Diagnostics
Diagnostic

Guignardia citricarpa

Specific scope

This standard describes a diagnostic protocol for *Guignardia citricarpa*.[1]

Specific approval and amendment

First approved in 2002–09.
Revised in 2009–09.

Introduction

Guignardia citricarpa is a damaging pathogen on *Citrus* spp., occurring in many areas where *Citrus* is cultivated including Asia, Australia, South America, Southern Africa, Central America and the Caribbean region (CABI/EPPO, 1998; EPPO/CABI, 1997; CABI, 2006). The disease has not been reported from Europe or North America. It is mainly a foliage and fruit disease. The pathogen has significant economic impact mainly due to the external blemishes that make citrus fruit unsuitable for the fresh market. 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, 2006). In addition, latently infected (asymptomatic) fruit at harvest may still develop symptoms during transport or storage (Kotzé, 1996).

In areas where only one disease cycle occurs annually, perithecia with ascospores, produced exclusively on leaf litter, are the main source of inoculum. However, in areas, where rain is not confined to a single season or citrus flowering occurs more than twice per year, conidia of the anamorph *Phyllosticta citricarpa* are as important as ascospores as inoculum sources (Spósito *et al.*, 2001). Ascospores released from the perithecia during rainfall or irrigation are carried by wind throughout the canopy and long distances beyond. The critical period for infection starts at fruit set and lasts for 4–5 months after which fruit becomes resistant (Kotzé, 2000). Leaves are susceptible for up to 10 months after development (Trute *et al.*, 2004). Following infection, the fungus remains in a quiescent state until the fruit becomes fully grown or mature, with disease symptoms being produced many months after infection has taken place (Kotzé, 2000). Pycnidia with conidia are produced on symptomatic citrus fruit and leaf litter (Kotzé, 2000). They may be splash-dispersed onto the canopy or washed off from infected late-hanging fruit onto young fruit and leaves that are still at the susceptible stage (Spósito *et al.*, 2001). Perithecia develop within 40–180 days after leaf drop (Kotzé, 2000).

Spread of *G. citricarpa* to continents previously free from the disease is assumed to have taken place mainly through infected plants for planting (nursery stock and other planting material, e.g. for botanical gardens), rather than through infected fruits.

Phytosanitary regulations cover fruits of *Citrus*, *Fortunella*, *Poncirus* and their hybrids[2], other than fruits of resistant *Citrus aurantium*. They should originate from countries or areas recognized to be free from 'pathogenic strains' of *G. citricarpa* or at least the orchard and the fruits should be free from symptoms caused by 'pathogenic strains' of *G. citricarpa*. The regulations refer to strains of *G. citricarpa* pathogenic to citrus because non-pathogenic, endophytic *G. citricarpa*-like strains have been reported from symptomless *Citrus* plants as well as from host plants other than *Citrus* (Chiu, 1955; McOnie, 1964). These non-pathogenic strains were considered in the past to be saprophytic forms (Sutton & Waterston, 1966) or avirulent strains of *G. citricarpa* (Kotzé, 2000), although McOnie (1964) reported that these strains belong to a distinct *Guignardia* species (or, perhaps, variety or form). Baayen *et al.* (2002) have recently confirmed the latter and have shown that such strains belong to a distinct species, *Guignardia mangiferae* (anamorph *Phyllosticta capitalensis*), a common endophyte in many plant families. *Guignardia mangiferae* can be distinguished from *G. citricarpa* by cultural, morphological and molecular characters, and has never been found associated with typical black spot symptoms (Baayen *et al.*, 2002). *Guignardia mangiferae* is sometimes isolated from

[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.

[2]*Poncirus* and its hybrids are citrus rootstocks, so that their fruits are not in practice traded.

citrus fruits with very small spots (<2 mm), but in these cases the fungus may have been present endophytically, prior to lesion formation by pathogenic Colletotrichum species. *Guignardia mangiferae* is also known as *Guignardia endophyllicola*, *Guignardia psidii*, *Phyllosticta anacardiacearum* and *Phyllosticta theaceae-rum*, all of which are junior synonyms of the same fungus.

This protocol is designed for the diagnosis of *G. citricarpa* the causal agent of citrus black spot and its distinction from *G. mangiferae* (previously referred to as 'non-pathogenic strains of *G. citricarpa*') and the newly described *Phyllosticta citriasiana* (Wulandari *et al.*, 2009).

Identity

Name: *Guignardia citricarpa* Kiely
Anamorph: *Phyllosticta citricarpa* (McAlpine) Van der Aa
Synonyms: *Phoma citricarpa* McAlpine
Phyllostictina citricarpa (McAlpine) Petrak
Synanamorph: *Leptodothiorella* sp.
Taxonomic position: Fungi: Ascomycota: Dothideales
EPPO code: GUIGCI.
Phytosanitary categorization: EPPO A1 list no. 194, EU Annex designation II/A1 (strains pathogenic to citrus).

Detection

Disease symptoms on fruits

Fruit symptoms vary to such an extent that confusion exists regarding their characteristics and description (Schüepp, 1961; Kotzé, 2000). The various types overlap, and the names used to indicate symptom types are not consistent. Symptoms are known as hard spot, shot-hole spot, false melanose, speckled blotch, freckle spot, virulent spot (described below), or types A, B, C, and D lesions (not described here). Four main types exist.

Hard spot/shot-hole spot: shallow lesions with a small central grey to tan crater usually with a dark brown rim, 3–10 mm in diameter (Fig. 1A,C,G). This is the most typical black spot symptom and it appears when fruit starts maturing, even before colour change (Kotzé, 1981; 2000). Often, but not always, pycnidia can be seen inside the spots as tiny and slightly elevated black dots in the grey to tan field (Fig. 1F). A magnifying glass or dissecting microscope is needed to see these clearly.

Freckle spot: on mature fruits, usually after harvest, small (1–3 mm in diameter), slightly depressed spots appear (Fig. 1B,D,E). These spots may be grey to tan, or reddish, or brownish, or not discoloured at all. Often, but not always, they have a dark red or brown rim. Pycnidia are only incidentally present in freckle spot lesions (Fig. 1A). Freckle spots often occur as satellite spots around hard-spot lesions. Many intermediates occur between these spots and the previous type.

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. 2A,B). The lesions are devoid of pycnidia and may coalesce as the season progresses (CABI, 2006). This type of symptoms is observed in citrus-growing areas where *G. citricarpa* has been present for a long time (FUNDECITRUS, 2005).

Virulent spot: spots may coalesce to form 'virulent' spots (Fig. 1H and the right hand orange lesion in Fig. 1A). This type of symptom, which appears towards the end of the season, is the most damaging form because it extends deeply into the peel causing premature fruit drop and serious post-harvest losses. Spots turn brown to black, develop a leathery texture and may cover the entire fruit. Pycnidia may be present in these spots (Kotzé, 1981).

Two additional types of symptoms have also been reported to occur on citrus fruit, though infrequently:

Lacey spot: superficial yellow to brown lesions with a smooth texture and no definite edges (Fig. 2C). The lesions appear when the fruit are still green and may cover a large part of their surface. Lacey spot is considered a variant of false melanose (Goes, 2001).

Cracked spot: superficial, slightly raised, cracked, variable in size lesions with irregular margins and devoid of pycnidia (Fig. 2D), which appear on fruit older than 6 months (Goes *et al.*, 2000). In Brazil, this type of symptom has been associated with the citrus rust mite (*Phyllocoptruta oleivora*) infestation (FUNDECITRUS, 2005).

Fruit lesions caused by *G. citricarpa* can be very similar to those caused by *Phyllosticta citriasisana, Alternaria alternata* pv. *citri*, *Colletotrichum* spp., *Diaporthe citri*, *Mycosphaerella citri*, *Septoria* spp., mechanical and insect damage (Snowdon, 1990). In particular, *A. alternata* pv. *citri* and *Colletotrichum* spp. may cause small, sharply black-rimmed, depressed lesions that are closely similar to tiny black spot lesions. By themselves, symptoms are not sufficiently distinctive. Therefore, diagnosis relies either on culturing the fungus followed by classical identification, or on direct PCR testing of the lesion.

Disease symptoms on leaves and twigs

Symptoms first appear on mature leaves and petioles as tiny circular red to red-brown spots, visible on both leaf surfaces. With time, the centres of the spots darken with an even darker brown to black ring. Spots remain small, not exceeding 3 mm. Leaf lesions often have yellow halos.

Identification

Procedures

The diagnostic procedure for *G. citricarpa* depends on the material that is to be examined. Plants for planting of *Citrus*, *Fortunella*, *Poncirus* and their hybrids, and also detached leaves or twigs, are examined by procedure A (below). Most EPPO countries prohibit the import of plants for planting of these genera from most origins, so in practice procedure A mostly refers to material detained in quarantine. Fruits are examined by procedure B when showing black spot-like symptoms indicative of *G. citricarpa* (e.g. hard spot, freckle spot, false melanose, virulent spot, etc.). A flow diagram of identification methods for *G. citricarpa* is presented in Fig. 2.

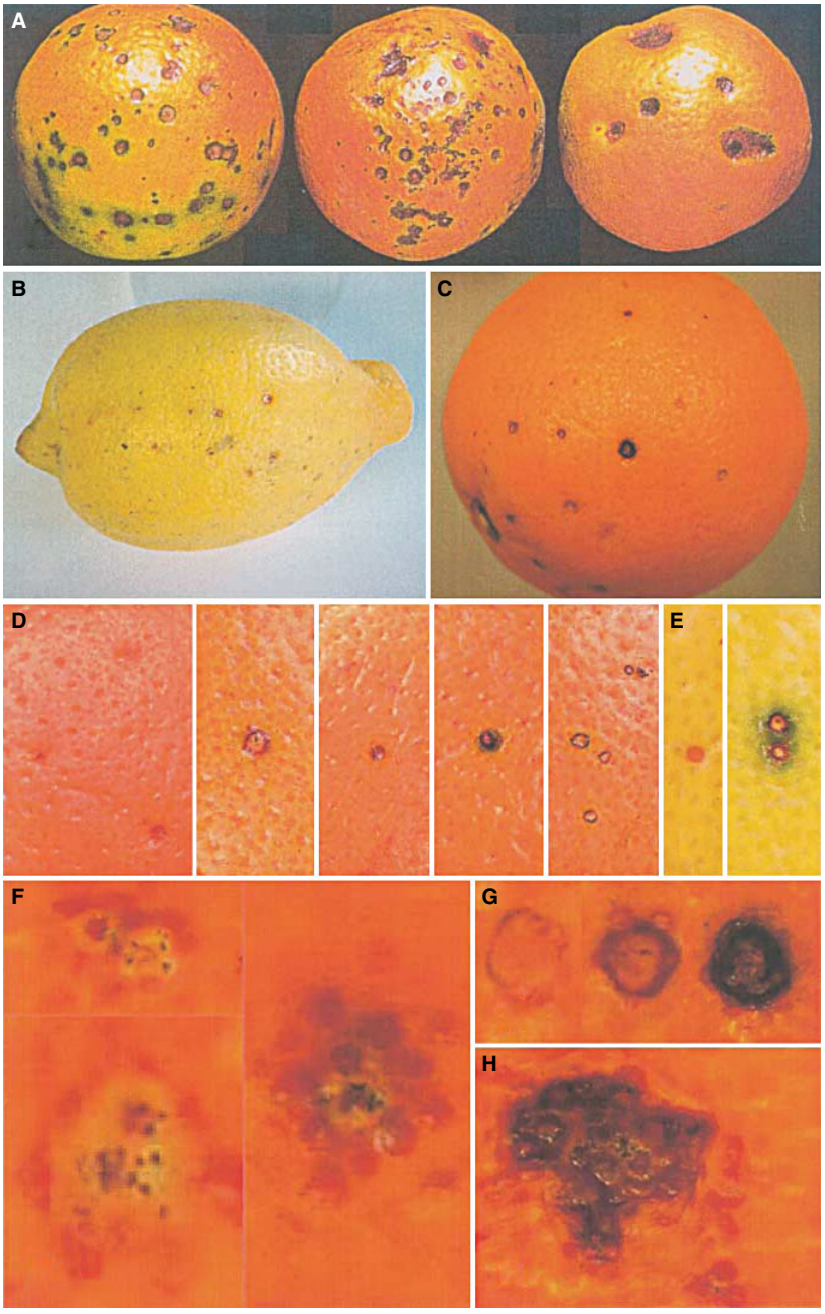


Fig. 1 Symptoms of citrus black spot, caused by *Guignardia citricarpa* on sweet orange (*Citrus sinensis*) and lemon (*Citrus limon*) fruits. (A) Sweet orange fruit with severe black spot symptoms (the lesions are of the hard spot type, except for those on the middle and right hand oranges which are beginning to spread, i.e. virulent spot). (B) Lemon fruit with predominantly freckle spot lesions (the larger lesions contain pycnidia). (C) Sweet orange fruit with freckle and hard spot lesions. (D and E) Freckle spot symptoms on sweet orange and lemon fruits (spots are always depressed in the peel, and may be concolorous with the healthy peel – left hand lesions in D and E – or have a dark brown rim – other lesions). (F) Lesions with pycnidia on sweet orange fruits. (G) Hard spot lesions on sweet orange fruits (pycnidia may or may not be present inside the lesions). (H) Virulent spot on sweet orange fruit (pycnidia are present in some areas). [(A) courtesy: P. Barkley, copyright Biological and Chemical Research Institute, Rydalmere (AU); (B) courtesy and copyright: Centrum voor Landbouwkundig Onderzoek, Merelbeke (BE); (C–G) courtesy and copyright: Plant Protection Service, Wageningen (NL)].

(A) Identification from plant material

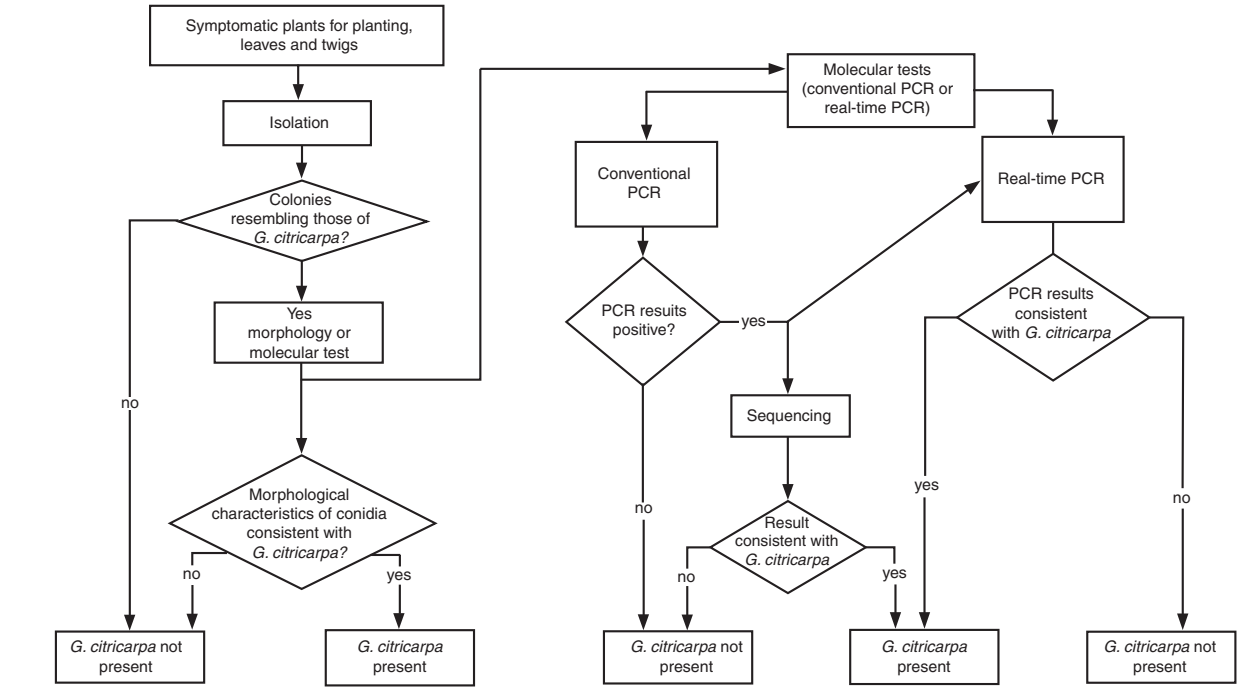
Leaf and twig lesions are cultured on appropriate media. The cultures obtained are identified using classical means (growth rate, pigment production, size and shape of the spores, thickness of the spore sheath) or PCR testing according to the methods given below. Classical identification of *Guignardia*-like cultures requires 7–14 days, and is possible as soon as mature pycnidia have been produced. Cultures of *G. citricarpa* produce the characteristic spores of the anamorph *P. citricarpa*, grow slowly on cherry decoction agar, and produce a yellow pigment on oatmeal agar (details on growth and morphology are given below). *Guignardia*-like cultures that do not produce mature pycnidia within 14 days are further analysed by PCR testing.

(B) Identification from fruits

Two methods are available for diagnosing citrus black spot from fruit lesions.

(1) Culturing of lesions on appropriate media, followed by classical identification of the fungus or PCR testing;

However, this approach is inferior compared to a direct PCR assay on fruit lesions. Culturing and classical identification will require 14 days, with an efficacy of <10% (Bonants *et al.*, 2003)



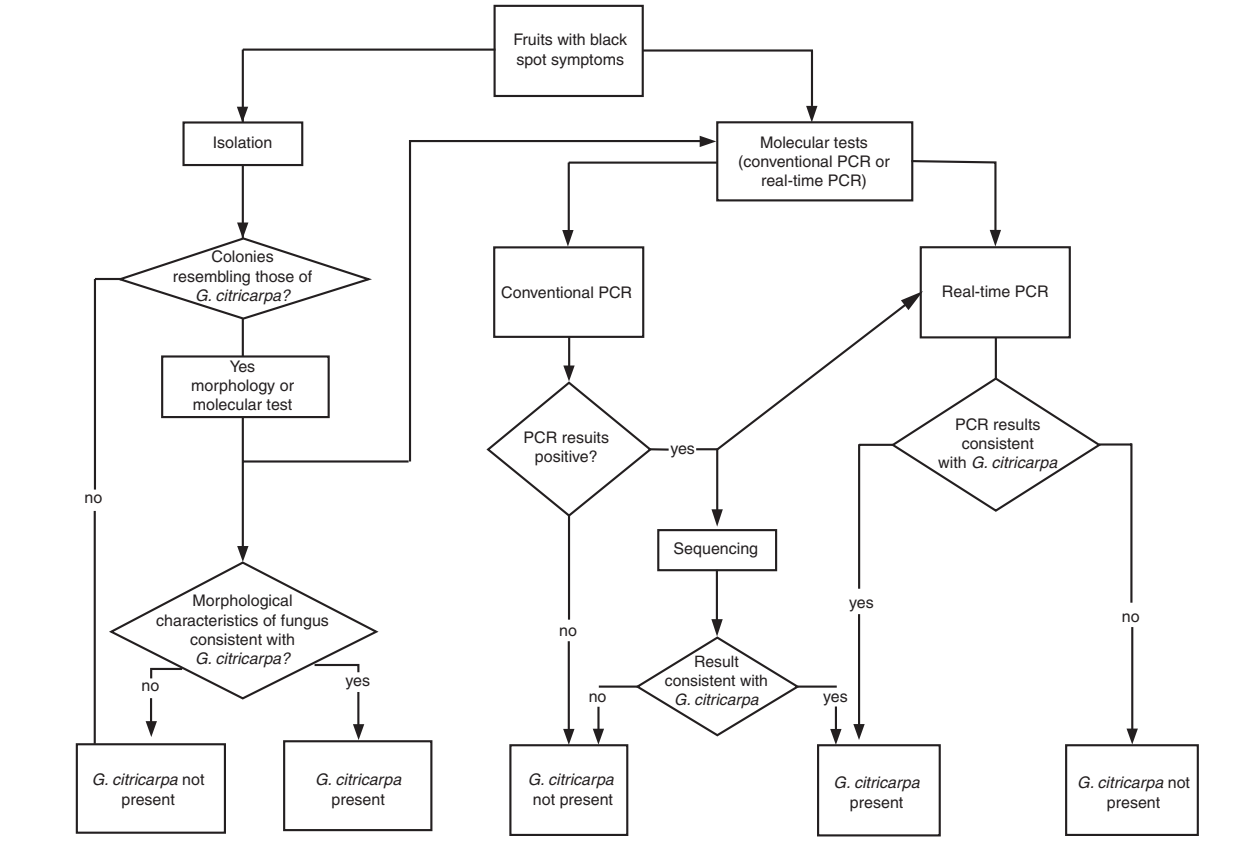


Fig. 2 Flow diagram for the identification of *Guignardia citricarpa*.

Culturing followed by PCR on cultures is more time-consuming and no more reliable than a direct PCR on fruit lesions (see below).

(2) The second method is a direct PCR test on the lesions: either a conventional PCR assay (Bonants *et al.*, 2003; Meyer *et al.*, 2006, Peres *et al.*, 2007) or a real-time PCR assay (van Gent-Pelzer *et al.*, 2007). A conventional PCR method, developed by Bonants *et al.* (2003), is described in Appendix 1 and a real-time PCR method, developed by van Gent-Pelzer *et al.* (2007), is described in Appendix 2.

Both assays are based on the use of ITS sequence-based primers. The conventional PCR assay detects *G. citricarpa* and the newly described *P. citriasiana,* consequently an additional test is necessary to identify *G. citricarpa* (real time PCR or sequencing). The real-time PCR assay is specific for *G. citricarpa.* Sequencing is described in Appendix 3. No cross-reactions with other *Guignardia* species or other citrus pathogens were observed. The methods are rapid (1 day) and have an efficacy of at least 90% per lesion. Reliability above 99% can easily be achieved by independent testing of multiple lesions.

Isolation and culturing of the fungus

Media

Cherry decoction agar (CHA). 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 water and 20 g Technical agar no. 3 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 (5), and resterilize for 5 min at 102°C.

Potato dextrose agar (PDA). Commercially available (Difco).

Oatmeal agar (OA). Commercially available (Difco), or wrap 30 g oatmeal flakes in cloth and hang in 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 Technical Agar no. 3 to 1 L of the oatmeal extract and sterilize 15 min at 121°C.

Malt extract agar (MEA). Commercially available.

Isolation

Plant material (leaves, twigs, and fruit) should be surface-disinfected with 70% ethanol (Bonants *et al.*, 2003) or sodium hypochlorite (NaOCl) (e.g. commercial bleach 2% of active chlorine) for 30 s, then dissected and aseptically placed on the isolation medium. Isolations are made on CHA, a medium on which pycnidia are readily formed, or PDA amended with 50 μg mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. Slow-growing, dark cultures, possibly representing *G. citricarpa,* are then transferred to CHA for growth rate testing, and to OA for evaluating yellow pigment production. The original cultures are incubated under near-ultraviolet (NUV) light at 22°C to induce pycnidia formation.

Examination of cultured isolates

Within 14 days, pycnidia should have been formed and a watery squash preparation of the pycnidium (or of the spores slime oozing from it) should be examined under the microscope. Cultures are identified as *G. citricarpa* when they produce the characteristic *P. citricarpa* spores, grow slowly on CHA, and produce a yellow pigment on OA (for details see below). *Guignardia*-like cultures that do not produce mature pycnidia within 14 days or do not fulfil all the above mentioned requirements should be further analysed by PCR testing. Cultures should be retained on CHA slants in a refrigerator (4°C) and transferred twice a year.

Growth characteristics in culture

Phyllosticta citricarpa colonies grow slowly on CHA at 22°C in darkness (16–33 mm diameter in 7 days) (Baayen *et al.*, 2002). The mycelium is submerged, dark, forming a plectenchymatous crust (Fig. 3D). Stromata develop within 8 days as hard, black masses, with one to numerous spore and spermatial cavities in the upper region. Mature pycnidia are generally formed within 10–14 days. On OA, a distinctive yellow pigment is produced that diffuses into the medium around the colony. The pigment is only weakly produced on other media.

In pure culture, *G. citricarpa* (Fig. 3D) closely resembles the common citrus endophyte *G. mangiferae* (Fig. 3E) and the newly described *Phyllosticta citriasiana*. Classical identification is possible by combining pigmentation on OA (a yellow pigment is produced by most strains of *G. citricarpa* but not by *G. mangiferae* and *Phyllosticta citriasiana*), growth rate on CHA (*G. citricarpa* colony <35 mm in diameter after 7 days on CHA; *G. mangiferae* colony generally >40 mm in diameter although the range of the latter overlaps with that of *G. citricarpa*), and thickness of the mucoid spore sheath [<1.5 μm in *G. citricarpa* (Fig. 3A); >1.5 μm in *G. mangiferae* (Fig. 3B,C)]. Upon prolonged culturing, only *G. mangiferae* will produce perithecia. *Phyllosticta citrisiana* can be distinguished from *G. mangiferae* by having smaller conidia, with a narrower mucoid sheath. Furthermore, it is distinguishable from *G. citricarpa* by having larger conidia, longer conidial appendages, and not producing any diffuse yellow pigment when cultivated on OA. In culture, colonies of *P. citriasiana* are also darker shades of grey and black on OA, MEA, PDA, and CMA than observed in the other two species.

Differentiating characteristics are listed in Table 1.

Morphology

Data on morphology of *G. citricarpa* vary considerably, partly because of the confusion that existed in the past about the identity of 'pathogenic' and 'non pathogenic' strains. The data given below are based on Sutton & Waterston (1966) and van der Aa (1973), as revised and amended by Baayen *et al.* (2002).

Ascocarps: perithecia exclusively formed on leaf litter, not on fruit or attached leaves or on media; solitary or aggregated, globose to pyriform, immersed, dark brown to black, 125–360 μm diameter; wall up to five cells thick, sclerotioid on the outside, pseudoparenchymatous and thin-walled within, ostiole papillate, circular, 10–17.5 μm diameter. Paraphyses and periphyses absent.

Asci: clavate cylindrical, shortly stipitate, 8-spored, 40–65 × 12–15 μm; ascus wall thick, bitunicate.

Ascospores: aseptate, hyaline, multiguttulate, cylindrical but swollen in the middle, ends obtuse, each with a colourless appendage, 12.5–16 × 4.5–6.5 μm.

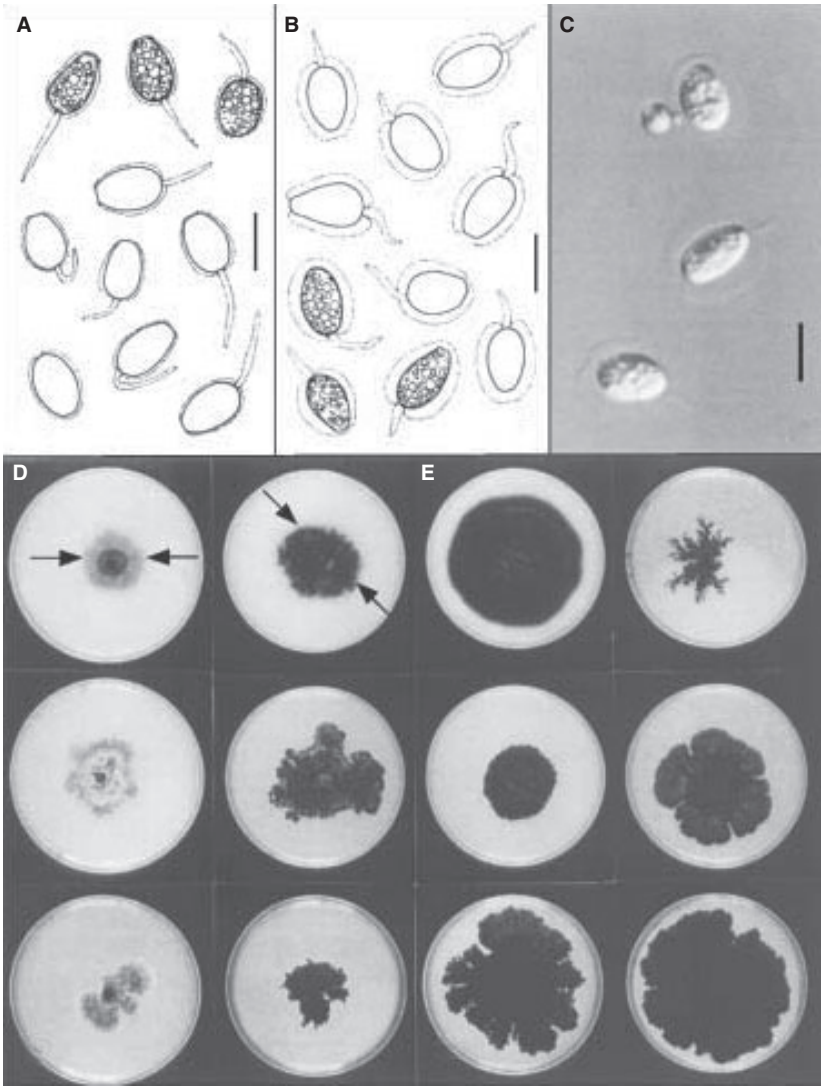


Fig. 3 Spores morphology and colony characteristics after 7 days growth on various media of *Guignardia citricarpa* and *G. mangiferae*. (A) Spores of *G. citricarpa* with thin mucoid sheath. (B and C) Spores of *G. mangiferae* with thick mucoid sheath (C – differential interference contrast – scale bar = 10 μm). (D) Colonies of *G. citricarpa* growing slowly on oatmeal agar (top), malt extract agar (middle), and cherry decoction agar (bottom) and showing the yellow pigment around the colony (arrows). (E) Colonies of *G. mangiferae* generally growing faster and without yellow pigment. [courtesy W. van Lienden, Plant Protection Service, Wageningen (NL) (cultures); G. Verkleij, Centraalbureau voor Schimmel-cultures, Utrecht (NL) (spores)].

Table 1 Main differentiating characteristics of *Guignardia citricarpa, Guignardia mangiferae* and *Phyllosticta citriasiana*

	Guignardia citricarpa	*Phyllosticta citriasiana*	*Guignardia mangiferae*
Average conidia size (μm)	10–12 × 6–7.5	12–14 × 6–7	11–12 × 6.5–7.5
Mucoid sheath (μm)	Thin, <1.5	Thin, <1.5	Thick, 1.5–3.0
Conidial appendage length (μm)	4–6 (10)	7–14	4–6 (10)
Ascospore size	Ascospores not produced in pure culture	Ascigerous state unknown	15–17.5 × 6.5–7.5 μm
Spermatia size (μm)	5–8 × 0.5–1	3–5 × 1–2	7–8 × 1.8–2.5
Maximum growth temperature (°C)	30–36	30–33	30–36
Production of yellow pigment in oatmeal agar (OA)	Yes	No	No

Pycnidia: formed in fruit and leaf lesions, also amphigenous on dead leaves, solitary, sometimes aggregated, globose, immersed, mid-to-dark brown, 70–330 μm diameter, wall up to four cells thick, sclerotioid on the outside, pseudoparenchymatous within, ostiole darker, slightly papillate, circular, 10–15 μm diameter.

Spores: obovate to elliptical, hyaline, aseptate, multiguttulate, apex slightly flattened with a colourless subulate appendage, base truncate, 9.4 –12.7 × 5.0–8.5 μm, surrounded by a barely visible (<1.5 μm thick) colourless gelatinous coat (Fig. 3A), formed as blastospores from hyaline, unicellular, cylindrical conidiophores up to 9 μm long. Spores and the spore sheath are best studied in slide preparations using a microscope equipped with differential interference contrast or, preferentially, using Indian Black ink, in which the sheath will be lucent against a black background.

Spermatial state: in the form-genus *Leptodothiorella*; formed both in pure culture and on the host; spermatia dumbbell-shaped, seldom cylindrical, straight or slightly curved, 5–8 × 0.5–1 µm.

Reference material

Reference cultures can be obtained from Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, 3584 CT Utrecht (NL). fax: +31 30 251 2097.

Internal controls for both PCR assays can be obtained from PJM Bonants, Plant Research International, Wageningen (NL) (for address see below).

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis* (EPPO, 2006).

Further information

Further information on this organism can be obtained from:

J de Gruyter, National Reference Laboratory (NRL), Plant Protection Service, PO Box 9102, 6700 HC Wageningen (NL) (Tel: +31 317 496 111, fax: +31 317 421 701; e-mail: j.de.gruyter@minlv.nl).

Inquiries about the availability of the PCR primers GcF3 and GcR7 should be directed to P Loux via e-mail (ploux@oregon.uoregon.edu) or by mail to the Office of Technology Transfer, 1238 University of Oregon, Eugene, OR 97403, (US) (attn of Dr Philip Loux).

The internal control for the conventional PCR and the Internal Amplification Control (IAC) for the real-time PCR assay can be obtained by writing to PJM Bonants, via e-mail (peter.bonants@wur.nl) or by mail to Plant Research International, PO Box 26, 6700 AA Wageningen (NL).

Acknowledgements

This protocol was originally drafted by: Baayen RP, Plant Protection Service, Wageningen (NL), Bonants PJM, Plant Research International, Wageningen (NL), Carroll GC, University of Oregon, Eugene, Oregon (US). It was revised by Meffert JP and Kox LFF (NL).

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Appendix 1 – Identification of *Guignardia citricarpa* by conventional PCR

1. General information
   - 1.1. Protocol developed by Bonants *et al.* (2003).
   - 1.2. Nucleic acid source: mycelium or dissected fruit lesions.
   - 1.3. The assay is designed to internal transcribed spacer (ITS) sequences producing an amplicon of 490 bp with *G. citricarpa* DNA and 230 bp for the internal control.
   - 1.4. Oligonucleotides used: Forward primer GcF3 (5′- AAA AAG CCG CCC GAC CTA CCT -3′) and reverse primer GcR7 (5′- TGT CCG GCG GCC AG - 3′), both at a final concentration of 0.60 μM.
   - 1.5. Taq DNA polymerase (5 U/μL; Roche) is used for PCR
   - 1.6. Nucleotides are used at a final concentration of 60 μM each.
   - 1.7. Buffers: 10× PCR buffer containing 15 mM $MgCl_2$ (Roche).
   - 1.8. Molecular grade water (MGW) is used to make up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.
   - 1.9. Amplification is performed in thin-walled PCR tubes in a Peltier-type thermocycler with heated lid, e.g. PTC 200 (MJ Research)
   - 1.10. The analytical specificity of the assay was assessed using *G. citricarpa, G. mangiferae, Phyllosticta citriasiana* and the citrus pathogens *Alternaria alternata, Colletotrichum acutatum, C. gloeosporioides, Diaporthe citri, Mycosphaerella citri* and *Penicillium digitatum*. *Guignardia citricarpa* and *P. citrirasiana* gave a positive reaction. The analytical sensitivity (detection limit) is 1 pg of DNA per reaction.
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 are dissected from the peel, 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 extraction buffer (0.02 M PBS, 0.05%, Tween T25, 2% polyvinylpyrolidone, 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 min^{-1}. 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.
   - 2.1.2. 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.
   - 2.1.3. The DNA is further purified using spin columns filled with polyvinylpyrolidone (PVPP). The columns are prepared by filling Axygen Multi-Spin columns (Dispolab, Asten, Netherlands) with 0.5 cm PVPP, placing it on an empty reaction tube, and washing twice with 250 μL MGW 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.
   - 2.1.4. Either use purified DNA immediately, store overnight at 4°C or at −20°C for longer periods.
   - 2.2. Polymerase chain reaction
   - 2.2.1. Master mix (concentration per 25 μL single reaction).
     1× PCR buffer containing 1.5 mM $MgCl_2$ (Roche)
     60 μM of dNTPs
     1 U Taq DNA polymerase (Roche)
     0.6 μM of each primer
     add MGW to a volume of 19.75 μL
     5.0 μL extracted DNA obtained as described above
     0.25 μL (10 pg) internal control DNA (see 3.2)
   - 2.2.2. PCR cycling parameters:
     94°C for 2 min,
     30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min,
     72°C for 10 min.
3. Essential procedural information
   - 3.1. Visualisation of products: After amplification, 10 μL of the PCR products are electrophorized on a 1% agarose gel according to standard methods (Sambrook *et al.*, 1989) along with a molecular weight marker (e.g. 100 bp DNA Ladder: Fermentas GmbH, St. Leon-Rot, DE) to size fragments. PCR products are viewed and photographed under UV light.
   - 3.2. The internal control, developed from heterologous *Lolium perenne* DNA, can be obtained from PJM Bonants, Plant Research International, Wageningen (NL).
   - 3.3. A negative control (no DNA target) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen). The positive control, but not the negative control, should yield an amplicon of 490 bp. Samples yielding an amplicon of this size can be identified as *G. citricarpa*. Samples not yielding such an amplicon can be considered negative for *G. citricarpa* only when the 230 bp internal control amplicon was properly produced. A control for extraction efficiency should also be included.

Appendix 2 – Identification of *Guignardia citricarpa* by real-time PCR

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 assay is designed to internal transcribed spacer (ITS) sequences producing an amplicon of 69 bp.
1.4 Oligonucleotides used: 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′) and Taqman probe GcP1 (5′- AAA AAG CCG CCC GAC CTA CCT TCA -3′), FAM label and TAMRA or Eclipse Dark quencher (Eurogentec)
1.5. 2× Premix Ex Taq (Takara) containing Taq polymerase, reaction buffer containing $MgCl_2$ and nucleotides (Applied Biosystems), is used for PCR.
1.6. ROX reference Dye (50× concentrated, Takara) is added to Premix Ex Taq.
1.7. Molecular grade water (MGW) is used to make up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 μm filtered) and nuclease-free.
1.8. Amplification is performed using a real-time PCR thermal cycler, e.g. 7700 Sequence Detector (Applied Biosystems).
1.9. The analytical specificity of the assay was assessed with *G. citricarpa, G. mangiferae*, *Phyllosticta citriasiana*, *Guignardia bidwellii* and 14 other citrus pathogens (*Alternaria* spp., *Penicillium* spp., *Colletotrichum* spp., and *Phyllosticta artocarpina*). Only *G. citricarpa* gave a positive reaction. The analytical sensitivity (detection limit) is 10 fg DNA per reaction and the diagnostic sensitivity is 100%.

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 1.
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. A DNA purification using spin columns filled with polyvinylpyrolidone (PVPP) is necessary for DNA isolated using the Puregene kit. The procedure is described in Appendix 1. For DNA isolated using the QuicPick kit no DNA purification is necessary.
2.1.4. Either use extracted DNA immediately, store overnight at 4°C or at −20°C for longer periods.
2.2. Polymerase chain reaction
2.2.1. Master mix (concentration per 30 μL single reaction).
1× Premix Ex Taq (Takara)
0.25 μM of each primer
0.1 μM of TaqMan probe
Add MGW to a volume of 29 μL
1× ROX Reference Dye (Takara)
1.0 μL extracted DNA obtained as described above.
2.2.2. PCR cycling parameters:
95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.
2.2.3. The limit of detection (LOD) positive control, but not the negative control, should yield a cycle threshold (Ct_{LOD}). Plant samples whose DNA extract yield a Ct lower or equal to Ct_{LOD} should be considered as infected by *G. citricarpa*, whereas samples whose DNA extract yields a $Ct > Ct_{LOD}$ 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).

3. Essential procedural information
3.1. A negative control (no DNA target) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen).
3.2. To check for false negative reactions caused by inhibition of the amplification reaction 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 (Klerks *et al.*, 2004) can be added to the reaction mixes. The IAC is a green fluorescent protein (GFP) plasmid construct. It can be obtained from PJM Bonants, Plant Research International, Wageningen (NL).

Appendix 3 – Identification by sequencing internal transcribed spacer (ITS) 1 and 2 of the nuclear ribosomal gene

1. General information
1.1. This PCR-sequencing protocol is used for the identification of *Guignardia citricarpa* using the internal transcribed spacer (ITS) 1 and 2.
1.2. Amplicons of 490 bp produced by the conventional PCR of Bonants *et al.* (2003)
1.3. The target region is the internal transcribed spacer region (ITS) 1 and 2 of the fungal ribosomal RNA gene
1.4. Amplicon location: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2; and 28S ribosomal RNA gene, partial sequence
1.5. Amplicon size (including primer sequences) is 632 bp for *Guignardia citricarpa*
1.6. Oligonucleotides: The forward primer is ITS1: 5′ TCC GTA GGT GAA CCT GCG G 3′ and the reverse primer is ITS4: 5′ TCC TCC GCT TAT TGA TAT GC 3′ (White *et al.*, 1990)
1.7. DNA Taq-polymerase at a final amount of 1.5 U (Roche, Cat. No. 11 146 165 001) is used for amplification
1.8. Molecular grade water is used for all reactions

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. DNA should be extracted from a 1 cm^2 plug taken from a pure culture of the test isolate

2.1.2. A suitable DNA extraction kit such as a QuickPick Plant DNA Kit (Isogen Cat. No. 53022) is used or DNA is extracted following a more traditional method such as described in Hughes *et al.* (2000)

2.1.3. Extracted DNA should then be stored at 4°C for immediate use or at −20°C if testing is not to be performed on the same day

2.2. Polymerase chain reaction (PCR)

2.2.1. Total reaction volume of a single PCR reaction is 50 µL

2.2.2. 37.5 µL of molecular grade water

2.2.3. 5.0 µL 10× of PCR reaction buffer (+15 mM $MgCl_2$) (Roche)

2.2.4. 4.0 µL dNTPs (10 mM each)

2.2.5. 0.6 µL primer ITS1 (10.0 µM)

2.2.6. 0.6 µL primer ITS4 (10.0 µM)

2.2.7. 0.3 µL DNA Taq-polymerase (5 U/µL)(Roche)

2.2.8. 1.0 µL of DNA extract

2.2.9. Perform amplification in PCR tubes in a thermocycler with heated lid programmed as follows: 30 sec 94°C, 40× (15 sec 94°C, 60 sec 55°C, 30 sec 72°C), 5 min 72°C, 1 min 20°C

2.3. Sequencing of amplicons

Run 5 µL of the amplified mixture on a 1.5% agarose gel to check for positive test reactions. Purify the remaining 45 µL from positive test reactions using a suitable PCR purification kit such as QIAquick PCR purification kit (Qiagen, Cat. no. 28106) following the manufacturers instructions. Sequencing with forward primer ITS1 and reverse primer ITS4.

3. Essential Procedural Information

3.1. Amplification and analysis

Defrost extracted DNA if necessary, prepare enough reaction mix for testing at least one sample of the unknown isolate, a positive control containing amplifiable DNA and negative control reactions of reaction mix loaded with water rather than DNA.

3.2. Resolve samples on a 1.5% agarose gel

3.3. Compare consensus sequences for test samples (excluding primer sequences) with a confirmed strain for *G. citricarpa* such as CBS 111.20 (GenBank ref FJ538314) on the NCBI database GenBank (http://www.ncbi.nlm.nih.gov). The level of identity should be between 99 and 100%.