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INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27 DIAGNOSTIC PROTOCOLS

DP 5: *Phyllosticta citricarpa* (McAlpine) Aa on fruit

(2014)

CONTENTS

1. Pest Information

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 *Citrus aurantium* and its hybrids and *Citrus latifolia*, all commercially grown *Citrus* species are susceptible (Aguilar-Vildoso *et al.,* 2002; Kotzé, 2000). *Citrus 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).

Citrus black spot was first recorded in Australia in 1895 on *Citrus sinensis* (Benson, 1895). It 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). The organism has not been reported from Europe, Central America or the Caribbean region (CABI, 2011; CABI/EPPO, 1998; EPPO/CABI, 1997; 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 pest 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). In areas where rain is confined to a single season, pseudothecia with ascospores, produced exclusively on leaf litter, are the main source of inoculum. Where rain is not confined to a single season, where out-of-season fruit with lesions remains on the trees after flowering and fruit set, or where successive and irregular flowering occurs in the cultivated citrus species and varieties, pycnidia with conidia of *P. citricarpa* are also important as inoculum sources (Kotzé, 1981; Spósito *et al*., 2008, 2011).

Pseudothecia develop 40–180 days after leaf drop, depending on the frequency of wetting and drying as well as on the prevailing temperatures (Kotzé, 1981). Citrus leaves drop all year round in some countries and seasonally in others, and this affects the availability of inoculum. 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 takes place during rainfall and occasionally during irrigation or when there is heavy dew (Kiely, 1949a; Kotzé, 2000). Ascospore discharges are closely influenced by the rainfall pattern (Kotzé, 1981). Ascospores are forcibly released up to a height of 1.2 cm above pseudothecia and are carried by air currents throughout the canopy and over long distances (Kiely, 1949a). The critical period for infection starts at fruit set and lasts 4–6 months, but the first symptoms on fruit do not appear until more than 6 months after fruit set (Baldassari *et al*., 2006). In Brazil, fruit of *C. sinensis* "Valencia" and "Natal" varieties are susceptible until at least 24 weeks after the fall of 75% of the petals, when they are 5–6 cm in diameter (Baldassari *et al*., 2006).

After infection, the fungus remains in a quiescent state until the fruit becomes fully grown or mature, with symptoms becoming apparent many months after infection has taken place (Kotzé, 2000). Leaves remain susceptible to infection from development up to 10 months of age (Truter *et al.*, 2007).

Pycnidia with conidia are produced on fruit, leaves, dead twigs, fruit pedicels and in abundance on leaf litter (Kotzé, 2000). They may be splash-dispersed onto the canopy or washed off infected late-hanging fruit onto younger fruit and leaves that are still at the susceptible stage (Agostini *et al*., 2006; Spósito *et al*., 2008). *P. citricarpa* also has a microconidial asexual state, described in the genus *Leptodothiorella* (Kiely, 1949a). This microconidial state, also referred to as the "spermogonial" state (Kiely, 1949a), usually appears on fallen leaves before pseudothecia develop. However, the role of microconidia in the biology of *P. citricarpa* is still unclear.

Symptom development on mature fruit is enhanced by rising temperature, high light intensity, drought and poor tree vigour. Older trees usually have more citrus black spot than younger trees (Kotzé, 2000). The spread of *P. citricarpa* to new areas is assumed to have taken place through infected nursery stock or other planting material rather than through citrus fruit (Kotzé, 2000; Timmer, 2004).

It should be noted that in symptomless citrus fruit or fruit with very small spots (<2 mm in diameter) without pycnidia, the non-pathogenic endophyte *Phyllosticta capitalensis* Henn (formerly incorrectly referred to as *Guignardia mangiferae* A.J. Roy) (Glienke *et al*., 2011), recorded in many plant families, may be present. The cultural, morphological and molecular characteristics that differentiate *P. capitalensis* from *P. citricarpa* have been described by Baayen *et al.* (2002). Furthermore, symptoms of *P. citricarpa* may be confused with those caused by *Phyllosticta citriasiana* Wulandari, Crous & Gruyter, a newly described pathogen that has so far been found only on *Citrus maxima* (Wang *et al*., 2012; Wulandari *et al*., 2009). The pathogenicity of *P. citriasiana* to other *Citrus* species is unknown. The cultural, morphological and molecular characteristics that differentiate *P. citriasiana* from *P. citricarpa*, the species pathogenic to citrus, have been described by Wulandari *et al.* (2009). Two *Phyllosticta* species have recently been described associated with *Citrus* spp. *Phyllosticta citrichinaensis* causes small sunken grey–brown spots with a dark brown margin and olive green halos on pomelo leaves. The pathogen also induces small brown to black spots similar to melanose on mandarin and orange fruits (Wang *et al.*, 2012). *P. citribraziliensis* has been found as an endophyte in healthy leaves of *Citrus* spp. in Brazil (Glienke *et al.*, 2011).

2. Taxonomic Information

Name:	*Phyllosticta citricarpa* (McAlpine) Aa, 1973
Synonyms:	*Phoma citricarpa* McAlpine, 1899
	Guignardia citricarpa Kiely, 1948
	Phyllostictina citricarpa (McAlpine) Petr., 1953
	Leptodothiorella sp. (spermatial state)
Taxonomic position:	Eukaryota, Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Botryosphaeriales, Botryosphaeriaceae
Common names:	Citrus black spot (for common names in other languages, see CABI (2011))
Reference:	MycoBank 320327

3. Detection

Fruit, pedicels, leaves and twigs of *Citrus*, *Poncirus* and *Fortunella* and their hybrids may potentially harbour *P. citricarpa* (CABI, 2011).

3.1 Symptoms on fruit

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 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 (Kotzé, 2000; Snowdon, 1990; L. Diaz, personal communication). The following four symptoms are widely recognized as described by Kiely (1949a, 1949b, 1960).

Hard spot. The most typical symptom of citrus black spot, consisting of shallow lesions, 3–10 mm in diameter, with a grey to tan centre and a dark brown to black margin (Figure 1A). 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 (Figure 1a) 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 (Figure 1B). The spots turn brown with age and are almost always devoid of pycnidia (Figure 1b). 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) (Figure 1C). Individual freckle spots may coalesce to form larger lesions that turn into virulent spots (Figure 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) (Figures 2A, 2a, 2B). 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 (FUNDECITRUS, 2005).

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 (Figure 2C). 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 four to five days. It is the most damaging symptom, because, unlike the other symptoms, it extends deeply into the mesocarp (albedo), occasionally involving the entire thickness of the rind, causing premature fruit drop and serious post-harvest losses (Kotzé, 1981).

Two 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) (Figure 2D). 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. Fruits showing lacy spot usually appear to be aggregated in the tree canopy (M. Spósito, personal communication).

Cracked spot. Superficial slightly raised dark brown to black lesions, variable in size, with a cracked surface and irregular margins (Goes *et al*., 2000) (Figure 2E). The lesions are devoid of pycnidia and appear on fruit older than six months. This symptom has been associated with the presence of *Phyllocoptruta oleivora* Ashmead (FUNDECITRUS, 2005; Spósito, 2003).

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 (Figure 1C, 1c).

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.

3.2 Symptoms on leaves and twigs

Citrus black spot usually occurs on leaves as quiescent infection without visible symptoms (Sutton and 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 in colour surrounded by a dark brown to black margin and a yellow halo (Kotzé, 2000) (Figure 3A). Pycnidia may occasionally be present in the centre of the lesions on the adaxial leaf surface.

Lesions similar to those on leaves may also occur on small twigs, more commonly on *C. limon* than on other citrus species (M. Truter, personal communication). Symptoms are small (0.5–2 mm in diameter) round slightly sunken lesions with a brown to black margin and a grey to light brown centre (Figure 3B). Pycnidia may occasionally be present in the centre of the lesions.

3.3 Comparison of citrus black spot symptoms with those caused by other organisms or abiotic factors

Symptoms on fruit are variable in appearance and often resemble those caused by other citrus pathogens (such as *P. citriasiana, P. citrichinaensis, Diaporthe citri*, *Mycosphaerella citri*, *Alternaria alternata* pv. *citri*, *Septoria* spp., *Colletotrichum* spp.) or by insect, mechanical or cold damage, particularly in the case of freckle spot (Bonants *et al*., 2003; Snowdon, 1990; Wang *et al*., 2012; Wulandari *et al*., 2009; L. Diaz, personal communication).

As the symptoms caused by *P. citricarpa* on citrus fruit are similar to those caused by other pathogens, reliable diagnosis can be made only by using the methods described below.

4. Identification

This protocol describes the detection and identification of *P. citricarpa* on symptomatic citrus fruit. Citrus fruit should be inspected for any symptoms typical of citrus black spot (see section 3). 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 and the morphological characteristics of the pycnidia and conidia are consistent with those in section 4.1.3, *P citricarpa* may be present. However, as the pycnidia and conidia of *P. citricarpa* are very similar to those of *P. citriasiana,* the recently described pathogen on *C. maxima* (Wulandari *et al.*, 2009), the identity of *P. citricarpa* can only be confirmed with certainty by applying the diagnostic methods described below (Figure 4). Diagnostic Method A (isolation and culturing) is used for the identification of *P. citricarpa* on citrus fruit, but can also be used on leaves, twigs and pedicels, whereas Method B (molecular assay) applies to citrus fruit only.

If after applying Method A the cultural characteristics of the colonies grown on cherry decoction agar (CHA) and oatmeal agar (OA) media are not consistent with those of *P. citricarpa* (see section 4.1.4, requirements (i), (ii), (iii) and (iv)) then the plant material is considered free of *P. citricarpa.* On *P. citricarpa*-like cultures that do not produce mature pycnidia within 14 days, application of conventional polymerase chain reaction (PCR) and internal transcribed spacer (ITS) sequencing (see section 4.2.1) or real-time PCR (see section 4.2.2) is recommended. However, isolation and culturing of the organism on appropriate media followed by a direct molecular test of the cultures is a time-consuming procedure and thus undesirable in time-critical diagnosis of consignments.

There are two PCR methods (conventional and real-time) available for the detection and identification of *P. citricarpa* on citrus fruit (see sections 4.2.1, 4.2.2). However, it has been recently observed during routine testing of *C. maxima* fruit showing typical symptoms that the real-time PCR method developed by Gent-Pelzer *et al*. (2007) gives no amplification (J.P. Meffert, personal communication). The reason is that the citrus black spot-like symptoms on *C. maxima* are caused by *P. citriasiana*, a newly described species closely related to *P. citricarpa* (Wulandari *et al.*, 2009). As it is not clear whether *P. citricarpa* is able to cause typical symptoms on *C. maxima*, fruit of this *Citrus* species showing citrus black spot-like symptoms should also be tested for the presence of *P. citricarpa.*

The real-time PCR method developed by Gent-Pelzer *et al.* (2007) (see section 4.2.2) can be used for a positive diagnosis of *P. citricarpa*, as it will give a positive signal only when *P. citricarpa* is present, and not for *P. citriasiana* or *P. capitalensis*. The conventional PCR method (as described in section 4.2.1) will give amplification when either *P. citricarpa* or *P. citriasiana* is present. In this case, after a positive signal, isolation and culturing (see section 4.1), real-time PCR (see section 4.2.2) or ITS

sequencing (see section 4.2.1) should be performed to discriminate between the two species. There are no data available on reactions of the recently described *P. citrichinaensis* from China in these molecular assays.

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).

In the present protocol, methods (including references to brand names) are described as published, as these define the original level of specificity achieved. Laboratory procedures presented may be adjusted to the standard of individual laboratories, provided that they are adequately validated.

4.1 Method A: Isolation and culturing of *P. citricarpa*

Fruit lesions are excised with a cork borer or scalpel, dipped in 70% ethanol for 30 s, surface disinfested with 1% sodium hypochlorite (NaOCl) for 2 min, rinsed twice in sterile distilled water and blotted dry (Peres *et al*., 2007). For increasing the isolation frequency, lesions must be excised carefully with any asymptomatic tissue being removed prior to plating (N.A. Peres, personal communication). Subsequently, the lesions are placed aseptically on Petri dishes (9 cm in diameter) with CHA or potato dextrose agar (PDA) (see section 4.1.1) or PDA with 50 µg/ml penicillin and 50 µg/ml streptomycin added (OEPP/EPPO, 2003). If PDA is used and slow-growing dark *P. citricarpa*-like cultures develop on it, they are subsequently transferred both to CHA dishes for testing the growth rate of the colonies and to OA (see section 4.1.1) dishes for evaluating the yellow pigment production. At the same time, the cultures grown on PDA medium should be placed under near-ultraviolet (NUV) light at 22 °C to facilitate the induction of pycnidia formation. Cultures that (i) grow slowly on CHA (see section 4.1.2); (ii) produce the characteristic pycnidia and conidia of *P. citricarpa* (see section 4.1.2); and (iii) produce a yellow pigment on OA – although not all *P. citricarpa* isolates produce such a pigment on OA (Baayen *et al*., 2002) – are identified as belonging to *P. citricarpa.*

The method has the following shortcomings: (a) *P. citricarpa* is a rather slow-growing fungus and is often overgrown by other fungi in culture (e.g. *C. gloeosporioides*) (Peres *et al*., 2007) as none of the culture media used is selective for *P. citricarpa*, and (b) it is a time-consuming method, as it requires 7–14 days for the production of pycnidia.

4.1.1 Culture media

Cherry decoction agar (CHA). Cherry juice is made by boiling 1 kg cherries, free of stones and petioles, in 1 litre tap water for approximately 2 h. The extract is filtered through cheesecloth, poured into bottles, sterilized for 30 min at 110 °C (pH 4.5) and stored until use. In a bottle containing 0.8 litres distilled water, 20 g technical agar no. 3 is added and the mixture is sterilized for 15 min at 121 °C. Immediately after sterilization, 0.2 litre sterilized cherry extract is added, mixed well and sterilized for 5 min at 102 °C (Gams *et al*., 1998).

Oatmeal agar (OA). OA is commercially available. Alternatively, it can be prepared by using the following method: 30 g oatmeal flakes is placed into cheesecloth and suspended in a pan containing tap water. After simmering for approximately 2 h, the flakes are squeezed, filtered through cheesecloth and the extract is sterilized for 15 min at 121 °C. In a bottle containing 1 litre oatmeal extract, 20 g of technical agar no. 3 is added and the mixture is sterilized for 15 min at 121 °C (Gams *et al*., 1998).

Potato dextrose agar (PDA). PDA is commercially available. Alternatively, it can be prepared according to the method described by Hawksworth *et al*. (1995).

4.1.2 Cultural characteristics

P. citricarpa colonies grow slowly on CHA; they have an average diameter of 25–30 mm after 7 days at 22 °C in darkness (Baayen *et al*., 2002). On PDA, the colonies have irregular margins lined by a much wider translucent zone of colourless submerged mycelium (Figure 5A). 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 (Figure 5B). On OA after 14 days at 25^0C 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). On OA a distinct yellow pigment is often produced that diffuses into the medium around the colony (Figure 6D, top row), although not all *P. citricarpa* isolates produce a yellow pigment (Baayen *et al*., 2002). This yellow pigment is weakly produced on CHA and PDA.

4.1.3 Morphology

Published data on the morphology of *P. citricarpa* vary considerably, partly because of the confusion about the identity of the different *Phyllosticta* species associated with *Citrus* (Baayen *et al*., 2002; Glienke *et al*., 2011; Wang *et al*., 2012; Wulandari *et al*., 2009). 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 and 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 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 µm × 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 µm × 4.5–6.5 µm, heteropolar with unequal obtuse ends. The smaller upper end has a truncate, non-cellular, mucoid cap-like appendage 1–2 µm long, and the lower end has an acute or ruffled appendage 3–6 µm long.

Pycnidia. Produced on fruit, attached leaves, 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 µm × (5.0–8.5) µm, with a colourless subulate appendage and a barely visible, colourless, gelatinous sheath <1.5 µm thick (Figures 5C, 5D, 6A). They are formed as blastospores from hyaline, unicellular, cylindrical conidiophores up to 9 µm long.

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 µm × 0.5–1 µm.

4.1.4 Comparison of *P. citricarpa* cultural and morphological characteristics with those of similar *Phyllosticta* species

Cultures of *P. citricarpa* are very similar to those of *P. citriasiana* (Wulandari *et al.*, 2009) and of the endophytic, non-pathogenic to citrus *P. capitalensis* (Baayen *et al*., 2002; Glienke *et al*., 2011).

Identification of *P. citricarpa* colonies is possible by combining:

(1) the colony growth on CHA (although the ranges may overlap)

(2) the thickness of the mucoid sheath surrounding the conidia (Figures 5C, 5D, 6A, 6B, 6C)

(3) the length of the conidial appendage

(4) the presence of yellow pigment on OA, although not all *P. citricarpa* isolates produce a yellow pigment (Baayen *et al*., 2002; Wulandari *et al*., 2009).

Detailed information of the distinctive characteristics of *P. citricarpa* and its related species are given in Table 1. In addition, *P. citrichinaensis* can be differentiated from *P. citricarpa* by its longer conidial appendage, 14–26 µm(Wang *et al*., 2012).

Table 1. Main cultural and morphological characteristics of *Phyllosticta citricarpa, Phyllosticta citriasiana* and *Phyllosticta capitalensis* (Baayen *et al.,* 2002; Wulandari *et al*., 2009)

Characteristic	*P. citricarpa*	*P. citriasiana*	*P. capitalensis*
Average conidia size (µm)	10–12 × 6–7.5	12–14 × 6–7	11–12 × 6.5–7.5
Mucoid sheath width (µm)	<1.5	1	1.5–2.5 (–3)
Apical appendage length (µm)	4–6 (–10)	7–10 (–14)	4–6 (–10)
Average ascospore size (µm)	12–16 × 4.5–6.5	Unknown	15–17.5 × 6.5–7.5
Average spermatia size (µm)	5–8 × 0.5–1	3–5 × 1–2	7–10 × 1.8–2.5
Average colony diameter (mm)*	25–30	18–-20	>40
Maximum growth temperature (°C)	30–36	30–33	30–36
Production of yellow pigment on oatmeal agar (OA) medium	Yes†	No	No

* On cherry decoction agar (CHA) medium after 7 days at 22 °C in darkness.

† It should be noted that not all *P. citricarpa* isolates produce a yellow pigment.

4.2 Method B: Molecular assays

Different molecular methods have been developed for the identification of *P. citricarpa* directly on pure cultures and fruit lesions (Bonants *et al*., 2003; Gent-Pelzer *et al*., 2007; Meyer *et al*., 2006, 2012; Peres *et al*., 2007; Stringari *et al*., 2009). Two methods, a conventional PCR assay, developed by Peres *et al*. (2007), and a real-time PCR assay, developed by Gent-Pelzer *et al*. (2007), are described for the identification of *P. citricarpa*. It is noted that the real-time PCR method will generate a positive signal from a single citrus black spot lesion on fruit, whereas, in some cases, the conventional PCR may give inconclusive results. It is also noted that there are no data available on positive reactions in molecular assays of *P. citrichinaensis*, recently described on fruits in China.

4.2.1 Identification of *P. citricarpa* by conventional PCR

Specificity (analytical specificity) was assessed in a study with 36 isolates of *P. citricarpa*, 13 isolates of *P. capitalensis* and isolates of common citrus pests, including *Alternaria alternata*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Diaporthe citri*, *Mycosphaerella citri* and *Penicillium digitatum*. Only *P. citricarpa* gave a positive reaction. Sensitivity (analytical sensitivity; detection limit) is 1 pg DNA/µl (Peres *et al.,* 2007). The method will amplify either *P. citricarpa* or *P. citriasiana* DNA. There are three methods available to discriminate between the two species after conventional PCR: isolation and culturing (see section 4.1), real-time PCR assay (see section 4.2.2) and ITS sequencing (see section 4.2.3).

4.2.1.1 General information

The protocol was developed by Peres *et al.* (2007). The nucleic acid source is mycelium or dissected fruit lesions. The assay is designed to amplify part of the ITS region producing an amplicon of 300 base pairs (bp). The oligonucleotide primers used are:

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').

2.5× Eppendorf®[1] MasterMix containing Taq DNA polymerase and reaction buffer containing Mg^{2+} and nucleotides is used for PCR amplification. Molecular grade water (MGW) is used to make up the reaction mixes: the MGW should be purified (deionized or distilled), sterile (autoclaved or filtered through 0.45 µm) and nuclease-free. Amplification is performed in a Peltier-type thermocycler with heated lid.

4.2.1.2 Methods

Nucleic acid extraction and purification

DNA is extracted either from fungal cultures grown for 7 days in potato-dextrose broth or from single fruit lesions. In the second case, the symptomatic tissue is dissected out, leaving behind as much mesocarp (albedo) and outer rind as possible.

DNA extraction from mycelium is done using commercially available DNA extraction kits (e.g. DNeasy Plant Mini Kit (Qiagen), QuickPick SML Plant DNA (Bio-Nobile), KingFisher® isolation robot (Thermo)) following the manufacturer's instructions. For the extraction of DNA from single fruit lesions, the following alkaline lysis DNA extraction protocol (Klimyuk *et al*., 1993) followed by purification using a dipstick method can be used as it has proven to be the most effective (Peres *et al*., 2007).

Alkaline lysis DNA extraction method. Symptomatic fruit tissue is placed into sterile 2 ml microtubes containing 40 µl 0.25 M NaOH and incubated in a boiling (100 °C) water bath for 30 s (critical period). The content of the tubes is neutralized by the addition of 40 µl 0.25 M HCl, 20 µl 0.5 M Tris-HCl, pH 8.0 and 0.25% (v/v) Nonidet P-40, and the tubes are placed again in the boiling water bath for 2 min. The material obtained can be either used directly for purification by applying the dipstick method (see below) or stored at 4 °C for several weeks. Prior to purification after storage, the samples are incubated in a boiling water bath for 2 min.

Dipstick DNA purification method. 150 µl 100% ethanol and a small piece of cellulose thin-layer chromatography plate (dipstick) are added to the 2 ml microtube after alkaline lysis (see above). Tubes are placed on their sides on ice and shaken for 30 min. The liquid is aspirated off and 500 µl wash buffer (10× (Tris, Na_2ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite NaClO, pH 7.0) and 95% ethanol) diluted to 25% is added and the tubes are inverted to mix the contents. Washing is repeated twice. The dipsticks are placed in new tubes and dried under vacuum. The tubes are then placed on their sides and 50 µl Tris-EDTA buffer is added to each tube. After incubation for 5 min, the tubes are spun for 10 s, the dipsticks are removed and discarded, and the DNA is recovered. The purified DNA can be used immediately or stored at 4 °C overnight or at −20 °C for longer periods.

Alternatively, DNA can be extracted from fruit lesions using commercially available DNA extraction kits, according to the manufacturer's instructions.

Polymerase chain reaction (PCR)

The master mix (concentration per 20 µl single reaction) is composed of the following reagents:

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
Molecular grade water	n/a	0.4	n/a
2.5× Eppendorf®[1] MasterMix (Taq DNA polymerase at 0.06 U/µl)	2.5×	8.0	1× (Taq 0.024 U/µl)
2.5× Taq reaction buffer (4 mM Mg^{2+}, 500 µM of each dNTP)	2.5×	8.0	1× (1.6 mM Mg^{2+}, 200 µM of each dNTP)

[1] The use of the brand Eppendorf® for PCR amplification in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Primer GCN	10 µM	0.8	0.4 µM
Primer GCMR	10 µM	0.8	0.4 µM
Subtotal	-	18.0	-
DNA	-	2.0	-
Total	-	20.0	-

The PCR cycling parameters are 94 °C denaturation for 2 min; 39 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 1 min; and 72 °C extension for 10 min. A PCR product of 300 bp indicates the presence of *P. citricarpa* DNA.

4.2.1.3 Essential procedural information

After amplification, 10 µl of the reaction mixture is mixed with 2 µl 6× DNA loading buffer (Promega) and loaded along with a molecular weight marker (100 bp DNA Ladder) onto a 1.5% agarose gel, separated by electrophoresis, stained with ethidium bromide or alternative reagents, and viewed and photographed under UV light (Sambrook *et al*., 1989).

DNA from a reference strain of *P. citricarpa* (positive control) must be included as an additional sample to ensure that amplification has been successful. PCR amplification must also be performed on a sample in which the *P. citricarpa* DNA extract has been replaced with the DNA extract of other related species or on a sample of healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control). It is advised to include an internal amplification control (IAC) to monitor inhibition.

4.2.2 Identification of *P. citricarpa* by real-time PCR

Specificity (analytical specificity) was assessed with the *P. citricarpa* reference strain CBS 111.20 (representative for 10 *P. citricarpa* isolates ITS sequence group I; Baayen *et al.*, 2002), the *P. capitalensis* reference strain GC14 (representative for 22 *P. capitalensis* isolates ITS sequence group II; Baayen *et al.*, 2002), 12 other citrus pests (*Alternaria* spp., *Penicillium* spp., *Colletotrichum* spp.), *Phyllosticta artocarpina* and *Guignardia bidwellii*. Only *P. citricarpa* gave a positive reaction. The sensitivity (analytical sensitivity; detection limit) is 10 fg DNA per reaction and the diagnostic sensitivity is 100% (Gent-Pelzer *et al*., 2007).

4.2.2.1 General information

The protocol was developed by Gent-Pelzer *et al.* (2007). The nucleic acid source is mycelium or dissected fruit lesions. The assay is designed to amplify part of the ITS region producing an amplicon of 69 bp. The oligonucleotide primers used are:

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').

Hydrolysis probe GcP1 (5'-AAA AAG CCG CCC GAC CTA CCT TCA-3') is labelled at the 5' end with the fluorescent reporter dye FAM (6-carboxy fluorescein) and modified at the 3' end with the dye TAMRA (6-carboxytetramethylrhod-amine) or Eclipse® Dark Quencher (Eurogentec).

2× Premix Ex Taq Master Mix (Takara)[2] containing Taq polymerase and reaction buffer containing $MgCl_2$ and nucleotides is used for PCR amplification. ROX Reference Dye (50× concentrated, Takara) is added to the Premix Ex Taq Master Mix. MGW is used to make up reaction mixes: the MGW

[2] The use of the brand Takara for the 2× Premix Ex Taq Master Mix in this diagnostic protocol implies no approval of it to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

should be purified (deionized or distilled), sterile (autoclaved or filtered through 0.45 µm) and nuclease-free. Amplification is performed using a real-time PCR thermal cycler.

4.2.2.2 Methods

Nucleic acid extraction and purification

DNA is extracted either from plugs of mycelium (0.5 cm in diameter) taken from the edges of a colony grown on CHA (see section 4.1.1) at 22 °C in darkness or from fruit lesions. Lesions are dissected from the peel, removing as much as possible of the surrounding albedo and peel tissue. Mycelium plugs or lesions are cut into small pieces and placed in a 1.5 ml microcentrifuge tube with a secure-fitting flat-top cap containing a stainless steel bead (3.2 mm in diameter) and 125 µl extraction buffer (0.02 M phosphate-buffered saline (PBS), 0.5% Tween 20, 2% polyvinylpyrrolidone (PVP), 0.2% bovine serum albumin). The tube is shaken in a bead beater for 80 s at 5 000 r.p.m. The mixture is centrifuged for 5 s at maximum speed (16 100 *g*) in a microcentrifuge and 75 µl of the resulting supernatant is used for DNA extraction. DNA can be extracted using commercially available DNA extraction kits, according to the manufacturer's instructions. The final volume of the DNA solution is 50 µl. The DNA is further purified over spin columns filled with PVP. The columns are prepared by filling Axygen Multi-Spin separation columns (Dispolab) with 0.5 cm polyvinylpolypyrrolidone (PVPP), placing it on an empty reaction tube and washing twice with 250 µl MGW by centrifuging the column for 5 min at 4 000 *g*. The DNA suspension is applied to a PVP column and centrifuged for 5 min at 4 000 *g*. The flow-through fraction is used as input for the PCR assay. Purified DNA can be used immediately or stored at 4 °C overnight or at −20 °C for longer periods. PVP is used as soluble compound in the extraction buffer. PVPP is cross-linked PVP and is used as insoluble filtration material.

Polymerase chain reaction

The master mix (concentration per 30 µl single reaction) is composed of the following reagents:

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
MGW	n/a	13.1	n/a
2× Premix Ex Taq Master Mix (Takara)[2]	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	-
Total	-	30.0	-

0.6 µl of 50× ROX Reference Dye can be added if applicable; in that case, 12.5 µl PCR grade water is used.

The PCR cycling parameters are 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The cycle cutoff value of 40 was obtained using the ABI PRISM® 7700 or 7900 Sequence Detection System (Applied Biosystems) and materials and reagents used as described above. It should be noted that:

- The amplification curve should be exponential.
- A sample will be considered positive if it produces a Ct value of <40, provided the contamination controls are negative.

- A sample will be considered negative if it produces a Ct value of ≥40, provided the assay and extraction inhibition controls are positive.

The cycle cutoff value needs to be verified in each laboratory when implementing the test for the first time.

4.2.2.3 Essential procedural information

DNA from a reference strain of *P. citricarpa* (positive control) must be included as an additional sample to ensure that amplification has been successful. PCR amplification must also be performed on a sample in which the *P. citricarpa* DNA extract has been replaced with the DNA extract of other related species (e.g. *P. citriasiana*) or on a sample of healthy exocarp (negative control). To monitor possible reagent contamination and false positives, a sample must be substituted by water (reaction control).

To check for false negative reactions caused by inhibition of the amplification reaction, 12.5 fg of an IAC, 75 nM IAC forward primer FIAC (5'-TGG CCC TGT CCT TTT ACC AG-3'), 75 nM IAC reverse primer RIAC (5'-TTT TCG TTG GGA TCT TTC GAA-3'), and 50 nM IAC MGB hydrolysis probe (5'-ACA CAA TCT GCC-3') labelled with the fluorescent reporter dye VIC™ (Eurogentec) and the quencher dye Eclipse® Dark Quencher (Eurogentec) can be added to the reaction mixes.

4.2.3 Identification of *P. citricarpa* by ITS sequencing

4.2.3.1 General information

The identity of positive samples obtained by conventional PCR can be confirmed by sequencing (Baayen *et al.,* 2002). The method for sequencing of the ITS 1 and 2 regions of the fungal ribosomal RNA gene is described below.

The oligonucleotide primers used are:

Forward primer: ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')

Reverse primer: ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990).

4.2.3.2 Methods

Nucleic acid extraction and purification

DNA should be extracted from a 1 cm^2 plug taken from a pure culture of the test isolate. A suitable DNA extraction kit is used or DNA is extracted following a more traditional method, such as that described in Hughes *et al.* (2000). Extracted DNA should be stored at 4 °C for immediate use or at –20 °C if testing is not to be performed on the same day.

Polymerase chain reaction (PCR)

The total reaction volume of a single PCR is 50 µl, and is composed of the following reagents:

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
MGW	n/a	37.5	n/a
10× PCR reaction buffer (+15 mM $MgCl_2$) (Roche)[3]	2×	5.0	1× (Taq 0.024 U/µl)
dNTPs	10 mM (each)	4.0	0.8 mM (each)
Primer ITS1	10 µM	0.6	0.12 µM

[3] The use of the brand Roche for the PCR reaction buffer and the DNA Taq polymerase in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Primer ITS4	10 µM	0.6	0.12 µM
DNA Taq polymerase (Roche)[3]	5 U/µl	0.3	0.03 U/µl
Subtotal	-	48.0	-
DNA	-	2.0	-
Total	-	50.0	-

The PCR cycling parameters are 94 °C for 30 s; 40 cycles of 94 °C for 15 s, 55 °C for 60 s and 72 °C for 30 s; and 72 °C for 5 min. The amplicon size is 550 bp (Baayen *et al*., 2002).

Sequencing of amplicons

The amplified mixture (5 µl of it) is run on a 1.5% agarose gel to check for positive test reactions. The remaining 45 µl from positive test reactions is purified using a suitable PCR purification kit, following the manufacturer's instructions. Sequencing is performed with forward primer ITS1 and reverse primer ITS4.

4.2.3.3 Essential procedural information

Amplification and analysis

Extracted DNA should be defrosted, if necessary. Enough reaction mix should be prepared for testing at least one sample of the unknown isolate, a positive control containing amplifiable DNA and a negative control loaded with water rather than DNA. Samples are resolved on a 1.5% agarose gel. Consensus sequences for test samples (excluding primer sequences) are compared with a confirmed strain for the ex-epitype of P. citricarpa CBS 127454 (GenBank accession number JF343583) on the National Center for Biotechnology Information (NCBI) database GenBank (http://www.ncbi.nlm.nih.gov/). The level of identity should be between 99% and 100%.

5. Records

The records and evidence detailed in section 2.5 of ISPM 27:2006 should be kept.

In cases where other contracting parties may be adversely affected by the results of the diagnosis, records and evidence of the results (in particular cultures, slides, photos of fungal structures, photos of symptoms and signs, photos of DNA extracts and separation gels) should be retained for at least one year.

6. Contact Points for Further Information

Further information on *P. citricarpa* and the methods for its detection and identification can be obtained from (in alphabetical order):

ARC-Plant Protection Research Institute, Biosystematics Division: Mycology, Private Bag x134, Queenswood 0121, South Africa (Dr Mariette Truter; tel.: +27 12 8088281; fax: +27 12 8088297; e-mail: truterm@arc.agric.za).

Plant Research International, PO Box 26, 6700 AA Wageningen, The Netherlands (Dr Peter J.M. Bonants; tel.: +31 31 7480648; fax +31 31 7418094; e-mail: peter.bonants@wur.nl).

Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz-ESALQ/USP, Piracicaba, São Paulo, Brazil (Dr Marcel B. Spósito; tel.: +55 19 34294190 ext. 4190; fax +55 19 34294414; e-mail: mbsposito@usp.br).

University of Florida, Citrus Research and Education Center (CREC), 700 Experiment Station Rd, Lake Alfred, FL 33850, USA (Dr Lavern W. Timmer; tel.: +1 863 9561151; fax: +1 863 9564631; e-mail: lwtimmer@ufl.edu).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on

Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel to develop Diagnostic Protocols (TPDP).

7. Acknowledgements

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9. Figures

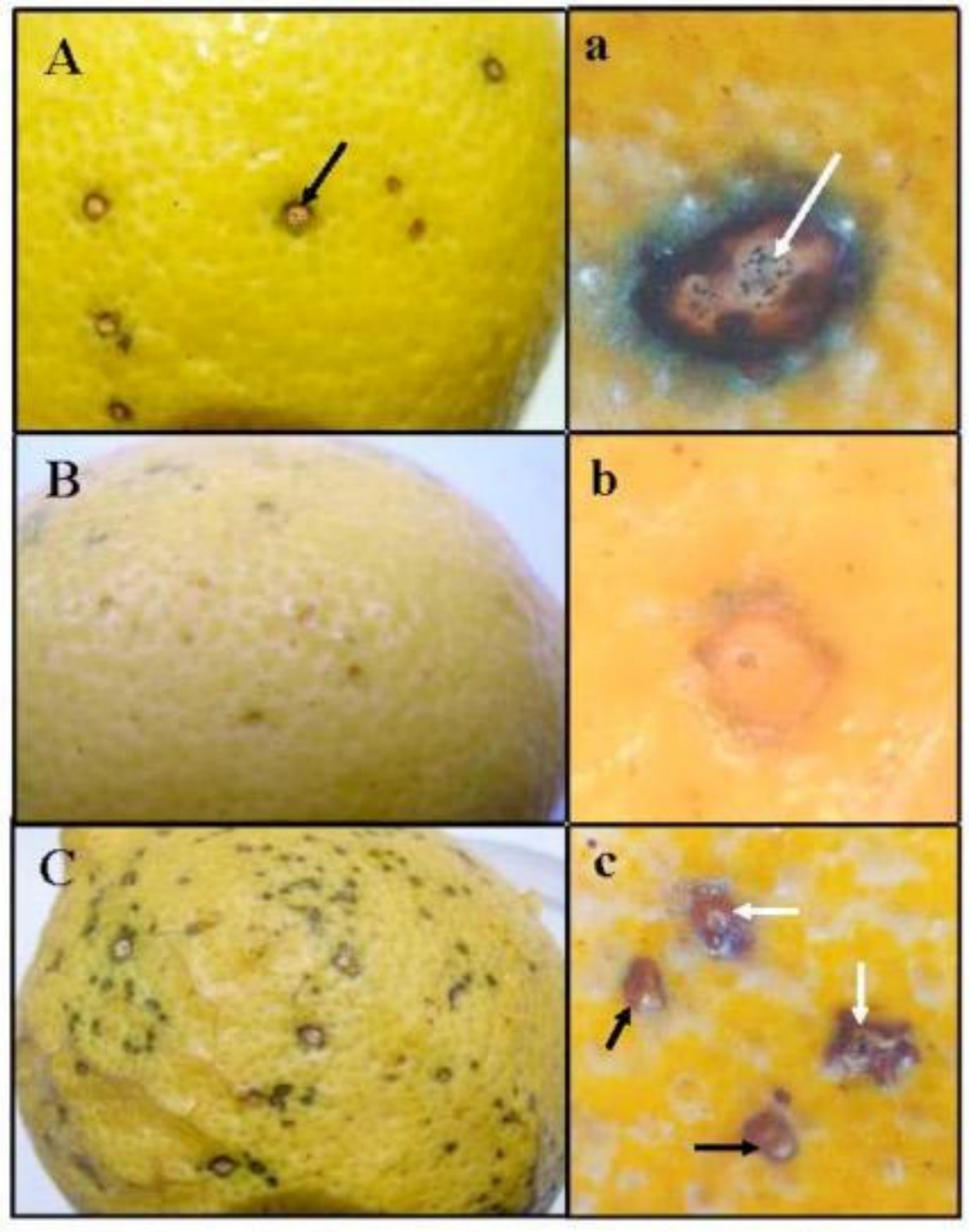

Figure 1. Hard spot and freckle spot symptoms caused by *Phyllosticta citricarpa* on sweet orange (*Citrus sinensis*) and lemon (*Citrus limon*) fruits: (A, a) hard spot lesions on sweet orange with the larger lesions containing pycnidia of the anamorph *Phyllosticta citricarpa* (arrows); (B) freckle 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.

Photos courtesy E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil.

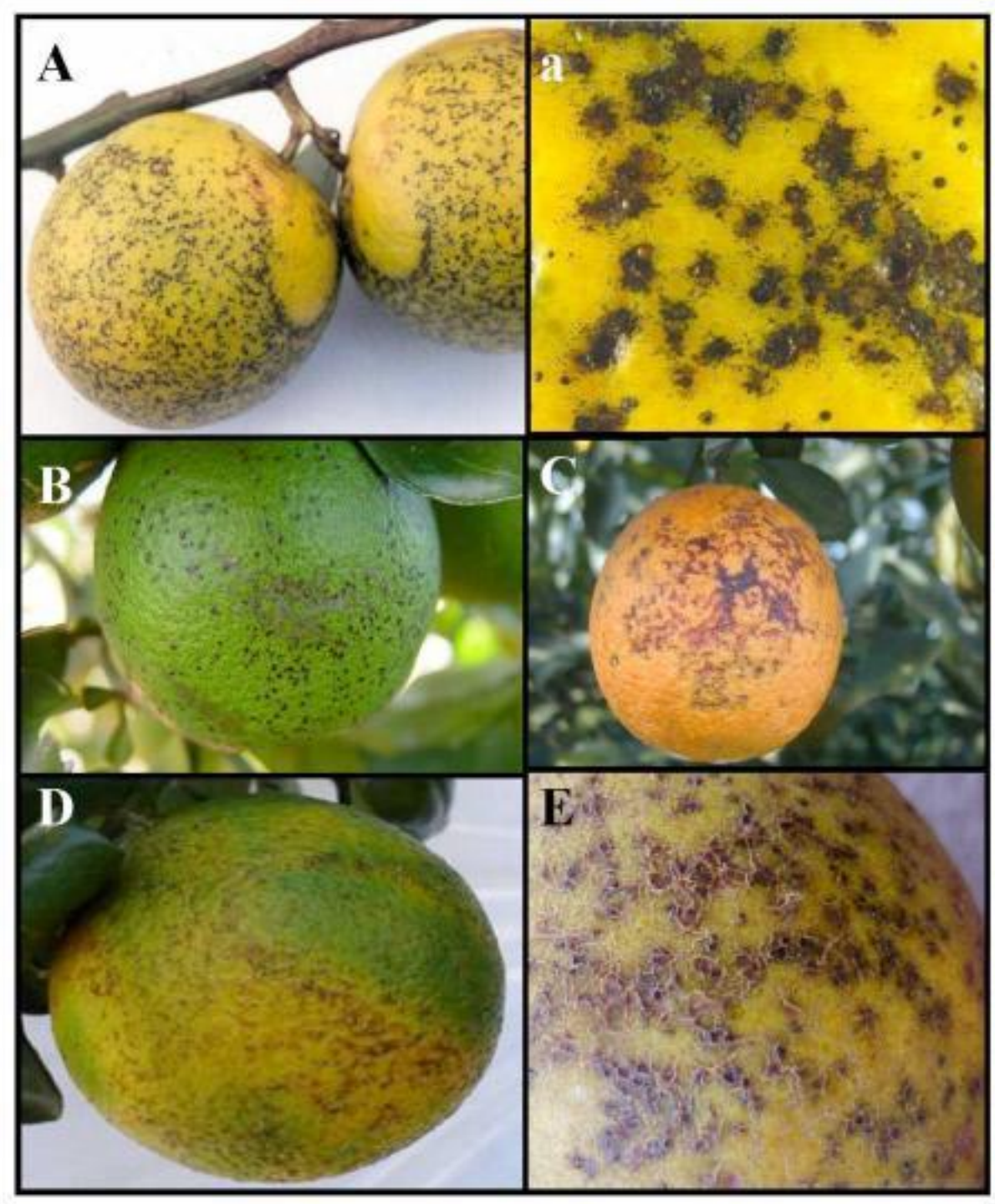

Figure 2. False melanose, virulent spot, lacy spot and cracked spot symptoms caused by *Phyllosticta citricarpa* on sweet orange (*Citrus sinensis*) and lemon (*Citrus limon*) fruits: (A) false melanose lesions on mature sweet orange; (a) false melanose lesions surrounded by dark specks on mature sweet 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).

Photos courtesy FUNDECITRUS (A, B, C, D, E) and E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (a).

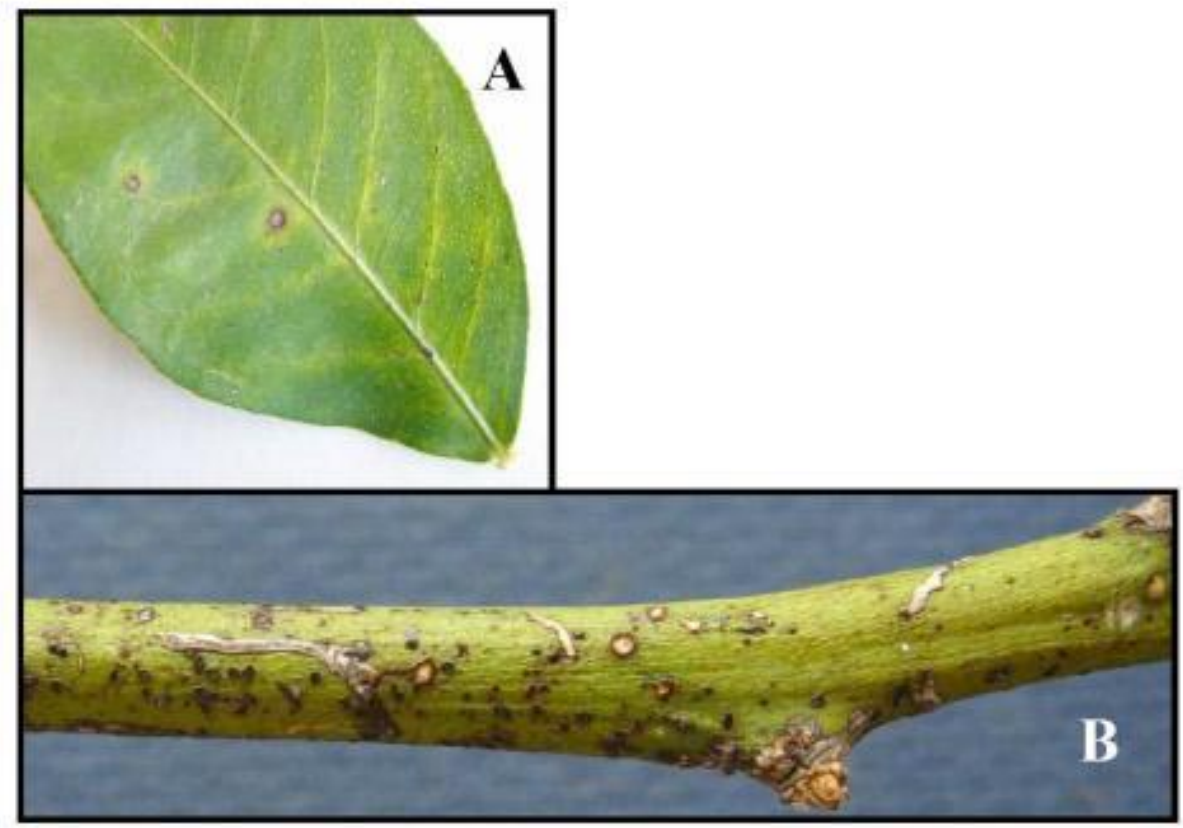

Figure 3. Symptoms of citrus black spot caused by *Phyllosticta citricarpa* on lemon (*Citrus limon*) leaves (A) and twigs (B).

Photos courtesy E. Feichtenberger, Instituto Biológico, Sorocaba, Brazil (A) and M. Truter, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa (B).

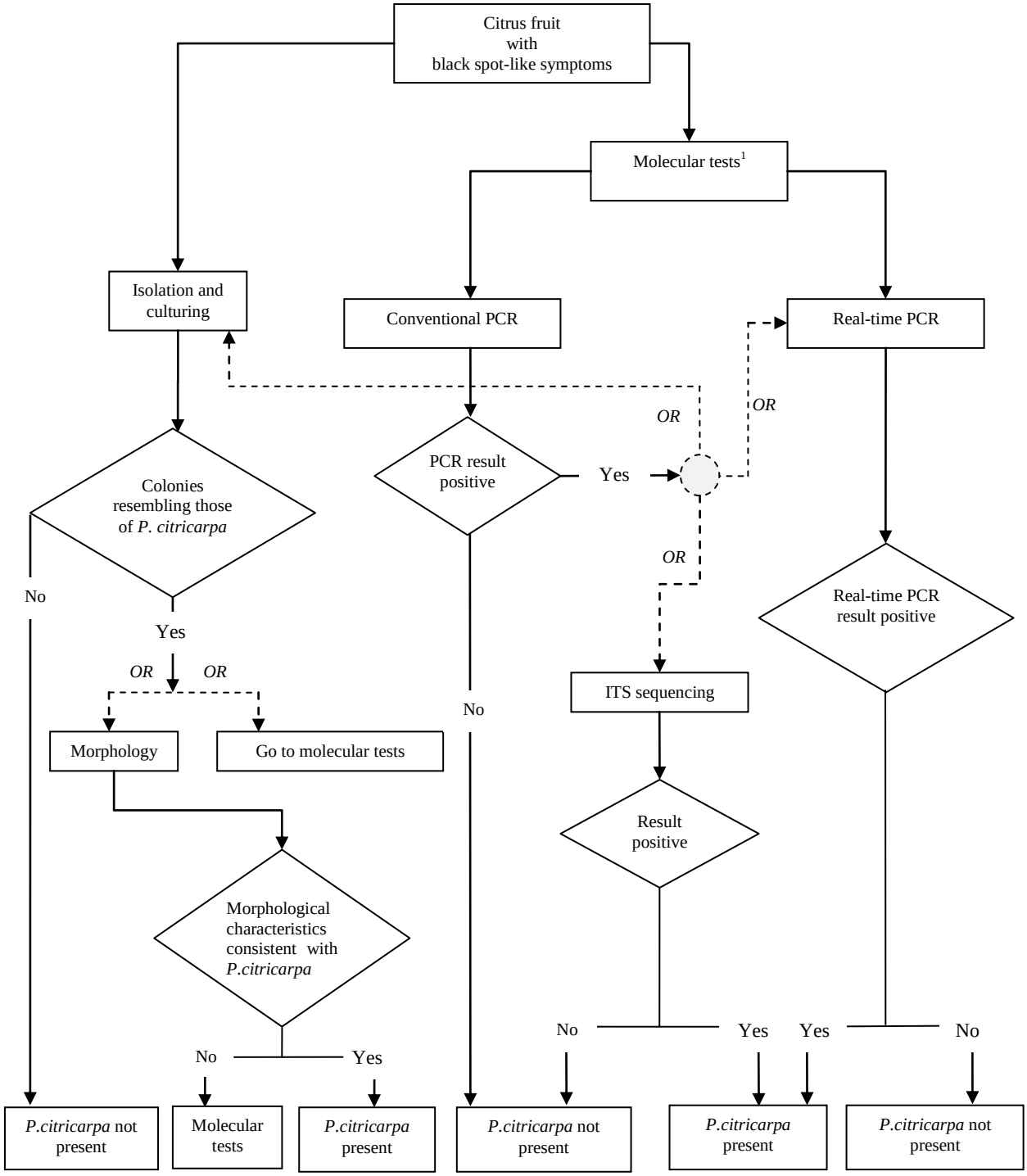

Figure 4. Flow diagram for the identification of *Phyllosticta citricarpa* on citrus fruit

[1]The molecular assays have been validated for the identification of the organism on pure cultures and fruit lesions and not on any other plant material (e.g. leaves, twigs). ITS, internal transcribed spacer; PCR, polymerase chain reaction.

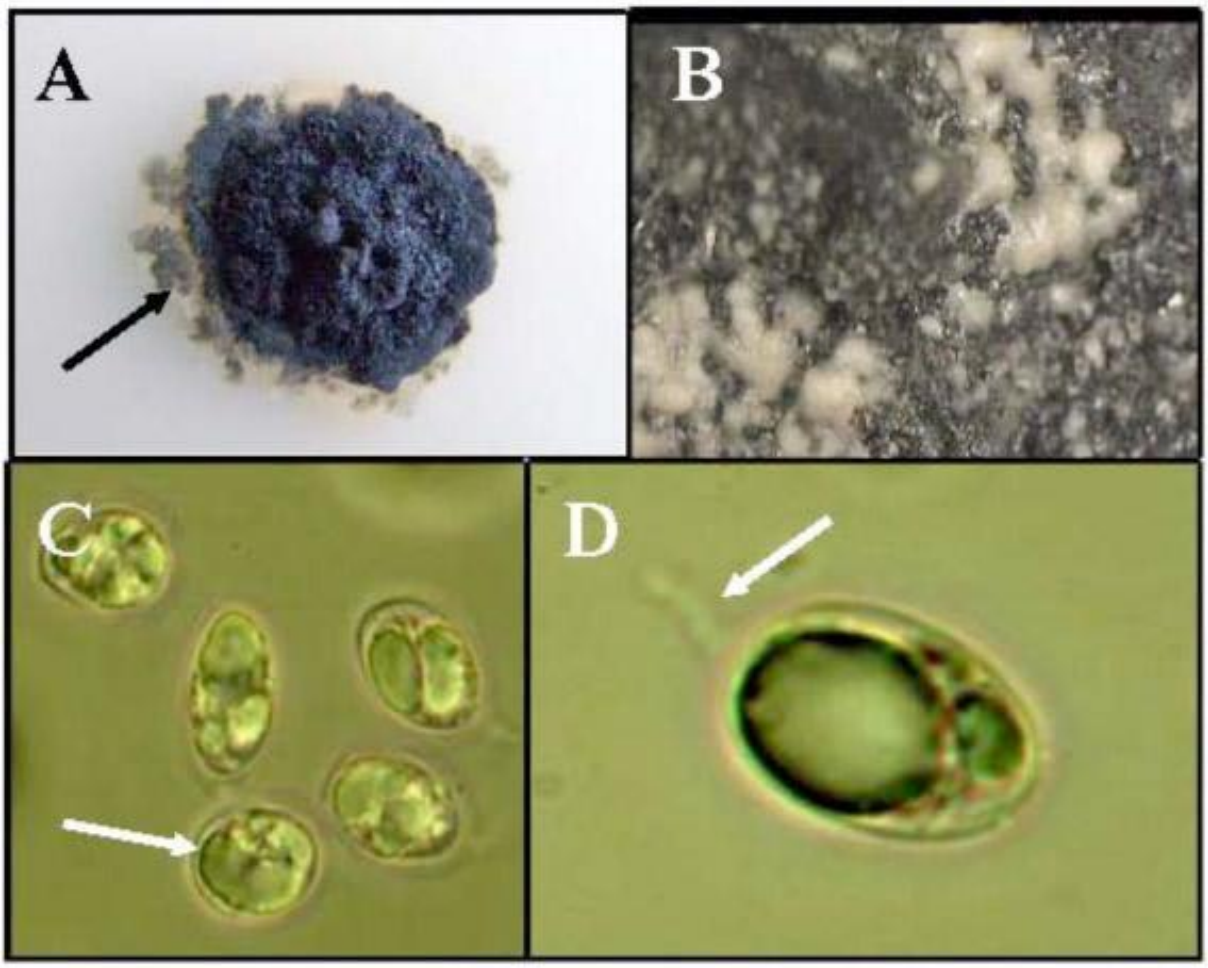

Figure 5. Colony characteristics and conidial morphology of *Phyllosticta citricarpa*: (A) colony with irregular margin surrounded by a translucent zone of colourless submerged mycelium (arrow) after 30 days of growth on potato dextrose agar (pH 5.5) at 25 °C and a 12 h photoperiod; (B) conidial slime oozing from mature pycnidia; (C, D) conidia with a thin mucoid sheath (C, arrow) and a colourless subulate appendage (D, arrow, magnification 1 000× with immersion oil).

Photos courtesy L.E. Diaz, Ministry of Husbandry, Agriculture and Fisheries, Montevideo, Uruguay.

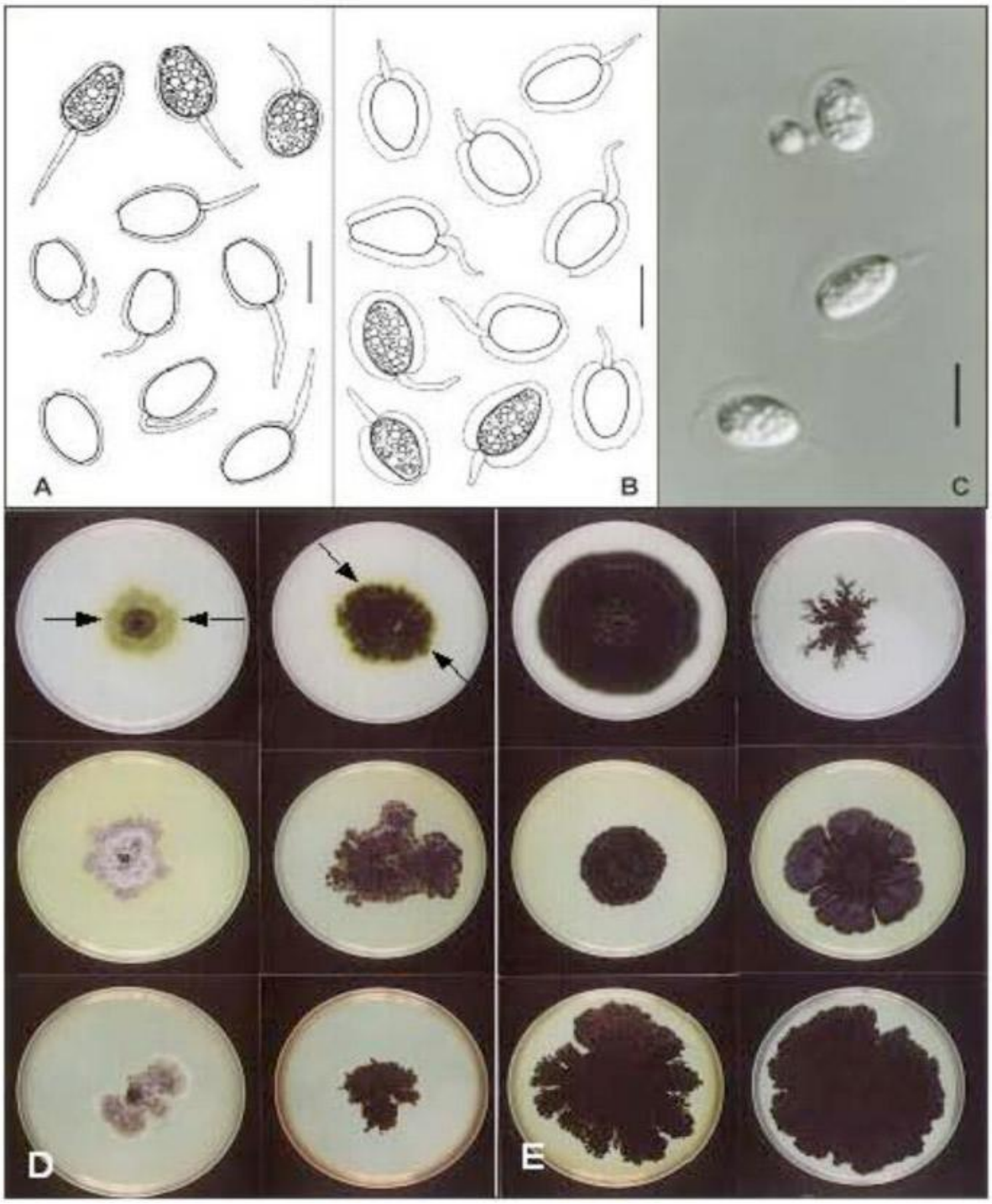

Figure 6. Conidial morphology and cultural characteristics of *Phyllosticta citricarpa* and *Phyllosticta capitalensis*: (A) conidia of *P. citricarpa* with thin (<1.5 µm) mucoid sheath; (B, C) conidia of *P. capitalensis* with thick (>1.5 µm) mucoid sheath (scale bar = 10 µm) (photo C was taken under a light microscope equipped with differential interference contrast); (D, E) colonies of *P. citricarpa* (D) and *P. capitalensis* (E) after 7 days of growth on oatmeal agar (top row), malt extract agar (middle row) and cherry decoction agar (bottom row) (note the production of a yellow pigment around the colony of *P. citricarpa* grown on oatmeal agar (D, arrows) and the absence of this pigment in cultures of *P. capitalensis* grown on the same medium (E)).

Photos courtesy G. Verkley, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (A, B, C) and W. van Lienden, Plant Protection Service, Wageningen, The Netherlands (D, E).

Publication history

This is not an official part of the standard

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ISPM 27. 2006: **Annex 5**. *Phyllosticta citricarpa* (McAlpine) Aa on fruit (2014). Rome, IPPC, FAO.

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