

EPPO Datasheet: *Phyllosticta citricarpa*

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IDENTITY

Preferred name: *Phyllosticta citricarpa*

Authority: (McAlpine) Aa

Taxonomic position: Fungi: Ascomycota: Pezizomycotina: Dothideomycetes: Botryosphaeriales: Phyllostictaceae

Other scientific names: *Guignardia citricarpa* Kiely, *Phoma citricarpa* McAlpine, *Phyllosticta paracitricarpa* Guarnaccia & Crous, *Phyllostictina citricarpa* (McAlpine) Petrák

Common names: CBS, black spot of citrus, freckle spot of citrus, hard spot of citrus, shot-hole of citrus, speckled blotch of citrus, virulent spot of citrus

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EPPO Categorization: A1 list

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EU Categorization: Emergency measures, Quarantine pest ((EU)

2019/2072 Annex II A)

EPPO Code: GUIGCI



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Notes on taxonomy and nomenclature

Citrus black spot disease was first described in Australia (Cobb, 1897; Kiely, 1948, 1949a; Chiu, 1955; McOnie, 1964a; McMillan, 1986; Lee, 1969; Kotzé, 1981). The asexual morph (anamorph) of the citrus black spot fungus was described by McAlpine (1899), initially under the name *Phoma citricarpa*, and then called *Phyllosticta citricarpa* (van der Aa, 1973). The sexual morph (teleomorph) was described by Kiely (1948) and designated *Guignardia citricarpa*. According to the new code for fungal nomenclature approved by the International Botanical Congress in Melbourne in July 2011, the dual nomenclature for fungi should not be used anymore ('One Fungus – One Name'), and gives priority to the oldest name irrespective of whether it is teleomorphic (sexual reproduction) or anamorphic (asexual reproduction) (Norvell, 2011). In the case of the citrus black spot pathogen, the name *P. citricarpa* has priority over *G. citricarpa* and should now be used as the only identifier of this species.

In the past, taxonomic confusion has characterized studies about *Phyllosticta/Guignardia* species found on citrus. For many years, the coexistence of pathogenic and non-pathogenic strains of *P. citricarpa* has been assumed, as *P. citricarpa* had been detected on asymptomatic citrus trees, as well as on other hosts in Australia and South Africa (Kiely, 1948; Wager, 1952). Based on pathogenicity tests, McOnie (1964a) demonstrated that the non-pathogenic strains belonged to other *Guignardia* species that did not play a role in citrus black spot disease. The non-pathogenic type could be distinguished by its faster growth, colony type, and production of pycnidia and ascocarps in culture, as well as by a wider geographical distribution than the citrus pathogenic type. Meyer *et al.* (2001) suggested that these two types represented distinct species. Baayen *et al.* (2002) used many of the above characteristics, as well as the presence or absence of a mucoid sheath on conidia, and DNA sequence data to show that the two types were distinct species, namely *G. mangiferae* (the widespread non-pathogenic type), and *G. citricarpa* (the citrus black spot pathogen). In subsequent studies about the identity and genetic diversity of *Phyllosticta* isolates (with emphasis on isolates from citrus), it was proposed that *P. capitalensis* (anamorph of *G. mangiferae*) was the name to be used to designate the taxon that is frequently isolated as an endophyte, with a wide host range and geographical distribution (Glienke *et al.*, 2011). Other *Phyllosticta* species have been associated with citrus, but in many cases confirmatory pathogenicity tests and evidence of re-isolation have not been published. Both *P. citriasiiana* and *P. citrimaxima* have been isolated from tan spots on pomelo (*Citrus maxima*) fruit from Asia (Wikee *et al.*, 2013, Wulandari *et al.*, 2009). *P. citribraziliensis* has been described from asymptomatic citrus leaves in Brazil (Glienke *et al.*, 2011). In extensive surveys conducted in China, *P. citrichinaensis* has been found in association with leaf and fruit spots of citrus (Wang *et al.*, 2012). Finally, *P. paracapitalensis* and *P. paracitricarpa* have been isolated in Europe from citrus leaves and leaf litter, respectively (Guarnaccia *et al.*, 2017).

Thus, while new knowledge on the *Phyllosticta* species associated with citrus is continuously emerging, the name *P. citricarpa* is exclusively applied to the fungus causing black spot of citrus.

HOSTS

P. citricarpa is a leaf spotting and fruit-blemishing fungus affecting *Citrus*, *Poncirus* and *Fortunella* and their hybrids. Except for *Citrus aurantium* (sour orange) and its hybrids, as well as *C. latifolia* (Tahiti lime), all commercially grown *Citrus* species are susceptible to *P. citricarpa* (Aguilar-Vildoso *et al.*, 2002; Kotzé, 2000). Milles *et al.* (2019), in a field inoculation test, showed that all mandarin, sweet orange, lemon and papeda types were susceptible; pomelo, lime, and sour orange types expressed immunity; while various different hybrids were susceptible, resistant and immune. *C. limon* (lemon) is particularly susceptible and thus it is usually the first *Citrus* species to show disease symptoms once the pathogen is introduced into a new area (Kotzé, 2000). However, citrus black spot emerged recently in Florida (USA) directly in *C. sinensis* (sweet orange) orchards (Schubert *et al.*, 2012). Late-maturing cultivars of *C. sinensis* were considered more susceptible than early-maturing ones (Timmer, 1999). However, cultivar field trials conducted in Brazil, as well as studies comparing the rate of disease progress, indicated that cultivar reaction to the disease is more linked to the interaction of environmental factors than with the dynamics of fruit maturation (Spósito *et al.*, 2004; Sousa and de Goes, 2010).

In the case of sour orange, *P. citricarpa* was isolated in Brazil from asymptomatic leaves, black spot lesions and other fruit blemishes (Baayen *et al.*, 2002; Wulandari *et al.*, 2009; Wickert *et al.*, 2009; Glienke *et al.*, 2011), although no evidence of reproduction on this citrus species was found. *C. latifolia* (Tahiti lime) is reported not to exhibit citrus black spot symptoms under field conditions, even in areas with high inoculum pressure. However, *P. citricarpa* was isolated in São Paulo, Brazil, from asymptomatic fruit and leaves of *C. latifolia* (Baldassari *et al.*, 2008; Wickert *et al.*, 2009). As *P. citricarpa* can colonise and form viable ascospores in *C. latifolia* leaves, this citrus species might be an asymptomatic host which could play a role in *P. citricarpa* epidemiology (Baldassari *et al.*, 2008).

Although it has been reported that *C. maxima* (pomelo) is not affected by *P. citricarpa* (Miles *et al.*, 2013; Wang *et al.*, 2012), more data as well as appropriate pathogenicity tests are needed to completely exclude this citrus species as a potential host of *P. citricarpa*.

With regard to *Fortunella* sp. (kumquat), this species was recorded by Kiely (1948) in Australia as moderately susceptible to *P. citricarpa* under conditions of natural infection, but no further experimental information is available.

No definitive information has been found on the susceptibility of *Poncirus* (trifoliate orange) to *P. citricarpa*.

In early studies, several non-citrus hosts have been reported to harbour *P. citricarpa*: almond (*Prunus dulcis*), avocado (*Persea americana*), cardamom (*Elettaria cardamomum*), *Cola nitida*, *Dioscorea pentaphylla*, *Eucalyptus deglupta*, guava (*Psidium guajava*, *P. montanum*), mango (*Mangifera indica*), passion fruit (*Passiflora edulis*), *Rubus* spp., sugarcane (*Saccharum officinarum*) and a variety of ornamentals such as *Caesalpinia pulcherrima*, *Callistemon citrinus*, *Camellia japonica*, *Dendrobium speciosum*, holly (*Ilex aquifolium*), *Magnolia* sp., *Smilax* sp. (Allen, 1971, FAO, 1960, Hudson, 1962, Kiely, 1948; Kiely, 1949b; McOnie, 1964a, Roy, 1965). However, this list of non-citrus hosts is controversial and doubtful for two main reasons: 1) adequate cross inoculation details are lacking, and 2) on *Camellia*, *Dioscorea*, *Ilex*, *Persea*, *Psidium*, *Mangifera* and *Smilax*, fungal species have been described under the names *Guignardia camelliae*, *G. dioscoreae*, *G. philoprina*, *G. perseae*, *G. psidii*, *G. mangiferae* and *G. smilacis*, respectively.

Host list: *Citroncirus webberi*, *Citrus maxima*, *Citrus medica*, *Citrus reticulata*, *Citrus tankan*, *Citrus x aurantiifolia*, *Citrus x aurantium* var. *paradisi*, *Citrus x aurantium* var. *sinensis*, *Citrus x aurantium*, *Citrus x limon*, *Citrus x limonia*, *Citrus x nobilis*, *Citrus*, *Fortunella*

GEOGRAPHICAL DISTRIBUTION

P. citricarpa is thought to originate in south-east Asia. Citrus black spot was first recorded in Australia in 1895 on *Citrus sinensis* (Benson, 1895). The disease has been present for decades in many humid subtropical citrus-

producing regions in Africa, Australia, Southeast Asia, and South America (CABI, 2011; Kotzé, 1981; Paul *et al.*, 2005.). In early 2010, *P. citricarpa* was discovered for the first time in the USA, in commercial citrus orchards in Southern Florida (NAPPO, 2010; Schubert *et al.*, 2012). In 2019, the presence of *P. citricarpa* was officially confirmed in the Mediterranean Basin, in Tunisia (Governorate of Nabeul) where official phytosanitary measures are being implemented to control the disease (EPPO, 2019).

Due to its complex and evolving taxonomy, the geographical distribution of *P. citricarpa* is difficult to establish with certainty. Past records of *Phyllosticta* on citrus in some countries may in fact correspond to the non-pathogenic and morphologically similar species, *P. capitalensis*. The latter being an endophyte commonly isolated from citrus and other hosts, and recorded on all continents (Wikee *et al.*, 2013). For example, *P. citricarpa* has been reported from Mexico and Japan (Stringari *et al.*, 2009), but its establishment is not confirmed by other publications. A record for New Zealand in CMI (1990) refers in fact to *G. mangiferae* (Everett and Rees-George, 2006). Old records in CMI (1990) for Burma, India, Iran, Israel, Korea (Republic of), Lebanon, Malaysia, Pakistan, Singapore, Sri Lanka, Thailand, Vietnam, Egypt, Tanzania, Cook Islands, Niue, Tonga, Western Samoa, Fiji, Hawaii (USA), Papua New Guinea, Georgia, Belize, Honduras, Jamaica, Trinidad, Peru, Vanuatu and Venezuela are most probably misidentifications. The situation in Hong Kong, Swaziland and Nigeria is still unclear. In 2017, Guarnaccia *et al.* reported the finding of *P. citricarpa* in Malta (Gozo and Zurrieq), Italy (Trebisacce) and Portugal (Monchique) however, neither symptoms nor the pathogen have been detected during official surveys in the areas where *P. citricarpa* was been reported by these authors. Current knowledge about the distribution of other *Phyllosticta* species reveals that most of them are present in Asia, where citrus originated (Wu *et al.*, 2018). *P. citrichinaensis*, *P. citriasihana* and *P. citrimaxima* were found only in Asia, and the endophyte *P. citribraziliensis* has been reported only in South America (Glienke *et al.*, 2011; Guarnaccia *et al.*, 2017; Wang *et al.*, 2012; Wikee *et al.*, 2013b; Wulandari *et al.*, 2009).

The countries, states and provinces listed below are locations where *P. citricarpa* is known to be present or where substantial evidence exists that citrus black spot occurs.



EPPO Region: Tunisia

Africa: Angola, Benin, Botswana, Ghana, Kenya, Mozambique, Namibia, South Africa, Tunisia, Uganda, Zambia, Zimbabwe

Asia: Bhutan, China (Chongqing, Fujian, Guangdong, Guangxi, Jiangsu, Jiangxi, Sichuan, Xianggang (Hong Kong), Yunnan, Zhejiang), India (Maharashtra), Indonesia (Java), Myanmar, Philippines, Taiwan

North America: United States of America (Florida)

Central America and Caribbean: Cuba

South America: Argentina, Brazil (Amazonas, Bahia, Espírito Santo, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Pernambuco, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, São Paulo, Tocantins),

Uruguay

Oceania: Australia (New South Wales, Queensland)

BIOLOGY

Life cycle

The natural disease cycle of the pathogen *P. citricarpa* has two infection cycles, a primary cycle driven by ascospores in the leaf litter and a secondary cycle involving pycnidiospores produced on lesions on fruit, twigs and leaves (EFSA, 2014; Guarnaccia *et al.*, 2019). The disease cycle stages include: the start of the ascospore season, the subsequent dynamics of ascospore production, ascospore release, infection by ascospores, production of pycnidia, dispersal of conidia (pycnidiospores) and finally infection by conidia.

Pseudothecia develop 40–180 days after leaf drop, depending on the frequency of wetness and dryness periods, 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. On germination, ascospores produce appressoria with infection pegs that penetrate the cuticle. At their tips, between the cuticle and the upper epidermal cells, the infection pegs produce knots of fungal tissue which are considered to establish latent infection (McOnie, 1967; Kotzé, 1981, Marques *et al.*, 2012).

Pycnidia with conidia (pycnidiospores) are produced on fruit (Kotzé 1981), leaves, dead twigs (Whitesite, 1967; Spósito *et al.*, 2011), fruit pedicels and leaf litter (Kotzé, 2000; Silva *et al.*, 2017; Guarnaccia *et al.*, 2017). They may be dispersed over short distances (less than 80 cm) by washing-down, being responsible for spread of the pathogen within a tree canopy (Kotzé, 1981; Agostini *et al.*, 2006; Spósito *et al.*, 2008, 2011) or inter-canopy (Hendricks *et al.*, 2017). Subsequent studies under laboratory conditions demonstrated that *P. citricarpa* pycnidiospores could reach longer distances than previously thought (Perryman and West, 2014; Perryman *et al.*, 2014). Pycnidiospores on germination enter both unwounded and wounded fruits, and through abrasions caused by hail or insect damage (Kiely, 1949a; Lee, 1969).

The presence of a spermatial state (*Leptothiorella* state) producing spermatia contained in spermagonia on fallen dead leaves and in pure culture has been recorded as a part of the *P. citricarpa* life cycle, although this state has not been formally described (Baayen *et al.* 2002; Kiely 1948). This state is sometimes referred to in the literature as the 'spermagonal' state and the dumb-bell shaped microconidia as spermatial cells (Kiely, 1949a; Van der Aa, 1973). Kiely (1948) observed that spermagonia always appeared prior to the formation of pseudothecia and hypothesised that spermatia were functioning as male gametes to fertilize a female organ of *P. citricarpa*. However, little is known about the role of spermatia of *P. citricarpa*, since they are incapable of germinating and forming colonies on media.

Tran *et al.* 2017 demonstrated that *P. citricarpa* is heterothallic and requires isolates of different MAT idiomorphs to be in direct physical contact, or for spermatia to fulfill their role as male elements to fertilize the receptive organs, in order to initiate the mating process. The heterothallic nature of *P. citricarpa* was recently confirmed through full sequencing of target loci, showing the presence of separated MAT1-1 or MAT1-2 idiomorphs (Amorim *et al.*, 2017; Wang *et al.*, 2016).

Epidemiology

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 stage of the citrus tree and age of the fruit and leaves (Kotzé, 1981, 2000).

The disease has been reported in most of the major citrus-producing countries, mainly in areas with warm climates with summer rainfall (Carstens *et al.*, 2012; Magarey *et al.*, 2015; Martínez-Minaya *et al.*, 2018; Paul *et al.*, 2005; Yonow *et al.*, 2013). Spore production and release occur during the rainy season (Dummel *et al.*, 2015; Fourie *et al.*, 2013; Kiely, 1948; Kotzé, 2000; Reis *et al.*, 2006).

In areas where rain is confined to a single season, pseudothecia with ascospores, produced exclusively on leaf litter, are the main source 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). Studies from South Africa and Taiwan indicated that maturation of ascospores occurs practically simultaneously in early summer on infected leaves abscised during late autumn, winter and early spring (Kotzé, 1963; McOnie, 1964b; Lee and Huang, 1973). Ascospore release takes place during rainfall and occasionally during irrigation or when there is heavy dew (Kiely, 1949a; Kotzé, 2000). 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). Windborne ascospores are generally dispersed under field conditions over longer distances and are associated with the *P. citricarpa* spread between trees (Spósito, *et al.*, 2007). Ascospores were infectious at 15 to 29.5°C and with 15 to 38 hours of wetness (Kiely, 1949a; Kotzé, 1963; McOnie, 1967; Reis, *et al.*, 2006). In Brazil, comparatively low to moderate numbers of ascospores were produced from October to March with peak production in January and February (Reis *et al.*, 2003) while in South Africa, ascospore release occurred mainly from November to March with the highest numbers from December to January (McOnie, 1964b, c).

Where rain is not confined to a single season, where out-of-season fruit with lesions remain 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 (pycnidiospores) of *P. citricarpa* are also important as inoculum sources (Kotzé, 1981; Spósito *et al.*, 2008, 2011). Under *in vitro* conditions, pycnidiospores of *P. citricarpa* can germinate and form appressoria between 10 and 40°C and 12–48 hours of wetness (Noronha, 2002). In Australia, freshly exuded mature pycnidiospores have been reported to lose their ability to germinate 1 month after they were produced (Kiely, 1949a) however, in South Africa pycnidiospores have been reported to retain their germinative capacity up to 5 months (Wager, 1952).

It has been observed that the pathogen may be present for many years in a region before the disease reaches epidemic proportions. In Mpumalanga province in South Africa, symptoms were present for over three decades before control measures became necessary (Kotzé, 1981). Infection is usually followed by a long period of latency which may last 12-36 months in Australia and about 3-12 months after anthesis for fruit infection in South Africa (McOnie, 1967; Kellerman & Kotzé, 1979). In artificial inoculations conducted under greenhouse conditions, the incubation period ranged from over 200 days for 3-cm-diameter sweet orange fruit to about 50 days for 7-cm-diameter fruit (Aguiar *et al.*, 2012). In field trials carried out in Australia, young fruits inoculated with conidial suspensions after petal fall in October produced black spot disease after nearly 1 year (Kiely, 1949a). In South Africa, young citrus fruits inoculated with high concentrations of conidia in mid-November showed speckled blotch lesions by the end of January the following year (McOnie, 1964e).

Epidemiological studies outside of North America indicate that the most important inoculum for disease spread is ascospores, which are solely produced in decomposing leaf litter under alternating wetting and drying cycles (Kotzé 1981; Reis *et al.*, 2006; Spósito *et al.*, 2008). However, evidence gathered from different parts of the world illustrates the importance of pycnidiospores during the early stages of invasion. At the beginning of the epidemics in Zimbabwe, it was shown that most infections originated from pycnidiospores, while ascospores were only found in very small numbers (Whiteside, 1967). In Argentina, Garrán (1996) indicated that attempts to detect the sexual stage by weekly sampling of dead leaves in plots affected by citrus black spot were unsuccessful. Epidemiological studies conducted in São Paulo, Brazil, found an aggregated spatial pattern of citrus black spot-affected trees in the orchard as well as diseased fruit in the canopy, suggesting that splash-dispersed conidia have an important role in this region (Spósito *et al.*, 2007, 2008, 2011), even in the presence of complementary mating types allowing for sexual reproduction through ascospores (Amorim *et al.*, 2017). In Florida, where citrus black spot was first observed in 2010 (Schubert *et al.*, 2012), disease establishment and spread were attributed to pycnidiospores since the population of *P. citricarpa* is clonal and only one mating type (MAT1-2) is present (Wang *et al.*, 2016; Hendricks *et al.*, 2017).

Flowers and fruits are susceptible to infection from anthesis for approximately 4–6 months (Kellerman & Kotzé, 1979) but the first symptoms on fruit do not appear for more than 6 months after fruit set (Baldassari *et al.*, 2006). According to Frare *et al.* (2019), the length of the latent period can be influenced by factors, such as the inoculum concentration and fruit diameter.

DETECTION AND IDENTIFICATION

Symptoms

P. citricarpa causes diverse symptoms such as hard spot, virulent spot, false melanose and freckle spot on fruit and necrotic lesions on leaves and twigs (Kotzé, 1981, 2000).

The presence of *P. citricarpa* on fruit is unlikely to be confirmed based on visual examination alone, since symptoms on fruit are variable in appearance and often resemble those caused by other citrus pathogens (such as *P. citriasianna*, *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).

The following four types of symptoms are widely recognized as associated with *P. citricarpa* and have been described by Kiely (1949a, 1949b, 1960):

Hard spot: the most typical symptom of citrus black spot, characterised by sunken, pale brown necrotic lesions, 3–10 mm in diameter, with a dark reddish-brown raised border, often containing pycnidia. A yellow halo, when the fruit is green, or a green halo, when the fruit is yellow or orange, may appear around these lesions. 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).

Freckle spot: cracked or speckled spots, grey, tan, reddish or colourless, 1–3 mm in diameter, slightly depressed at the centre, with no halo around them and almost always devoid of pycnidia. Freckle spots mostly develop after the fruit has changed colour (Bonants *et al.*, 2003).

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). The lesions are devoid of pycnidia and may coalesce as the season progresses (CABI, 2019). 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 necrotic lesions without defined borders mostly on mature fruit. Numerous pycnidia eventually develop in these lesions under conditions of high humidity (Kotzé, 2000). Virulent 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 postharvest losses (Kotzé, 1981).

Two additional types of symptoms have also been reported to occur infrequently on citrus fruit: *lacy spot*, superficial yellow lesions with a dark yellow to brown centre, a smooth texture and no defined margins (Aguilar-Vildoso *et al.*, 2002) and *cracked spot*, superficial slightly raised dark brown to black lesions, variable in size, with a cracked surface and irregular margins (Goes *et al.*, 2000).

Leaf and twigs symptoms rarely occur on orange, mandarin and other commercial citrus species, but they are frequently present on lemons. They appear as round, small, sunken necrotic lesions with a yellow halo (Kotzé, 1981).

Morphology

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).

Pycnidia produced on fruit, attached leaves, dead twigs and leaf litter as well as in culture are solitary or occasionally aggregated, globose, immersed, mid- to dark brown, and 70–330 μm in diameter. They contain aseptate, hyaline conidia, multiguttulate, 9.4–12.7 $\mu\text{m} \times$ (5.0–8.5) μm , with a colourless subulate appendage and a barely visible, colourless, gelatinous sheath <1.5 μm thick.

The sexual morph presents erumpent, globose to pyriform ascomata, often irregularly shaped, unilocular and with a central ostiole. Ascii are eight-spored, bitunicate, clavate to broadly ellipsoid, with a wide, obtusely rounded or slightly square apex. Ascospores are ellipsoid to limoniform, sometimes slightly elongated, aseptate, hyaline, swollen in the middle, slightly curved, 12–16 $\mu\text{m} \times$ 4.5–6.5 μm , showing a large central guttule and a mucoid cap at both ends. Spermatia produced both on hosts and in pure culture are hyaline, aseptate, cylindrical to dumbbell-

shaped with guttules at each end, 5–8 ?m × 0.5–1 ?m.

Detection and inspection methods

Identification of *P. citricarpa* is difficult. The distinction between this and similar species based on morphological examination is very difficult and additionally most of the molecular tests available are not specific to *P. citricarpa*. Several of the tests available are described below and these should be combined as recommended in the the EPPO Standard PM 7/17 to have a reliable identification (EPPO, 2020).

Phyllosticta species from citrus requires isolation of the slow-growing fungus and comparison of several traits (cultural and morphological characteristics). For isolation and culturing of the fungus the following media are usually used: cherry decoction agar (CHA), potato dextrose agar (PDA) or PDA amended with 50 ?g/mL penicillin and 50 ?g/mL streptomycin, oatmeal agar (OA) and malt extract agar (MEA). *P. citricarpa* colonies grow slowly on CHA (Baayen *et al.*, 2002) and usually produce a yellow pigment on OA that diffuses into the medium around the colony. Proof of pathogenicity requires inoculation of fruit or leaves and an incubation period of weeks or months before symptoms appear (Kotzé, 2000). Discrimination between *P. citricarpa* and other *Phyllosticta* species based on morphological features is difficult, time consuming and requires considerable taxonomic expertise, hence a range of molecular tests have been developed to distinguish *P. citricarpa* from other species present on citrus fruits.

Bonants *et al.* (2003) developed a PCR-based detection method. Stringari *et al.* (2009) used random amplified polymorphic DNA (RAPD) markers to develop specific primers for the identification of *P. citricarpa* with PCR. Gent-Pelzer *et al.* (2007) developed a TaqMan PCR method for the diagnosis of *P. citricarpa* on citrus fruit which was more sensitive than conventional PCR, while Hu *et al.* (2014) used quantitative polymerase chain reaction (qPCR) tests based on internal transcribed spacer (ITS)-1 genes for detection and quantification *P. citricarpa* and *P. capitalensis* in leaf litter samples. A real?time PCR test specific for *P. citricarpa* which did not amplify *P. citriasihana* or *P. capitalensis* DNA, was designed by Schirmacher *et al.* (2019). Several studies have been performed with multigene sequencing of a large number of *Phyllosticta* species (Glienke *et al.*, 2011; Wang *et al.*, 2012; Wulandari *et al.*, 2009). Species-specific primers were used to amplify the internal transcribed spacer region (ITS) (de Hoog & Gerrits van den Ende, 1998; White *et al.*, 1990; Guarnaccia *et al.* 2017), part of the translation elongation factor 1-? gene (*tef1*) (Carbone & Kohn, 1999; O'Donnell *et al.*, 1998; Guarnaccia *et al.* 2017), part of the actin gene (*actA*) (Carbone & Kohn, 1999; Guarnaccia *et al.* 2017), the 28S large subunit nrDNA (**LSU**) (Moncalvo *et al.*, 1995; Vilgalys & Hester, 1990; Guarnaccia *et al.* 2017), the RNA polymerase II second largest subunit (*rpb2*) (Sung *et al.*, 2007; Liu *et al.*, 1999; Guarnaccia *et al.* 2017) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (Myllys *et al.*, 2002; Guerber *et al.*, 2003; Glienke *et al.*, 2011; Guarnaccia *et al.*, 2017). A multilocus phylogenetic analysis, using partial DNA sequences from genes encoding the above loci, was carried out on many *Phyllosticta* species (Sanders *et al.*, 2003; Guarnaccia *et al.*, 2017).

In addition to improved methods for laboratories (e.g. high throughput qPCR), in recent years a lot of research has been focused also on improved methods for inspection services in the field (e.g. Lateral Flow Devices and LAMP methods). As an aid to visual inspection of symptoms, Tomlinson *et al.* (2013) have developed a method for detection of *P. citricarpa* using loop-mediated isothermal amplification (LAMP) which can be used to test black spot lesions, including those lacking pycnidia.

Guidance on the detection and identification of *P. citricarpa* can be found in the EPPO Standard PM 7/17 and ISPM 27 (EPPO, 2020; FAO, 2016).

PATHWAYS FOR MOVEMENT

Natural spread of *P. citricarpa* is mainly ensured by its airborne ascospores over short distances. Conidia (pycnidiospores) produced on fruit, leaves, dead twigs, fruit pedicels and on leaf litter may be also dispersed over short distances (less than 80 cm) by washing-down or rain splashed, being responsible for spread of the pathogen within a tree canopy (Kotzé, 1981; Agostini *et al.*, 2006; Spósito *et al.*, 2008, 2011) or inter-canopy (Hendricks *et al*., 2017).

Over long distances, the pathogen is very likely to be spread with human assistance by the international trade of citrus fruit and plants for planting (if not prohibited). Current knowledge about the distribution of *Phyllosticta*

species reveals that most of them are present in Asia, where citrus originated (Wu *et al.*, 2018). In addition, MAT genotyping of populations demonstrates that several incursions have occurred along with the global establishment of citrus cultivation and given the long-range dispersal of these species via infected citrus plant propagation material (Amorim *et al.*, 2017; Carstens *et al.*, 2017; Guarnaccia *et al.*, 2017; Hendricks *et al.*, 2017; Tran *et al.*, 2017; Wang *et al.*, 2016; Zhang *et al.*, 2015). The ‘plants for planting’ pathway from a citrus production area where both mating types occur would be in general the most likely route of introduction for both mating types, as the plant will be a persistent source of inoculum and sexual ascospores can be produced on the citrus leaves in the leaf litter.

In the EPPO region, *P. citricarpa* is regularly intercepted on citrus fruit imports from countries where citrus black spot occurs (Europhyt). However, it is generally considered that the risk of transferring *P. citricarpa* from infested fruit to citrus plants is relatively low (EFSA, 2014). Citrus fruit will be more likely to introduce a single mating type clonal genotype of *P. citricarpa*, compared to an infected plant for planting. This is because *P. citricarpa* only reproduces on fruit through asexual pycnidiospores (Kotzé, 2000). More recently, Tran *et al.* (2017) suggested that only one mating type is present in a single disease lesion in the above ground parts. Moreover, fruit is short-lived compared to an infected plant and so the chance of an infection establishing by splash dispersal from a single lesion of a transient fruit would be lower. In addition to that, citrus black spot lesions on fruit or discarded peel segments have a very low reproductive potential (Korf *et al.*, 2001; Schreuder *et al.*, 2018; Schutte *et al.*, 2014).

PEST SIGNIFICANCE

Economic impact

In most of its current distribution, *P. citricarpa* is reported to cause severe quality and yield losses to citrus fruit production. Several symptoms including hard spot, virulent spot, and false melanose occur on the rind of affected fruit, reducing its commercial value for the fresh market (Kotzé, 2000). Premature fruit drop due to *P. citricarpa* causes significant yield loss in Brazil, and probably in other citrus regions of the world (Reis *et al.*, 2006; Spósito *et al.*, 2011; Araújo *et al.*, 2013).

In the Windsor and Hawkesbury River areas of Australia in 1931, all orchards of sweet orange cvs Washington Navel, Joppa and White Siletta were severely affected and losses of 80% were common in individual orchards (Kiely, 1960). Before the adoption of control measures, heavy losses in sweet orange cv. Valencia had been reported in the coastal orchards in New South Wales (Kiely, 1949a; 1949b). In South Africa 90% of fruits from unprotected trees were claimed to be unfit for export (McOnie, 1964b) and losses of more than 80% of unprotected fruits were reported to be common (McOnie, 1964d). The apparent absence of severe impact at specific locations, e.g. in Addo, Eastern Cape, South Africa, where the pathogen is reported to ‘persist but not flourish’ (Yonow *et al.*, 2013), could be due to the relatively recent emergence of the disease, as well as to the fungicide schedules currently in place.

Citrus black spot is expected to affect mainly lemons and late-maturing sweet orange and mandarin varieties, with moderate negative consequences for the production of fresh fruit. There would be a potential for reduction in disease incidence by chemical treatments, but this would cause environmental impacts because in most European and Mediterranean countries fungicides are not widely applied, and the most effective fungicide products are not currently registered for use in citrus. In addition, to export citrus fruit to areas where *P. citricarpa* is regulated, additional fungicide treatments in the orchards, official inspections, quality controls in packing houses and/or establishment of pest-free areas might be needed to meet the phytosanitary requirements of these countries. The consequences would be minimal for citrus fruit intended for processing, as external lesions or spots on citrus fruit are not a quality issue for citrus for processing.

Control

Preventing the introduction into new areas

Since eradication and containment are difficult, phytosanitary measures should focus on preventing the introduction of the disease into new areas. Fruit is not considered to be a likely pathway for spread of *P. citricarpa* to new areas (USDA APHIS, 2010). Trees from certified *P. citricarpa*-free nurseries should be used for the establishment of new orchards, (Whiteside, 1965; Kiely, 1948b; Kotzé, 1981; Marchionatto, 1926; McOnie, 1964b; Silva-Junior *et al.*,

2016a;). It is also important to prevent the movement of leaf litter from infected orchards through vehicle/ machine movement (Dewdney *et al.*, 2018; Silva-Junior *et al.*, 2016a).

Cultural measures

In areas where *P. citricarpa* is present, sanitation practices in the orchard are the most important cultural management practices for *P. citricarpa* inoculum reduction. These include the removal and destruction of leaf litter from the orchard floor as fallen leaves may harbour spores (Scaloppi *et al.*, 2012; Spósito *et al.*, 2011; Truter, 2010, Bellotte *et al.*, 2013; Schutte and Kotzé, 1997). Removal of late-hanging fruit may prevent *P. citricarpa* infection of new fruit flushes, especially in citrus varieties where new and old fruit flushes can overlap (Calavan, 1960; Kiely, 1969; Kotzé, 1996; Dewdney *et al.*, 2018).

Maintaining tree vigour during fruit growth, using timely fertilization and irrigation regimes, may reduce *P. citricarpa* incidence (Calavan, 1960; Dewdney *et al.*, 2018; Kotzé, 1981). Declining trees are more susceptible and thus management practices for pest and disease control should be used.

Pruning of dead twigs remove *P. citricarpa* inoculum (Silva *et al.*, 2017; Silva-Junior *et al.*, 2016a) while pruning the tree canopy increases airflow which helps reduce leaf wetness periods, disease incidence and pycnidiospore dissemination (Calavan, 1960; Kotzé, 1981; Dewdney *et al.*, 2013).

Mulching with plants that grow between rows of orchards to cover leaf litter reduced disease incidence (Bellotte *et al.*, 2013; Schutte and Kotzé, 1997).

Host-plant resistance

Attempts have been made to produce tolerant hybrids using sour orange (*C. aurantium*) as a source of resistance (Anon., 1974). Rodriguez *et al.* (2018) produced transgenic sweet orange trees with reduced production of d-limonene in the fruit flavedo and enhanced resistance against *P. citricarpa*, but these materials need to be assessed under field conditions. According to Miles *et al.* (2019) hybrid progeny from crosses using pomelo as a parent is preliminary evidence of segregation for citrus black spot immunity. These preliminary results could open new perspectives for breeding *P. citricarpa* -resistant citrus cultivars.

Chemical control

Effective chemical control relies heavily on well-timed applications of protectant and systemic fungicides (Kellerman and Kotzé, 1979; Lanza *et al.*, 2018; Makowski *et al.*, 2014; Schutte *et al.*, 2003) integrated with cultural practices. Spore trapping, as well as rainfall and dew measurements have been helpful in determining the timing of ascospore release and the need for fungicide applications in South Africa (Kotzé, 2000). In Brazil, infections seem to occur to varying degrees throughout the susceptible period and fruit must be protected from petal fall to mid-summer (Reis, 2002). Disease management decisions should rely on seasonal dynamics studies of the *P. citricarpa* in environmental samples such as leaf litter. Hu *et al.* (2014) developed a real-time PCR test for this purpose.

For many years, protective products such as copper and dithiocarbamates were the basis for the control program (Bertus, 1981; Kiely, 1950, 1976; Kotzé, 1981; Tsai, 1981). Subsequently, mid-summer post-infectious applications of benzimidazoles were sufficient to control *P. citricarpa* in many areas (Kellerman and Kotzé, 1973, 1979). With the development of resistance (Herbert and Grech, 1985) many growers have returned to the use of protectant sprays or combinations of systemic and protectant products (Kotzé, 2000; Goes *et al.*, 2000; Goes, 2002). Use of strobilurins may reduce citrus black spot symptoms by almost 100%, (Dewdney *et al.*, 2018; Fogliata *et al.*, 2011; Miles *et al.*, 2004; Schutte *et al.*, 2003; Silva-Junior *et al.*, 2016a). However, there is a potential problem with strobilurins resistance (Hincapie *et al.*, 2014; Stammler *et al.*, 2013). Rotations of strobilurin fungicides with copper-based fungicides or mancozeb are recommended for effective *P. citricarpa* control (Schutte *et al.*, 2003; Miles *et al.*, 2004).

Postharvest

Fruit from citrus black spot-infected groves often bear quiescent infections that may later develop into black spot lesions in transport or at the final destination (Hall, 1973).

After harvest, hot water treatments or application of water-wax emulsions have been shown to reduce the development of citrus black spot during storage (Korf *et al.*, 2001; Seberry *et al.*, 1967; Wild, 1981). Storage in the dark and at low temperatures tends to reduce the development of disease symptoms (Kiely, 1970; Korf *et al.*, 2001; Agostini *et al.*, 2006; Brodrick and Rabie, 1970). On the other hand, if it is desirable that black spot symptoms are expressed as soon as possible for detection of the disease, then fruit should be held at 27°C under continuous light. In Brazil, Uruguay and South Africa, ethephon has been used to induce symptom expression in citrus fruits intended for export (Baldassari *et al.*, 2007)

Schreuder *et al.* (2018) demonstrated that the combination of standard packhouse treatments (including pre-packhouse drench with guazatine or propiconazole in combination with pyrimethanil, thiabendazole and 2,4-D; chlorine wash; dip treatment in imazalil; and brush application of a wax coating incorporated with imazalil, thiabendazole and 2,4-D) consistently showed moderate to high levels of control of *P. citricarpa* in lemons and oranges while cold storage subsequent to packhouse treatments (as is common shipping protocol) further improved the levels of control. According to Yan *et al.* (2016), postharvest fungicide applications could reduce *P. citricarpa* severity, but not incidence, on orange fruit that are still asymptomatic at harvest. Agostini *et al.* (2006) also reported no significant control with postharvest fungicide treatments and suggested that the most effective means to reduce postharvest development of symptoms is through preventive application of fungicides during the fruit growing season and cold storage of harvested fruit.

Phytosanitary risk

Though of tropical origin, the fungus has established itself and causes serious damage in subtropical climates, e.g. China, New South Wales (Australia) and South Africa. The detection of *P. citricarpa* in 2019 in citrus orchards in Tunisia confirms that the pathogen has the potential to enter and possibly establish in the EPPO region. The origin of this outbreak is unknown, but it is suspected that infected plant material had been brought illegally (import of citrus plants for planting is prohibited in Tunisia) and that the floods which occurred in 2018 in the infected area facilitated fungal dispersal (EPPO, 2019). Although, there are some uncertainties about the potential of establishment of *P. citricarpa* in citrus-growing countries (EFSA, 2014), it is recognized that this pathogen has the potential to cause direct and indirect losses (i.e. reduced market access) to citrus production in the EPPO region.

PHYTOSANITARY MEASURES

In citrus-producing countries of the EPPO region, it is usually prohibited to import citrus plants for planting from outside the region. This measure effectively covers the risk of introducing *P. citricarpa* (and other citrus pests) via imports of planting material. In some cases (e.g. valuable breeding material), certification and pre- and post-entry quarantine systems can be envisaged. *P. citricarpa* can readily be carried on imported citrus fruits, but the risk of spread from these is considered to be relatively low. However, fruits from infested countries should come from orchards found free from, or be treated against the pathogen. The induction of precocious symptoms expression can be applied at the point of entry to help detecting latent infections. As explained above, in countries where *P. citricarpa* is present, exporters are performing an ethephon treatment to enhance symptom expression on exported fruits. The application of strict waste processing measures would probably be effective in reducing the transfer of *P. citricarpa* from infected citrus fruit to citrus orchards, but this would probably be difficult to apply in practice. After establishment, *P. citricarpa* has not been eradicated anywhere and is reported to be very difficult to contain. Therefore, risk reduction options to prevent the entry of the pathogen have been evaluated as being the most effective (EFSA, 2014).

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