CHAPTER 1

General introduction

Citrus is the most important fruit crop in the world in terms of production, with 122 million metric tonnes (Mt) produced in 2008 (FAO, 2010a). Citrus is grown in more than 100 countries all over the world in tropical and subtropical areas, located within 40° north and south of the equator (Davies & Albrigo, 1994; Spiegel-Roy & Goldschmidt, 1996). Major citrus producing countries include China, Brazil, United States of America (USA), Mexico, India, Spain, Italy, Iran, Egypt and Turkey (FAO, 2010a). South Africa (SA) is the 12th largest producer of citrus world-wide with 2.2 Mt during 2008, consisting of sweet orange (*Citrus sinensis* Osbeck) (66% of production), grapefruit (*Citrus paradisi* Macf.) (17%), lemon (*Citrus limon* (L.) Burn. f.) and lime (*Citrus aurantifolia* Christm.) (11%) and mandarin (*Citrus deliciosa* Ten., *Citrus reticulata* Blanco and *Citrus unshiu* Marc.) (6%) (FAO, 2010a).

Citrus production in SA is largely limited to irrigation areas and takes place in Limpopo (16 255 ha), Mpumalanga (11 681 ha), Eastern Cape (12 923 ha), KwaZulu-Natal (4 004 ha), Western Cape (9 524 ha) and Northern Cape Province (639 ha) (Burger, 2009). SA's citrus industry are export-oriented with total exports averaging at about 65% of total production, while processing and local consumption are at about 25% and 10%, respectively (Siphugu, 2009). In 2007, SA was world-wide the second largest exporter of fresh citrus, after Spain, at 1.4 Mt (FAO, 2010b). Although production is relatively small compared to other countries, the citrus industry significantly contributes to the economy. In the 2007/2008 season, income from citrus showed the biggest increase of 35% from the previous year and amounted to R5 013 million (Burger, 2009).

The genus *Citrus* L. belongs to the subfamily Aurantiodeae, within the family Rutaceae. The family contains about 150 genera and 1 600 species but true citrus and related genera all belong to Aurantioideae (Spiegel-Roy & Goldschmidt, 1996; Mukhopadhyay, 2004). The taxonomy of *Citrus* are complex and confusing, and complicated by several factors such as a long history of cultivation of over 4000 years, a high frequency of bud mutation, ability to reproduce asexually by seed through nucellar embryony, sexual compatibility between *Citrus* and related genera and the ability of species to hybridise naturally (Barrett & Rhodes, 1976; Federici *et al.*, 1998; Nicolosi *et al.*, 2000; Moore, 2001). Currently two different classification systems are used for citrus taxonomy, the

system of Swingle (1943, 1967) recognising 16 species and that of Tanaka (1954, 1961) recognising 162 species.

Hybridisation has played an important role in the evolution of many, or even most, *Citrus* species. Many of the named species are clonally propagated hybrids and there is genetic evidence that even some wild, true-breeding species are of hybrid origin (Nicolosi *et al*., 2000; Moore, 2001; Nicolosi, 2007). Phylogenetic analyses, supported by biochemical and molecular markers, suggested that there are only three true species within the cultivated *Citrus*, i.e. *Citrus medica* L. (citron), *Citrus reticulata* Blanco (mandarin) and *Citrus grandis* (L.) Osb. (pummelo) (Scora, 1975; Barrett & Rhodes, 1976; Federici *et al*., 1998; Nicolosi *et al*., 2000; Moore, 2001; Barkley *et al*., 2006; Nicolosi, 2007).

Spread of citrus from its origin in the tropical and subtropical regions of Asia and the Malay Archipelago to other parts of the world occurred mainly through migration and trade (Reuter *et al*., 1967). The most ancient *Citrus* species, citron, is probably native to India, while pummelo originated in Malaysia, Indonesia and Vietnam, and mandarin in southern China and Japan (Mukhopadhyay, 2004; Nicolosi, 2007). Citrus appears to have spread relatively slowly over thousands of years south-east through the Philippines and the Pacific Islands and was subsequently introduced to Europe around 310 B.C., America in 1493, southern Africa in 1654 and Australia in 1788 (Reuter *et al*., 1967; Spurling, 1969). Worldwide trade in citrus fruit did not appear until the 1800's and trade in orange juice developed as late as 1940 (Reuter *et al*., 1967).

Today there are five major citrus groups that are world-wide of commercial significance, viz. grapefruit, lemon, lime, mandarin and sweet orange (Davies & Albrigo, 1994; FAO, 2010a, b). Various cultivars within each species have developed, which differ in fruit size, shape, seed content, quality and season of maturity. Sweet orange is the most widely distributed and produced citrus crop in the world, consisting of 55.5% of world production in 2008, followed by mandarin (23.4%), lemon and lime (11.0%) and grapefruit (4.1%) (FAO, 2010a).

As with most agricultural crops, many factors are known to limit the production and quality of citrus. Major constraints to citrus production involve management inefficiencies, susceptibility to pests and diseases and environmental challenges. Citrus diseases can have a profound impact on citrus production by not only leading to increasing production costs, but also resulting in large losses of harvestable and/or marketable crop. One of these diseases that has a profound influence on the marketability of citrus fruit, is citrus

black spot (CBS) caused by *Guignardia citricarpa* (Kiely) (anamorph *Phyllosticta citricarpa* (McAlpine) Aa).

G. citricarpa occurs for a large part of its life cycle in an endophytic state and has been extensively isolated from healthy citrus tissue (Azevedo *et al.*, 2000; Araújo *et al.*, 2001; Baayen *et al.*, 2002; Glienke-Blanco *et al.*, 2002; Durán *et al.*, 2005; Baldassari *et al.*, 2008). The pathogen can cause a variety of cosmetic and superficial lesions on citrus fruit, leaves and twigs under favourable conditions. Single lesions remain small and do not negatively influence the quality of fruit but symptomatic fruit are unacceptable to the fresh and export markets (Kotzé, 1981).

Almost all commercial citrus species are susceptible to CBS, and lemons are the most susceptible. When CBS is found in a new area, it is usually first observed on lemons before other citrus is affected (Kiely, 1948; Kotzé, 1981). Sour orange (*Citrus aurantium* L.) and its hybrids, rough lemon (*Citrus jambhiri* Lish.) and Tahiti acid lime (*Citrus latifolia* Tan.) are insensitive to the pathogen (Wager, 1952; Kotzé, 1981; Baldassari *et al.*, 2008).

CBS originated in South East Asia (Smith *et al.*, 1997), but the symptoms were first described from infected sweet orange fruit by Benson (1895) in Australia. Today the disease is widespread and occurs in Argentina, Australia, Bhutan, Brazil, China, Ghana, India, Indonesia, Kenya, Mozambique, Nigeria, Philippines, SA, Swaziland, Taiwan, USA, Uruguay, West Indies, Zambia and Zimbabwe (European Union, 1998; Baayen *et al.*, 2002; Paul *et al.*, 2005; Lemon & McNally, 2010; Schubert *et al.*, 2010). The global distribution of the disease appears to partially follow citrus production patterns but is restricted by specific climatic parameters, of which cold wet conditions during winter were indicated as the main restrictive parameters (Paul *et al.*, 2005; Yonow & Hattingh, 2009). CBS has not been recorded in citrus producing Mediterranean and European countries, or in Chile, Japan and New Zealand (European Union, 1998; Baayen *et al.*, 2002; Paul *et al.*, 2005; Everett & Rees-George, 2006).

The disease has resulted in barriers to trade, due to the potential phytosanitary risk associated with the export of fruit from CBS positive production areas to particularly the European Union (EU) and USA (European Union, 1998; Baayen *et al.*, 2002). Although CBS has recently been recorded in Florida, USA, trade restrictions regarding imports to the USA still apply (Lemon & McNally, 2010). In addition to the phytosanitary trade barriers, economic losses attributed to CBS includes premature fruit drop in heavy infected orchards, lower market value of symptomatic fruit and higher production costs

due to extensive control programmes (Wager, 1952; Kellerman & Kotzé, 1973, 1977). If not controlled, CBS may cause total loss of the marketable crop in some areas, and without effective CBS control programmes, citrus production will be unfeasible (Kotzé, 1981; Smith, 1996). The extent of post-harvest losses are not always apparent as latently infected, asymptomatic export fruit may develop CBS symptoms while in transit and may be rejected upon arrival (Kiely, 1948; Loest, 1958; Smith, 1962; Brodrick, 1969). Whole consignments of fruit may be rejected at packinghouses or ports if, during inspection, they are found to contain affected fruit (Bonants *et al*., 2003). Consequently, CBS has a great impact on global trade of citrus, and is of great concern to affected growers.

Phytosanitary barriers to trade play a vital role in protecting a country from introduction of alien species by restricting the movement of plant material world-wide (European Union, 1998; Baayen *et al*., 2002). However, countries may not impose unnecessary restrictions on traded commodities and restrictions can only be imposed if based on scientifically justifiable principles (WTO, 1993). Ideally, the potential risks of introduction and establishment of a pathogen or pest into a new geographical location should be determined through a Pest Risk Assessment (PRA) that is supported by scientific research (IPPC, 1996; Rafoss, 2003). In PRA studies the life cycle, host specificity, and current and potential geographical distribution of the organism is considered (McKenney *et al*., 2003). If findings suggest that the risk of introduction is very low, phytosanitary measures may be removed in part or all together.

A PRA on the potential risk of CBS introduction into European countries through commercial citrus fruit exports were presented by SA to the European Commission in 2000 in a request to amend the current phytosanitary regulations (Hattingh *et al*., 2000). The PRA suggested that the risk of introducing CBS based on the aetiology of the pathogen and epidemiology of the disease is very low. In response, the European Commission stated that there is not enough scientific evidence to support a final decision to amend current phytosanitary regulations (European Union, 2001). More research was then required on various epidemiological aspects of the disease and in particular on the risk of infected fruit as inoculum source for CBS free areas. This study was designed to address this question as well as other epidemiological aspects of CBS that needed clarification.

The main aim of this study was to further elucidate some of the epidemiology of CBS, including inoculum production on infected fruit and leaf litter, susceptibility of citrus leaves

and leaf litter to infection, detection and monitoring methods as well as non-chemical control.

The approach was to:

1. review our current knowledge of the pathogen and disease (Chapter 2);
2. evaluate the likelihood of infection of leaf litter by symptomatic fruit (Chapter 3);
3. evaluate susceptibility of citrus leaves to the CBS pathogen from emergence to fully developed (Chapter 4);
4. evaluate ascospore production on leaf litter (Chapter 5);
5. develop and standardise a method to detect the pathogen in symptomless leaves (Chapter 6);
6. evaluate effect of leaf litter management on inoculum levels in a commercial orchard (Chapter 7).

A summary of the conclusions is presented in Chapter 8.

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CHAPTER 2

Review of *Guignardia citricarpa* Kiely, the causal agent of citrus black spot

2.1 The pathogen, *Guignardia citricarpa*

2.1.1 Origin and distribution of *Guignardia citricarpa*

Guignardia citricarpa Kiely originated collectively with its host, *Citrus* L., from South East Asia (Smith *et al*., 1997). The asexual form of the fungus was first described by McAlpine in 1899 as *Phoma citricarpa* McAlpine from symptomatic citrus fruit in Australia. Since then it had two name changes and *Phyllosticta citricarpa* (McAlpine) Aa is currently the accepted name (Van der Aa, 1973; Van der Aa & Vanev, 2002). The sexual form was described by Kiely (1948b) as *G. citricarpa* from citrus leaf litter in Australia. The spermatial state or synanamorph is a *Leptodothiorella* and the species has not been described (Van der Aa, 1973; Baayen *et al*., 2002).

Today, the citrus pathogen is widespread and occurs in Argentina, Australia, Bhutan, Brazil, China, Ghana, India, Indonesia, Kenya, Mozambique, Nigeria, Philippines, South Africa (SA), Swaziland, Taiwan, United States of America (USA), Uruguay, West Indies, Zambia and Zimbabwe (European Union, 1998; Baayen *et al*., 2002; Paul *et al*., 2005; Lemon & McNally, 2010; Schubert *et al*., 2010). *G. citricarpa* has not been recorded in Mediterranean and European countries, or in Chile, Japan and New Zealand (European Union, 1998; Baayen *et al*., 2002; Paul *et al*., 2005; Everett & Rees-George, 2006a).

2.1.2 *Guignardia* species on citrus

There are two main morphologically similar *Guignardia* species occurring on *Citrus*, *G. citricarpa*, causing black spot or symptomless infections in *Citrus*, and *Guignardia mangiferae* A.J. Roy, non-pathogenic to *Citrus*, causing only symptomless infections that remains latent (Meyer *et al*., 2001; Baayen *et al*., 2002; Bonants *et al*., 2003). The endophytic nature of the fungi on citrus caused confusion in the past, since all isolates of *Guignardia* obtained from *Citrus* was considered to be the citrus pathogen, *G. citricarpa*. The latent or endophytic nature of *G. citricarpa* was first recognised by Cobb (1897), and the pathogen has ubiquitously been isolated from healthy citrus tissue (McOnie, 1964a, d; Araújo *et al*., 2001; Glienke-Blanco *et al*., 2002; Bonants *et al*., 2003; Baldassari *et al*., 2008).

Both species of *Guignardia* may simultaneously colonise the same citrus tissue, being either symptomatic or symptomless leaves, twigs or fruit (McOnie, 1964a, d; Baayen *et al*., 2002; Bonants *et al*., 2003; Baldassari *et al*., 2006) and have been reported to coexist in a single black spot lesion (Baldassari *et al*., 2008). Furthermore both species have been reported from cultivars not susceptible to CBS, including Seville sour orange (*Citrus aurantium* L.) and Tahiti acid lime (*Citrus latifolia* Tan.) (McOnie, 1964d; Baldassari *et al*., 2008), contributing further to the uncertainty surrounding the identity of the pathogen for so many years.

Apart from pathogenicity, these species differ in culture characteristics and host range. Isolates of *G. citricarpa* can be distinguished from *G. mangiferae* by a combination of several characteristics (Table 2.1), although none of the characteristics on its own was found to separate both species unambiguously (Baayen *et al*., 2002). One of the more useful characteristics is the yellow pigment production at the edge of colonies on Oats agar (OA). Only isolates of *G. citricarpa* produce a yellow pigment on OA and it is reported to be a consistent trait in *G. citricarpa* isolates from various citrus materials (Baayen *et al*., 2002; Baldassari *et al*., 2008). However, Wulandari *et al*. (2009) reported three isolates of *G. mangiferae* producing yellow pigment on OA. Also, sporulation is required for confirmation as other fungi may resemble *G. citricarpa* while still sterile.

Another important characteristic is the production of spores in culture and although the feature is consistent in fresh isolates, there are numerous conflicting reports. Isolates from *G. citricarpa* never produces ascospores in culture, irrespective of what growth media are used, and infertile pseudothecia has been reported to occur rarely (McOnie, 1964b, d; Korf, 1998; Baayen *et al*., 2002; Baldassari *et al*., 2008). Isolates of *G. mangiferae* produces both pycnidiospores and ascospores in culture, although not all isolates formed fertile pseudothecia (Kiely, 1948b; Baayen *et al*., 2002; Baldassari *et al*., 2008). All reports on isolates of *G. citricarpa* producing ascospores in culture (Frean, 1964; Brodrick, 1969; Wager, 1952) are believed to be erroneous. Results of Lemir *et al*. (2000), who claimed to have produced pseudothecia of *G. citricarpa* in culture, could not be repeated (Baayen *et al*., 2002; Baldassari *et al*., 2008; M. Truter, unpublished data).

Molecular studies on *Guignardia* isolates from *Citrus* and other hosts indicated that *G. citricarpa* could clearly distinguished morphological similar isolates as a separate species (Meyer *et al*., 2001; Baayen *et al*., 2002; Wulandari *et al*., 2009). Meyer *et al*. (2001)

Table 2.1. Characteristics differing between *Guignardia citricarpa* and *Guignardia mangiferae*

Characteristic	*Guignardia citricarpa*	*Guignardia mangiferae*	Reference
Growth rate in culture	Slow growing, ca. 25-30 mm in 7 days	Fast growing, ca. ≥ 40 mm in 7 days	McOnie, 1964d; Baayen *et al.*, 2002
Colony colour	Dark brown with a wider translucent outer zone and lobate margin	Dark brown, although darker than *G. citricarpa*; margin entire	McOnie, 1964d; Baayen *et al.*, 2002; Baldassari *et al.*, 2008
Yellow pigment on Oats agar	Present	Absent	Baayen *et al.*, 2002; Baldassari *et al.*, 2008
Sporulation in culture	Produce pycnidia and pycnidiospores and rarely infertile pseudothecia (never ascospores)	Produce both pycnidia with pycnidiospores and pseudothecia with ascospores	McOnie, 1964d; Kotzé, 1963; Baayen *et al.*, 2002; Baldassari *et al.*, 2008
Symptoms	Spots on fruit, leaves and twigs of citrus only	Small spots in guava and mango	Baayen *et al.*, 2002; Baldassari *et al.*, 2008
Host range	*Citrus*, symptomatic and symptomless material	Endophytic in all woody plants, including *Citrus*	Baayen *et al.*, 2002
Distribution	Argentina, Australia, Bhutan, Brazil, China, Ghana, India, Indonesia, Kenya, Mozambique, Nigeria, Philippines, South Africa, Swaziland, Taiwan, United States of America, Uruguay, West Indies, Zambia and Zimbabwe	World-wide	European Union, 1998; Baayen *et al.*, 2002; Paul *et al.*, 2005; Lemon & McNally, 2010

used restriction enzyme digestion fingerprints of the polymerase chain reaction (PCR) product of a portion of the internal spacer region (ITS) to indicate the two species, while Baayen *et al.* (2002) used ITS sequence analysis and amplified fragment length polymorphic fingerprint patterns. These and other molecular studies on *Guignardia* isolates resulted in development of species-specific PCR primers that provided fast, accurate and reliable techniques to distinguish and detect the species without reservation (Meyer *et al.*, 2001; Baayen *et al.*, 2002; Bonants *et al.*, 2003; Meyer *et al.*, 2006; Everett & Rees-George, 2006b; Peres *et al.*, 2007; Van Gent-Pelzer *et al.*, 2007; Stringari *et al.*, 2009).

It has been suggested that a third *Phyllosticta* species is associated with *Citrus*, but only as symptomless infections (Van der Aa & Vanev, 2002; Baayen *et al.*, 2002). Stringari *et al.* (2009) recently indicated that isolates from symptomless *C. limon* in Brazil belonged to *Phyllosticta spinarum* (Died.) Nag Raj & M. Morelet based on sequence data. Wulandari *et al.* (2009) also referred to one of these isolates from Brazil, and subported that it could be *P. spinarum*. Besides Possiede *et al.* (2009) referring to the same *P. spinarum* isolates on citrus as Stringari *et al.* (2009), no further record(s) of this fungus on citrus are known.

A fourth *Phyllosticta* species, *Phyllosticta citriasiana* Wulandari, Crous & Gruyter, has recently been described from pummelo, *Citrus maxima* Merr., causing citrus tan spot (Wulandari *et al.*, 2009). The teleomorph was indicated as unknown. All isolates from the newly described species were obtained from spotted fruit of *C. maxima* from China, Thailand and Vietnam (Wulandari *et al.*, 2009). Fruit symptoms are similar to those produced by *G. citricarpa*, consisting of shallow lesions with a small central grey to tan crater usually with a dark brown rim, 3-10 mm in diameter (Wulandari *et al.*, 2009). *P. citriasiana* can be distinguished from *G. mangiferae* by having smaller conidia with a narrower mucoid sheath, and from *P. citricarpa* by having larger conidia, longer conidial appendages and not producing any diffuse yellow pigment when cultivated on OA (Wulandari *et al.*, 2009). In culture, colonies of *P. citriasiana* are also darker shades of grey and black on OA, malt extract agar, potato-dextrose agar and cornmeal agar than observed in the other two species (Wulandari *et al.*, 2009).

2.1.3 Morphology of *Guignardia citricarpa*

Pseudothecia are produced solitary (125-135 µm in diameter) or in groups of two (220-240 µm) and three (340-360 µm). Pseudothecial wall are 20-22 µm thick, carbonaceous dark brown by transmitted light and globose. Pseudothecia are sub-epidermal, finally erumpent, no stroma present nor distinct beak, but an ostiole of 14-16 µm in diameter are

present at maturity. Paraphyses and periphyses are absent. Pseudothecia are produced on the ventral and dorsal surfaces of decaying citrus leaves, but have never been found on fruit (Kiely, 1948b; Van der Aa, 1973).

Asci (50-85 x 12-15 µm) are produced from the base of a pseudothecium, 45 to 60 in number, clavate; cylindrical, eight spored and uniseriate (Kiely, 1948b). Ascopsores are hyaline to granular grey, usually with one large central guttule at maturity. Ascospores are non-septate but occasionally with septum near one end of the spore, 8.0-17.5 x 3.3-8.0 µm with a small round clear gelatinous cap at each end (Kiely, 1948b).

Pycnidia are produced on citrus leaves, petioles, twigs and fruit (Van der Aa, 1973). Pycnidia are 70-330 µm in diameter, subhyaline to brownish on leaves, brown to almost black on fruit, globose or depressed on leaves, pyriform on fruit, flat or conspicuously papillate with a circular pore of 10-15 µm diameter. Stroma developed on fruit only, are subhyaline to dark brown and 5-18 µm in diameter. Conidiogenous cells are cylindrical and 4-8 x 2-3.5 µm. Under ideal conditions for their development, pycnidia are closely studded over the entire leaf surface. They can occur on either the dorsal or ventral surfaces of the leaf, but are usually thickest on the one side only, the side or portion of the leaf exposed to the sun's radiation (Darnell-Smith, 1918; Kiely, 1948b).

Pycnidiospores still attached to the sporophore possess a terminal gelatinous cap, which later shrink to form the appendage, 5-15 µm in length. Pycnidiospores are one-celled, obovoidal, ellipsoidal or subglobose, somewhat clavate when young, with a truncate base, broadly rounded apically and slightly indented, 6-13 x 5-9 µm, usually 9-10 x 6-7 µm (Van der Aa, 1973). They may have one or two nuclei, generally two (Darnell-Smith, 1918). Pycnidiospores are usually hyaline with granular contents and sometimes having a greenish hue. More than one crop of pycnidiospores can be produced as the sporogenous layer is regenerative (Kiely, 1948b).

Spermatial state occurs both in pure culture and on the host and usually develops simultaneously with the conidial state, but is much more scarcely found (Van der Aa, 1973). Fruiting bodies are similar to those of the conidial state. Spermatiogenous cells are elongated cylindrical and 4-10 x 0.5-2 µm. Spermatia are dumb-bell shaped, seldom cylindrical, straight to slightly curved and 5-8 x 0.5-1 µm.

The mycelium exhibits much diversity. The extreme tips may be pointed or round, hyphae being thin, hyaline, and almost devoid of septa (Darnell-Smith, 1918). Older hyphae

become thicker, septa more numerous and olive-green in colour. In the older hyphae, septa are numerous, dark greenish-brown in colour, and the contents of the cells granular. The cells may be oblong or round and often carry numerous short, round, protuberances. Hyphae anastomose readily with one another (Darnell-Smith, 1918).

Cultures of *G. citricarpa* on potato-dextrose agar are dark brown to black; mycelium is mostly submerged, thick and prostrate. Colonies are slow growing, reaching a diameter of 70 mm in 20 days on various media at 24°C (Van der Aa, 1973). Stromata develop within eight days as hard, black masses, resembling those on fruits, pyriform, globose or cylindrical, with one to numerous conidial and spermatial cavities in the upper region (Van der Aa, 1973).

2.1.4 Sporulation

All attempts to promote pseudothecial development of *G. citricarpa in vitro* were unsuccessful (McOnie, 1964d; Korf, 1998; Baayen *et al.*, 2002; Baldassari *et al.*, 2008) and although Lemir *et al.* (2000) claim to have produced pseudothecia in culture, their results were never repeated. With our current knowledge about *G. mangiferae*, we can conclude that reports on *in vitro* ascospore production of *G. citricarpa* (Frean, 1964, 1966; Brodrick, 1969; Wager, 1952) are erroneous. Other methods for the production of pseudothecia on water agar medium augmented with leaf pieces were described, but for members of the genus *Guignardia* and not for *G. citricarpa* specifically (Petrini *et al.*, 1991; Furukawa & Kishi, 2002).

Brodrick and Rabie (1970) investigated the effects of light and temperature on the sporulation on artificial culture medium. Incubation under continuous light resulted in significantly higher counts of pycnidiospores produced than under alternating light/dark or continuous dark. Incubation at 27°C resulted in significantly more pycnidiospores produced on flavedo pieces than at 20°C, whereas the reverse was true for pycnidiospore production on Potato Dextrose Agar. Numbers of pycnidiospores produced were significantly higher in all the treatments after 15 days than after 10 and 20 days. At 20 days, it was possible that the pycnidiospores remained embedded in the gelatinous matrix in the pycnidium and were not released under the conditions of the experiment.

2.1.5 Spore germination

Since ascospores of *G. citricarpa* cannot be produced *in vitro*, very few studies have investigated the germination of ascospores. According to Kiely (1948b) ascospores take more than 24 h to germinate *in vitro* at 25°C and 4 days to reach 98% germination. In

another study, germination was investigated *in vitro* and *in plantae* and germination of ascospores on lemon (*Citrus limon* (L.) Burn. f.) leaves varied from 14 to 91% after 24 h and most did not show an increase after 48 h compared to 24 h (McOnie, 1967).

In vitro germination of pycnidiospores of *P. citricarpa* has been reported to be very slow, with only a few spores germinating after several days (Darnell-Smith, 1918). Germination of pycnidiospores in tap water has been reported, albeit at varying degrees (Kiely, 1948b; Wager, 1952). Spore germination was stimulated by extracts of orange peel or citric acid solutions at concentrations of 0.1-0.5% (Darnell-Smith, 1918; Kiely, 1948b). Maximum germination of nearly 80% has been obtained using 0.3% citric acid solution and incubating spores for 4 days at 25°C in a damp chamber (Kiely, 1948b). Freshly exuded mature pycnidiospores have been reported to lose their ability to germinate in about one month after they were produced (Kiely, 1948b). Darnell-Smith (1918) also showed that the rapidity with which spores germinate depended largely on the age of the spores (time since released from pycnidia) with young spores germinating within 12 h and older spores taking several days to germinate while many failed to germinate.

An extensive investigation on the germination of pycnidiospores of *Phyllosticta ampelicida* (Engleman) Van der Aa (teleomorph *Guignardia bidwellii* (Ellis) Viala & Ravaz) was undertaken mainly by K. Huo, H.C. Hoch and B.D. Shaw. They indicated that pycnidiospores did not germinate readily unless they are attached to a hydrophobic surface (Kuo & Hoch, 1995, 1996a, b; Shaw & Hoch, 1999, 2000; Shaw *et al*., 1998, 2006). The requirement for pycnidiospore attachment to trigger germination was indicated to be pervasive to the genus *Phyllosticta* (Shaw *et al*., 2006). Similar to other fungi where spores require attachment for germination, additional nutrients (e.g. host leaf extract) can overcome this requirement and germination on hydrophilic surfaces were improved (Darnell-Smith, 1918; Kiely, 1948b; Kuo & Hoch, 1996a; Shaw & Hoch, 1999, 2000). Since pycnidiospores are negatively charged, low pH reduces the inherent electro-negativity of the surface components, thus reducing electrostatic repulsive forces and enhancing attachment (Shaw & Hoch, 1999).

Pycnidiospore germination of *P. ampelicida* can be described by a sequence of events. Once spores came into contact with a hydrophobic surface, such as a leaf, spores attached passively to the surface in less than 0.03 s (Shaw & Koch, 2000). Dead spores attached equally well to the substrate as viable ones and spore attachment to the host surface involved the surrounding extracellular matrix, consisting of carbohydrates, proteins and glycoproteins (Kuo & Hoch, 1995, 1996a; Shaw & Hoch, 1999). Spores

germinated usually 40-60 min after attachment by forming a germ tube on either side of the spore (Kuo & Hoch, 1996b; Shaw & Hoch, 2000). Appressoria started to form after 2-3 h after attachment and mature, highly melanised appressoria were observed after 6 h following initial spore attachment (Kuo & Hoch, 1996b; Shaw & Hoch, 2000). Germ tubes were mostly short (5 µm) on host leaves while longer germ tubes (20-40 µm) developed *in vitro* (Kuo & Hoch, 1995, 1996b; Shaw *et al.*, 1998). Although the last work of Shaw *et al.* (2006) included 14 species of *Phyllosticta*, *G. citricarpa* was not included as sporulation of available isolates was reported to be insufficient. Nevertheless, it is likely that pycnidiospores of *P. citricarpa* would germinate in a similar manner than described for *P. ampelicida*.

2.2 The host, *Citrus*

Almost all commercial citrus species are susceptible to CBS, and lemons are the most susceptible. When CBS is found in a new area, it is usually first observed on lemons before other citrus is affected (Kiely, 1948b; Kotzé, 1981). The disease can be serious on sweet orange (*Citrus sinensis* Osbeck), which is a late maturing cultivar (Kiely, 1948b; Wager, 1952). It may also cause significant losses on grapefruit (*Citrus paradisi* Macf.) and lime (*Citrus aurantifolia* Christm.) (Brodrick, 1969) and has been reported to occur on citron (*Citrus medica* L.), pummelo (*Citrus grandis* (L.) Osbeck) and mandarin (*Citrus reticulata* Blanco) (Kiely, 1948a; Brodrick, 1969; Kiely, 1970). Seville sour orange (*Citrus aurantium* L.) and its hybrids, rough lemon (*Citrus jambhiri* Lish.) and Tahiti acid lime (*Citrus latifolia* Tan.) is regarded as insensitive to the pathogen (Wager, 1952; Kotzé, 1981; Baldassari *et al*, 2008). Although no CBS symptoms have ever been observed on sour orange and acid lime, the pathogen has been isolated from the cultivars and spores can be produced on the leaf litter (Baldassari *et al.*, 2008). The importance of these insensitive cultivars in disease dissemination and inoculum production should be investigated further.

Various other woody plants were reported to carry latent infections of *G. citricarpa* and that these plants may act as a source of inoculum after the leaves die (Kiely, 1948a, b; Wager 1952). It was first proved by McOnie (1964d; 1965a) with conventional methods and later by Baayen *et al*. (2002) and others with molecular techniques, that the isolates from the alternative hosts belonged to the non-pathogenic *G. mangiferae* and not G. *citricarpa*. However, there has been one exception to this rule when Bonants *et al*. (2003) identified *G. citricarpa* from leaves of an unidentified *Sapotaceae* using a PCR-test. The finding was not confirmed with subsequent supporting data and accuracy of the PCR-test is questionable. Also, whether the pathogen could grow and sporulate within this host to

form a reservoir for inoculum of CBS is unknown. This new finding may be of particular importance in the context of quarantine regulations and calls for the screening of non-citrus hosts in the proximity of citrus orchards for the presence of *G. citricarpa*. Various highly specific PCR-tests are available (Bonants *et al*., 2003; Meyer *et al*., 2006; Peres *et al*., 2007; Van Gent-Pelzer *et al*., 2007) that could facilitate such research.

Citrus fruit are susceptible to infection by either asco- or pycnidiospores for 20 to 24 weeks after petal fall, after which time the fruit become resistant regardless of the prevailing weather conditions (Kotzé, 1981). This is as a result of an increase in fruit resistance, rather than a decrease in inoculum (Whiteside, 1965). Similarly, the susceptibility period of citrus leaves to infection by *G. citricarpa* was originally reported to be five weeks (Kiely, 1948b; McOnie, 1967), although subsequent field observations suggested that it could be five months (Kotzé, 1981).

2.3 The disease, citrus black spot

2.3.1 Origin and distribution of citrus black spot

CBS originated in south east Asia (Smith *et al*., 1997), but the symptoms were first described from infected sweet orange fruit by Benson (1895) in Australia. CBS occurs in all citrus producing countries where the pathogen has been recorded (see section 2.1.1 Origin and distribution of *Guignardia citricarpa*). The global distribution of the disease appears to partially follow citrus producing patterns but is restricted by specific climatic parameters, of which cold wet conditions during winter were indicated as the main restrictive parameter (Paul *et al*., 2005; Yonow & Hatting, 2009).

Various citrus-growing areas within countries where the disease has been recorded have remained free of CBS. In Australia, areas free of CBS include Sunraysia and mid-Murray areas of Victoria and NSW, Emerald in Queensland, as well as the two states Western Australia and South Australia (European Union, 1998; Paul, 2006). In Brazil, CBS has only been recorded from the state of Rio de Janeiro, Rio Grande do Sul and São Paulo (European Union, 2000), whereas in China the distribution is restricted to the provinces of Fujian, Guangdong, Sichuan, Yunnan and Zhejiang (European Union, 1998). In SA, citrus producing regions in the Northern Cape, Free State, North West and all the citrus producing regions within the south-western Western Cape Province are free of CBS (European Union, 1998; Mabiletsa, 2003; APHIS, 2009, Shea, 2010). In the USA, CBS was recorded for the first time in March 2010 in Florida (Lemon & McNally, 2010; Schubert *et al*., 2010) and it is still uncertain if the disease can be contained or if it will spread to other citrus producing regions in the USA with suitable climates.

2.3.2 Economic importance of citrus black spot

One of the first records of the economic impact of CBS is that of Benson (1895) indicating the disease caused great losses in many orange growing districts throughout Australia. In 1945, 90% of citrus fruit produced in unsprayed orchards in Northern and Mpumalanga Provinces, SA, were rendered unfit for export (Sutton & Waterson, 1966). This resulted in an oversupply of unwanted CBS infected fruit on the local market. However, with the advent of the general application of fungicides for the control of fungal diseases in the early 1970's (Brandes, 1971), major losses due to fruit symptoms have not again been reported in literature. CBS control programmes are costly (Cobb, 1897; Kotzé, 1961), but necessary as total loss in exportable fresh fruit may be experienced in uncontrolled orchards (Seberry *et al*., 1967; Smith, 1996).

Pre-harvest fruit drop due to excessive CBS infection do not readily occur within orchards where proper pre-harvest control is applied, but have been reported (McCleery, 1939; Wager, 1945, 1949, 1952). Post-harvest CBS losses are not always apparent as infected, asymptomatic fruit may develop CBS symptoms while in transit to the markets resulting in possible rejection at local or overseas harbours when exported to CBS-sensitive markets (Brodrick, 1969; Kiely, 1948b; Loest, 1958; Smith, 1962; Kotzé, 1996).

CBS gained prominence as a disease of great economical importance in recent years because of phytosanitary restrictions on the movement of fruit from CBS infected areas. Although the European Union allow import of fresh citrus fruit from CBS-positive areas, the presence of any symptomatic fruit at inspection results in the rejection of whole consignments, leading to great economical losses. Even in local markets, CBS lesions significantly lowered the market value of fruit and resulted in the product being re-directed for processing (Calavan, 1960; Cobb, 1897; Kellerman & Kotzé, 1977; Wager, 1945).

2.3.3 Inoculum

2.3.3.1 Ascospores

Windborne ascospores are seen as the primary source of inoculum in countries with only one fruit set per season, such as Australia and SA (Kiely, 1948b; Kotzé, 1963; Sutton & Waterson, 1966). Ascospores are produced in pseudothecia only on leaf litter and these fruiting bodies have never been found on fruit, twigs or attached leaves (Kotzé, 1963; McOnie, 1965a; Truter *et al*., 2007). Mature pseudothecia can be detected on leaf litter in 30 to 180 days after leaf fall, depending on the prevailing temperature and the frequency of wetting (Kiely, 1948b; McOnie, 1964b; Lee & Huang, 1973).

Temperature influences the rate of pseudothecia maturation as well as the release of mature ascospores (Kotzé, 1963; Fourie *et al*., 2009). Maturation of pseudothecia is seasonal, and mature spores are found within leaf litter mainly during summer months (Kotzé, 1963; McOnie, 1964b, c). Data from spore traps combined with on-site weather stations indicated that most ascospores of *Guignardia* spp. are release when temperatures are 18°C or above (Fourie *et al*., 2009).

Mature ascospores are forcibly released from the pseudothecia to a height of about 12 mm during rainfall (Kiely, 1948b; Kotzé, 1963; McOnie, 1964b), sprinkler or micro-jet irrigation (Smith, 1996), heavy dew (Lee & Huang, 1973) or high humidity (Swart & Kotzé, 2007) and are carried on air currents throughout the canopy (Kotzé, 1963; McOnie, 1964c, 1965a; Sutton & Waterston, 1966). Although ascospores are windborne, their ejection from the mature pseudothecia is dependent on wetting. Therefore, the onset of rain, temperatures of 18°C or above, ascospore discharge and the infection period are closely related (Kotzé, 1963; McOnie, 1964b; Fourie *et al*., 2009).

2.3.3.2 Pycnidiospores

In addition to pseudothecia, pycnidia containing pycnidiospores are produced on dead leaves beneath trees (Kiely, 1948b). Pycnidia may also occur in fruit lesions, on dead twigs, and sparsely within lesions on attached leaves or on fruit stalks. Production and maturation of pycnidia on leaf litter is considerable faster than pseudothecia and mature pycnidia can be detected on leaf litter weeks before the first pseudothecia are mature (McOnie, 1964b). In wet weather, mature pycnidiospores ooze as a gelatinous mass from pycnidia contained in lesions on the rind of infected mature fruit hanging on the tree. These spores require water for dispersal (Sutton & Waterson, 1966; Whiteside, 1967). Similarly, masses of gelatinous pycnidiospores are produced from pycnidia on fallen leaves (McOnie, 1964b; Kotzé, 1996).

Alternate wetting and drying of fallen leaves and variations in temperature provide optimal conditions for asco- and pycnidiospore formation and maturation (Kiely, 1948a, b; Lee & Huang, 1973). Pseudothecia and pycnidia will not mature in areas where the leaf litter is either constantly dry or constantly wet (Kiely, 1948b; Wager, 1949; Lee & Huang, 1973). Maturation of pseudothecia and pycnidia is seasonal, and mature spores are found mainly during summer months (Kotzé, 1963; McOnie, 1964b, c). In production areas with mild winters such as Tzaneen and Letsitele in SA and various areas in Australia, ascospores can be detected throughout the year (Kiely, 1948b; Swart & Kotzé, 2007). In areas with

lower winter temperatures, maturation of spores was retarded and no or few spores were detected during late autumn to early spring (Kiely, 1948b; Kotzé, 1963; Smith, 1996).

2.3.3.3 Symptomless infection

Mycelium latently present in citrus trees may be a source of inoculum (Kiely, 1949). If the CBS pathogen in such trees is introduced to new, uninfected citrus production areas, CBS might successfully establish in the new area (Calavan, 1960). In the past, CBS have been transmitted to uninfected areas through infected, but symptomless nursery trees (Kiely, 1949; Wager, 1952). Symptomless infected fruit are not a source of inoculum as the latent infection remains localised within the fruit tissue for the lifespan of the fruit. Furthermore, pycnidiospores are only produced within lesions on fruit and never on symptomless fruit (Kotzé, 1981). Symptoms may develop on fruit after harvest, but symptomatic fruit are not regarded as an important inoculum source.

2.3.4 Infection

Infection of susceptible citrus material takes place when a viable spore (either asco- or pycnidiospore) lands on suitable host material, attaches to the surface, and germinates. An appresorium may form sessile on the germinating spore or at the end of a short germ tube. The appressorium attaches to the plant surface and a thin infection peg forms between the appressorium and plant tissue. Penetration of the infection tube is by both mechanical pressure and enzymatic degrading of the cell wall (McOnie, 1967). After penetrating the tissue, the fungus forms a resting body within the rind tissue of fruit, or just below the cuticula of leaves. This resting body remains dormant until tissue maturity when conditions are conducive for further growth and spore production (Kiely, 1948b, 1970; Kotzé, 1963). This kind of infection is known as a latent or quiescent infection and the latent period may last several months (Kotzé, 1963; Kiely, 1969; Cook, 1975). Consequently, *G. citricarpa* may be isolated from apparently healthy citrus fruit tissues (Yin *et al.*, 1981; Baldassari *et al.*, 2008).

It is widely accepted that ascospores are the major source of inoculum. The critical period for ascospore infection is approximately within a single five-month window period when fruit set coincides with rainfall. Late-hanging infected mature fruit are removed from trees a month before the new season's fruit sets (Kiely, 1948b, 1970; Kotzé, 1963, 1996; McOnie, 1965a). Therefore, pycnidiospores are not a major source of inoculum for fruit infection as mature CBS infected fruit and susceptible young fruit never occur simultaneously on the same trees. However, this is not true for citrus produced in Brazil

where rain is not so confined to a single season and flowering may occur more than twice a year.

Ascospore infection frequency is determined by the rainfall pattern whereas climatic conditions greatly influence the intensity of infection (Wager, 1952; Whiteside, 1967). If conditions are not favourable for the development and maturation of the pathogen's fruiting bodies, citrus fruit and leaves may escape ascospore infection (Whiteside, 1967). Additionally, availability of spore inoculum during the time when young fruit and leaves are susceptible has an important influence on the rate of infections and disease severity (Whiteside, 1965, 1967). Any new leaf flushes that coincide with wet weather may become infected (Whiteside, 1965). Leaf infections remain predominantly latent until leaf drop and desiccation, although lesions may appear on mature attached leaves, especially lemon leaves (Whiteside, 1965). Infected leaves fall to the ground a year or longer after infection and eventually produce mature ascospores, which are forcefully released from pseudothecia and may infect young fruit and leaves and so complete the infection cycle (Whiteside, 1965).

Infection by pycnidiospores happens when spores from late-hanging, infected, mature fruit are washed down to young susceptible leaves and fruit (Sutton & Waterson, 1966; Whiteside, 1965, 1967). Pycnidiospores from fallen leaves and fruit are not thought to readily cause infection of fruit, since their dispersal to fruit hanging on the trees, unless splashed by raindrops, seems unlikely (McOnie, 1964b; Kotzé, 1996). In rare cases a tear stain pattern of black spots are observed on infected fruit, indicating pycnidiospores rather than ascospores as source of infection (Fig. 2.1). Pycnidiospores, although not important for fruit infections, may significantly contribute to leaf infections and play a part in the life cycle of the pathogen.

2.3.5 Symptoms

G. citricarpa mainly causes symptoms on citrus fruit and to a lesser extent on leaves and twigs. Symptoms on fruit, leaves and twigs usually remain small and do not significantly reduce yield, but spotted fruit are unacceptable to fresh markets (local and export), resulting in reduction in marketable fruit.

Figure 2.1. Tear stain pattern of hard spot lesions on a mature Valencia orange fruit, typically formed from pycnidiospore infections of *Guignardia citricarpa*.

2.3.5.1 Fruit symptoms

Disease symptoms usually starts to develop around colour break and are most noticeable on fully matured fruit (Kiely, 1969), although symptoms may appear on immature fruit, especially lemons (Wager, 1952; Whiteside, 1965). Symptoms are confined to the surface of the fruit (Wager, 1952; Kotzé, 1981) and lesions may appear as a single spot or up to a thousand spots per fruit (Calavan, 1960). The disease rarely causes post harvest decay, even though the rind of infected fruit may become severely necrotic (Kotzé, 1981). Severely infected immature fruit have been reported to drop prematurely and go to waste (Wager, 1952).

Disease expression (pre- or postharvestly) may be enhanced by numerous factors inducing stress on the host, e.g. heat, poor soil conditions, improper irrigation, nematodes and other diseases. Expression is generally promoted by relatively high temperatures (>26°C) and high light intensities (Kotzé, 1963; Whiteside, 1967; Kiely, 1969; Brodrick & Rabie, 1970; Kotzé, 1971; Kellerman, 1976; Kellerman & Kotzé, 1977). Temperatures below 21°C reduce the rate of fruit symptom development (Brodrick, 1969) while temperatures below 5°C could prevent symptom development for duration of cold storage (Korf, 1998; Korf *et al.*, 2001).

Pre-harvest symptom development on fruit is dependent on weather conditions, and on the age and condition of the host tree (Kiely, 1969; Kotzé, 1996). Consequently, trees older than 10 years (Kiely, 1948b), trees suffering from root rot (Whiteside, 1965), wilting, or element deficiencies (Kotzé, 1961); and trees affected by drought (Kiely, 1969) or hail damage (Kellerman, 1975) seems more susceptible to CBS. Symptoms also develop more rapidly as the rind matures. Thus, factors that influence rind maturation, such as soil moisture, can also influence the occurrence of symptoms (Kiely, 1969).

Lesions are well defined and four kinds of symptoms are widely recognised viz. red spot (not formally described), hard spot, first described by Cobb (1897); freckle spot and virulent spot, both first described by Kiely (1948b). Two other symptoms, speckled blotch and cracked spot are not as widely recognised and were reported from South Africa (McOnie, 1965b) and Brazil (De Goes *et al.*, 2000), respectively.

2.3.5.1.1 Red spot

Reference to red spots has been made in the past, but it has not been formally described (Kotzé, 1963; McOnie, 1967; Korf, 1998; Bonants *et al.*, 2003; Meyer *et al.*, 2006; Truter *et al.*, 2007). Lately, the use of red spots as a CBS symptom category has increased, mainly due to the phytosanitary restrictions on trade of symptomatic fruit and increased attentiveness to the presence of red spots on fruit at inspection sites. Although all symptom types can develop postharvestly, red spot is often the first postharvest symptom to develop and development in transport is more common as the other symptoms require higher temperature and a longer incubation period for development. Lesions appear as minute, round, sunken, reddish depressions on the fruit surface (Fig. 2.2). Lesions are mostly 1 mm in diameter, never larger than 2 mm and about 1 mm deep. Pycnidia seldom develops in red spots. The pathogen can be readily isolated from this symptom and the isolation success from red spots is almost twice as high as compared to hard spots (Kotzé, 1963; M. Truter, unpublished data). A single red spot is also sufficient to positively detect the pathogen with molecular methods (Meyer *et al.*, 2006). Red spot symptoms may later develop into the first developmental stage of hard spots (McOnie, 1967).

2.3.5.1.2 Hard spot

Hard spot are sometimes referred to as shot hole, and is the most typical CBS fruit symptom (Fig. 2.3). It is a circular brown lesion, originating from an initial slight depression. Lesions tend not to increase in diameter, but sink in the centre to form a crater-like depression. The tissue in the centre turns grey-white and pycnidia may develop therein (Kiely, 1948b; Korf, 1998). The rim of these lesions is typically black, but

brown and red margins have been reported (Korf, 1998). On green fruit a yellow halo sometimes surrounds the rim of lesions and on mature fruit a green halo surround it. Pseudothecia never develop within hard spot lesions (Kotzé, 1981; Bonants *et al*., 2003). Generally hard spot lesions are few in number per fruit, but more than 50 lesions per fruit have been observed (Kiely, 1948b). These lesions mostly appear with the onset of fruit maturation preharvestly, but can also be found on immature fruit, especially lemons, or develop postharvestly (Kotzé, 1981).

2.3.5.1.3 Freckle spot

Multiple (up to several hundred), separate, deep orange to brick red lesions may appear simultaneously on a portion of the fruit surface, usually the side that is more exposed to the sun (Kiely, 1948b) (Fig. 2.4). Lesions develop preharvestly and are about 1 mm in diameter and slightly depressed at the centre. Lesions grow fast and reach 2-3 mm in diameter before turning brown and ceasing growth. The depth of the lesion might increase, depending on the thickness of the rind. These symptoms are generally devoid of pycnidia (Bonants *et al*., 2003). Fruit with freckle spot are usually more unsightly than those with only hard spot (Kiely, 1948b). Following period of hot weather, the growth of the fungus in the lesions can suddenly increase and lesions rapidly enlarge. Individual lesions may coalesce to form a tearstain lesion similar to melanose (*Diaporthe citri* F.A. Wolf) or develop further into virulent spot (Kiely, 1948b; Baayen *et al*., 2002). This symptom mostly appears after the fruit have undergone colour change from green to orange (Kotzé, 1981).

2.3.5.1.4 Virulent spot

Virulent spot may develop from coalesce freckle spot lesions (Fig. 2.5) (Kiely, 1948b) or on fruit without any other CBS symptoms. In the latter case, lesions originate as small sunken red to brown spots or as irregularly depressed centres approximately 6 mm in diameter showing no colour change (Calavan, 1960). Infection centres develop rapidly and black pycnidia may develop inside these centres (Kiely, 1948b; Calavan, 1960). Lesions appear typical black in the centre due to multiple pycnidia and brown further out due to necrosis of rind tissue. Lesions have a narrow brick-red active peripheral area several millimetres wide, forming the margin of the sunken lesion (Kiely, 1948b). Lesions assume irregular shapes and develop late in the season on fully mature fruit. Compared to the previous lesions, virulent spot extends more deeply into the tissue of the albedo, even to the extent of involving the entire thickness of the rind tissue. These lesions could

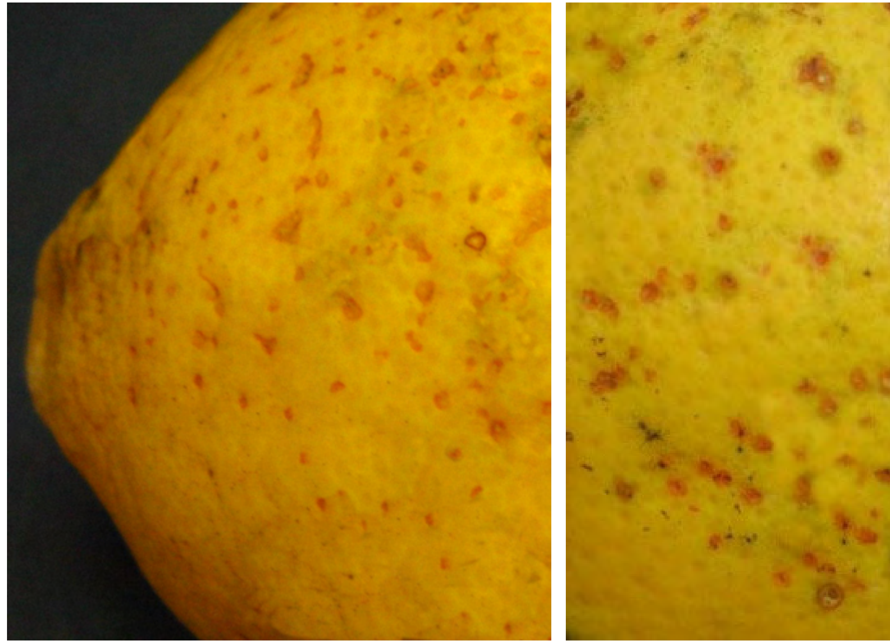

Figure 2.2. Red spot lesions caused by *Guignardia citricarpa* on mature Eureka lemon fruit.

Figure 2.3. Hard spot lesions caused by *Guignardia citricarpa* on a mature Eureka lemon fruit.

Figure 2.4. Freckled spot lesions caused by *Guignardia citricarpa* on a mature Eureka lemon fruit.

Figure 2.5. Virulent spot lesions caused by *Guignardia citricarpa* on a mature Eureka lemon fruit.

be surrounded by brown necrotic tissue and cause post-harvest losses (Kiely, 1948b; Kotzé, 1981).

2.3.5.1.5 Speckled blotch

Speckled blotch occurs infrequently on fruit and develops early on immature green fruit. It was first thought to be melanose, but later it was concluded that the causal organism was *G. citricarpa* (McOnie, 1965b). Blotching consists of separate, roughly circular spots, 1-2 mm in diameter, either depressed or slightly raised. At first appearance the spots are brick red but turn dark brown in colour over a period of two weeks (Kiely, 1960). Speckled blotch may develop into hard spot as the season progresses (Kotzé, 1981). These lesions are usually devoid of pycnidia (Bonants *et al.*, 2003).

2.3.5.1.6 Cracked spot

Cracked spot appears in fruit older than six months and is characterized by the presence of superficial lesions which are variable in size and appear cracked. The symptoms are slightly salient, can occur individually or in groups and do not contain any pycnidia (De Goes *et al.*, 2000).

2.3.5.2 Citrus tan spot

A new disease on *C. maxima*, caused by *P. citriasiana*, was recently described from Asia, causing similar fruit symptoms than *G. citricarpa* (Wulandari *et al.*, 2009). Citrus tan spot usually appears after the fruit has started to ripen and lesions sometimes contain pycnidia. Lesions are shallow with a small central grey to tan crater usually with a dark brown rim and are 3-10 mm in diameter (Wulandari *et al.*, 2009). Another symptom variation of citrus tan spot can sometimes develop after harvest, consisting of small (1-3 mm diameter), slightly depressed spots. These spots may be grey or tan, or reddish, or brownish, or not discolour at all. Often they have a dark red or brown rim. Pycnidia are only incidentally present in these lesions (Wulandari *et al.*, 2009). Citrus tan spot may be mistaken for CBS lesions, especially red and black spots. Since these lesions are so similar to CBS the correct identification of the causal organism on spotted citrus fruit with molecular techniques is essential in future studies and surveys.

2.3.5.3 Leaf symptoms

Symptoms (Fig. 2.6) occur more frequently on the leaves of lemon trees than on those of oranges (Kiely, 1949). Leaf infection within a tree varies considerably, and the number of lesions per leaf may range from a few to numerous spots (Wager, 1952). Lesions on immature leaves are extremely scarce (Kiely, 1949). Symptoms first start to appear

several months after initial infection (Wager, 1952). Small pin-point sunken lesions are visible on both sides of the leaf (Kiely, 1948b; Wager, 1952). These lesions are perfectly round, have a grey or light brown centre, a black to reddish circumference and are mostly surrounded by a yellow halo. Sometimes pycnidia can be seen in the centre of the lesion on the upper side of the leaf (Wager, 1952). Further colonisation of the leaf only happens after leaf drop, where the pathogen eventually produces pseudothecia and pycnidia over the surface of the dead leaf amongst the leaf litter (Fig. 2.7) (Kotzé, 1996).

2.3.5.4 Twig symptoms

Lesions on twigs have not been described formally, but occur commonly in South Africa on lemons (J.M. Kotzé, 2004, personal communication; M. Truter unpublished data). In contrast, pycnidia of the anamorph have been reported on mostly dead twigs and the pycnidiospores produced on these twigs can be a source of inoculum (Kiely, 1948b; McOnie, 1964c; Whiteside, 1967).

Symptoms are small (0.5-2 mm in diameter), round, slightly sunken and occur on the surface of active growing twigs (Fig. 2.8). The lesions typical have a brown to black margin and a grey to light brown centre. Pycnidia can be produced in the centre of the lesion, but never pseudothecia. *G. citricarpa* was positively identified from the lesions on the twigs in Fig. 2.8 with a PCR-based method (M. Truter, unpublished data).

2.3.6 Control

2.3.6.1 Chemical control

Control of CBS greatly relies on preventative fungicide sprays applied during the period of fruit susceptibility (Garrán, 1996; Schutte *et al.*, 1997). Timely application of appropriate fungicides is essential to protect fruit, eradicate infections and prevent symptom development (Kellerman, 1976; Kellerman & Kotzé, 1977). However, the degree to which fungicides can control CBS is highly variable (Calavan, 1960) and requires a comprehensive strategy (Kiely, 1969, 1970). The effectiveness of fungicide applications is particularly reliant on the number and timing of applications (Kellerman, 1976). Generally, control of CBS has mostly relies on continuous protection of young citrus fruit during the potential infection period when the host is most susceptible and inoculum are present (McOnie & Smith, 1964).

The earliest method of controlling CBS was by applying a Bordeaux mixture as a preventative measure (Benson, 1895; Cobb, 1897; Kiely, 1948b, 1950), which was later

Figure 2.6. Lesions on Eureka lemon leaves caused by *Guignardia citricarpa*.

Figure 2.7. Fructification of *Guignardia citricarpa* on Eureka lemon leaf litter.

Figure 2.8. Lesions of *Guignardia citricarpa* on an infected Eureka lemon twig.

found to result in copper toxicity (Kotzé, 1964). Other formulations of copper fungicides also resulted in rind stippling (Schutte *et al*., 1997). In 1964, dithiocarbamates were introduced as preventative control measure by first applying zineb (active ingredient (a.i.) zinc ethylene bisdithio-carbamate) and later mancozeb (a.i. manganese ethylene bisdithio-carbamate) (Kotzé, 1964). These proved superior to copper based products (Kellerman, 1976; Kellerman & Kotzé, 1977), as they did not retard fruit colouration or result in dark rind injuries (McOnie & Smith, 1964). Oil additives, which increased the penetration of fungicides into the plant tissues, were often added to these fungicides to enhance fungicide efficacy (Kellerman, 1976; Kellerman & Kotzé, 1977; McOnie & Smith, 1964).

The carbamate chemicals were replaced by benomyl [a.i. methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate] having a preventative and curative approach (Kiely, 1971; Kellerman & Kotzé, 1973, 1977). However, by the early 1980's the CBS pathogen developed resistance to benomyl due to frequent and almost exclusive use of the fungicide (Herbert & Grech, 1985; De Wet, 1987). A few years later, strobilurins were indicated to be a good replacement for benomyl in orchards with known resistance of the CBS pathogen to benomyl (Schutte *et al*., 1996; Tollig *et al*., 1996; Schutte *et al*., 2003; Miles *et al*., 2004). The strobilurins have protective, curative and eradicative activities and provides long-lasting residual disease control (Gold & Leinhos, 1995) and is recommended in rotation or combination with other fungicides such as mancozeb or copper to control CBS (Schutte *et al*., 2003; Miles *et al*., 2004).

Postharvest treatment of citrus fruit in the packhouse focuses mainly on preventing postharvest decay by various spoilage fungi and not *G. citricarpa* specifically. In the packing line, fruit are subjected to various treatments, including hot water (42-42°C), fungicides such as imazalil and thiabendazole, and waxing (Seberry *et al*., 1967; Eckert & Brown, 1986; Rappussi *et al*., 2009). Although these fungicides do not inhibit formation of new lesions or eradicate *G. citricarpa* from lesions, it did reduce the viability of the pathogen in black spot lesions and reduce pycnidiospore viability to zero (Korf *et al*., 2001).

2.3.6.2 Non-chemical control

Preharvestly, the main non-chemical control measure consists of sanitation practices, although one study showed that biocontrol agents have the potential to control CBS. Biofertiliser, generated from the anaerobic and aerobic fermentation of cattle manure and applied as a spray to trees, seem to hold potential for the pre-harvest control of CBS in

commercial orchards (Kupper *et al.*, 2006). Control achieved with the biofertilisers was less effective than the industry standard fungicides, but use of the biofertiliser as a protective biofungicide to replace copper oxychloride in organic production have potential (Kupper *et al.*, 2006).

As trees that are in a poor condition are more susceptible to CBS, maintaining tree vigour can reduce the incidence of CBS (Calavan, 1960; Kotzé, 1961; Loest, 1968; Kiely, 1971; Kellerman, 1975). However, the most important non-chemical approach in CBS control is to use cultural techniques to reduce transmission. Sources of pycnidiospore inoculum may be removed by removal of diseased mature, late-hanging fruit before the new crop sets (Calavan, 1960; Kiely, 1969, 1970; Kotzé, 1996). Similarly, ascospore inoculum can be removed by the removal of leaf litter from the orchard floor or confinement of ascospore inoculum by mulching (Kotzé, 1996; Schutte & Kotzé, 1997). Efforts to breed resistant varieties have not been successful (Calavan, 1960).

Postharvestly, control measures are directed at preventing symptom development rather than eradicating symptomless infection. A water-wax emulsion can be applied to harvested fruit to reduce the development of CBS during storage at 16-27°C (Seberry *et al.*, 1967). Light and temperature affect the development of symptoms on fruit, so fruit should be moved as quickly as possible into the packhouse and stored in darkness at low temperatures (Calavan, 1960; Smith, 1962; Brodrick, 1969; Kiely, 1970; Korf, 1998). Postharvest application of chitosan, *Bacillus thuringiensis* var. *kurstaki* and harpin, a bacterial hypersensitive response elicitor, reduced the number of new developed CBS lesions on Valencia orange fruit as well as reduced the number of pycnidia produced in the CBS lesions (Rappussi *et al.*, 2009; Lucon *et al.*, 2010).

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