendage (Fig. 10B).

Conidial size will give no resolution between the four species mentioned in Table 1.

It should be noted that preparing the conidial suspension with water makes the mucoid sheath swollen or thicken and this can lead to misidentifications (J. Hubert, pers. comm., 2020)

Distinction between *P. citricarpa*, *P. paracitricarpa*, *P. citriasiana* and *P. capitalensis* based on morphology is very difficult

Jin (2011) recommends using phase contrast and differential interference contrast optics to observe clearly appendices and sheath. However, when microscopes do not have these options it is possible to use black India ink to provide a negative image (the ink will not cross the sheath). It should be noted, however, that it will not be possible to measure the width of the sheath, and that the appendices will sometimes remain inconspicuous.

Published data on the morphology of *P. citricarpa* vary considerably, partly because of the confusion about the identity of several other *Phyllosticta* species associated with *Citrus* (Baayen *et al*., 2002; Wulandari *et al*., 2009; Glienke *et al*., 2011; Wang *et al*., 2012). The following morphological and morphometric characteristics refer to fructifications and spores of *P. citricarpa* produced mainly in culture; they are based on data from Sutton & Waterston (1966) and van der Aa (1973), as revised and amended by Baayen *et al*. (2002).

Ascocarps. Pseudothecia are formed on leaf litter and in culture (De Holanda Nozaki, 2007) but not on any other plant material (e.g. attached leaves, fruit). They are solitary or aggregated, globose to pyriform, immersed, dark-brown

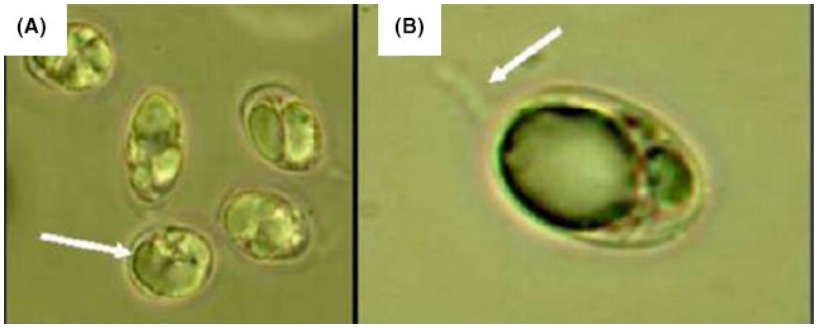

Fig. 12 Conidial morphology of *P. citricarpa*: (A) and (B) conidia with a thin mucoid sheath (A, arrow) and a colourless subulate appendage (B, arrow, magnification 1000 × with immersion oil). Photographs courtesy of L.E. Diaz, Ministry of Husbandry, Agriculture and Fisheries, Montevideo, Uruguay.

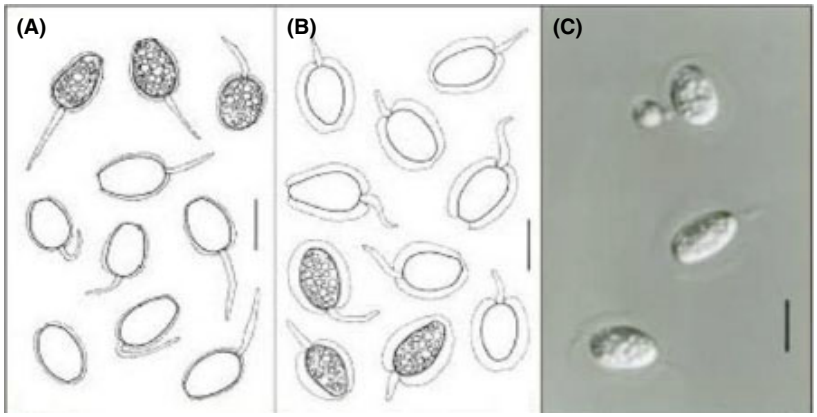

Fig. 13 Conidial morphology of *P. citricarpa* and *P. capitalensis*: (A) conidia of *P. citricarpa* with thin (<1.5 µm) mucoid sheaths; (B) and (C) conidia of *P. capitalensis* with thick (>1.5 µm) mucoid sheaths (scale bar = 10 µm) (photograph C was taken under a light microscope equipped with differential interference contrast). Photographs courtesy of G. Verkley, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands.

to black, 125–360 µm, with a single papillate to rostrate ostiole, and their surface is often covered with irregular hyphal outgrowths. The outer wall layer is composed of angular cells with brown thickened walls, whereas the inner layer is composed of angular to globose cells with thinner colourless walls.

Asci. Fasciculate, bitunicate, clavate, eight-spored with a rounded apex. Their dimensions are 40–65 × 12–15 µm before the rupture of the outer wall, and they become cylindrical-clavate and extend in length to 120–150 µm prior to dehiscence.

Ascospores. Short, aseptate, hyaline, cylindrical, swollen in the middle, slightly curved, 12–16 × 4.5–6.5 µm, heteropolar with unequal obtuse ends. The smaller upper end has a truncate, non-cellular, mucoid, 1–2 µm long, cap-like appendage, and the lower end has an acute or ruffled, 3–6 µm long appendage.

Pycnidia. Produced on fruit, attached leaves, twigs (although more pycnidia are produced on dead twigs) and leaf litter as well as in culture. They are solitary or occasionally aggregated, globose, immersed, mid- to dark-brown and 70–330 µm in diameter. The pycnidial wall is up to four cells thick, sclerotioid on the outside, pseudoparenchymatous within, with ostiole darker, slightly papillate, circular and 10–15 µm in diameter.

Conidia. Obovate to elliptical, hyaline, aseptate, multiguttulate, 9.4–12.7 × 5.0–8.5 µm, with a colourless, subulate appendage and a barely visible, colourless, gelatinous sheath <1.5 µm thick (Figs 10C and 11A). They are formed as blastospores from hyaline, unicellular, cylindrical, up to 9 µm long conidiogenous cells.

Spermatial state. Described in the form genus *Leptodothiorella*, formed both on hosts and in pure culture. Spermatia dumbbell-shaped, rarely cylindrical, straight or slightly curved, 5–8 × 0.5–1 µm.

If the cultural characteristics of the colonies are consistent with those of *Phyllosticta* spp., sequencing should be performed (see Section 4.2).

If the cultural characteristics of the colonies are not consistent with those of *Phyllosticta* spp. then the plant material is considered free from the pests.

Isolation and culturing of the organism on appropriate media is a time-consuming procedure and thus undesirable in time-critical diagnosis of consignments.

12.1.1. Possible confusion with other species: wInformation on characteristics of *P. citricarpa* and its related species are given in Table 2. Distinction between *P. citricarpa*, *P. paracitricarpa*, *P. citriasiana* and *P. capitalensis* based on morphology is very difficult.

P. citrichinaensis can be differentiated from *P. citricarpa* by its longer conidial appendage, 14–26 µm (Wang *et al*., 2012).

It should be noted that occasionally acervuli of the common endophytic fungi *Colletotrichum* spp. may be present and may look similar to pycnidia of *P. citricarpa*. However, *Colletotrichum* spp. can be differentiated by the presence of setae in their acervuli, the production under humid conditions of pink or salmon-coloured masses of conidia on the surface of the lesions and the morphology of their conidia (Kotzé, 2000).

Table 2. Main morphological characteristics of *P. citricarpa*, *P. citriasiana*, *P. capitalensis* and *P. paracitricarpa* (data from Baayen *et al*. (2002), Wulandari *et al*. (2009) and Guarnaccia *et al*. (2017)

Characteristic	*P. citricarpa*	*P. citriasiana*	*P. capitalensis*	*P. paracitricarpa*
Average conidia size (µm)	10–12 × 6–7.5	12–14 × 6–7	11–12 × 6.5–7.5	11–13 × 7–8
Mucoid sheath width (µm)	<1.5	1	1.5–2.5 (–3)	1–1.5
Apical appendage length (µm)	4–6 (–10)	7–10 (–14)	4–6 (–10)	(8-)10–12 (–15)
Average ascospore size (µm)	12–16 × 4.5–6.5	Unknown	15–17.5 × 6.5–7.5	Unknown
Average spermatia size (µm)	5–8 × 0.5–1	3–5 × 1–2	7–10 × 1.8–2.5	

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CORRIGENDUM

PM 7/017 (3) *Phyllosticta citricarpa* (formerly *Guignardia citricarpa*)

It has been brought to our attention that there is an error in the Acknowledgments section of the protocol on *Phyllosticta citricarpa* (formerly *Guignardia citricarpa*) (EPPO, 2020).

In the Acknowledgments section the name J. Pages should be replaced by J. Armengol Forti (IAM-UPV, ES).

The EPPO Secretariat would like to apologise for this error.

REFERENCE

EPPO (2020) PM 7/017 (3) *Phyllosticta citricarpa* (formerly *Guignardia citricarpa*). *EPPO Bulletin* 50, 440–461.

