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Epidemiology of citrus black spot disease in South Africa and its impact on phytosanitary trade restrictions

by

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This thesis is dedicated to Drikus Truter

Epidemiology of citrus black spot disease in South Africa and its impact on phytosanitary trade restrictions

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Abstract

Citrus black spot (CBS), caused by *Guignardia citricarpa* Kiely, occurs in various citrus producing regions of the world. Due to the potential phytosanitary risk associated with the export of fruit from CBS positive production areas to CBS-free countries, restrictive trade barriers have been introduced. This study aimed to further elucidate some epidemiological aspects of CBS that can be used to address critical questions identified in the pest risk assessment submitted by South Africa to the World Trade Organisation to address phytosanitary trade restrictions.

Results indicated that Eureka lemon leaf litter exposed to viable pycnidiospores under controlled conditions or in the field in different production regions of South Africa, were not infected and colonised by *G. citricarpa*. Symptomatic CBS fruit or peel lying on the ground underneath citrus trees therefore can not lead to infection and colonisation of freshly detached leaves or leaf litter, or represent a source of inoculum in citrus orchards. Symptomatic fruit therefore pose no danger for the establishment of the pathogen in CBS-free orchards and are not considered to be a pathway for the pathogen.

The period of leaf susceptibility to *G. citricarpa* was indicated to be maximum eight and ten months from development, for Valencia orange and Eureka lemon, respectively, in a greenhouse study. The susceptibility period of citrus leaves to infection by the black spot pathogen could be longer than previously perceived.

Ascospores were captured, using the newly developed Kotzé Inoculum Monitor (KIM), from natural Valencia orange and Eureka lemon leaf litter during October to March with peak ascospore availability between December to February. The KIM is the first sampler designed to capture fungal spores directly from plant material in the laboratory without environmental influences and was effectively used to confirm that ascospores production

is seasonal. The KIM in combination with environmental data can be used to improve control through more targeted fungicide applications.

Techniques such as isolations and DNA amplification with species-specific primers to detect the pathogen directly from symptomless green leaves have a low success rate due to the restricted growth of the pathogen in latently infected tissue. Artificial leaf wilting enhanced the detection of *G. citricarpa* from symptomless leaves. Leaf wilting is a reliable, fast and effective method to detect the CBS pathogen and can be applied to monitor citrus nurseries and orchards throughout the year. It can also be applied to monitor pest-free orchards to maintain its CBS pest-free status.

This study confirmed that sanitation practices, such as leaf litter removal and mulching of leaf litter with wheat straw can decrease the primary inoculum, ascospores, of CBS and contribute to better management of the disease in a commercial orchard. Regardless of the prevailing climatic conditions each year, control achieved through leaf litter management resulted in >95% clean fruit and are equal to the control achieved with industry standard fungicides. This approach provided improved integrated disease control and an alternative to chemical control.

DECLARATION

I, the undersigned, hereby declare that this thesis, submitted for the degree of Doctor of Philosophy, Plant Pathology, is my own and original work except where acknowledged. This work has not been submitted for a degree at any other tertiary institution.

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Mariette Truter

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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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CHAPTER 3

Failure of *Phyllosticta citricarpa* pycnidiospores to infect Eureka lemon leaf litter

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3.1 Abstract

Pycnidiospores of ***Phyllosticta citricarpa*** from pure cultures, symptomatic black spot Valencia orange fruit and peelings were evaluated for their potential to infect and colonise citrus black spot-free Eureka lemon leaf litter in a controlled environment and in the field in different production regions of South Africa. Leaf litter, consisting of freshly detached mature green and old brown leaves that were exposed to viable pycnidiospores under controlled conditions or in the field underneath citrus trees, were not infected and colonised by ***P. citricarpa***. Ascospores, conforming to ***Guignardia citricarpa***, the pathogen, or ***Guignardia mangiferae***, a cosmopolitan endophyte, were collected with a Kotzé Inoculum Monitor from leaves placed in the field only at Tzaneen and Burgersfort. Distinguishing between these two species on ascospore morphology alone is not reliable. A diagnostic polymerase chain reaction conducted on representative leaf material from all the treatments revealed the presence of only ***G. mangiferae*** on 12.5% of the treatments. This study demonstrated the failure of ***P. citricarpa*** pycnidiospores to infect mature detached green leaves or leaf litter under controlled and field conditions. Symptomatic citrus black spot fruit or peel lying on the ground underneath citrus trees therefore cannot lead to infection and colonisation of freshly detached leaves or natural leaf litter or represent a source of inoculum in citrus orchards for these leaves.

3.2 Introduction

Citrus black spot (CBS), caused by ***Guignardia citricarpa*** Kiely (anamorph ***Phyllosticta citricarpa*** (McAlpine) Aa), represent superficial cosmetic fruit spots that are unacceptable in global fresh fruit trade and pose a phytosanitary risk. Symptoms can develop on more than 90% of the fruit produced from unsprayed orchards, ranging from one up to a thousand spots per fruit (Calavan, 1960). Three kinds of symptoms are widely recognised, viz. hard, freckle and virulent spot (Cobb, 1897; Kiely, 1948). Two other symptoms, speckled blotch and cracked spot, occurs predominantly in South Africa

(Kotzé, 1963; McOnie, 1963; Brodrick, 1969) and Brazil (De Goes ***et al***., 2000), respectively. Of these symptoms, hard and virulent spot may contain pycnidia within the lesions, although freckle spot may turn into virulent spot and speckled blotch may turn into hard spot as the season progresses (Kotzé, 1981).

Black spot is an economically important disease of citrus in summer rainfall regions of South Africa and various other subtropical countries. Although the disease has spread to most of the summer rainfall areas in South Africa since its first reported occurrence in 1929 (Doidge, 1929), it has not established in predominantly winter rainfall areas. These areas have official CBS-free status and consist of the citrus production regions of Northern Cape and Western Cape Provinces and some regions in the Free State and North West Provinces (European Union, 1998; Mabiletsa, 2003; APHIS, 2009; Shea, 2010). Confirmation of this distribution pattern in South Africa was recently illustrated using global modelling of weather patterns to map CBS occurrence (Paul ***et al***., 2005; Yonow & Hatting, 2009). 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).

Environmental conditions required for successful infection of susceptible citrus material include the presence of adequate moisture and relative high temperatures ranging between 18 and 30°C for at least 15 hours (Kotzé, 1963; McOnie, 1967). These conditions usually prevail in the summer rainfall areas of South Africa from late spring to autumn. The critical infection period is usually from October until January, as fruit susceptibility and main ascospore release coincides (Kotzé, 1981, 1996). The critical infection period may start and end a month earlier and/or later depending on prevailing rainfall and mean temperature.

Fruit remains susceptible to infection from fruit set up to five months later, whereas leaves remain susceptible from development up to 10 months of age (Kiely, 1948, 1950; Kotzé, 1963; McOnie, 1964c; Truter ***et al***., 2004b). Two types of spores produced by the pathogen can infect susceptible citrus material (Kiely, 1948; McOnie, 1964c; Whiteside, 1967; Kotzé, 1996). The airborne ascospores from pseudothecia are only produced on leaf litter and are the main source of inoculum and dissemination of the disease (Kiely, 1948; McOnie, 1964c; Kotzé, 1981; Korf, 1998). Pycnidiospores of the anamorph are produced in pycnidia on symptomatic fruit, leaf litter and with the highly susceptible cultivar, Eureka lemon, on petioles and small twigs (Kiely, 1948; McOnie, 1964c;

Whiteside, 1967). In general, the water-borne pycnidiospores are regarded as unimportant in the dissemination of the disease, mainly due to the limited spread of the pathogen by means of water and the short viability period of the pycnidiospores (Kiely, 1948; McOnie, 1964c; Korf, 1998).

Asco- and pycnidiospores require moisture for production and discharge. In the presence of adequate moisture, ascospores are forcibly released from pseudothecia to a height of about 12 mm to be dispersed by air currents, while masses of gelatinous pycnidiospores ooze from pycnidia to be dispersed by water (Kiely, 1948; Kotzé, 1963; McOnie, 1964b, c). Viable ascospores and pycnidiospores landing on young attached citrus fruit and leaves will usually lead to successful infection under favourable environmental conditions (Kiely, 1948; Kotzé, 1963; McOnie, 1964c; Whiteside, 1967).

Following successful infection, the pathogen remains latent in the fruit and leaves for several months as a small knot of mycelium between the cuticle and epidermis. The latent period in fruit usually lasts until fruit maturity, although several factors regarding the host and environment can influence symptom expression. Leaf infections can remain latent for up to 36 months before leaf fall and under favourable conditions, production of pycnidio- and ascospores on the leaf litter (Kiely, 1948; Whiteside, 1965; McOnie, 1967; Kotzé, 1996). Alternate wetting and drying of leaves and temperature fluctuations provide optimal conditions for maturation of pseudothecia on leaf litter (Kotzé, 1996).

Pycnidiospores produced on symptomatic fruit or peel representing an inoculum source in a citrus orchard has not yet been proven. This raises the concern that symptomatic fruit and/or peelings discarded in a citrus orchard could lead to new infections. The concern that symptomatic fruit may introduce the pathogen into CBS-free areas has led to more restrictive requirements for market access and trade. The premise of this approach was that only attached green leaves can be infected and will eventually add to the inoculum load produced on leaf litter. The aim of this investigation was, therefore, to determine whether pycnidiospores from an active growing culture and from symptomatic CBS fruit or peelings could infect and colonise both freshly detached CBS-free mature, green leaves and natural leaf litter from Eureka lemon under controlled and field conditions.

3.3 Materials and methods

Pycnidiospores from three different sources were used as inoculum in separate experiments, viz. pure culture, infected fruit and peelings of infected fruit.

3.3.1 Pure culture

A ***P. citricarpa*** isolate (PPRI 8774), originally obtained from naturally infected Valencia fruit from Burgersfort (Mpumalanga) during July 2002 was preserved at -80°C and plated from storage as required without repeated sub-culturing. The culture was plated onto 2% potato dextrose agar (PDA) (Biolab, Merck) and incubated for 21 days under continuous fluorescent light at 25°C. Pycnidiospores produced were harvested by repeatedly rolling a sterile cotton swab over the culture and rinsing the spores from the swab in 15 ml sterile tap water. Rolling and rinsing were continued until spores from the whole culture were harvested. The spore suspension was filtered through four layers of sterile gauze to remove mycelial fragments. The concentration of the spore suspension was determined with a haemocytometer and the final concentration adjusted to 10^4 spores ml^{-1} with sterile tap water. The spore suspension was kept at 15°C until used (within 4 to 6 h). A dilution series from the final spore suspension in sterile 0.3% orange juice was plated to PDA and incubated at 25°C. Colony forming units ml^{-1} of the pycnidiospore suspension were determined by counting the developing ***G. citricarpa*** colonies after seven days.

Mature, green leaves (older than one year) were picked from 25 five-year-old CBS-free Eureka lemon trees. The trees were originally obtained from Stargrow nursery in the CBS-free citrus production region, Western Cape, and maintained in a greenhouse at the University of Pretoria for the duration of the study. Trees received regular insecticide but no fungicide sprays for the duration of the study. Detached leaves were secured between two circular plastic grid sheets (350 mm diameter, 10 mm mesh size) with cable ties. Each grid set contained between 20 to 25 leaves. Ten prepared leaf grids were sprayed with the spore suspension on both sides until run-off and were then individually enclosed in plastic bags to maintain high moisture content conducive for pycnidiospore germination and infection. Ten control leaf grids were prepared and processed as described but were sprayed with sterile tap water instead. All leaf grids were removed from the plastic bags after 48 h at 25°C. Five of the control and pathogen inoculated leaf grids were further incubated in a growth chamber at 25°C, 90% relative humidity (RH) and a 14:10 h light:dark cycle, whereas the remaining grid sets were placed underneath citrus trees in Pretoria, Gauteng Province. Prevailing minimum and maximum temperature and total rainfall were recorded in all the field experiments for the duration of each trial. All leaf grids were moistened on both sides three times a week with a fine mist of tap water until run-off. The leaf grids were removed from the growth chamber after eight weeks, before the onset of leaf degradation, whereas the field exposed leaf grids were removed after 12 weeks. Leaf degradation within the growth chamber was enhanced by the constant high humidity of 90% RH. The leaves were prepared for polymerase chain reaction (PCR) and

ascospore capturing with the Kotzé Inoculum Monitor (KIM) within a week from collection. The experiment was done during May to July and repeated during September to November 2003.

The same procedures as described for the mature green leaves were followed using leaf litter collected from an orchard in Paarl, Western Cape Province. Each grid set contained ca. 30 g of dry Eureka lemon leaf litter and five grid sets per treatment were maintained in the growth chamber and placed in the field from May to July and was repeated from September to November 2003.

3.3.2 Infected fruit

Another similar experiment was done using CBS symptomatic fruit as a natural pycnidiospore inoculum source instead of spraying leaves and litter with a pycnidiospore suspension. Valencia oranges with at least 20 red or hard spot symptoms per fruit were collected from a CBS affected orchard in Nelspruit, Mpumalanga Province. Fruit was submerged in tap water for 30 min, removed and incubated in a moist chamber at 25°C for 24 h to stimulate release of mature pycnidiospores and production of new viable pycnidiospores (Kiely, 1948). Lesions on selected infected fruit were microscopically examined to confirm the presence of pycnidia and pycnidiospores before being used. Isolations were made from selected CBS lesions as described by Meyer ***et al.*** (2006), deviating only by plating tissue onto 2% PDA supplemented with 50 mg l^{-1} rifampicin to confirm the viability and identity of the pathogen present. Identities of retrieved cultures were confirmed by PCR. Disease-free Valencia orange fruit from Citrusdal, Western Cape Province, were used as control. The fruit was visually inspected to confirm its CBS-free status and rinsed with sterile tap water to ensure that it contained no traces of inoculum before being used.

Mature, green CBS-free leaves (older than one year) were picked from 40 15-year-old Eureka lemon trees in Paarl. Leaves (20-25) were secured between two circular plastic grid sheets with cable ties as described for the first experiment. Three black spot infected fruit was placed in a plastic mesh and secured directly on top of each prepared leaf grid (Fig. 3.1). Disease-free fruit was similarly prepared representing the control treatment. This time three fruit/leaf grids were used for each set of exposure conditions. Three incubation temperature conditions were selected, viz. 20, 25 and 30°C in different growth chambers at 90% RH with a 14:10 h light:dark cycle. The fruit/leaf grids were sprayed on both sides with a fine mist of tap water until run-off three times a week. Grid sets were

Figure 3.1. Valencia orange fruit with hard spots, tied to a grid containing mature, green, citrus black spot-free Eureka lemon leaves.

also placed on the ground underneath citrus trees in CBS affected regions viz. Pretoria (Gauteng Province), Tzaneen (Limpopo Province), Burgersfort (Mpumalanga Province) and Brits (North-West Province) and a CBS-free region viz. Bellville, Constantia and Stellenbosch (Western Cape Province). Localities for the field treatments were selected to include areas with summer rainfall with moderate to high levels of CBS and a CBS-free area with winter rainfall in Western Cape Province. None of the citrus orchard blocks selected had received any chemical sprays against CBS for at least five years before commencement and for the entire duration of the study. The fruit/leaf grids in the field were moistened by hand with tap water on both sides until run-off on a weekly basis. The grids in the growth chamber and field were collected after eight and 12 weeks, respectively. Fruit with plastic mesh were removed from the grids and the leaves prepared for PCR and ascospore capturing. The removed fruit were microscopically examined for the presence of fruiting bodies and segments of the peel selected for PCR to confirm the presence of ***G. citricarpa***. The experiment was done between May and July and repeated between September and November 2003.

The same procedures described for the fruit and mature green leaves were again followed, this time using leaf litter instead of mature green leaves. The leaf litter was collected underneath the same Eureka lemon trees in Paarl as the green leaves. The leaf litter was secured between two plastic grid sheets with cable ties and treated the same as

before. Three fruit/leaf litter grids per treatment were used between May and July and repeated between September and November 2003.

3.3.3 Peelings of infected fruit

Naturally infected Valencia oranges from Nelspruit with at least 20 red or hard spot symptoms per fruit were rinsed with sterile tap water and air-dried on paper towel. Ten randomly selected fruit were kept separate for microscopic examination, whereas the remaining fruit were peeled. Lesions on selected infected fruit were microscopically examined to confirm the presence of pycnidia and pycnidiospores. Isolations were made from selected CBS lesions as described previously. The identities of retrieved cultures were confirmed by PCR. Disease-free Valencia orange fruit from Citrusdal were treated similarly and were included as controls.

Mature, green CBS-free leaves (older than one year) were picked from 40 15-year-old Eureka lemon trees in Paarl. Leaves were secured between two circular plastic grid sheets with cable ties as described for the first and second experiments. The peel from four infected fruit were placed in a plastic mesh and secured directly on top of each prepared leaf grid. Peel from disease-free fruit was treated in the same way. The peel/leaf grids were incubated at 25°C in a growth chamber at 90% RH with a 14:10 h light:dark cycle. Peel and leaf grids were also placed on the ground underneath citrus trees in Pretoria. All grids were sprayed on both sides with a fine mist of tap water until run-off three times a week. The peel and leaf grids were removed from the growth chamber and field after eight and 12 weeks, respectively. Peelings and the plastic mesh were removed from the grids and the leaves prepared for PCR and ascospore capturing. The removed peelings were microscopically examined for the presence of fruiting bodies and segments were selected for PCR to confirm the presence of ***G. citricarpa***. Five peel and leaf grid sets were prepared per exposure condition and used from January until March 2004.

In the last experiment the transfer of natural pycnidiospore inoculum from CBS infected fruit peelings to leaf litter was investigated. Natural leaf litter was collected under CBS-free Eureka lemon trees in Paarl. The leaf litter was secured between two plastic grid sheets with cable ties, about 30 g per grid, and treated as described for the peel and green leaf grids. Five peel and leaf grids per exposure condition were used between January until March 2004.

3.3.4 Polymerase chain reaction

Twenty leaf pieces (8 mm diameter) were selected from all the previously described treatments before being prepared for the ascospore capturing and incubated in moist chambers for 14 days at 28°C to induce development of fungal fruiting structures. The leaf pieces were microscopically examined for the presence of ***G. citricarpa***-like pycnidia or pseudothecia. DNA was extracted from 100 mg selected leaf material from each treatment by grinding in liquid nitrogen and using the DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Polymerase chain reactions were done to confirm the presence of ***G. citricarpa*** and/or ***Guignardia mangiferae*** A.J. Roy with the primers CITRIC1 and CAMEL2 in conjunction with ITS4 primer as described by Meyer ***et al***. (2006).

3.3.5 Ascospore capturing

The grids were submerged in water at 40°C for 5 min to induce ascospore release, followed by drainage for 10 min to remove excess water. Each grid pair with leaves was placed in the KIM, previously referred to as the Kotzé-Quest Inoculum Monitor (Truter ***et al***., 2004a), and a microscope slide coated with a thin layer of Vaseline was used to collect spores. Grids were processed separately using one microscope slide for each grid. After the two-hour KIM operation at room temperature, the slide was removed, stained with lactofuchsin and examined with a compound microscope at x400 magnification. Each slide was divided into three 5 mm sections along the width of the slide. ***G. citricarpa***-like ascospores were counted along four lanes, covering the width of the microscope field within the centre longitudinal 5 mm transect. These lanes ran across the length of the microscope slide from the starting point to where the trapping process stopped.

3.4 Results

Harvesting of spores with a swab was superior to other methods tested, including the method described by Korf (1998), being less time consuming and resulting in improved spore yield. Sufficient numbers of pycnidiospores were produced in culture on a single 2% PDA dish (90 mm diameter) in 21 days to prepare a spore suspension of 10^4 spores ml^{-1} with which to inoculate all the treatments. More than 80% of the pycnidiospores in the final spore suspensions prepared in May and September germinated, leaving 3.6 x 10^4 and 5.2 x 10^4 colony forming units ml^{-1} for infection, respectively. Black spot-infected Valencia orange fruit yielded pycnidiospores in 78% of all the selected hard spot lesions that were examined microscopically. Fungal isolates retrieved from the selected lesion pieces yielded 64% ***G. citricarpa***, confirmed by PCR, 35% ***Colletotrichum gloeosporioides***

(Penz.) Penz. & Sacc., confirmed by morphological characteristics and 1% unidentified fungi.

Microscopic examination of selected leaves from all the treatments after the treatment period, revealed the presence of pycnidia and pseudothecia, but morphological characteristics of these fruiting bodies could not be confirmed to be that of ***Guignardia*** without the presence of spores. Other fungi fruiting on the leaf material that were identified included ***Alternaria alternata*** (Fr.) Keissl., ***Aspergillus*** sp., ***Cladosporium*** spp., ***C. gloeosporioides***, ***Eudarluca caricis*** (Biv.) O.E. Erikss. and ***Phoma*** spp. PCR tests conducted on the selected leaf pieces were negative for ***G. citricarpa*** for all treatments (Table 3.1). Seven samples tested positive for the endophyte ***G. mangiferae*** with PCR. After the first detection of ***G. mangiferae*** additional leaf samples were collected from the same orchard where the leaves were originally collected to verify the natural occurrence of the endophyte. Of the 25 samples randomly collected from the same trees in this orchard, al 10 green leaf samples tested negative, whereas two of the leaf litter samples tested positive for ***G. mangiferae***.

In the experiments using symptomatic CBS fruit as inoculum source, both infected and non-infected fruit as well as peelings were observed to have severe superficial microbial growth after the incubation period. Most of the fruit were mummified at this stage and all the peelings were dry and brittle. No pycnidia and/or pycnidiospores could be discerned by microscopic examination in the CBS lesions of the infected fruit or peel after the treatment period. No evidence was also found that ascospores were able to develop on the fruit or peel of infected and non-infected fruit after the treatment. PCR tests conducted on selected fruit and peel segments of the used infected and non-infected fruit were negative for both ***G. citricarpa*** and ***G. mangiferae***.

Ascospores, resembling those of ***G. citricarpa*** or ***G. mangiferae*** were captured with the KIM from four treatments, viz. i) detached green leaves placed in Tzaneen with and ii) without infected fruit, iii) detached green leaves exposed to infected fruit and iv) leaf litter exposed to clean fruit placed in Burgersfort. In each of the four treatments, ascospores were captured from only one grid pair incubated during the summer months (January to March). Since PCR on selected leaf material from these grids tested positive for ***G. mangiferae*** and no ***G. citricarpa*** could be found on any of the leaf pieces used for PCR confirmation, ascospores captured, therefore, represented ***G. mangiferae*** and not the pathogen.

Table 3.1. Presence of ***Guignardia citricarpa*** or ***Guignardia mangiferae*** on black spot free Eureka lemon leaves after exposure to pycnidiospores under controlled conditions (growth chambers) and in the field

Treatment	Prevailing temperature (°C)[a]	Detection of ***Guignardia*** spp. on citrus leaves (no. of ascospores / PCR results)[b]			
		Freshly detached mature green leaves		Leaf litter collected from orchard floor	
		Treated	Control	Treated	Control
Pure culture					
Growth chamber	25	0 / Neg[c]	0 / Neg	0/ Neg	0 / Neg
Field: Pretoria (Gauteng)	5.8-20.0; 13.2-26.9	0 / GM[d]	0 / Neg	0 / Neg	0 / Neg
Symptomatic fruit					
Growth chamber	20	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Growth chamber	25	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Growth chamber	30	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Field					
Pretoria (Gauteng)	5.8-20.0; 13.2-26.9	0 / Neg	0 / Neg	0 / GM	0 / Neg
Tzaneen (Limpopo)	12.6-22.0; 15.7-26.2	75 / GM	142 / GM	0 / Neg	0 / Neg
Brits (North-West)	4.7-21.9; 13.1-29.5	0 / Neg	0 / GM	0 / Neg	0 / Neg
Burgersfort (Mpumalanga)	6.6-17.5; 10.8-22.1	35 / GM	0 / Neg	0 / Neg	104 / GM
Bellville (Western Cape)	7.8-19.6; 11.5-22.3	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Constancia (Western Cape)	7.8-19.6; 11.5-22.3	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Stellenbosch (Western Cape)	7.3-20.9; 12.4-24.2	0 / Neg	0 / Neg	0 / Neg	0 / Neg
Peelings of symptomatic fruit					
Growth chamber	25	0 / Neg	0 / Neg	0/ Neg	0 / Neg
Field: Pretoria (Gauteng)	15.4-25.7	0 / Neg	0 / Neg	0 / Neg	0 / Neg

[a]Leaf exposure to pycnidiospores from pure culture and symptomatic fruit were carried out from May to July (first temperature range) and repeated from September to November 2003 (second temperature range), whereas leaf exposure to peelings of symptomatic fruit were carried out from January to March 2004.

[b]Mean of five replicates for pure culture and three replicates for symptomatic fruit, each repeated twice; Mean of five replicates for peelings of symptomatic fruit; Mean ascospore count per replicate with Kotzé Inoculum Monitor.

[c]Neg = negative for ***Guigarndia citricarpa*** and ***Guignardia mangiferae***.

[d]GM = positive for ***G. mangiferae***.

3.5 Discussion

This study demonstrated that viable pycnidiospores from a culture, symptomatic fruit or peel were not able to infect and colonise freshly detached green leaves or natural leaf litter from Eureka lemon under controlled and field conditions. Even after exposure of the leaves to high inoculum pressure under highly favourable environmental conditions, ***G. citricarpa*** did not colonise any of the leaves. As Eureka lemon is the most susceptible cultivar to CBS, we can deduce that the same results will be achieved on other susceptible cultivars.

In a concurrent study, leaves on Eureka lemon trees were spray-inoculated with a pycnidiospore suspension from the same pathogen isolate as the present study (Truter ***et al***., 2004b). The leaves were inoculated at different ages, ranging from one to 14 months, to determine the susceptibility period of green leaves. Symptomless infections established in one- to 10-month-old leaves, demonstrated the effectiveness of the inoculation technique as well as the conduciveness of the controlled environment to infection. Favourable infection conditions were also present in the field as the mean maximum temperatures were above 18°C during both trial periods in all the localities selected in this study. Infection conditions in the field were furthermore not dependant on rainfall since all grid pairs were wetted weekly. The presence of favourable infection conditions in the field was accentuated by abundant black spot symptoms on fruit in the orchards in the summer rainfall production areas during the trial period as these blocks received no chemical treatment for CBS control.

Leaf inoculations with pycnidiospores from infected fruit and ascospores from leaf litter have only been reported for attached young green leaves (Kiely, 1948; Wager, 1952; McOnie, 1967) and no reports were found on leaf litter inoculations. Wager (1952) placed symptomatic black spot fruit in a wire basket and hung it in a citrus tree in a CBS-free orchard to determine if the infected fruit could act as an inoculum source. Symptoms developed after several months on the fruit and similar to the current study leaf infections remained latent. Leaf infections usually remain latent, although symptoms can be produced on very old leaves or on younger leaves from trees under stress.

Another critical element for successful infection is the presence of ample viable inoculum. The inoculum load applied to the CBS-free leaves was quantified by determining the cfu ml^{-1} of the pycnidiospore suspension and by microscopic examination of the fruit lesions. Pycnidiospores produced on fruit were described as short-lived, with pycnidiospores older than three to 14 days failing to germinate, depending on the technique used (Wager,

1949; Kiely, 1948; Korf, 1998). Despite the short viability period of pycnidiospores, symptomatic CBS fruit can be a source of viable pycnidiospore inoculum for several months as the sporogenous layers in pycnidia are regenerative and numerous crops of pycnidiospores can be produced following regular wetting of the fruit (Kiely, 1948; Wager, 1952).

In a recent study, the viability of ***G. citricarpa*** was evaluated over time in peel and fruit under different temperature and humidity combinations (Agostini ***et al***., 2006). The viability was determined by isolation of the pathogen from the fruit tissue, but unfortunately no attention was given to the vitality of pycnidiospores. Also, no PCR-based diagnostics were conducted to verify the identity of the retrieved cultures. Despite inconsistent results obtained from fruit isolations, ***Guignardia*** was recovered over 40 days as long as the lesion was intact on peel or fruit, irrespective of the storage conditions. This is in agreement with previous reports (Kiely, 1948; McOnie, 1964c; Korf, 1998). Although the pathogen can remain viable in symptoms on infected citrus fruit, the successful isolation frequency decline with storage time (Kiely, 1948; Wager, 1952; McOnie, 1967; Agostini ***et al***., 2006). In the current study, the pathogen could not be detected with PCR from infected fruit or peelings after eight to 12 weeks due to severe host tissue degradation and subsequent breakdown of the pathogen.

Of the three detection methods used on leaf litter, fruit and peelings, PCR with the species selective primers, were the most sensitive and enable one to distinguish accurately between ***G. citricarpa*** and ***G. mangiferae***. Furthermore, ***G. mangiferae*** was detected from leaf litter from which no ascospores were captured, indicating that the leaf litter was not devoid of ***Guignardia*** spp, and that the ascospores were perhaps not matured at the time of evaluation. The endophyte, ***G. mangiferae***, occurs worldwide on citrus and other woody plants and is of no phytosanitary concern (Baayen ***et al***., 2002; Meyer ***et al***., 2006). Our detection of ***G. mangiferae*** from leaves collected in Paarl is in accordance with the reported occurrence of the endophyte from CBS-free regions of Western Cape and other areas in South Africa (McOnie, 1964a). Dual infections by ***G. citricarpa*** and ***G. mangiferae*** have also been reported on citrus leaves and fruit (McOnie, 1964c, d; Baayen ***et al***., 2002; Meyer ***et al***., 2006; Baldassari ***et al***., 2008).

This is the first report on artificial inoculation of leaf litter with pycnidiospores of ***G. citricarpa***. The study evidently showed that ***G. citricarpa*** artificially inoculated or through natural inoculum exposure could not infect freshly detached mature green leaves or natural leaf litter. The detached leaves, either fresh or old, were not susceptible to

pycnidiospore infection. The inoculum produced on the leaf litter, thus depends on the level of infection of young leaves while attached to the tree (Kiely, 1948; Wager, 1952; Kotzé, 1963; McOnie, 1964c; Whiteside, 1967). There is no evidence that viable pycnidiospores produced on infected fruit could infect freshly detached mature green leaves and natural leaf litter and in practice lead to the production of inoculum in an orchard. Pycnidiospores produced on infected fruit or leaf litter on the orchard floor do not contribute to production of pseudothecia with ascospores on leaf litter and therefore do not increase inoculum levels in an orchard.

Commercial fruit are not considered to be a high risk for introduction of the pathogen into new areas, as the presence of susceptible host tissue in close proximity to the source is required. Further, the present study clearly showed that waterborne pycnidia cannot infect mature detached green leaves or old litter. The likelihood that infected fruit or peel will come in direct contact with attached young leaves and that viable pycnidiospores will be washed down onto the leaves is implausible. There is no evidence that infected fruit lying on the ground in a CBS-free orchard will be able to infect detached leaves and contribute to the spread of the disease. Infected citrus fruit or peel poses no danger for the establishment of the pathogen in CBS-free orchards when exposed to detached leaves only.

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CHAPTER 4

Susceptibility of citrus leaves to *Phyllosticta citricarpa* relative to leaf age and phenolic acid content

4.1 Abstract

The period of susceptibility of green citrus leaves to ***Phyllosticta citricarpa*** was investigated in healthy, citrus black spot-free, Eureka lemon and Valencia orange trees in the greenhouse. Infections were successfully established in Eureka lemon and Valencia orange leaves artificially inoculated with ***P. citricarpa*** at the age of up to 10 and eight months old, respectively, but not in older leaves. The pathogen could be reisolated monthly for a maximum of five months from leaves inoculated at the age of one to four months. The recovery rate of ***P. citricarpa*** was significantly higher from infected Eureka lemon leaves than Valencia orange each month except month seven, when the opposite was observed. Inoculated Valencia orange leaves produced significantly higher levels of both gallic and ferulic acid esterified to the cell walls than uninoculated leaves, but differences in the total soluble glycoside-bound, non-conjucated soluble and soluble esterified phenolic content of inoculated and unioculated leaves were inconsistent. Results from the phenolic acids indicate host response to infection and no conclusions can be made on the resistance of leaves to ***P. citricarpa*** developing over time. The scientifically-founded evidence provided by this study suggest that the susceptibility period of citrus leaves to infection by the black spot pathogen could be longer than previously perceived.

4.2 Introduction

Citrus black spot (CBS), caused by ***Guignardia citricarpa*** Kiely (anamorph ***Phyllosticta citricarpa*** (McAlpine) Aa), is an economically important disease in summer-rainfall regions of South Africa and various other subtropical countries. Symptomatic fruit are unacceptable in global fruit trade and represent a perceived phytosanitary risk, as pycnidiospores may be produced in lesions on fruit (Kotzé, 1981). Dissemination of the pathogen does not primarily occur through pycnidiospores but mainly by infected nursery trees and airborne ascospores originating from infected leaf litter (Kiely, 1948; McOnie, 1964b; Kotzé, 1981).

Ascospores are only produced on infected leaf litter under favourable environmental conditions and mature ascospores, discharged mainly during spells of summer rain, are

dispersed by air currents (Kiely, 1948; Kotzé, 1963; McOnie, 1964a, b). By contrast, pycnidiospores of the anamorph are produced in pycnidia on symptomatic fruit, green leaves and leaf litter (Kiely, 1948; Kotzé, 1981). Pycnidiospores can also be produced on petioles and small twigs of the highly susceptible cultivar, Eureka lemon (***Citrus limon*** (L.) Burn. f.) (Kiely, 1948; McOnie, 1964b; Whiteside, 1967). Masses of gelatinous pycnidiospores are released under moist conditions and are dispersed by water. In general, ascospores are regarded as the main source of inoculum in an orchard (Kiely, 1948; McOnie, 1964b; Kotzé, 1981; Korf, 1998). Pycnidiospores are regarded as unimportant in the dissemination of the pathogen and epidemiological development of the disease, mainly due to the limited spread of the pathogen by means of water and the short viability period of the pycnidiospores (Kiely, 1948; McOnie, 1964b; Korf, 1998). Viable asco- and pycnidiospores landing on susceptible young citrus fruit and leaves may lead to successful infection under favourable environmental conditions (Kiely, 1948; Kotzé, 1963; McOnie, 1964b, Whiteside, 1967).

Previous studies on the susceptibility of citrus to ***G. citricarpa*** were mainly directed at fruit. Infection of fruit occurs within the first five months of their development after which they become resistant to new infections (Kiely, 1948, 1950; Kotzé, 1963; McOnie, 1964b). The duration of the susceptibility period depends on the age and condition of the tree (Kiely, 1950). Infection remains latent within the rind tissue as a small knot of mycelia until fruit maturity. In South Africa, fruit maturity normally occurs up to 10 months after the initial infection (Kotzé, 1963; McOnie, 1967).

The susceptibility period of citrus leaves to infection by ***G. citricarpa*** was originally reported to be five weeks (Kiely, 1948; McOnie, 1967), although subsequent field observations suggested that it could be five months (Kotzé, 1981). Leaf infection may remain latent until leaf drop and the pathogen may then only produce pycnidio- and ascospores on leaf litter. Leaf symptoms on green leaves were reported mainly on Eureka lemon (Kiely, 1948; Wager, 1952; Whiteside, 1965; McOnie, 1967; Kotzé, 1996). Leaf infections can occur throughout the year under favourable conditions, as several new, susceptible leaf flushes are produced during the year.

Phenolic compounds, present in virtually all types of plants, are an integral component of their natural defence system (Harborne, 1984; Nicholson & Hammerschmidt, 1992). This highly diversified group of phytochemicals are derived from phenylalanine and tyrosine, synthesised via the shikimic acid pathway, during normal plant development and in response to various stress conditions such as infection, wounding, low temperatures,

some fungicides and UV radiation (Nicholson & Hammerschmidt, 1992; Harborne, 1993; Kuć, 1995; Beckman, 2000; De Ascensao & Dubery, 2003; Naczk & Shahidi, 2006; Charles ***et al***., 2008). The level of phenolics also depends on factors such as growth and storage conditions, cultivation techniques, cultivar and ripening processes (Naczk & Shahidi, 2006). Most phenolic compounds are present in conjugated form, i.e. linked to a sugar through one or more of the phenolic hydroxyl groups, or as conjugated esters (Harborne, 1984; Antolovich ***et al***., 2000; De Ascensao & Dubery, 2003).

Some phenolics, such as phytoanticipins, function as pre-infection inhibitors to plant pathogens, while phytoalexins accumulate rapidly in response to microbial infection or specific elicitors (Nicholson & Hammerschmidt, 1992; Harborne, 1993; De Ascensao & Dubery, 2003). Responses associated with pathogen infection include cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic compounds or physical barriers and the synthesis of specific antimicrobial compounds such as phytoalexins (Nicholson & Hammerschmidt, 1992).

Leaf infections present a critical component of the life cycle of ***G. citricarpa*** and with proper orchard sanitation where old fruit are removed from the orchard before onset of the next crop, infected leaves provide the only means of survival for the pathogen until the next crop. As infected leaves can be a significant inoculum source for the following year's crop, it is important to investigate the period for which new leaves are susceptible to infection. The aim of this study was to determine the duration of susceptibility of Eureka lemon and Valencia orange (***Citrus sinensis*** Osbeck) leaves to ***P. citricarpa*** infection, and to investigate the chemical nature and levels of associated soluble free phenolic acids (non-conjugated), wall-bound phenolic acids as well as phenolic polymers (ester-bound and phenolic glycosides) in Valencia orange leaves inoculated at different stages of development with ***P. citricarpa*** pycnidiospores.

4.3 Materials and methods

4.3.1 Leaf inoculation

Two-year-old CBS-free Eureka lemon and Valencia orange trees on Rough lemon (***Citrus jambhiri*** Lush.) rootstock were obtained from Stargrow nursery, Western Cape Province, and maintained in a greenhouse at the University of Pretoria. Mean temperatures within the greenhouse ranged between 18°C ±2°C (night) and 26°C ±2°C (day) throughout the study. Seventy-two trees of each cultivar were manually defoliated and petioles of new leaf flushes were labelled when ca. 10 days old. The leaves were inoculated monthly with a pycnidiospore suspension of ***P. citricarpa*** (see below), using three new trees per

treatment each month in order to ensure that leaves that were used represented one to 12 months old. The experiment was done from September 2002 until August 2003 and was repeated from August 2004 until July 2005 using new two-year-old trees prepared and maintained as above.

P. citricarpa isolate PPRI 8790, originally obtained from a symptomatic Valencia orange fruit from Burgersfort, Mpumalanga Province during July 2002, was maintained in sterile water at 15°C and in 15% glycerol at -80°C. The culture was sub-cultured onto 2% potato-dextrose agar (PDA) (Biolab, Merck) each month to prepare fresh inoculum. Inoculated PDA dishes were incubated at 25°C for 21 days under continuous fluorescent light. Pycnidiospores were harvested as described by Truter ***et al.*** (2007). The concentration of the spore suspension was determined with a haemacytometer and the final concentration adjusted to about 6 x 10^4 spores ml^{-1} with sterile tap water. The colony forming units (cfu's) ml^{-1} of the final spore suspension was determined by plating a dilution series of the suspension in sterile 0.3% orange juice on PDA. Developing ***P. citricarpa*** colonies were counted after seven days at 25°C and cfu's ml^{-1} calculated. The spore suspension was kept at 15°C until used (within 4 to 6 h).

Three new replicate trees of each citrus cultivar were marked and inoculated each month. The entire canopy of each tree was covered with a clear plastic bag and the leaves sprayed abaxially and adaxially within the bag with the prepared pycnidiospore suspension until run-off. The bag was closed directly after spraying and removed after 48 h. Three trees serving as control were treated similarly, but were sprayed with sterile tap water instead of a pycnidiospore suspension. Five leaves representative of the relevant age at inoculation (i.e. one to 12 months) were collected four weeks after inoculation from each replicate inoculated and control tree. A further five leaves inoculated when one to four months old, were collected for six months after inoculation from each of three inoculated trees to determine the recovery rate of the pathogen at different time intervals after inoculation.

Five leaf segments (ca. 25 mm^2) were aseptically cut from each collected leaf, surface-disinfested for 1 min in 1.5% sodium hypochlorite, rinsed with sterile tap water and blotted dry on sterile filter paper. The 25 leaf segments from each tree were randomly plated on 2% PDA, with five leaf segments per dish. Dishes were incubated for four weeks at 25°C and developing colonies recorded each week. Fungi that developed were morphologically identified and the identity of representative isolates confirmed by means of polymerase chain reaction (PCR) with species-specific primers as described by Meyer ***et al***. (2006).

4.3.2 Extraction and quantification of phenolic acids

Five leaves were collected from each replicate inoculated and control Valencia orange tree four weeks after inoculation for each time interval and freeze-dried for 48 h. The freeze-dried leaf material was ground, passed through a 0.08-mm-mesh sieve and stored at room temperature until analysed. Phenolic acids were extracted from 50 mg leaf material in duplicate with 1 ml methanol:acetone:water (7:7:1 v/v) (Régnier, 1994). The suspension was homogenised for 1 min and agitated for 1 h at 4°C at 200 rpm on an orbital shaker. The suspension was centrifuged at 12000 ***g*** for 5 min and the supernatant collected and stored. The remaining precipitate was re-homogenised and centrifuged as described above. Extraction was repeated three times to ensure complete recovery of the soluble phenolic acids. The four supernatants were pooled and concentrated to 1 ml under vacuum. The two duplicate extracts per sample were combined and aliquoted into four microcentrifuge tubes (0.5 ml per tube) to determine total soluble, non-conjugated, methanol:acetone soluble ester-bound and glycoside-bound phenolic acids. The remaining alcohol insoluble residue was dried overnight at 55°C and used to extract the ester-bound cell wall phenolic acids (De Ascensao & Dubery, 2003).

The concentration of phenolic acids in each fraction was determined using Folin-Ciocalteau reagent (Sigma) (Swain & Hillis, 1959). Volumes were modified to facilitate the use of ELISA-plates. Four replicates of the extract (5 µl) were diluted to 175 µl with distilled water, added to 25 µl of Folin-Ciocalteau reagent and mixed. After 3 min, 50 µl of aqueous sodium carbonate (20% m/v) was added, mixed thoroughly and incubated at 40°C for 30 min. A blank of 5 µl methanol was used instead of the sample. The absorbance was read using an ELISA reader (Muliskan Ascent V1.24354 – 50973, Version 1.3.1). Gallic acid was used as phenolic standard to construct a standard curve ranging from 0 to 40 mg, $r^2 = 0.9989$. The concentration of phenolics in the various extracts was calculated from the standard curve and expressed as mg gallic acid equivalent g^{-1} dry mass.

The second supernatant aliquat was used to determine the amount of non-conjugated phenolic acids. The aliquot was acidified with 50 µl of 1 M trifluoro-acetic acid (Sigma) and the solution was extracted three times with 1 ml anhydrous diethyl ether (Cvikrová ***et al.***, 1993). The ether extract was dried under vacuum and the resulting precipitate was re-suspended in 0.25 ml pure methanol. This solution was used to determine the free phenolic content with Folin-Ciocalteau reagent.

The third aliquoted supernatant for soluble glycoside-bound phenolic content determination was hydrolysed in 50 µl concentrated HCl for 1 hour at 96°C and then extracted three times with 1 ml anhydrous diethyl ether. The ether extract was dried and the resulting precipitate was re-suspended in 0.25 ml pure methanol. This solution was used to determine the phenolic glycoside content using Folin-Ciocalteau reagent.

The fourth aliquoted supernatant was used to extract soluble ester-bound phenolic acids, after alkaline hydrolysis under mild conditions. Thereafter, 125 µl 2 M NaOH was added and the tubes were sealed and allowed to stand for four hours at room temperature in the dark. After hydrolysis the tubes were cooled at 4°C for 30 min before addition of 60 µl 1 M HCl. The phenolics were then extracted three times with 1 ml anhydrous diethyl ether. The ether extract was evaporated to dryness and the resulting precipitate was re-suspended in 250 µl pure methanol. This solution was used to determine the phenolic ester content using the Folin-Ciocalteau reagent.

Ester-bound phenolic acids incorporated in the cell wall were extracted after alkaline hydrolysis. The remaining alcohol insoluble residue was weighed into a glass tube (50 mg) and re-suspended in 0.5 M NaOH (1 ml) before being sealed. The tubes were then placed in a water bath for 1 hour at 96°C. Under these conditions, wall-esterified hydroxycinnamic acid derivatives were selectively released (Régnier, 1994). The tubes were then cooled at -10°C for 30 min before addition of 40 µl concentrated HCl. The phenolic acids were extracted three times with 1 ml anhydrous diethyl ether. The ether extract was reduced to dryness and the resulting precipitate was re-suspended in 250 µl pure methanol. This solution was used to determine the cell wall-bound phenolic acid content with the Folin-Ciocalteau reagent. Extracts were diluted five times before being analysed by High-Performance Liquid Chromatography (HPLC).

Phenolic compounds were analysed on a Varian HPLC (9012) equipped with a 20 µl loop injection valve connected to a Spectra 6000 LP UV diode array detector at 280 and 325 nm. A Malsil C18 reverse-phase column (250 x 4.6 mm, 5 µm particle size) was used. Data were analysed by OS/2 WARP system software. Acetonitrile and 0.01 M phosphoric acid (H_3PO_4) were used as eluents with a gradient programme from acetonitrile per 0.01 M H_3PO_4 at a ratio 7:93 for 2 min, increasing to 70:30 for 50 min and decreasing to 24:76 for 5 min. The flow rate was 1 ml min^{-1}. Ferulic acid was confirmed by co-elution with a standard. A standard of ferulic acid (Sigma) was used to construct a standard curve ranging from 0 to 6 mg ml^{-1}, $r^2_=$ 0.9983. The concentration of ferulic acid esterified to the

cell wall was calculated from the ferulic standard curve and expressed as mg ferulic acid g^{-1} dry mass.

4.3.3 Statistical analysis

Reisolation frequencies of ***P. citricarpa*** from leaves were angularly transformed prior to statistical analysis to stabilise treatment variances. All data were analysed according to GenStat (2000). Analysis of variance was used to test for differences between variables and means were separated by Fisher's protected ***t***-test least significant difference.

4.4 Results

Pycnidiospore suspensions that were prepared each month contained between 4.8 and 6.2 x 10^4 cfu ml^{-1}. The inoculation technique was highly effective, resulting in successful establishment of leaf infections in both cultivars tested in both trials. The pathogen could be reisolated after inoculation from up to 10-months-old Eureka lemon leaves and eight-months-old Valencia orange leaves (Table 4.1). From the ANOVA results, the ***P***-value for reisolation frequency per month, cultivar and month*cultivar was <0.001, <0.001 and 0.070, respectively. The least significant difference (lsd) of means at 5% level was 9.98 for month, 4.46 for cultivar and 14.11 for month*cultivar. Reisolation frequency of ***P. citricarpa*** was significantly the highest in one-month-old Eureka lemon leaves, with 72.7% of the plated leaf segments yielding the pathogen. All colonies of the pathogen developed from the cut edge of the leaf segment rather than from the intact surface of both cultivars. Growth of ***P. citricarpa*** usually became visible within two weeks after plating, although some colonies (less than 5%) developed only after four weeks.

In Valencia orange, reisolation of the pathogen was significantly the highest in leaves inoculated when one and seven months old, although the reisolation rate at month seven was not consistent between the two trials, viz. 76.0% in trial 1 (March 2003) and 41.3% in trial 2 (February 2005). The recovery rate of ***P. citricarpa*** from infected leaves was significantly higher in Eureka lemon than in Valencia orange for each month, except for month seven where the opposite was observed. Infections remained latent throughout the study and no leaf symptoms developed on any of the inoculated or control leaves.

The ***P***-value for reisolation of ***P. citricarpa*** over a period of six months after inoculation was <0.001 for both cultivars and the lsd of means at 5% level was 8.6 for Eureka lemona and 9.4 for Valencia orange. Reisolation frequency of ***P. citricarpa*** over a period of six months after inoculation from leaves inoculated when one to four months old was highest at one month after inoculation, irrespective of the cultivar or age of the leaves at time of

inoculation (Table 4.2). In general, the frequency of reisolation decreased each month until the pathogen could no longer be recovered from the inoculated leaves after three to five months following inoculation.

P. citricarpa could not be isolated from any of the control leaves. Very few to no other fungi commonly associated with citrus leaves in the field such as ***Alternaria alternata*** (Fr.) Keissl., ***Colletrotrichum gloeosporioides*** (Penz.) Penz. & Sacc. and ***Cladosporium*** species were isolated from the plated leaf segments of inoculated and control trees. All isolates of ***P. citricarpa*** subjected to verification of identity by PCR using the species-specific primer CITRIC1 in combination with ITS4 tested positive for ***P. citricarpa***.

The level of total soluble and soluble glycoside-bound phenolic acids measured monthly in inoculated Valencia orange leaves did not differ significantly from the control at any stage (Table 4.3). Total soluble phenolics increased over time, with the highest concentration recorded in month 11 for inoculated leaves and months seven and eight for control leaves. The highest levels of soluble glycoside-bound phenolic acids were recorded in months six and eight for inoculated leaves and in months seven and eight for control leaves.

Non-conjugated soluble phenolic acid levels were significantly lower in inoculated than in control leaves, except for months two and 12 when an opposite trend was evident (Table 4.3). Levels of non-conjugated soluble phenolic acids in inoculated leaves were variable from month to month, with the highest level recorded in month eight, albeit not significantly different from months two, three, seven, 11 and 12. Levels recorded in control leaves showed a similar tendency than total soluble and soluble glycoside-bound phenolic acids, with the highest levels being recorded in months seven and eight.

Levels of soluble ester-bound phenolic acids in inoculated leaves were initially lower than, or did not differ significantly from, those in control leaves, but the levels were significantly higher in the inoculated leaves from month six onwards compared to the control leaves. The highest levels of soluble ester-bound phenolic acids were recorded in months six to eight in inoculated leaves and in months three, four, six and seven in control leaves.

Levels of ester-bound phenolic acids incorporated in the cell wall and ferulic acid esterified to the cell wall were significantly higher in inoculated than in control leaves from month five and three onwards, respectively. Ferulic acid esterfied to the cell wall was significantly the highest in months seven, eight and 11 in inoculated leaves and in months

Table 4.1. Reisolation frequency (RF) of ***Phyllostica citricarpa*** from Eureka lemon and Valencia orange leaves artificially inoculated at advancing states of development with a pycnidiospore suspension of the pathogen

Leaf age at time of inoculation (months)	RF of ***P. citricarpa*** from inoculated leaves (%)[a]	
	Eureka lemon	Valencia orange
1	72.7 a A	65.3 b A
2	42.0 a C	32.0 b C
3	46.0 a BC	26.0 b C
4	54.0 a B	43.3 b B
5	47.3 a BC	24.0 b C
6	34.0 a CD	23.3 b C
7	45.3 b BC	58.7 a A
8	29.3 a DE	6.7 b D
9	20.3 a E	0 b D
10	3.3 a F	0 a D
11	0 a F	0 a D
12	0 a F	0 a D

[a]Reisolations were done four weeks after inoculation; Values are the mean of six replicates, each replicate comprising a total of 25 leaf segments (25 mm^2) excised from five leaves collected uniformly from each of three trees in each of two trials; RFs were angularly transformation prior to statistical analysis to stabilise treatment variances; Values followed by the same letter in rows (lower case) and columns (upper case) do not differ significantly according to Fisher's protected ***t***-test least significant difference (***P***≤0.05).

Table 4.2. Reisolation frequency (RF) of ***Phyllostica citricarpa*** over a period of six months from Eureka lemon and Valencia orange leaves artificially inoculated with a pycnidiospore suspension of the pathogen when one to four months old

Leaf age at time of inoculation (months)	Time of reisolation after inoculation (months)	RF of ***P. citricarpa*** from inoculated leaves (%)[a]	
		Eureka lemon	Valencia orange
1	1	72.7 a	65.3 a
	2	46.8 bc	58.3 a
	3	65.2 a	38.6 bc
	4	27.2 d	42.1 b
	5	0 f	8.4 de
	6	0 f	0 e
2	1	42.0 c	32.0 c
	2	18.5 e	28.2 c
	3	21.4 de	25.9 c
	4	4.0 f	0 e
	5	0 f	0 e
	6	0 f	0 e
3	1	46.0 bc	26.0 c
	2	34.0 cd	31.5 c
	3	16.1 e	29.5 c
	4	20.5 de	22.8 cd
	5	5.0 f	16.3 d
	6	0 f	0 e
4	1	54.0 b	43.3 b
	2	42.6 c	27.2 cd
	3	49.2 bc	28.4 c
	4	20.4 de	11.4 de
	5	6.8 ef	2.6 e
	6	0 f	0 e

[a]Values are the mean of six replicates, each replicate comprising a total of 25 leaf segments (25 mm^2) excised from five leaves collected uniformly from each of three trees in each of two trials; Values followed by the same letter in columns do not differ significantly according to Fisher's protected ***t***-test least significant difference (***P***≤0.05).

Table 4.3. Soluble and cell-wall bound phenolic compounds in Valencia orange leaves artificially inoculated or not inoculated at advancing stages of development with a pycnidiospore suspension of ***Phyllosticta citricarpa***

Leaf age at time of inoculation (months)	Phenolic acid determined as gallic acid equivalent per dry leaf mass (mg g^{-1})[a]					
	Total soluble		Soluble glycoside-bound		Non-conjugated soluble	
	Inoculated	Control	Inoculated	Control	Inoculated	Control
2	8.1698 a F	8.2241 a D	3.4997 a D	3.6475 a D	1.5702 a AB	1.2363 b CD
3	7.7361 a F	10.4665 a C	4.9203 a BC	5.7117 a BC	1.4669 b ABC	1.6608 a BC
4	12.2653 a D	10.5302 a C	5.4230 a B	5.5358 a BC	1.3226 b BC	1.5359 a C
5	10.3728 a E	11.0391 a C	4.4607 a C	5.2152 a C	1.2316 b C	1.5957 a BC
6	14.3205 a B	12.9258 a B	6.5393 a A	4.4479 a C	1.2788 b C	1.8610 a B
7	14.1515 a BC	15.6118 a A	1.0223 a E	6.1500 a AB	1.3731 b ABC	2.0587 a AB
8	12.7385 a CD	15.3604 a A	7.3376 a A	6.6257 a A	1.6250 b A	2.3600 a A
11	17.0558 a A	13.6995 a B	3.6019 a D	1.1673 a E	1.3893 b ABC	1.8351 a B
12	11.3538 a DE	7.0757 a D	5.3811 a B	4.6369 a C	1.5521 a AB	1.0958 b D

Table 4.3. Continued

Leaf age at time of inoculation (months)	Phenolic acid determined as gallic acid equivalent per dry leaf mass (mg g^{-1}) [a]				Cell wall ferulic acid determined as ferulic acid equivalent per dry leaf mass (mg g^{-1}) [a]	
	Soluble ester-bound		Cell wall ester-bound			
	Inoculated	Control	Inoculated	Control	Inoculated	Control
2	2.6779 a C	2.8392 a C	11.7485 a CD	9.4702 b BC	0.3137 a F	0.2846 a C
3	2.5300 b C	3.9839 a AB	11.7567 a CD	11.3276 a B	0.3696 a EF	0.2397 b C
4	3.0623 b BC	3.8931 a AB	12.8232 a C	12.0107 a AB	0.4087 a E	0.2440 b C
5	3.4541 a B	3.5972 a B	15.5978 a B	11.9201 b AB	0.6260 a D	0.4011 b B
6	5.1125 a A	4.2585 b A	20.9575 a A	12.6192 b AB	0.7881 a C	0.3353 b BC
7	5.0361 a A	4.0775 b A	21.1799 a A	10.7023 b B	1.2480 a A	0.3378 b BC
8	5.0188 a A	3.2569 b BC	20.4513 a A	13.5464 b A	1.3333 a A	0.5367 b A
11	1.4310 a D	0.9258 b E	15.1512 a B	8.4486 b C	1.2667 a A	0.5132 b A
12	2.7616 a C	1.6700 b D	10.8265 a D	6.3663 b D	0.9936 a B	0.4582 b AB

[a]Phenolic acids were extracted and quantified one month after inoculation, each value is the mean of five leaves pooled, each from three replicate trees; values followed by the same letter in rows within a phenolic group (lower case) or in columns (upper case) do not differ significantly according to Fisher's protected ***t***-test least significant difference (***P***≤0.05).

eight, 11 and 12 in control leaves. Ester-bound phenolic acids incorporated in the cell wall were significantly the highest in months six to eight in inoculated leaves and in months four to six and eight in control leaves. Cell wall-bound phenolic acids represented 56% to 48% of the total phenolic acids in two- to 12-month-old control leaves, and 60% to 62% of the total phenolic acids in two- to 12-month-old inoculated leaves (results not shown).

4.5 Discussion

The current study provided the first scientifically founded data, substantiated by molecular identification of the pathogen, on the duration of the susceptibility to CBS of newly emerging citrus leaves monitored over time. Leaves were regarded as susceptible when the pathogen could be reisolated from the leaf tissue four weeks after inoculation. Eureka lemon and Valencia orange leaves remained susceptible to new infections by ***P. citricarpa*** for up to 10 and eight months, respectively. This refutes the susceptibility period of five weeks previously reported by Kiely (1948), as well as the five month period stated by Kotzé (1981).

No report(s) could be found indicating that susceptible citrus material reacts differently to infection by ascospores of the CBS pathogen than by pycnidiospores and conditions required for infection have been reported as similar for both spore types (Kiely, 1948; Wager, 1952; McOnie, 1964c). Results obtained with pycnidiospore infections in the current study should therefore apply to ascospore infections under similar conditions. Pycnidiospores were used in the current study since, unlike ascospores, they can be mass-produced with relative ease in culture, hence ensuring a continuous supply of viable spores in high concentrations. All previous attempts at ***in vitro*** production of ascospores of ***G. citricarpa*** failed in our (unpublished data) and other (Kiely, 1948; Kotzé, 1963; McOnie, 1964c; Baayen ***et al.***, 2002; Baldassari ***et al***., 2008) studies. Although there were some confusion in the past about distinguishing between the CBS pathogen and a cosmopolitan saprophyte, ***Guignardia mangiferae*** A.J. Roy, in culture, physiological studies with correctly identified ***P. citricarpa*** indicated that it produces pycnidiospores with relative ease in culture, but never ascospores (Van der Aa, 1973; Baldassari ***et al***., 2008). Reports on ascospore production of ***P. citricarpa in vitro*** (Wager, 1952; Frean, 1966; Brodrick, 1969; Lemir ***et al***., 2000) were probably misidentified isolates that were actually ***G. mangiferae***.

Factors influencing disease expression of CBS on fruit are well documented (Kiely, 1948; Wager, 1952; Kotzé, 1963; Brodrick, 1969; Kiely, 1969), but no data are available on the factors influencing disease expression on leaves since leaf infections have previously not

been considered to be of real economical concern. It is not surprising that the inoculated leaves remained symptomless throughout the current study as infected citrus leaves usually remain latent until leaf-drop. Leaf symptoms are rare on most ***Citrus*** species, but may be present on mature to old lemon leaves (Kiely, 1948; Wager, 1952; Kotzé, 1963; Whiteside, 1965; McOnie, 1967), although seldom on those of Valencia orange (Wager, 1952).

The reisolation frequency of ***P. citricarpa*** from infected leaves in the current study was similar to a study in Brazil on naturally-infected mature 'Pêra' sweet orange leaves, where 33 to 58% of the plated leaf segments yielded the pathogen (Schinor ***et al***., 2002). In the current study, all the retrieved ***P. citricarpa*** isolates started to grow from the cut edge of the plated leaf segments. It is therefore assumed that percentage reisolation reflects the internal colonisation frequency of the pathogen in the leaves and not spores and/or appressoria attached to the leaf surface. The higher incidence of ***P. citricarpa*** in leaves of the highly-susceptible Eureka lemon than in that of Valencia orange is also in accordance with previous reports about the occurrence and incidence of CBS (Whiteside, 1965; McOnie, 1967; Kotzé, 1981; Schinor ***et al***., 2002; Truter ***et al***., 2004).

P. citricarpa could not be isolated after four to six months from leaves inoculated when one to four months old, although the pathogen initially established at high levels particularly in the case of Eureka lemon leaves. All the leaf segments plated on PDA in the current study contained no to very few other fungi normally co-isolated with ***Phyllosticta***, indicating a general loss of endophytes over time. This is in accordance with a previous study indicating that citrus trees maintained in a greenhouse remained free of natural endophytes (Gongui ***et al***., 1981). Reduction in reisolation of ***P. citricarpa*** over time can probably be attributed to the greenhouse conditions and is not likely a result of the host reacting to infections, as latent leaf infections in the field remain viable until leaf fall (Kotzé, 1981). Therefore conditions in the current greenhouse study do not represent natural conditions in the field and could have been more optimal for infection by ***P. citricarpa*** with less or no competition to the pathogen, than under natural conditions.

In general, soluble and cell wall-bound phenolic acids in Valencia orange leaves gradually increased, followed by a decrease towards the end of the evaluation period. Maximum phenolic acid levels were recorded between month six and 11, although the period when maximum levels occurred differed between phenolic acid types. This trend agrees with the observation by Castillo ***et al.*** (1992) that the phenolics reached a maximum concentration in leaves of Seville orange (***Citrus aurantium*** L.) during the logarithmic

phase of growth, where after levels gradually decreased until the leaves were fully developed. The decrease was attributed to the dilution of metabolites due to cell growth.

An increase in phenolic acid content not attributed to natural fluctuations was evident in the case of soluble ester-bound, esterified cell wall-bound phenolic acids, and cell wall-bound ferulic acid in inoculated, but not uninoculated leaves. The difference between inoculated and control leaves indicates that Valencia orange leaves responded to ***P. citricarpa*** infection by a significant increase in the amount of phenolic material bound to the cell wall. Esterification of phenols to cell wall material is a common host response to microbial attack and is generally regarded as an expression of resistance (Bolwell ***et al***., 1985; Fry, 1987; Grand ***et al***., 1987; Matern & Kneusel, 1988; Nicholson & Hammerschmidt, 1992).

Esterification of phenolic acids to cell-wall material forms part of a plant's defence system and forms an integrated process with the formation of lignin-like polymer systems and accumulation of lignin and/or suberin in the cell walls (Vance ***et al***., 1980; Grand ***et al***., 1987; Bolwell, 1988; Beckman, 2000; El Modafar ***et al***., 2000; De Ascensao & Dubery, 2003; Menden ***et al***., 2007). The accumulation of both phenolic and lignin compounds in cell walls provides both a physical and chemical barrier to invading pathogens (Ampomah & Friend, 1988). Besides providing physical strength to the cell walls, the abundance of phenols in cell walls renders polysaccharides less sensitive to the cell wall-degrading enzymes of pathogens (El Modafar ***et al***., 2000).

No single compound or mechanism explains disease resistance in plants (Kuć, 1995). Various factors contributing to resistance have been described, including accumulation of inhibitory substances excreted by the tissue on the surface, thickening of the wax layer and accumulation of inhibitory substances in the wax, less nutrients excreted on the leaf surface, increased cell wall thickness, accumulation of phenolic compounds in the cell wall and enhanced competition by resident epiphytes and endophytes (Blakeman & Atkinson, 1976; Allen ***et al.***, 1991; Juniper, 1991; Petrini, 1991). Results of the current study do not indicate any development of resistance over time, but rather host response to infection. Further research is required to investigate the possible involvement of other mechanisms in the resistance of citrus leaves and fruit to CBS.

4.6 References

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CHAPTER 5

Monitoring *Guignardia citricarpa* ascospores from citrus leaf litter in commercial orchards

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5.1 Abstract

A volumetric spore sampler, the Kotzé Inoculum Monitor (KIM), was used to determine the presence of dischargeable ascospores of ***Guignardia citricarpa*** in citrus leaf litter in the laboratory. Different soaking conditions at 30, 35 and 40°C for 5, 10 and 20 min were compared to induce spore release from Eureka lemon and Valencia orange leaf litter. Most ascospores were captured after submerging the leaves for 5 min in 40°C water. Ascospore development on naturally infected mature green Eureka lemon and Valencia orange leaves was investigated by placing detached leaves under tree canopies (artificial leaf litter). No ascospores were captured from this artificial leaf litter, even after six months exposure. The leaf decomposition rate of these detached leaves varied with temperature and rainfall and was more rapid in the summer than winter months. In contrast to the artificial leaf litter, ascospores were captured from naturally formed leaf litter collected monthly, for 31 months, in four commercial Valencia orange and two Eureka lemon orchards. Ascospores were captured from leaf litter collected during October to March each year with peak ascospore availability between December to February. The KIM is the first sampler designed to capture fungal spores directly from plant material without environmental influences and was effectively used to indicate the period of available ascospores of ***G. citricarpa*** in commercial orchards.

5.2 Introduction

Guignardia citricarpa Kiely, the causal agent of citrus black spot (CBS), infects susceptible citrus tissue by means of ascospores or pycnidiospores (Kiely, 1948; McOnie, 1964b; Whiteside, 1967; Kotzé, 1996). Airborne ascospores released from pseudothecia produced only on citrus leaf litter are the main source of inoculum and dissemination of the disease (Kiely, 1948; McOnie, 1964b; Kotzé, 1981; Korf, 1998). Pycnidiospores of the anamorph are produced in pycnidia on symptomatic fruit, leaf litter and in the case of the highly susceptible cultivar, Eureka lemon, also on petioles and small twigs (Kiely, 1948;

McOnie, 1964b; Whiteside, 1967). The water-borne pycnidiospores are generally regarded as unimportant in the dissemination of the disease, mainly due to the limited spread of the pathogen by means of water and the short viability of pycnidiospores (Kiely, 1948; McOnie, 1964b; Korf, 1998).

Asco- and pycnidiospores require moisture and moderate temperature for production and discharge. Pseudothecia of the fungus develop on dead infected leaves on the orchard floor within 40 to 180 days after leaf drop, depending on the temperature and frequency of wetting (Kotzé, 1981). Alternate wetting and drying of the fallen leaves and variations in temperature provide optimal conditions for ascospore formation and maturation (Kiely, 1948). Pseudothecia will not develop or mature in areas where the leaf litter is either too dry or too wet (Wager, 1949).

Once mature, ascospores are discharged during and shortly after spells of rain (Kiely 1948; Kotzé 1963; McOnie 1964a, b), irrigation (Kotzé, 1963; McOnie, 1964a; Smith, 1996) or heavy dew (Kiely, 1948; Lee & Huang, 1973). Once released, ascospores are highly dependent on convection currents and favourable environmental conditions to reach a suitable host substrate, since the maximum distance of ascospore ejection from a pseudothecium is only 10-12 mm (Kotzé, 1963).

Currently, ascospore release is recorded with the aid of commercially available volumetric spore traps, such as the Hirst and Burkhard versions (Gregory, 1973; Dhingra & Sinclair, 1995b; Lacey, 1996). These spore traps collect air-borne particles for up to eight successive days by passing 10 l of air per min past a slowly rotating collection disk coated with a sticky substrate. These volumetric spore traps can provide data on when ascospore release occurred and the number of spores captured per day or per hour (Dhingra & Sinclair, 1995b).

These spore traps proved ineffective for studying the availability of ascospores, their stage of development and the potential inoculum load on infected leaf litter at a specific time in an orchard. To address these aspects of the disease, a new sampler was developed by J.M. Kotzé and manufactured by Interlock Systems, Pretoria. The aim of this study was to evaluate the leaf litter preparation and use of the Kotzé Inoculum Monitor (KIM) in capturing ascospores, and to determine development of dischargeable ascospores from manually detached green leaves as well as from naturally-infected citrus leaf litter under controlled conditions over time in commercial orchards.

5.3 Materials and methods

5.3.1 Evaluation of the Kotzé Inoculum Monitor

Leaf litter was collected from two citrus estates in South Africa, situated ca. 380 km apart, with a known history of CBS. One orchard near Mooinooi in North West Province, comprised 28-year-old Eureka lemon trees on Rough Lemon rootstock and the other near Burgersfort in Mpumalanga Province, comprised 36-year-old Valencia orange trees on Rough Lemon rootstock. In each orchard, leaf litter was collected during October 2003 underneath ten randomly selected trees and pooled. The leaves were examined under a stereo-microscope and those that contain fungal fruiting bodies resembling pseudothecia of ***Guignardia***, were selected. The selected leaves (ca. 20 for each plastic grid) were rinsed for 30 sec in tap water to remove excess soil and dirt before being secured with cable ties between two circular plastic grids (350 mm diameter, 10 mm mesh size). Litter was placed between the grids so that most of the pseudothecia faced in one marked direction. Prepared grids were submerged in water at 30, 35 or 40°C for 5, 10 or 20 min, followed by draining on paper towels for 5 min to remove excess water. The prepared grid with leaves was placed on the grid support in the hopper so that the marked side of the grid face downwards into the KIM (Fig. 5.1). A microscope slide coated with silicone spray (Perrin, 1977; Galán & Domingues-Vilches, 1997; Alcázar ***et al***., 2003) was placed in the slide holder to collect spores. For a description of the operational procedures of the KIM, see Appendix 1.

After the two-hour KIM operation at room temperature, the slide was removed, stained with lactofuchsin (Dhingra & Sinclair, 1995a) and examined under a compound microscope at 400x magnification. The 30 x 25 mm area on the slide that passed the orifice was divided into 25 mm^2 sections in which the total number of ascospores resembling those of ***G. citricarpa*** was counted along the centre longitudinal transect, with total spore counts per section. Each 25 mm^2 section correlated to ca. 20 minutes of KIM operation. Each temperature and time combination was replicated three times.

Petri dishes containing nutrient medium were also evaluated for effectiveness in capturing spores and subsequent culturing of ***Guignardia*** isolates from captured spores. Ten millilitres of 2% potato dextrose agar (Biolab, Merck) was dispensed into each 65 mm Petri dish. Dishes were allowed to stand for one day at room temperature before being used. Selected leaf litter was rinsed and secured between grids as described before, followed by submersion for 10 min in 30°C water. The specific submersion condition was randomly selected for this experiment and five replicate grids were used. Dishes were

Figure 5.1. The Kotzé Inoculum Monitor illustrating the position of the prepared leaf grid in the hopper (Courtesy of T.N. Janse van Rensburg).

examined directly after exposure in the KIM without any stain under a compound microscope at 100x magnification and the number of ascospores recorded. Following microscopic examination of each dish, dishes were incubated at 25°C in the dark. Developing fungal colonies were examined after seven days.

5.3.2 Rate of ascospore maturation and leaf decomposition

The rate of ascospore maturation and leaf decomposition were evaluated in the same commercial Valencia orange and Eureka lemon orchards as mentioned before. Mature to old green leaves were randomly collected from 20 trees in each orchard and secured between two circular plastic grids, using 20-25 leaves per grid. Eighteen leaf-grids per orchard were prepared monthly from October 2003 to March 2005 and placed randomly underneath the canopy of selected trees. Grids were placed in such a manner as not to be wetted by irrigation water. Three replicate grids per time interval were collected monthly for six months. A grid with leaf litter was submersed in water at 40°C for 5 min, excess water removed and exposed to the KIM as described before. A standard microscope slide coated with a smooth, thin layer of petroleum jelly was used to collect

spores. Petroleum jelly instead of silicon spray was preferred to coat the slides in this and the following experiment seeing that applying a uniform layer of Vaseline was more consistent and the optical quality of the slides was improved. After the two-hour KIM operation, the slide was stained with lactofuchin and ascospores resembling those of ***G. citricarpa*** were counted in four longitudinal rows in the centre of the slide. Each row consisted of a microscope field, 450 µm in diameter, 45 mm long and separated by 2 mm.

The leaf decomposition level was determined monthly by examination of all the collected leaves and using the following formula:

$$\text{Leaf decomposition level} = 100 \times (0n_0 + 0.25n_1 + 0.5n_2 + 0.75n_3 + 1n_4)/n_{total}$$

where n represents the total number of leaves in each of the categories: 0 = Leaves fully intact; 1 = More than 75% of leaf material remained; 2 = 51 to 75% of leaf material remained; 3 = 26 to 50% of leaf material remained; and 4 = Less than 25% of leaf material remained (mostly veins).

5.3.3 Ascospore capturing from naturally produced leaf litter

The presence of dischargeable ascospores on Eureka lemon and Valencia orange leaf litter was assessed from three commercial Valencia orange orchards near Burgersfort (ca. 10 to 30 km apart), and one Valencia orange and two Eureka lemon orchards near Mooinooi (ca. 10 km apart). Eureka orchard A and Valencia orchard A were the same commercial orchards used in the previous two experiments. Eureka orchard B and Valencia orchard D near Mooinooi comprised of 18- and 22-year-old trees on Rough Lemon rootstock, respectively. Valencia orchard B and C near Burgersfort comprised of 20- and 26-year-old trees on Rough Lemon rootstock, respectively. Natural leaf litter was collected monthly from October 2003 to April 2006 underneath the canopy of at least 20 randomly chosen trees within the specific orchard and pooled. Leaves were selected by giving preference to ones with visible pseudothecia. Selected leaves were thoroughly mixed by hand and packed between two circular plastic grids and secured with cable ties. Three replicate grids per orchard, each grid containing 20 to 25 leaves, were prepared and processed for spore capturing with the KIM as described for the artificial leaf litter.

5.3.4 Statistical analysis

Spore capturing data from each orchard were analysed separately using the statistical program, GenStat (2000). One-way analysis of variance (ANOVA) was used to test for differences in total spore counts per slide section in the evaluation of the KIM and per

slide for the rest. Treatment means were separated using Fishers' protected ***t***-test least significant difference at 5% level of significance.

5.4 Results

5.4.1 Evaluation of the Kotzé Inoculum Monitor

More ascospores (Fig. 5.2) were retrieved from Eureka lemon than Valencia orange leaf litter, with a mean of 70.37 compared to 8.48 ascospores per 25 mm^2, respectively (Table 5.1). The trend also correlated with the greater number of ***Guignardia***-like fruiting bodies present on the Eureka lemon leaf litter as observed by stereo-microscope examination (results not shown). The only treatment that resulted in significantly more discharged ascospores was the submersion of Eureka lemon leaf litter for 5 min at 40°C. Leaf samples from both cultivars yielded more ascospores when leaf litter was submerged in water at 40°C for 5 min, compared to the other submerging treatments.

The main release of ascospores from the leaf litter occurred in the fourth section examined, correlating to ca. 61 to 80 minutes of KIM operation (Fig. 5.3). The centre longitudinal transect was found to be more accurate than the edges to determine amount of ascospores on the slide, since spore losses occurred near the edges of the capturing surface (results not shown).

Petri dishes with 2% potato dextrose agar were less effective in capturing spores than silicon coated microscope slides. A maximum of 18 ascospores were observed on one dish directly after spore capturing and no ***Guignardia*** isolates could be discerned from the incubated dishes after seven days. Main problems encountered with the Petri dishes were moisture loss from the agar, low number of spores captured on the agar and saprophytes overgrowing the dishes following incubation.

5.4.2 Rate of pseudothecium maturation and leaf decomposition

No ascospores were captured with the KIM from any of the detached leaves placed monthly on the orchard floor from October 2003 to March 2005 and collected after exposure for one to six months. No fruiting bodies conforming morphologically to ***G. citricarpa*** could be detected after microscopic examination of randomly selected treated leaves.

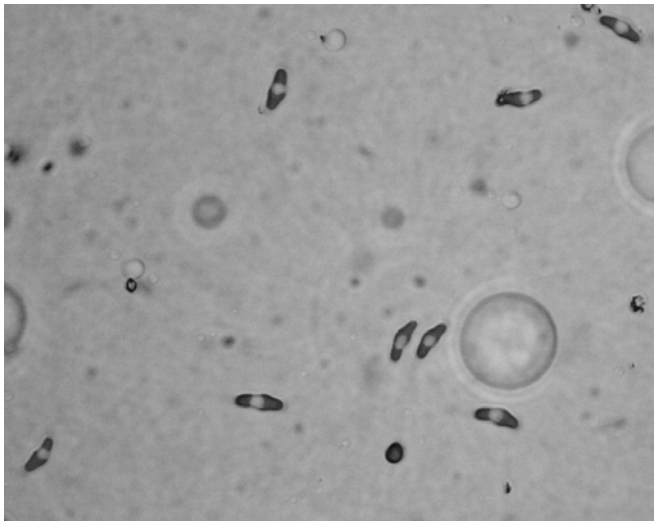

Figure 5.2. Ascospores of ***Guignardia citricarpa*** collected from Eureka lemon leaf litter on silicone-coated microscope slide with the Kotzé Inoculum Monitor.

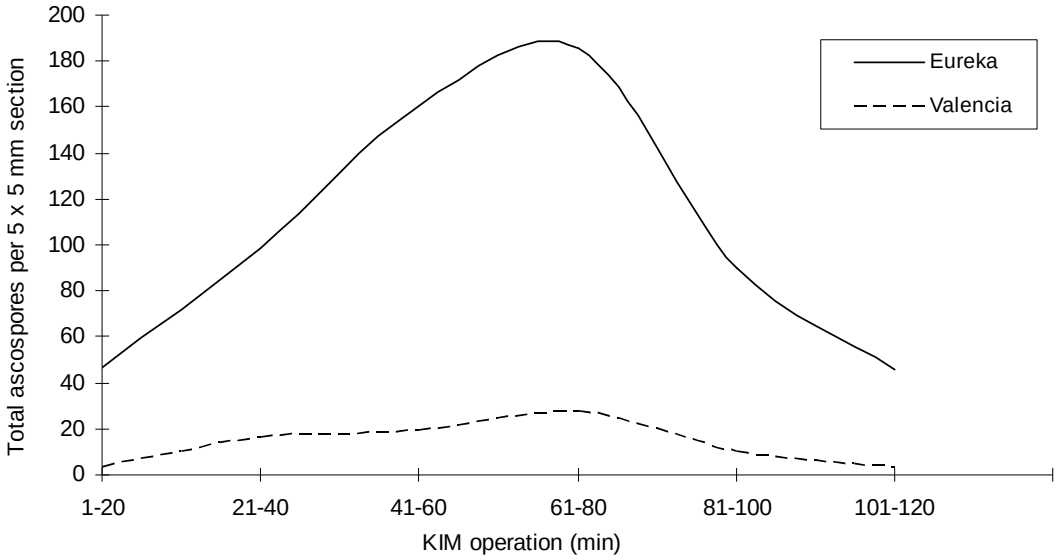

Figure 5.3. ***Guignardia citricarpa*** ascospores collected with the Kotzé Inoculum Monitor (KIM) from Eureka lemon and Valencia orange leaf litter. Total ascospore counts were done along the centre transect row (30 x 5 mm) in 25 mm^2 sections of a microscope slide coated with silicon. Each 25 mm^2 section correlates to ca. 20 minutes of KIM operation.

The aging and decomposition of the leaves were noted monthly and the decomposition rate was faster in the summer than in the winter months (Tables 5.2 and 5.3). Leaves detached in December were completely decomposed after only three months. Leaves decomposed slower in Mooinooi compared to Burgersfort despite having a slightly higher mean temperature and total rainfall (Figs 5.4 and 5.5). In Mooinooi more than 50% of the leaves detached in March to August remained intact after six months, whereas in Burgersfort only leaves detached in June and July remained more than 50% intact after six months.

5.4.3 Ascospore capturing from naturally produced leaf litter

More than 8 000 ascospores were captured per leaf grid from natural Eureka lemon leaf litter (Table 5.4). Accurate spore counts were not possible when high spore numbers of more than 50 spores per microscope view were present and mean ascospore count was estimated based on total spores counted on 20% of four longitudinal rows. Ascospores were captured from both Eureka lemon and Valencia orange leaf litter between October and May. The most ascospores were captured from leaf litter that were collected in February (56%), followed by December (28%) and January (16%).

Large variation in available ascospores occurred between replicate grids from the same sample as well as between orchards in the same area on the same sampling date. During February 2005, as an example, spores captured from three replicate grids collected in Valencia orange orchard B were 0, 980 and 460, respectively while three replicate grids collected in Valencia orange orchard A resulted in 0, 50 and 112 spores. Due to this large variation in ascospore numbers, none of the collected Valencia orange leaf litter resulted in significant differences between numbers of ascospores captured on the various collection dates. The ***P***-value for Valencia A, B, C, D, Eureka A and B orchard was 0.4234, 0.2501, 0.3744, 0.4234, 0.005 and 0.001, respectively. The least significant difference for means at 5% level was 1750 and 1640 for Eureka A and B, respectively. Significantly more ascospores were captured from Eureka lemon leaf litter collected during January to March compared to the other months (Table 5.4).

5.5 Discussion

The KIM, a new volumetric spore sampler, was successfully applied to capture ascospores of ***G. citricarpa*** released from citrus leaf litter in a laboratory. The KIM has the advantage over field-based volumetric sucking-type spore traps, such as the Hirst and Burkard versions, of providing information on the presence of mature, ready to be

Table 5.1. ***Guignardia citricarpa*** ascospores collected with the Kotzé Inoculum Monitor from Eureka lemon and Valencia orange leaf litter

Water (ºC)	Leaf submersion (min)	Total ascospore count[a]	
		Eureka lemon	Valencia orange
30	5	58.00 (±7.94)[b]	5.33 (±2.47)
	10	62.67 (±9.05)	9.67 (±3.09)
	20	63.33 (±8.30)	13.67 (±3.29)
35	5	53.67 (±8.46)	8.67 (±3.08)
	10	76.33 (±8.34)	3.67 (±1.78)
	20	73.00 (±7.95)	5.67 (±2.20)
40	5	106.33***** (±9.16)	14.67 (±3.53)
	10	62.33 (±10.47)	6.00 (±2.06)
	20	77.67 (±10.35)	9.00 (±2.67)

[a] Total ascospore counts were done along the centre longitudinal transect (30 x 5 mm) of a microscope slide coated with silicone; values are the mean of three replicates.

[b] Standard deviation.

* Differs significantly from other counts in the column according to Fisher's protected ***t***-test least significant difference (***P***≤0.05).

Table 5.2. Leaf decomposition level (%) of Eureka lemon leaves detached monthly and exposed to the environmental conditions in an orchard near Mooinooi in North West Province for one to six months

Date collected (year and month)		Date detached and placed in the orchard (month and year)									
		October 2003	November 2003	December 2003	January 2004	February 2004	March 2004	April 2004	May 2004	June 2004	July 2004
2003	November	0	-	-	-	-	-	-	-	-	-
	December	7.9	0.8	-	-	-	-	-	-	-	-
2004	January	38.3	15.0	13.2	-	-	-	-	-	-	-
	February	71.2	28.8	30.4	15.7	-	-	-	-	-	-
	March	100	57.4	71.5	21.3	7.9	-	-	-	-	-
	April	100	64.0	100	67.6	12.4	10.4	-	-	-	-
	May	-	100	100	71.7	23.6	21.6	0	-	-	-
	June	-	-	100	82.0	36.5	27.7	0	0	-	-
	July	-	-	-	100	41.8	35.5	17.7	0	0	-
	August	-	-	-	-	62.8	41.8	23.2	0	0	0
	September	-	-	-	-	-	47.9	31.5	17.4	6.5	0
	October	-	-	-	-	-	-	37.9	21.6	17.6	8.4
	November	-	-	-	-	-	-	-	27.5	31.8	14.8
	December	-	-	-	-	-	-	-	-	48.3	26.3
2005	January	-	-	-	-	-	-	-	-	-	50.5

- = Not assessed.

Table 5.2. Continued

Date collected (year and month)		Date detached and placed in the orchard (month and year)							
		August 2004	September 2004	October 2004	November 2004	December 2004	January 2005	February 2005	March 2005
2004	September	0	-	-	-	-	-	-	-
	October	4.7	0	-	-	-	-	-	-
	November	12.6	5.7	1.4	-	-	-	-	-
	December	18.5	21.6	12.6	7.5	-	-	-	-
2005	January	29.4	61.6	26.4	18.4	12.6	-	-	-
	February	46.4	100	42.6	31.6	28.4	1.4	-	-
	March	-	100	68.6	53.7	46.6	24.8	0	-
	April	-	-	89.5	72.2	89.5	51.9	10.6	0
	May	-	-	-	100	100	70.7	22.4	7.6
	June	-	-	-	-	100	100	34.6	22.4
	July	-	-	-	-	-	100	47.2	36.2
	August	-	-	-	-	-	-	51.4	48.6
	September	-	-	-	-	-	-	-	57.7

- = Not assessed.

Table 5.3. Leaf decomposition level (%) of Valencia orange leaves detached monthly and exposed to the environmental conditions in an orchard near Burgersfort in Mpumalanga Province for one to six months

Date collected (year and month)		Date detached and placed in the orchard (month and year)									
		October 2003	November 2003	December 2003	January 2004	February 2004	March 2004	April 2004	May 2004	June 2004	July 2004
2003	November	0	-	-	-	-	-	-	-	-	-
	December	3.5	6.2	-	-	-	-	-	-	-	-
2004	January	14.6	21.5	11.6	-	-	-	-	-	-	-
	February	40.4	43.6	35.5	13.6	-	-	-	-	-	-
	March	84.4	63.2	70.6	32.5	11.4	-	-	-	-	-
	April	100	100	100	54.8	27.6	3.5	-	-	-	-
	May	-	100	100	73.6	31.6	15.4	0	-	-	-
	June	-	-	100	100	63.8	23.6	5.7	0	-	-
	July	-	-	-	100	100	45.7	14.4	5.4	0	-
	August	-	-	-	-	100	65.8	23.5	14.3	24.2	0
	September	-	-	-	-	-	100	41.6	27.7	27.6	17.6
	October	-	-	-	-	-	-	57.0	47.4	31.6	25.4
	November	-	-	-	-	-	-	-	71.6	37.5	31.6
	December	-	-	-	-	-	-	-	-	43.2	40.8
2005	January	-	-	-	-	-	-	-	-	-	47.8

- = Not assessed.

Table 5.3. Continue

Date collected (year and month)		Date detached and placed in the orchard (month and year)							
		August 2004	September 2004	October 2004	November 2004	December 2004	January 2005	February 2005	March 2005
2004	September	0	-	-	-	-	-	-	-
	October	5.5	4.6	-	-	-	-	-	-
	November	24.8	7.6	7.6	-	-	-	-	-
	December	41.1	16.6	24.4	12.5	-	-	-	-
2005	January	51.3	27.1	31.6	24.4	3.6	-	-	-
	February	84.6	31.6	73.8	43.2	32.4	15.8	-	-
	March	-	-	100	65.4	62.8	30.6	0	-
	April	-	-	100	100	100	73.2	13.6	0
	May	-	-	-	100	100	89.5	30.3	7.5
	June	-	-	-	-	100	100	53.7	13.6
	July	-	-	-	-	-	100	100	23.8
	August	-	-	-	-	-	-	100	30.5
	September	-	-	-	-	-	-	-	73.2

- = Not assessed.

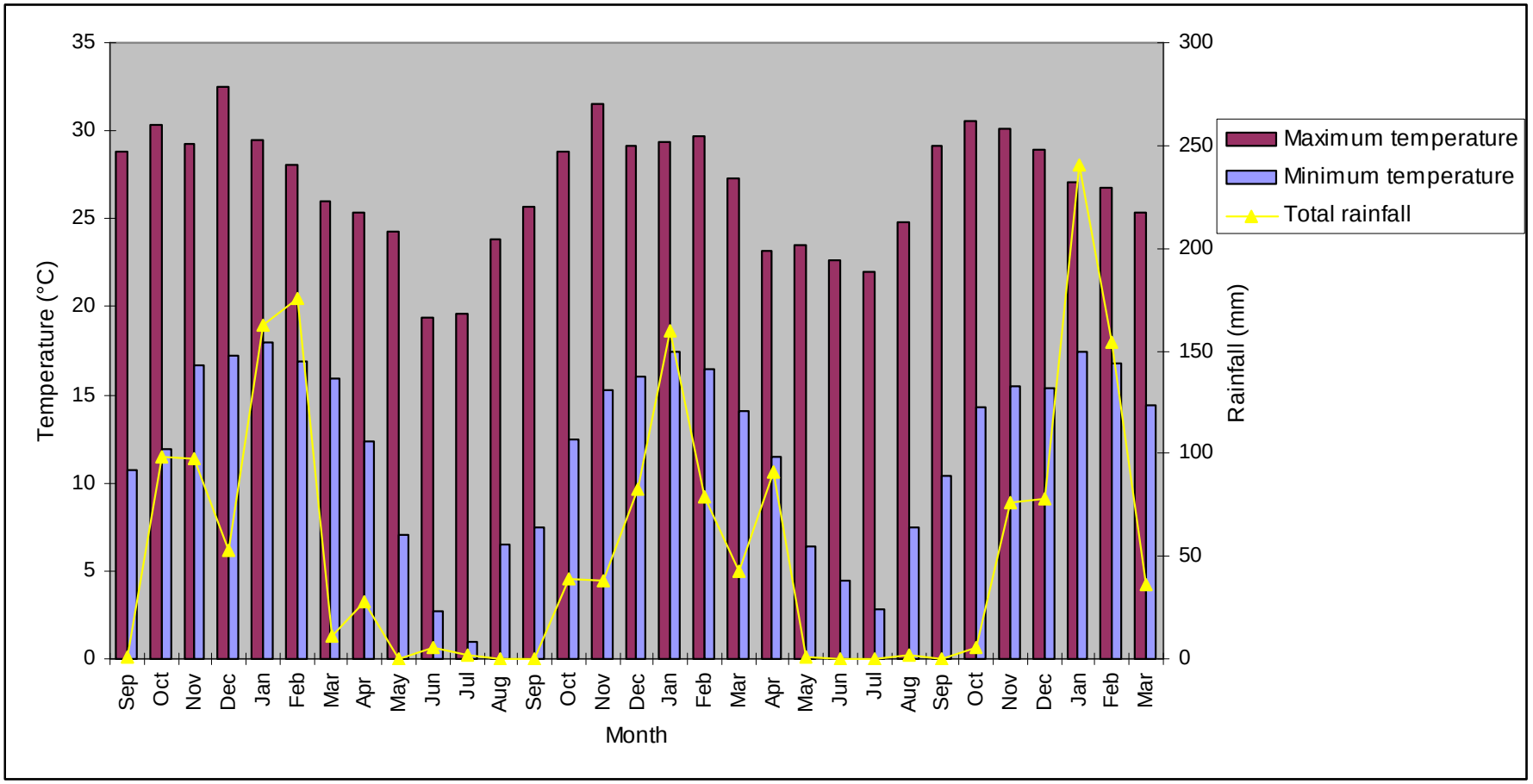

Figure 5.4. Mean maximum and minimum temperature and total rainfall per month recorded in Mooinooi in North West Province during September 2003 to March 2006. Data obtained from the South African Weather Service.

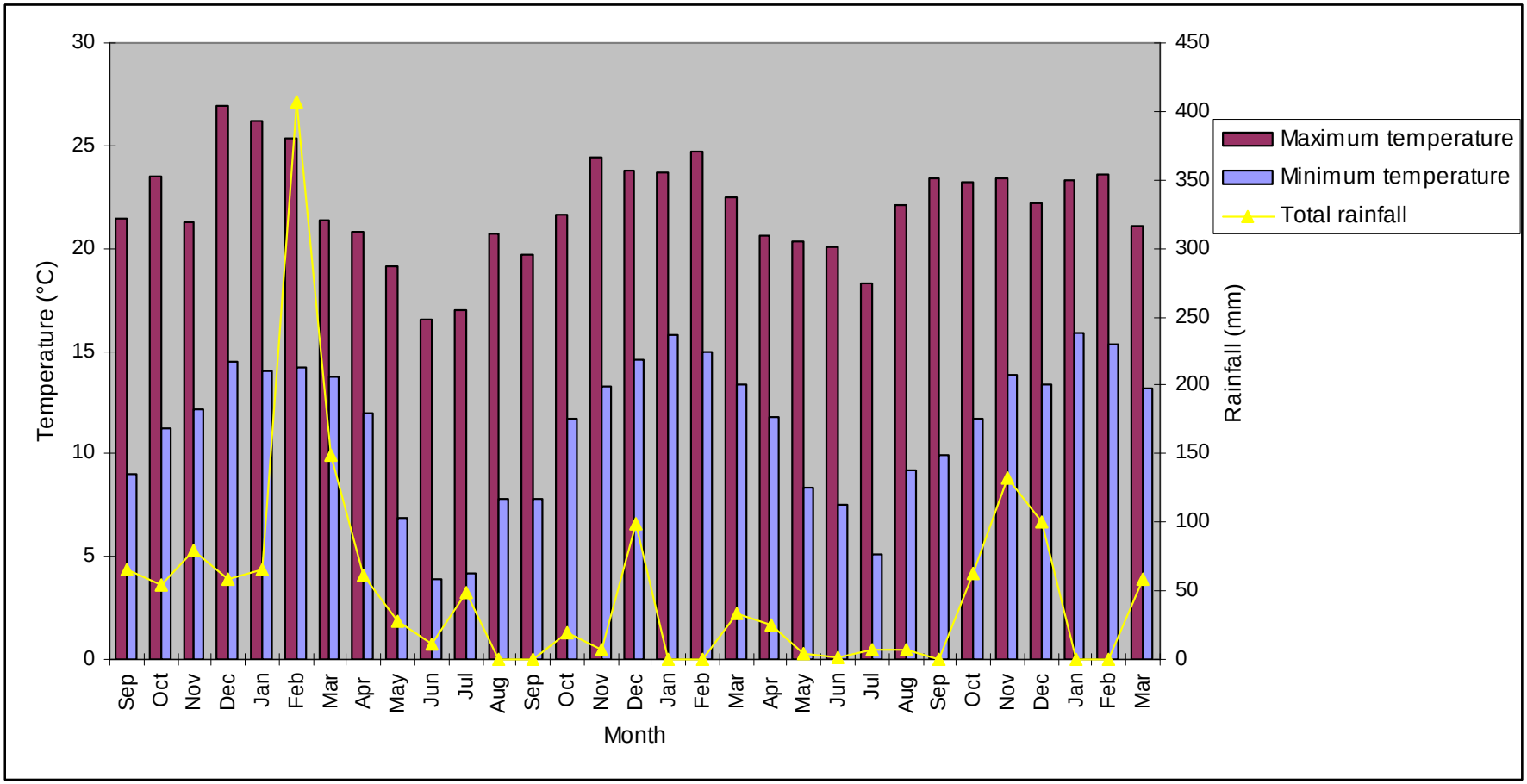

Figure 5.5. Mean maximum and minimum temperature and total rainfall per month recorded in Burgersford in Mpumalanga Province during September 2003 to March 2006. Data obtained from the South African Weather Service.

Table 5.4. Number of ascospores captured with the Kotzé Inoculum Monitor from naturally produced leaf litter collected monthly from the orchard floor

Time of collection (year and month)		Orchards where leaf litter were collected[a]					
		Burgersfort			Mooinooi		
		Valencia A	Valencia B	Valencia C	Valencia D	Eureka A	Eureka B
2003	October	0 a	0 a	0 a	0 a	98 d	356 cd
	November	83 a	89 a	0 a	136 a	672 d	792 cd
	December	363 a	231 a	182 a	87 a	± 2800[b] cd	± 1800 c
2004	January	61 a	124 a	409 a	382 a	± 3000 c	± 4200 b
	February	13 a	0 a	657 a	490 a	± 4800 bc	± 3700 b
	March	0 a	0 a	282 a	3 a	± 3500 c	± 2600 bc
	April	0 a	0 a	5 a	22 a	498 d	425 cd
	May	0 a	0 a	18 a	0 a	27 d	0 d
	June	0 a	0 a	0 a	0 a	0 d	0 d
	July	0 a	0 a	0 a	0 a	0 d	0 d
	August	0 a	0 a	0 a	0 a	0 d	0 d
	September	0 a	0 a	0 a	0 a	2 d	0 d
	October	0 a	0 a	38 a	41 a	65 d	168 d
	November	0 a	81 a	0 a	72 a	327 d	472 cd
	December	16 a	309 a	363 a	451 a	± 2100 cd	964 cd
2005	January	8 a	268 a	185 a	376 a	± 6900 ab	± 3200 bc
	February	54 a	480 a	243 a	581 a	± 8000 a	± 5900 a
	March	0 a	0 a	0 a	72 a	± 5800 b	± 3800 b
	April	0 a	0 a	0 a	0 a	± 1200 d	853 cd
	May	0 a	0 a	0 a	0 a	0 d	0 d
	June	0 a	0 a	0 a	0 a	0 d	0 d
	July	0 a	0 a	0 a	0 a	0 d	0 d
	August	0 a	0 a	0 a	0 a	0 d	0 d
	September	0 a	0 a	0 a	0 a	0 d	0 d
	October	125 a	0 a	0 a	0 a	281 d	0 d
	November	86 a	268 a	156 a	73 a	685 d	316 cd
	December	267 a	367 a	167 a	268 a	± 4200 bc	674 cd
2006	January	164 a	285 a	469 a	489 a	± 3800 c	± 4600 ab
	February	56 a	68 a	268 a	358 a	± 6800 ab	± 4900 ab
	March	0 a	0 a	0 a	0 a	± 5100 bc	± 3600 b
	April	0 a	0 a	0 a	0 a	1160 d	492 cd

[a]Mean ascospore count from three replicate grids; values followed by the same letter in a column do not differ significantly according to Fisher's protected ***t***-test least significant difference (***P***≤0.05).

[b]Mean ascospore count were estimate based on total spores counted on 20% of four longitudinal rows.

dispersed, ascospores on leaf litter before a natural spore release event. The detection of mature ascospores from leaf litter before natural spore release in the orchard may indicate the density of available inoculum, improve prediction of possible infection periods and the timing of application of preventative chemicals (Swart & Kotzé, 2007). Another advantage of the KIM is that variations in external factors such as temperature, water (dew/rain) and wind are eliminated, making data from different samples from the same orchard or from different orchards more comparable. Samples of leaf litter can also be collected from various geographic sites and evaluated by one operator under constant conditions without moving the trap. Therefore the KIM can be used to compare the inoculum densities between different orchards, indicating potential CBS risks in each orchard, which in turn will contribute to improved management of the disease.

Most of the dirt on the leaf surface was removed with submersion in water before placing in the KIM, reducing collection of interfering particles, such as dust, pollen and other fungal spores, on the microscope slide. With the KIM recognising and counting ascospores was easier than trapping with conventional field operated spores traps. It can be extremely difficult to distinguish and recognise ascospores on the trapping surface due to the presence of dust and numerous other particles (Gregory, 1973; Pazoti ***et al***., 2005). Slides used for trapping spores in the KIM can also be used in conjunction with computer software designed to recognise ascospores based on shape analysis, making it less time consuming and labour intensive (Pazoti ***et al***., 2005).

Apart from the difficulty in recognising the shape of ***G. citricarpa*** ascospores among other interfering particles, it is almost impossible to distinguish it from the ascospores of ***Guignarida mangiferae*** A.J. Roy on spore morphology alone. ***G. mangiferae*** is a cosmopolitan saprophyte reported from numerous woody hosts that co-exists with the CBS pathogen on citrus (McOnie, 1964c; Baayen ***et al***., 2002; Meyer ***et al***., 2006; Baldassari ***et al***., 2008). Therefore, the results obtained in the present and other CBS spore trapping studies must be seen as the presence of ***Guignardia*** ascospores and do not necessarily reflect the situation of ***G. citricarpa***. The accurate identification of ***G. citricarpa*** from collected spores can only be determined by amplification of a species-specific DNA sequence in the internal transcribed spacer region (Baayen ***et al***., 2002; Bonants ***et al***., 2003; Meyer ***et al***., 2006).

The main release of ascospores from the leaf litter occurred between approximately 61 and 80 minutes of KIM operation, which coincided with results from Kotzé (1963) where discharge of ascospores was monitored in Petri dishes and the main release period

occurred within an hour after wetting, which decreased thereafter. The centre longitudinal transect was found to be more accurate to determine amount of ascospores on the slide, since spore losses occurred near the edges of the capturing surface as proposed by Molina ***et al.*** (1996) with pollen grains captured with a Hirst volumetric trap and Irdi ***et al***. (2002) with various fungal spores captured with a Burkhard volumetric trap.

The failure of ascospores to develop on the detached green leaves placed in grids on the orchard floor for up to six months is a clear indication of the complexity of CBS. In a separate study, the same procedure was followed where detached green leaves were placed underneath trees in orchards in Letsitele, Hoedspruit and Tzaneen in Limpopo Province (Swart & Kotzé, 2007). In contrast to the current study, ascospores were produced on artificial leaf litter within four months, irrespective during which month of the year the leaves were detached. The faster decomposition rate of the artificial leaf litter in the summer, compared to the winter months were similar than the study in Limpopo Province, where detached leaves were totally decomposed within two to three months in the summer (Swart & Kotzé, 2007).

High numbers of ascospores were captured from natural leaf litter collected in the same orchards and during the same time as when the green leaves were detached for production of artificial leaf litter, indicating that prevailing environmental conditions were conducive for sporulation and maturation of ***Guignardia***. With regular rainfall in the summer months the required alternate wetting and drying of the fallen/detached leaves and variations in temperature required for ascospore formation and maturation (Kiely, 1948; Wager, 1949; Kotzé, 1981) were present. Therefore results from the current study cannot be explained in terms of our current knowledge on conditions required for sporulation and more work is required on specific conditions required for spore production on leaf litter.

Ascospores from natural leaf litter collected monthly were produced seasonal with most spores captured from leaf litter collected between October and February each year and no spores during the winter months. This seasonal production and maturation of spores has been reported for ***G. citricarpa*** as well as numerous other fungi (Pady, 1957; Kotzé, 1963; McOnie, 1964a, b; Chatterjee & Hargreave, 1974; Smith, 1996; Guerin ***et al***., 2001; Rossi ***et al***., 2001; Swart & Kotzé, 2007). In South African production areas such as Tzaneen and Letsitele in the Limpopo Province, and various areas in Australia which all have moderate winters, ascospores can be detected throughout the year, but numbers of ascospores produced in the winter is considerable less than in the summer since

pseudothecia ripen slower in winter than in summer (Kiely, 1948; Kotzé, 1963; Swart & Kotzé, 2007). In support of this, Lee & Huang (1973) reported the production of ascospores only after 42 days at 14°C, whereas ascospores were produced in 27 days at 21-28°C.

Ascospores captured from natural leaf litter represented spores produced on leaves of various ages. The January to March peak in available ascospores can be explained in terms of the continued production of ascospores on leaves shed before October being augmented by those which developed rapidly on younger fallen leaves. This is in agreement with a study by McOnie (1964a) on ascospore development in the orchard. McOnie (1964a) captured peak ascospores numbers in December to January. He also reported that pseudothecia on leaves which abscised during April took about 24 weeks to mature, whereas those on leaves collected in December took six weeks. Although McOnie (1964a) found no correlation between ascospore maturity by leaf examination and number of captured discharged ascospores, the rate of ascospore production and maturity in different production regions provides valuable information to improve prediction of critical infections periods.

The KIM provided a fast and repeatable means to determine the available ascospore inoculum present on citrus leaf litter. The KIM in combination with environmental data can be applied to establish the potential inoculum load available to cause new infections by ***G. citricarpa***. The same principles can be applied to determine the inoculum load of other related diseases, such as apple scab, caused by ***Venturia inaequalis*** (Cooke) Wint.

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CHAPTER 6

Artificial wilting of symptomless green citrus leaves to enhance detection of *Guignardia citricarpa*

6.1 Abstract

The citrus black spot pathogen, ***Guignardia citricarpa***, can remain latent in infected green leaves until leaf fall and senescence. Detection techniques such as isolations and DNA amplification with species-specific primers to detect the pathogen directly from symptomless green leaves have a low success rate due to the restricted growth of the pathogen in symptomless tissue. Different wilting treatments of green symptomless leaves have been tested with regard to time to fructification on leaf tissue and ability to detect ***G. citricarpa*** with microscopic examination and polymerase chain reaction (PCR). The leaves were artificially wilted by exposing detached leaves to sunlight or heat followed by alternating wetting and drying on consecutive days. Formation of visual fungal fruiting structures on treated leaves developed after six to 14 days, depending on the initial level of infection. Detection of the pathogen by PCR after leaf wilting was improved between 12 and 83%, compared to untreated green leaves. A standardised protocol for artificial wilting of citrus leaves to enhance detection of ***G. citricarpa*** from symptomless leaves is proposed.

6.2 Introduction

Citrus black spot (CBS), caused by ***Guignardia citricarpa*** Kiely, is a foliage and fruit disease of citrus occurring in subtopical regions with summer rainfall (Sutton & Waterson, 1966). CBS has not been recorded in Mediterranean and European countries, or in Chile, United States of America (USA) (except Florida), Japan and New Zealand (European Union, 1998; Baayen ***et al***., 2002; Paul ***et al***., 2005; Everett & Rees-George, 2006; 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 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. Countries in which certain production areas have remained CBS-free include Australia, Brazil, China, South Africa (SA) and USA (European Union, 1998, 2000b; Paul, 2006; Lemon & McNally, 2010; Schubert ***et al***., 2010). In SA some of the

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 officially recognised as being free of CBS (European Union, 1998; Mabiletsa, 2003; APHIS, 2009; Shea, 2010).

The presence of CBS in an orchard can be monitored by inspection of fruit before harvest, during harvesting or packing, spore trapping in the orchard with volumetric spore traps or directly from leaf litter with the Kotzé Inoculum Monitor (KIM), microscopic examination of leaf litter, twigs and symptomatic leaves (Kotzé, 1981; Truter ***et al***., 2004). Highly sensitive and fast detection of ***G. citricarpa*** in symptomatic citrus material with species-specific DNA primers have been recently described but most failed to detect the presence of the fungus in symptomless tissue (Bonants ***et al***., 2003; Everett & Rees-George, 2006; Meyer ***et al***., 2006; Peres ***et al***., 2007). Polymerase chain reaction (PCR) with species-specific primers is preferred above other methods such as microscopic examination and isolations since it, besides being faster, also has the advantage of being able to distinguish between the pathogen and the morphological similar saprophyte ***Guignardia mangiferae*** A.J. Roy (Bonants ***et al***., 2003; Meyer ***et al***., 2006; Peres ***et al***., 2007). ***G. mangiferae*** is a cosmopolitan saprophyte of woody hosts and it co-exists with ***G. citricarpa*** on citrus. The two morphological similar fungi have been isolated from the same lesion and have caused confusion in the past on many epidemiological aspects of CBS (McOnie, 1964b; Kotzé, 1981; Baayen ***et al***., 2002; Baldassari ***et al***., 2008).

Leaf infections can stay latent for two to 36 months before leaf senescence and in favourable conditions, leaf wilting and production of pycnidio- and ascospores (Kiely 1948; Whiteside 1965; McOnie 1967; Kotzé 1996). Pycnidia and/or pseudothecia of the pathogen develop on dead infected leaves on the orchard floor within 40 to 180 days after leaf drop, depending on the temperature and frequency of wetting (Kotzé, 1981). Alternate wetting and drying of the leaves and variations in temperature provide optimal conditions for sporulation of ***G. citricarpa*** on infected leaves (Kiely 1948; Whiteside 1965; McOnie 1967; Kotzé 1981). Spore production on leaf litter is seasonal, making it very difficult to detect the pathogen during winter months (McOnie, 1964a; Swart & Kotzé, 2007).

Due to the endophytic nature of the pathogen, infected fruit, leaves and twigs can remain symptomless making it difficult to detect CBS irrespective of what detection method is used. Kiely (1948) described an artificial wetting and drying technique to induce sporulation of the CBS pathogen on freshly detached mature green leaves. The

technique was vaguely described and only a few researchers could apply the technique with success (Wager, 1952; Kotzé, 1963; McOnie, 1964b, 1967; Whiteside, 1967).

Since ***G. citricarpa*** is an important quarantine organism and has resulted in a phytosanitary barrier to trade from CBS positive countries to especially the USA and European Union (European Union, 2000a), an improved method to detect the pathogen in symptomless orchards is required. For SA to maintain its CBS pest-free status in four of its provinces, IPPC standards require that an intensive continuous monitoring programme are in place (Shea, 2010). Therefore the aim of the investigation was to develop a standardised protocol for artificially wilting of citrus leaves to enhance detection of ***G. citricarpa*** on the leaves and to evaluate the effectiveness of microscopic examination of fungal fruiting bodies and PCR-based detection of ***G. citricarpa*** and ***G. mangiferae*** from wilted leaves.

6.3 Materials and methods

6.3.1 Optimisation of leaf wilting

Mature Eureka lemon leaves were randomly collected from a heavy Black spot-infected orchard near Mooinooi in North-West Province. Trees received no chemical treatments for the control of CBS for the past 20 years. The leaves were randomly divided into groups, 20 leaves per group, surface disinfected with 1.5% sodium hypochlorite for 2 min, rinsed twice with sterile tap water, drained on paper towel to remove excess water and subjected to 57 different treatments (Table 6.1) with no replications. Clear plastic bags (250 x 380 mm, 20 µm thick) were used to create a moist environment during the incubation step. A treatment consisted of a once-off pre-incubation step and an incubation step repeated each day for 21 days. All treated leaves were visually inspected after 21 days for extent of browning, flexibility and presence of ***Guignardia***-like pycnidia and/or pseudothecia. Selected leaves with visible fungal fruiting bodies were microscopically examined.

Treatments 1, 3, 6, 9, 11, 14, 17, 23, 32, 36, 40, 43, 46, 49 and 52 from Table 6.1 were repeated once as described before deviating by using 40 µm thick clear plastic bags (250 x 380 mm) instead of 20 µm bags. All treated leaves were visually inspected for extent of browning, flexibility and presence of ***Guignardia***-like fruiting structures after 21 days of treatment. Selected leaves with visible fungal fruiting structures were microscopically examined.

Eight treatments (32, 33, 40, 41, 46, 47, 52 and 53) which resulted in the best leaf wilting and/or fungal fructification were selected and replicated in four blocks in a randomised complete block design as described before using 20 µm clear plastic bags. Leaves were visually inspected for extent of browning, flexibility and formation of fungal fruiting structures after 10 and 21 days. All leaves with visible fungal fruiting structures were microscopically examined to distinguish between ***Guignardia*** and other fungi. A rating system was used based on estimation to evaluate leaf browning and formation of ***Guignardia***-like fruiting structures. Browning was scored on a five point ordinal scale (0 = 0%, 1 = 1-25%, 2 = 25-50%, 3 = 51-75% and 4 = 76-100% brown) and the presence of ***Guignardia***-like fruiting structures was scored on a four point ordinal scale (0 = 0%; 1 = 1-10%; 2 = 11-25%; and 3 = >25%). Most leaves remained flexible with very small variation between leaves and a rating system was not required to evaluate it.

6.3.2 Field samples

Mature, green leaves (older than one year) were collected randomly from 20 trees in six commercial orchards. Two orchards consisted of Eureka lemon on Rough lemon rootstock trees (Mooinooi and Paarl in Western Cape Province), and four orchards of Valencia orange on Rough lemon rootstock trees (Mooinooi, Burgersfort and Nelspruit in Mpumalanga Province and Tzaneen in Limpopo Province). Leaves from two Eureka lemon trees also on Rough lemon rootstock in a residential garden in Pretoria (Gauteng Province) were also included. All trees were approximately between 15 and 35 years old. Freshly detached leaves were maintained in paper bags between 5 and 12°C during transport and processed upon arrival (within a day). The final wilting treatment was based on the combination of treatments conducted during optimisation that resulted in the best enhancement of the fructification of ***Guignardia***.

The freshly detached mature green leaves were surface disinfected with 1.5% sodium hypochlorite for 2 min, followed by rinsing twice in sterile tap water and draining on paper towel to remove excess water. The leaves were randomly divided into groups, 20 per group, and placed in brown paper bags at 22-26°C overnight (16 to 18 h) as a once-off pre-incubation step. The leaves were removed from the paper bag, submerged in tap water at 35°C for 30 min, drained and placed into a plastic bag (250 x 380 mm, 20 µm thick). Each bag with leaves was closed and incubated at 42°C for 6 h in the dark, after which the bags with leaves were removed, opened and the leaves placed in the open bag under fluorescent lights for about 18 h at room temperature (22-26°C). The treatment of wetting, incubation at 42°C and air-drying of the leaves was repeated daily for up to 21 days. Leaves of each sample were visually inspected for extent of browning and

flexibility, and leaves with visible fungal fruiting structures were microscopically examined every seven days for 21 days.

The same rating system was used to evaluate leaf browning and formation of ***Guignardia***-like fruiting structures as described before. In addition, all leaves were tested for presence of ***G. citricarpa*** and ***G. mangiferae*** with species-specific PCR primers, CITRIC1 (5'-GAA AGG TGA TGG AAG GGA G-3') and CAMEL2 (5'-AGT ATA CAA AAC TCA AGA ATT C-3') (Meyer ***et al***., 2006), together with ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White ***et al***., 1990), before commencement of treatment and every seven days thereafter up to 21 days. Four to eight batches of leaf samples were collected on separate dates from each orchard.

6.3.3 Microscopic examination

Leaves were examined for the formation of fungal fruiting structures under a stereo microscope at 50x magnification. Microscope slides were prepared from structures resembling those of ***G. citricarpa*** (Kiely, 1948b), stained with lactofuchsin or Trypan blue (Dhingra & Sinclair, 1995) and examined under a compound microscope at 400x magnification.

6.3.4 DNA extraction

DNA of the leaf samples were extracted from 20 leaf disks (2 mm in diameter) collected per sample using a Harris Uni-core (Whatman), giving preference to leaves and leaf areas showing discolouration and/or fungal fructification. No more than five disks per leaf were collected. In cases where the leaves remained green, one disk per leaf was randomly collected. Collected leaf disks per sample were placed in a 1.5 ml microcentrifuge tube, frozen in liquid nitrogen for 15 s and grinded using a hand held micro-pestle. Total genomic DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The elution volume was reduced to 75 µl. Successful DNA extractions were confirmed by visualisation on a 1.0% (w/v) agarose gel in TAE buffer.

6.3.5 PCR condition

PCR reactions were performed in 50 µl volumes, each reaction containing 2 µl template DNA, 20 pmol of each primer (CITRIC1, CAMEL2 and ITS4), 5 µl recommended 10x buffer (supplied with Taq polymerase), 2 mM $MgCl_2$, 200 µM of each dCTP, dGTP, dATP and dTTP (Bioline) and 0.5 U Taq polymerase (Bioline). Following an initial denaturation

Table 6.1. Treatments applied for 21 days on mature green Eureka lemon leaves during optimisation of leaf wilting process

Treatment no	Pre-incubation step[a]	Incubation step repeated daily[b]
1	N	C at RL for 23.3 h; W
2	N	O at RL for 23.3 h; W
3	N	C at RL for 15.3 h; O at RL for 8 h; W
4	N	C at RL for 19.3 h; O at RL for 4 h; W
5	N	C at RL for 22.3 h; O at RL for 1 h; W
6	N	C at RL for 8 h; O at RL for 15.3 h; W
7	N	C at RL for 4 h; O at RL for 19.3 h; W
8	N	C at RL for 1 h; O at RL for 22.3 h; W
9	N	C at RS for 23.3 h; W
10	N	O at RS for 23.3 h; W
11	N	C at RS for 8 h; O at RL for 15.3 h; W
12	N	C at RS for 4 h; O at RL for 19.3 h; W
13	N	C at RS for 1 h; O at RL for 22.3 h; W
14	N	O at RS for 8 h; W; C at RL for 15.3 h
15	N	O at RS for 4 h; W; C at RL for 19.3 h
16	N	O at RS for 1 h; W; C at RL for 22.3 h
17	AL for 8 h	W; C at RL for 8 h; O at RL for 15.3 h
18	AL for 8 h	W; C at RL for 4 h; O at RL for 19.3 h
19	AL for 8 h	W; C at RL for 1 h; O at RL for 22.3 h
20	AL for 4 h	W; C at RL for 8 h; O at RL for 15.3 h
21	AL for 4 h	W; C at RL for 4 h; O at RL for 19.3 h
22	AL for 4 h	W; C at RL for 1 h; O at RL for 22.3 h
23	AS for 4 h	W; C at RL for 8 h; O at RL for 15.3 h
24	AS for 4 h	W; C at RL for 4 h; O at RL for 19.3 h
25	AS for 4 h	W; C at RL for 1 h; O at RL for 22.3 h
26	AS for 2 h	W; C at RL for 8 h; O at RL for 15.3 h
27	AS for 2 h	W; C at RL for 4 h; O at RL for 19.3 h
28	AS for 2 h	W; C at RL for 1 h; O at RL for 22.3 h
29	AS for 1 h	W; C at RL for 8 h; O at RL for 15.3 h
30	AS for 1 h	W; C at RL for 4 h; O at RL for 19.3 h
31	AS for 1 h	W; C at RL for 1 h; O at RL for 22.3 h

Table 6.1. Continued

Treatment no	Pre-incubation step[a]	Incubation step repeated daily[b]
32	N	C in I for 6 h at 42°C; O at RL for 17.3 h; W
33	N	C in I for 4 h at 42°C; O at RL for 19.3 h; W
34	N	C in I for 2 h at 42°C; O at RL for 21.3 h; W
35	N	C in I for 1 h at 42°C; O at RL for 22.3 h; W
36	N	C in I for 6 h at 35°C; O at RL for 17.3 h; W
37	N	C in I for 4 h at 35°C; O at RL for 19.3 h; W
38	N	C in I for 2 h at 35°C; O at RL for 21.3 h; W
39	N	C in I for 1 h at 35°C; O at RL for 22.3 h; W
40	AL for 8 h	C in I for 6 h at 42°C; O at RL for 17.3 h; W
41	AL for 8 h	C in I for 4 h at 42°C; O at RL for 19.3 h; W
42	AL for 8 h	C in I for 2 h at 42°C; O at RL for 21.3 h; W
43	AL for 8 h	C in I for 6 h at 35°C; O at RL for 17.3 h; W
44	AL for 8 h	C in I for 4 h at 35°C; O at RL for 19.3 h; W
45	AL for 8 h	C in I for 2 h at 35°C; O at RL for 21.3 h; W
46	AS for 4 h	C in I for 6 h at 42°C; O at RL for 17.3 h; W
47	AS for 4 h	C in I for 4 h at 42°C; O at RL for 19.3 h; W
48	AS for 4 h	C in I for 2 h at 42°C; O at RL for 21.3 h; W
49	AS for 4 h	C in I for 6 h at 35°C; O at RL for 17.3 h; W
50	AS for 4 h	C in I for 4 h at 35°C; O at RL for 19.3 h; W
51	AS for 4 h	C in I for 2 h at 35°C; O at RL for 21.3 h; W
52	AS for 2 h	C in I for 6 h at 42°C; O at RL for 17.3 h; W
53	AS for 2 h	C in I for 4 h at 42°C; O at RL for 19.3 h; W
54	AS for 2 h	C in I for 2 h at 42°C; O at RL for 21.3 h; W
55	AS for 2 h	C in I for 6 h at 35°C; O at RL for 17.3 h; W
56	AS for 2 h	C in I for 4 h at 35°C; O at RL for 19.3 h; W
57	AS for 2 h	C in I for 2 h at 35°C; O at RL for 21.3 h; W

[a]N = no treatment; AL = air-dried on paper towel on laboratory bench (out of direct sunlight); AS = air-dried on paper towel on greenhouse bench in direct sunlight.

[b]C = in closed bag; O = removed from bag; RL = room temperature (22-26°C) under fluorescent light; RS = ambient temperatures (26-32°C) in direct sunlight on greenhouse bench; I = incubator in darkness; W = remove leaves from bag, soak in sterile tap water for 30 min and drain on paper towel for 5 min.

step of 95°C for 2 min, 35 PCR cycles were performed on a Perkin-Elmer 2400 thermocycler using the following conditions: a denaturation step of 94°C for 30 s followed by annealing at 56°C for 45 s and extension at 72°C for 90 s, followed by a final extension of 72°C for 7 min. Water was used instead of DNA as a negative control. The amplified DNA fragments were visualized on a 1.2% (w/v) agarose gel in TAE buffer.

6.3.6 Statistical analysis

A frequency per rating class contingency table was constructed and a Chi-Squared test for association was performed to test for pattern differences over classes (Snedecor & Cochran, 1967). The frequency data were subjected to a generalised linear model technique with a logistic link function. The maximum likelihood estimators (Xbeta's) were calculated on an underlying scale (McCullagh & Nelder, 1989) and called location values. The percentages that occurred within each class and the overall browning (class 1-4) and fruiting (class 1-3) were calculated. These percentages and the location data were subjected to a split-plot analysis of variance using the repeated measurements over time as a sub-plot factor (Little & Hills 1972). Shapiro-Wilk's test was performed on the standardised residuals to test for non-normality (Shapiro & Wilk, 1965). If deviation from normality was caused by outliers they were identified and removed until the standardized residuals had a symmetrical distribution. In cases where deviation from normality was caused by kurtosis and not skewness we considered the data as reliable and continued with interpretation (Glass ***et.al***., 1972). The Student's ***t***-least significant difference (LSD) was calculated at the 5% level of significance to compare means of significant effects. All the above statistics were done with SAS statistical software version 9.2 (SAS, 1999).

6.4 Results

6.4.1 Optimisation of leaf wilting

Treated Eureka lemon leaves showed a large variation in response to the 57 wilting treatments (Table 6.2). Generally the leaves that were air-dried in direct sunlight and out of direct sunlight (treatment 1 to 31) remained green or remained more than 50% green. Leaves that were kept in closed plastic bags for more than 15 h were too moist and some started to rot within 21 days (treatments 1, 5, 9 and 16) (Fig. 6.1), while others were colonised by other fungi, mainly ***Colletotrichum gloeosporioides*** (Penz.) Penz. & Sacc., and no ***Guignardia***-like fruiting structures were observed (treatments 3-4, 36-39, 43-45, 49-51, 55-57). Leaves that were left open to air-dry for more than 15 h per day became dried and brittle with no fungal fruiting bodies (treatments 2, 6-8, 10-13, 19, 22, 25, 28, 31 and 35) (Fig. 6.2).

Table 6.2. State of mature green Eureka lemon leaves, collected in Mooinooi, North-West Province, 21 days after leaf wilting treatment commenced using 20 µm thick clear plastic bags during optimisation of the leaf wilting process

Treatment no[a]	Description of leaves in relation to leaf colour, flexibility/brittleness of leaves and presence of ***Guignardia***-like pycnidia and/or pseudothecia or other fungal structures
1, 5, 9	Mostly green, wet and flexible with no fungal fruiting structures, some bacterial decay
16	Mostly green, wet and flexible with limited fungal fruiting structures, some bacterial decay
3, 4	Mostly green and flexible with limited fungal fruiting structures
36-39, 43-45, 49-51, 55-57	Mostly green and flexible with fungal fruiting structures
15, 34	Mostly green, dry and brittle with limited fungal fruiting structures
14, 17, 20, 23, 26, 29	Mostly green, dry and brittle with fungal fruiting structures
2, 6-8, 10-13, 19, 22, 25, 28, 31, 35	Green, dry and brittle with no fungal fruiting structures
18, 21, 24, 27, 30	Green, dry and brittle with limited fungal fruiting structures
32	Mostly brown and flexible with limited ***Guignardia***-like fruiting structures
33	Mostly brown and flexible with fungal fruiting structures
40-41, 46-47, 52-53	Brown and flexible with ***Guignardia***-like fruiting structures
42, 48, 54	Brown and flexible with limited ***Guignardia***-like fruiting structures

[a]Refer to Table 6.1 for the full descriptions of treatments 1 to 57.

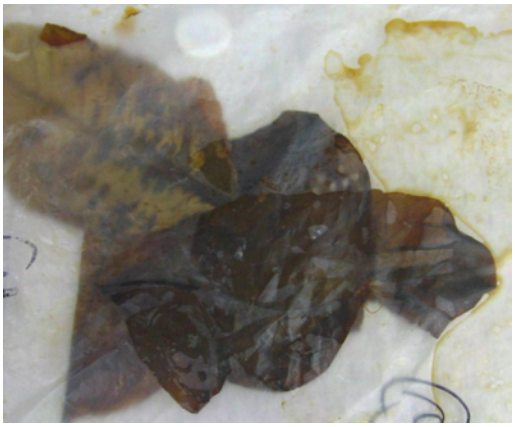
A  B 

Figure 6.1. Green mature Eureka lemon leaves that were kept in closed bags for more than 15 hours remained too moist while some started to rot. A: leaves in a 20 µm thick bag, B: leaves in a 40 µm thick bag.

Figure 6.2. Green mature Eureka lemon leaves dried by direct sun light remained mainly green while becoming dry and brittle within 21 days. No ***Guignardia***-like structures formed consequently.

Leaves wilted faster when incubated in an incubator at 35 or 42°C than at room temperature. Best results in terms of leaf de-colouration and flexibility were obtained when leaves were incubated at 42°C for at least 4 h with no pre-incubation treatment or at 42°C for at least 2 h with a pre-incubation treatment (treatments 32, 33, 40-42, 46-48, 52-54) (Table 6.2). Only treatments 32, 40, 41, 42, 46, 47, 48, 52, 53 and 54 resulted in the formation of ***Guignardia***-like fruiting structures on the wilted leaves. No ***Guignardia***-like fruiting structures were produced on green leaf tissue. ***Guignardia***-like fruiting structures were only observed on fully brown leaves (Fig. 6.3B) or on brown areas of leaves that were partially brown (Fig. 6.3A).

The identity of the fruiting structures resembling those of pycnidia and/or pseudothecia of ***Guignardia*** was very difficult to verify and in most cases impossible. Mature pycnidiospores were seldom observed within 21 days of treatment and ascospores, even immature ones did not develop on the treated leaves within the evaluation time. Ascospores only developed on the treated leaves after additional two weeks of wilting. Therefore the production of fruiting structures resembling those of ***Guignardia*** was only indicated as ***Guignardia***-like fruiting structures.

All the treatments that were repeated with the 40 µm thick plastic bags gave similar results as the 20 µm thick bags (Table 6.3). Although, in general, the extent of development of fungal fruiting structures on the leaves was less with the 40 µm thick bags compared to the 20 µm bags. With the use of the 40 µm thick bags only treatments 40, 46, and 52 resulted in the formation of some ***Guignardia***-like fruiting structures.

Significantly more ***Guignardia***-like fruiting structures were observed after 10 and 21 days when leaves received a pre-incubation step (treatments 40, 41, 46, 47, 52 and 53) compared to no pre-incubation step (treatments 32 and 33) (Table 6.4). In the same way, leaves also become brown at a significantly higher rate after 10 and 21 days when leaves received a pre-incubation step compared to no pre-incubation step (Table 6.5). Treatment 40 consisting of air-drying leaves out of direct sunlight for 8 h as an once-off pre-incubation step, followed by daily wetting, incubation at 42°C in a closed 20 µm thick bag for 6 h, followed by air-drying at room temperature under fluorescent lights, resulted in significantly more ***Guignardia***-like fruiting structures developing on treated leaves (Table 6.4).

6.4.2 Field samples

The extent of leaf browning did not differ significantly between leaves collected from different orchards, after 7, 14 or 21 days of leaf wilting treatment (Fig 6.4). The mean frequency of leaves per browning category was 0 for category 0 to 2, 0.95 for category 3 and 99.05 for category 4 after three weeks of wilting treatment.

All leaf samples, except one sample from Burgersford and one from Paarl, had some ***Guignardia***-like fruiting structures developing on one or more of the treated leaves after seven days. Leaves from Paarl developed significantly less ***Guignardia***-like fruiting structures compared to leaves from the other orchards (Fig. 6.5). A mean of 60% of leaves collected in Paarl had no ***Guignardia***-like fruiting structures after 21 days of wilting treatment. Leaves from Paarl tested negative for ***G. citricarpa*** in all the samples with species-specific DNA primers, although ***G. mangiferae*** was detected in 50% of the samples (Table 6.6, Fig. 6.6).

Leaves from all CBS-positive orchards from at least one sample tested positive with DNA amplification either before treatment, with the exception of Burgersford, or after treatment (Table 6.6). Green untreated leaves from Burgersford tested negative for ***G. citricarpa*** and ***G. mangiferae*** with PCR, but tested positive for ***G. citricarpa*** in 83% of the samples after 14 or more days of wilting (Table 6.6). Detection of ***G. citricarpa*** with PCR was improved by 83% when green symptomless citrus leaves were artificially wilted. Although wilted Eureka lemon leaves from Mooinooi did not differ significantly from other CBS-positive orchards in terms of fruiting body formation, better amplification of target DNA from untreated and treated leaves were obtained (Figs 6.6 and 6.7).

Variation in amplification of target DNA from samples collected on different dates from the same orchard was obtained. The PCR products from the Eureka lemon leaves from Pretoria after 21 days of wilting resulted in detection of ***G. citricarpa*** and ***G. mangiferae*** in two samples, while only ***G. citricarpa*** in the remaining two samples were detected (Fig. 6.7 lanes 13 to 16).

Table 6.3. State of mature green Eureka lemon leaves, collected in Mooinooi, North-West Province, 21 days after leaf wilting treatment commenced using 40 µm clear plastic bags during initial optimisation of the leaf wilting process

Treatment no[a]	Description of leaves in relation to leaf colour, flexibility/brittleness of leaves and presence of ***Guignardia***-like pycnidia and/or pseudothecia or other fungal structures
1, 9	Mostly green, wet and flexible with no fungal fruiting structures, some bacteria decay
3	Mostly green and flexible with limited fungal fruiting structures
36, 43, 49	Mostly green and flexible with fungal fruiting structures
14, 17, 23	Mostly green, dry and brittle with fungal fruiting structures
6, 11	Green, dry and brittle with no fungal fruiting structures
32	Mostly brown and flexible with fungal fruiting structures
40, 46, 52	Brown and flexible with limited ***Guignardia***-like fruiting structures

[a]Refer to Table 6.1 for the full descriptions of treatments 1, 3, 6, 9, 11, 14, 17, 23, 32, 36, 40, 43, 46, 49 and 52.

Table 6.4. Formation of ***Guignardia***-like fruiting structures on treated mature green Eureka lemon leaves collected in Mooinooi, North-West Province, expressed as frequency of leaves in each rating scale (0 to 3), 10 and 21 days after wilting treatment commenced using 20 µm thick clear plastic bags[a]

Treatment	10 days				21 days			
no[b]	0[c]	1	2	3	0	1	2	3
32	72.50 a	26.25 bc	1.25 d	0 a	48.75 a	37.50 b	13.75 c	0 c
33	67.50 a	32.50 ab	0 d	0 a	52.50 a	47.50 a	0 d	0 c
40	17.50 c	37.50 a	45.00 a	0 a	2.50 c	8.75 d	33.75 b	55.00 a
41	46.25 b	31.25 ab	21.25 c	1.25 a	30.00 b	11.25 d	17.50 c	41.25 b
46	45.00 b	26.25 bc	28.75 bc	0 a	28.75 b	30.00 b	41.25 ab	0 c
47	55.00 b	20.00 c	25.00 bc	0 a	37.50 b	23.75 c	38.75 ab	0 c
52	47.50 b	18.75 c	33.75 b	0 a	30.00 b	23.75 c	46.25 a	0 c
53	50.00 b	18.75 c	31.25 b	0 a	36.25 b	20.00 c	43.75 a	0 c
lsd[d]	18.906	9.440	11.184	3.246	11.392	8.669	8.701	5.370
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a]Mean of four replicates, each consisting of 20 leaves; values followed by the same letter in a column do not differ significantly according to Students ***t***-least significant difference (***P*** ≤ 0.05).

[b]Refer to Table 6.1 for the full descriptions of treatments.

[c]***Guignardia***-like fruiting structures was scored as a four point ordinal scale as percentage of total leaf area, with 0 = 0%; 1 = 1-10%; 2 = 11-25% and 3 = >25%.

[d]lsd = least significant difference.

Table 6.5. Leaf browning of treated mature green Eureka lemon leaves collected in Mooinooi, North-West Province, expressed as frequency of leaves in each rating scale (0 to 4), 10 and 21 days after wilting treatment commenced using 20 µm thick clear plastic bags[a]

Treatment	10 days					21 days				
no[b]	0[c]	1	2	3	4	0	1	2	3	4
32	1.25 ab	55.00 a	43.75 a	0 c	0 c	0 a	0 a	21.25 a	30.00 a	48.75 c
33	2.50 a	58.75 a	38.75 ab	0 c	0 c	0 a	0 a	17.50 ab	26.25 a	56.25 bc
40	0 b	0 b	28.75 abc	36.25 a	35.00 b	0 a	0 a	0 b	6.25 b	93.75 a
41	0 b	0 b	31.25 abc	48.75 a	20.00 bc	0 a	0 a	13.75 ab	12.50 b	73.75 ab
46	0 b	0 b	13.75 c	38.75 a	47.50 ab	0 a	0 a	5.00 b	7.50 b	87.50 a
47	0 b	1.25 b	22.50 bc	16.25 b	60.00 a	0 a	0 a	7.50 b	5.00 b	87.50 a
52	0 b	0 b	35.00 ab	8.75 bc	56.25 a	0 a	0 a	2.50 b	8.75 b	88.75 a
53	0 b	0 b	38.75 ab	11.25 bc	50.00 ab	0 a	0 a	16.25 ab	2.50 b	81.25 a
lsd[d]	1.320	6.628	16.410	11.171	21.068	0	0	18.671	12.715	20.952
P-value	0.0010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0055	<0.0001	<0.0001

[a]Mean of four replicates, each consisting of 20 leaves; values followed by the same letter in a column do not differ significantly according to Students ***t***-least significant difference (***P*** ≤ 0.05).

[b]Refer to Table 6.1 for the full descriptions of treatments.

[c]Leaf browning was scored on a five point ordinal scale as percentage of total leaf area, with 0 = 0%, 1 = 1-25%, 2 = 25-50%, 3 = 51-75% and 4 = 76-100%.

[d]lsd = least significant difference.

A

B

Figure 6.3. Fruiting bodies of ***Guignardia*** spp. on artificially wilted mature Eureka lemon leaves after 7 (A) and 14 (B) days of treatment.

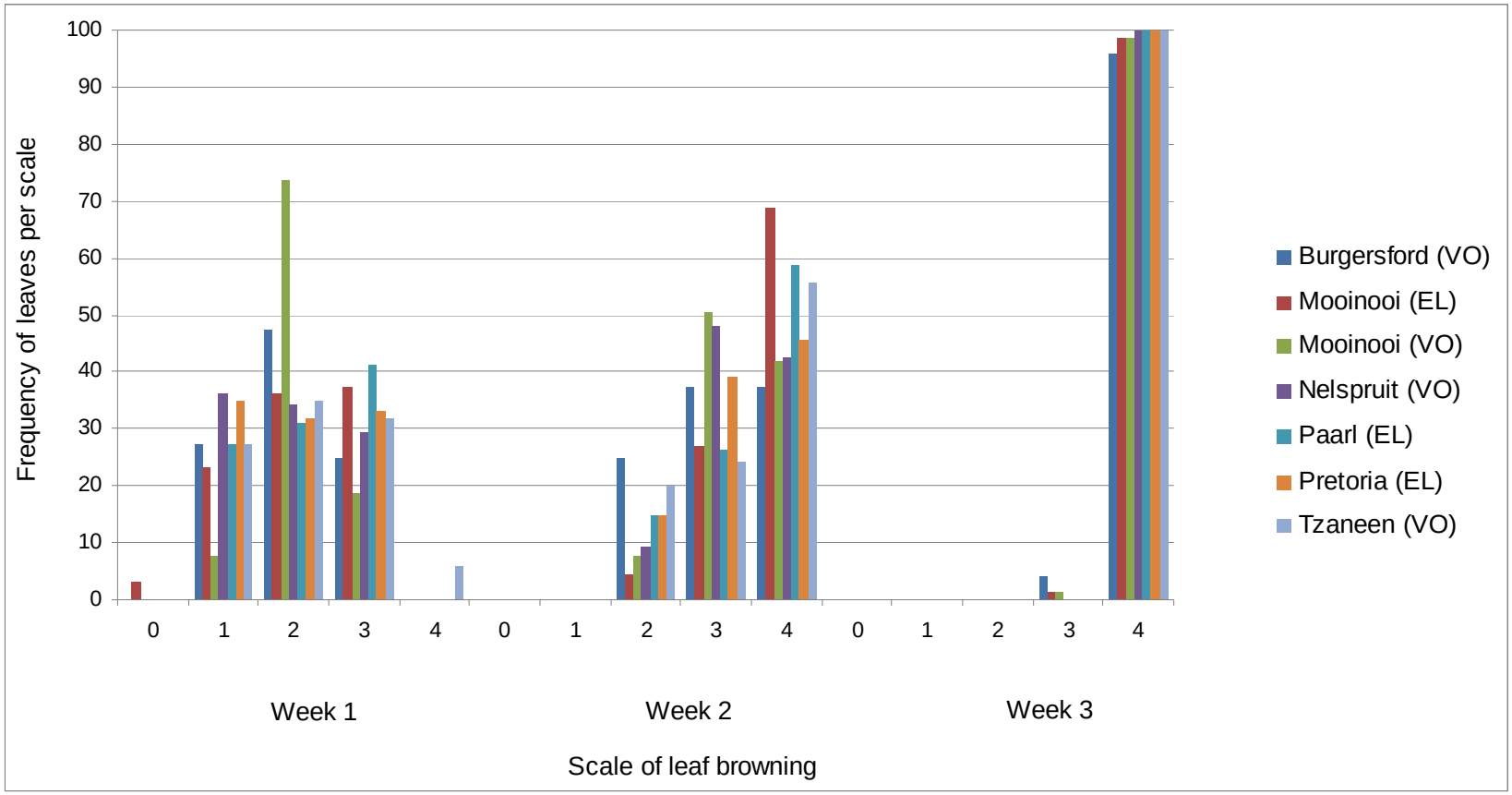
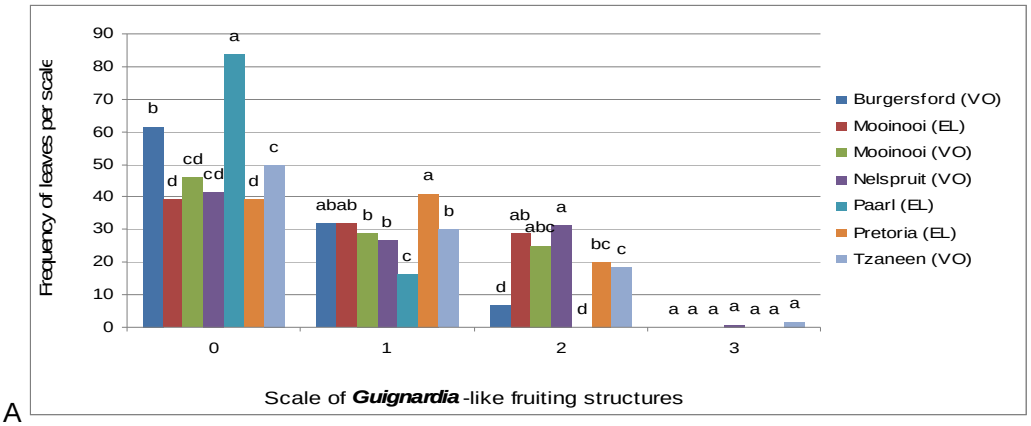
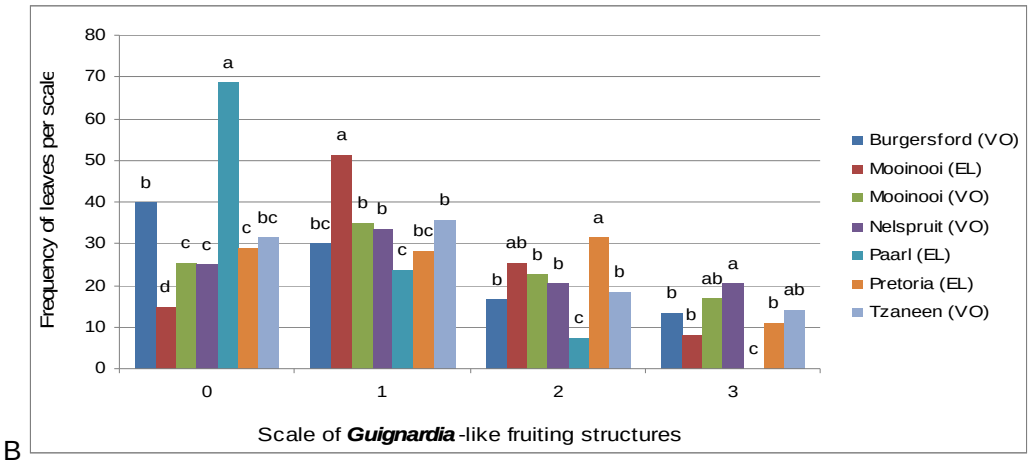


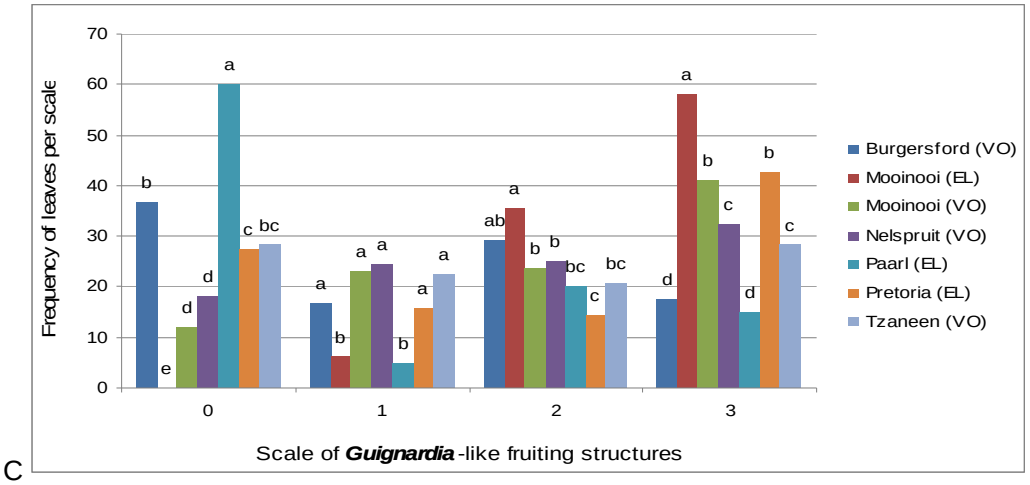
Figure 6.4. Leaf browning of treated mature green Eureka lemon (EL) and Valencia orange (VO) leaves expressed as frequency of leaves in each rating scale (0 to 4), 7, 14 and 21 days after wilting treatment commenced using 20 µm thick clear plastic bags. Leaf browning was scored on a five point ordinal scale as percentage of total leaf area, with 0 = 0%, 1 = 1-25%, 2 = 25-50%, 3 = 51-75% and 4 = 76-100%. Mean of four to eight replicates, each consisting of 20 leaves; bars do not differ significantly according to Students ***t***-least significant difference (***P***≤0.05).



A



B



C

Figure 6.5. Formation of ***Guignardia***-like fruiting structures on treated mature green Eureka lemon (EL) and Valencia orange (VO) leaves expressed as frequency of leaves in each scale (0 to 3); A=7, B=14 and C=21 days after wilting treatment commenced using 20 µm thick clear plastic bags. Scale of visual assessment of ***Guignardia***-like fruiting structures as percentage of total leaf area, with 0 = 0%; 1 = 1-10%; 2 = 11-25% and 3 = >25%. Mean of four to eight replicates, each consisting of 20 leaves; bars followed by the same letter do not differ significantly according to Students ***t***-least significant difference (***P***≤0.05).

Table 6.6. Detection of ***Guignardia citricarpa*** in naturally infected green symptomless citrus leaves before and 7, 14 and 21 days after wilting treatment commenced using primer set CITRIC1 and ITS4 (amplicon 580 bp)

Area (cultivar)[a]	Frequency of PCR-positive samples[b]			
	0 days	7 days	14 days	21 days
Burgersfort, Mpumalanga (VO)	0	33	83	83
Mooinooi, North West (EL)	88	100	100	100
Mooinooi, North West (VO)	88	88	100	100
Nelspruit, Mpumalanga (VO)	38	50	100	100
Paarl, Western Cape (EL)	0	0	0	0
Pretoria, Gauteng (EL)	17	17	33	83
Tzaneen, Limpopo (VO)	17	33	100	100

[a]VO = Valencia orange, EL = Eureka lemon.

[b]Mean of four to eight replicates, each consisting of 20 leaves; leaf material from 20 leaves pooled for one DNA extraction.

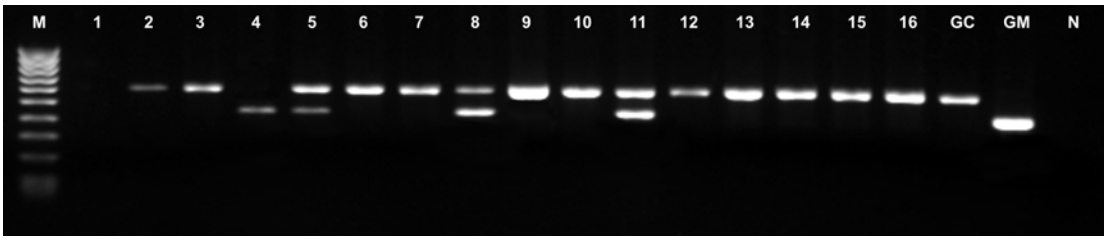


Figure 6.6. PCR amplicons of ***Guignardia citricarpa*** and ***Guignardia mangiferae*** from DNA extracted from symptomless citrus leaves. 1 to 7 = green, un-wilted leaves, 1 = Valencia Orange (VO) from Burgersford, 2 = VO from Nelspruit, 3 = VO from Tzaneen, 4 = Eureka lemon (EL) from Paarl, 5 = VO from Mooinooi, 6 = EL from Mooinooi, 7 = EL from Pretoria. 8 to 14 = leaves wilted for seven consecutive days, 8 = VO from Nelspruit, 9 = EL from Mooinooi, 10 = VO from Mooinooi, 11 = VO from Tzaneen, 12 = VO from Burgersford, 13 = EL from Pretoria. 14 to 16 = leaves wilted for 14 consecutive days, 14 = VO from Nelspruit, 15 = VO from Tzaneen, 16 = EL from Pretoria. M = DNA marker Hyperladder IV (Bioline), GC = ***Guignardia citricarpa*** positive control, GM = ***Guignardia mangiferae*** positive control, Neg = negative control with no DNA added.

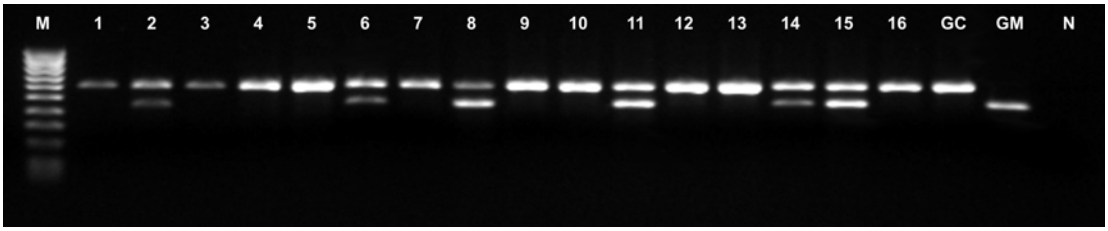


Figure 6.7. PCR amplicons of ***Guignardia citricarpa*** and ***Guignardia mangiferae*** from DNA extracted from symptomless artificially wilted citrus leaves. 1 to 6 = leaves wilted for seven consecutive days, 1 = Valencia Orange (VO) from Nelspruit, 2 = VO from Burgersford, 3 = VO from Tzaneen, 4 = Eureka lemon (EL) from Pretoria, 5 = EL from Mooinooi, 6 = VO from Mooinooi. 7 to 12 = leaves wilted for 14 consecutive days, 7 = VO from Nelspruit, 8 = VO from Burgersford, 9 = VO from Tzaneen, 10 = EL from Pretoria, 11 = VO from Mooinooi, 12 = EL from Mooinooi. 13 to 16 = EL leaves from Pretoria wilted for 21 consecutive days, 13 = sample 1, 14 = sample 2, 15 = sample 3, 16 = sample 4. M = DNA marker Hyperladder IV (Bioline), GC = ***Guignardia citricarpa*** positive control, GM = ***Guignardia mangiferae*** positive control, Neg = negative control with no DNA added.

6.5 Discussion

Artificial wilting of green citrus leaves is a reliable, fast and effective method to detect the CBS pathogen and can be applied to monitor citrus nurseries and orchards throughout the year. Leaf treatment was 100% effective in wilting Eureka lemon and Valencia Orange leaves collected from five Provinces in South Africa. All treated leaves became brown while remaining leathery and fungal fruiting structures developed on most of the leaves.

Although ***Guignardia***-like fruiting structures were observed on leaves from every sample after the wilting treatment, four of the 46 samples tested negative for either ***G. citricarpa*** or ***G. mangiferae*** with PCR. The results indicate that either not all ***Guignardia***-like fruiting structures observed in fact belonged to ***Guignardia*** or that the PCR did not always detect the pathogen. Each method has its pros and cons and should be used in combination with each other. The four samples with fruiting structures that tested negative with the PCR, can be regarded as negative, since from experience, molecular detection of ***Guignardia*** was more effective than microscopic examination.

Microscopic examination of leaves for pycnidia and/or pseudothecia of ***Guignardia*** are labour intensive, time consuming and a high level of expertise is required. To positively identify the fungal fruiting structures on the leaves as ***Guignardia*** requires a longer treatment period than what is required to achieved positive results with PCR. Production of spores on the treated leaves was slower than the evaluation period, thus little to no pycnidiospores were observed whereas no ascospores were observed up to 21 days. Furthermore, one cannot accurately distinguish between the citrus pathogen and the saprophyte, ***G. mangiferae***, on morphology alone.

The main advantage of microscopic examination over PCR is that it is considerably cheaper and besides a microscope, no other expensive equipment or consumables are required. PCR is less labour intensive, requires a shorter leaf treatment period, and is more accurate to detect ***G. citricarpa*** than microscopic examination. Results of PCR tests are less variable between people as there is less room for subjective interpretation as long as proper positive and negative controls are included. Some level of expertise is still required to select the correct type or part of plant material for DNA extraction, since even with the wilting treatment growth of ***G. citricarpa*** is still localised within the leaf tissue (Kiely, 1948; Kotzé, 1963).

The method for leaf wilting as described by Kiely (1948) could not be successfully replicated in a pilot study (data not shown). Only after a detailed description and

demonstration provided by Prof J.M. Kotzé (personal communication, September 2005), was some success achieved, although with variable results. Leaf wilting in sunlight was attempted numerous times without much success, with the main problem being over or under exposure to sunlight. Leaf wilting in sunlight was found to be more labour intensive than when using an incubator as the leaves have to be monitored several times per day due to natural variations in sunlight (clouds, shadows, seasonal variation). The duration of incubation of leaves in direct sunlight depended on the intensity and quality of sunlight. In summer, leaves should be left in the sun for about 3 h while in the winter, a whole day might be required (J.M. Kotzé, personal communication, September 2005).

In another pilot study, leaves were incubated without bags in a growth chamber at 30°C and relative humidity above 80%. Leaves mainly dried out without proper wilting and browning, and in cases where the leaves did turn brown, the rate of browning was about twice as slow as when leaves were incubated in plastic bags in an incubator. Observations from the current study indicate that heat could be more important than light to accelerate leaf browning. After leaf browning was achieved, regular wetting and proper drying of leaves was crucial for development of ***G. citricarpa***. Regular and proper drying of leaves reduced the growth of other fungi, such as ***Colletotrichum***, in relation to ***Guignardia*** (Kotzé, 1963).

The artificial wilting of naturally infected citrus leaves can be an effective method for not only detecting the pathogen, but also producing spores in sufficient quantities for inoculation studies. Although various researchers have used naturally infected leaf litter as inoculum in infection studies (McOnie, 1964a; 1967), based on observations from the current study, artificially wilted leaves had more fruiting structures that were at the same level of maturity than on natural leaf litter. Mature ascospores did develop on the treated leaves after three to five weeks.

The artificial leaf wilting technique was very reliable, fast and effective in enhancing growth and sporulation of the CBS pathogen in latently infected citrus leaves. The method when used in conjuction with a suitable PCR-test can be used to monitor orchards to maintain its CBS pest-free status. Larger samples rather than smaller ones should be used due to natural variation in level of infection in leaves. This is the only detection method not depended on season for sample collection and can greatly enhance the detection of the CBS pathogen throughout the year.

6.6 References

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CHAPTER 7

Leaf litter management as a non-chemical means of reducing citrus black spot

7.1 Abstract

A four year study was carried out in a commercial Valencia orange orchard to assess the effect of leaf litter removal and mulching on citrus black spot inoculum and disease development on fruit. All leaves on the orchard floor were manually removed and burned between August and early October each year. Eight rows of 18 to 20 trees each were used for the treatments and received no chemical spray for citrus black spot control. The soil surface in four of the eight rows was mulched with a layer of wheat straw in October, whereas leaves were removed once again from the non-mulched area in the other four rows, a month later. Twelve Valencia orange trees in an adjacent orchard used as control, received no chemical spray for citrus black spot control or any leaf litter removal or confinement. No ascospores of *G. citricarpa* could be trapped with a volumetric spore trap in the orchard receiving leaf litter treatments during October to February for the 2001/2002, 2002/2003 and 2003/2004 seasons. Evaluation of fruit at harvest, indicated a mean citrus black spot severity index over four years of 0.4, 1.5 and 12.2 in the mulched, non-mulched and control rows, respectively. Except for the 2001/2002 season, there were no significant differences of citrus black spot infected fruit between trees where leaves were removed or removed and mulched with wheat straw. Results showed that leaf litter management by leaf removal and mulching allowed a reduction of up to 97% in citrus black spot development compared to control.

7.2 Introduction

South Africa (SA) is the second largest exporter of fresh citrus fruit in the world after Spain, although citrus growing in SA is a relative small industry compared to other countries (FAO, 2010b). During 2008, SA produced 2.2 million metric tonnes of fruit (FAO, 2010a) of which 64% were exported (FAO, 2010b). *Guignardia citricarpa* Kiely, the causal agent of citrus black spot (CBS), is an important quarantine organism that has resulted in sanitary and phytosanitary trade barriers for countries with CBS exporting to especially the European Union and United States of America (USA) (European Union, 1998). Furthermore, control of CBS contributes significantly to the production cost of citrus, contributing to the economic importance of CBS.

Control of CBS to a large extent relies on preventative fungicide sprays applied up to six times during the period of fruit susceptibility, from October to January (Kotzé, 1981;

Schutte *et al*., 1997, 2003; Miles *et al*., 2004). Since intensive fungicide spray programmes are expensive and have resulted in development of resistance in *G. citricarpa* to benomyl (Herbert & Grech, 1985), alternative non-chemical control measures are needed. Furthermore, environmental and human health concerns have led to increasing restrictions on the use of chemical fungicides (Janisiewicz & Korsten, 2002). This shift has resulted in greater emphasis in agriculture on adopting alternative approaches and use of integrated control measures.

Sanitation practices can contribute to disease control and resistance management in an integrated approach. Orchard sanitation whereby infected late hanging fruit are removed before the new crop sets and pruning of dead and possible infected twigs are widely practiced within SA (Kotzé, 1981). These sanitation practices effectively remove or reduce pycnidiospore inoculum in the trees, but no sanitation practices are currently employed to reduce inoculum from leaf litter, especially airborne ascospores that are considered the main inoculum source of CBS (Kotzé, 1981).

Pseudothecia of the pathogen develop on dead infected leaves on the orchard floor within 40 to 180 days after leaf drop, depending on the temperature and frequency of wetting (Kotzé, 1981). Once mature, ascospores are discharged mainly during spells of rain (Kotzé, 1963). Ascospore production, maturation and release are seasonal with most spores captured during October to February in summer rainfall regions (Swart & Kotzé, 2007). The most critical period for infection occurs at fruit set and can persist for four to five months (Kotzé, 1981). Reduction or removal of CBS inoculum from the orchard floor should significantly reduce infection of fruit. Therefore infected leaf litter must be removed before and during this critical infection period, to reduce the available CBS inoculum.

Mulching is an ancient technique and has many advantages such as reduction in water use due to limited evaporation, improved water infiltration, increased soil fertility, structure, porosity and aeration, reduced fertilizer use, less temperature fluctuations of soil, control of soil-borne diseases and reduced weed growth (Casale *et al*., 1995; Wolstenholme *et al*., 1996; Faber *et al*., 2003). The net result is improved root growth and reduction in physiological stress, resulting in better fruit set, larger fruit and higher yields (Casale *et al*., 1995; Wolstenholme *et al*., 1996; Faber *et al*., 2003). Disadvantages of mulching include that suitable material is either not available or too expensive, can create a fire hazard in dry winter months, may house insect pests and is labour intensive. Under certain conditions it can be toxic to plants, releasing toxic amount of ammonia upon degradation (Casale *et al.,* 1995; Wolstenholme *et al*., 1996; Faber *et al*., 2003).

Mulching has been used to control fungal foliar and fruit diseases by reducing the release of airborne ascospores from infected leaf or fruit litter. Mulching significantly reduced the release of ascospores of *Guignardia bidwellii* (Ellis) Viala & Ravaz from over wintering mummified grape berries (Becker & Pearson, 1993). A significant reduction in *Venturia inaequalis* (Cooke) G. Winter inoculum and scab symptoms on apple was achieved with various leaf litter management strategies, including mulching (Sutton *et al*., 2000; Vincent *et al*., 2004; Holb *et al*., 2006; Gomez *et al*., 2007). In citrus, mulching with grass resulted in a reduction of CBS at harvest although mulching on its own was not as effective as fungicidal sprays or mulching combined with sprays (Schutte & Kotzé, 1997).

Since mulching without fungicidal sprays did not reduce CBS to acceptable levels in a two-year study (Schutte & Kotzé, 1997), the effectiveness of leaf litter sanitation over a longer period needed to be investigated. Therefore, the aim of this study was to evaluate the effect of leaf litter removal and mulching with wheat straw in a commercial citrus orchard on CBS incidence over four seasons.

7.3 Materials and methods

The experiment site comprised an orchard near Burgersfort, Mpumalanga planted with Valencia Orange on Rough Lemon rootstock. All the leaves on the orchard floor from the entire estate were manually removed and burned between August and early October each year for three years before commencement of the study and for the duration of the study. Trees used during the 2001/2002 season were 31 years old. Four adjoining rows of 16 trees each were selected in each of two adjacent orchard blocks, 1.36 and 1.03 ha in size, respectively. All trees in the two sets of four rows of trees including those in an additional border row on each side received no chemical spray for CBS during 2001 to 2002. Late October 2001 the entire orchard floor in four rows in one block was mulched with a layer of wheat straw, whereas leaves were again removed from the non-mulched area in the other four rows in the adjacent block. The wheat used for mulching was cultivated on a nearby field on the same estate. The wheat straw was spread under the trees selected for mulching in such a way that the entire orchard floor was covered with a layer *ca*. 20 cm thick. All the trees in the two blocks used during the 2001/2002 season were removed and burned during 2003 due to generally poor tree condition and yield.

Valencia Orange on Rough Lemon rootstock trees used during the 2002/2003, 2003/2004 and 2004/2005 seasons were 34 years old at commencement of treatment. During 2002 to 2005 a total of eight adjacent rows of 20 trees each were used in a 5.56 ha block. All

trees, including those in an additional border row on each side of the eight rows of trees used for the study, received no chemical spray for CBS during 2002 to 2005. Late October the surface under the trees in four of the eight rows, was mulched with a layer of wheat straw, whereas leaves were again removed from the non-mulched area in the other four rows. Twelve Valencia Orange on Rough Lemon rootstock trees in an adjacent orchard served as control during 2001 to 2005. Control trees received no chemical treatment for CBS or leaf removal and mulching for the duration of the study and were less than 250 m from the treatment trees.

Trees were evaluated in July the following year shortly before harvest. Forty-eight evenly-distributed fruit on each tree were assessed for CBS severity. Fruit were randomly selected to include 12 fruit per wind direction. From each wind direction, four fruit were from the top (top 33% of trees), the middle (middle 33% of tree) and the bottom (bottom 33% of tree) of the tree. From the four fruit per wind direction and horizontal position, two fruit were on the outside of the tree (peripheral) and two fruit on the internal side of the tree. Fruit were assessed according to a rating scale of 0 to 3, where 0 = clean; 1 = 1-5 spots per fruit; 2 = 6-50 spots per fruit; 3 = > 50 spots per fruit (Fig. 7.1). A severity index was calculated for each tree by means of the following formula, adapted from De Wet (1987):

$$\text{CBS-index} = 100 \times (0n_0 + 0.25n_1 + 0.5\, n_2 + 0.75n_3) / n_{total}$$

Where n represents the total number of infected fruit in each of the categories.

A Quest volumetric spore trap (Interlock Systems, Pretoria) was operated in the same block used for mulching treatments during October to February for 2001/2002, 2002/2003 and 2003/2004 seasons, but not for the 2004/2005 season. The spore trap was placed on a platform to ensure that the orifice is about 1 m from the soil surface (Fig. 7.2). The eight-day rotating disk was sprayed with a thin layer of petroleum jelly (Interlock Systems) to capture spores and replaced every seven days with a new petroleum-coated disk. Disks were stained with Trypan blue and the whole capturing surface was systematically examined at 100X and 400x magnification using a compound light microscope.

Data were analysed separately using the statistical program, SAS 9.2. One-way analysis of variance (ANOVA) was used to test for differences between values. The Student's *t*-least significant difference (LSD) was calculated at the 5% level of significance to compare means of significant effects.

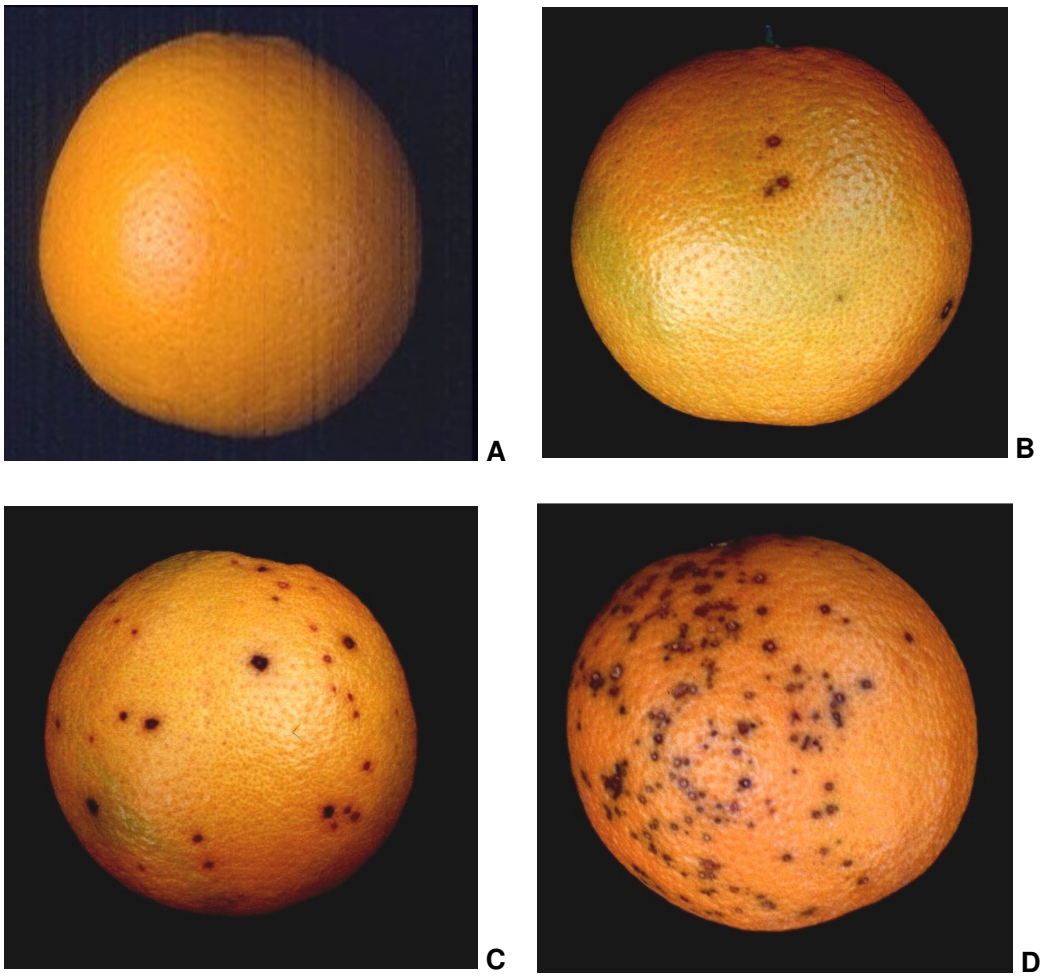


Figure 7.1. Rating used to evaluate level of fruit infection, where A: 0 = clean; B: 1 = 1-5 spots per fruit; C: 2 = 6-50 spots per fruit; D: 3 = > 50 spots per fruit.

Weather data consisting of mean monthly maximum and minimum temperature and total monthly rainfall for the Burgersfort area was obtained from the South Africa Weather Service.

7.4 Results

No ascospores resembling those of *G. citricarpa* could be discerned on the discs of the spore trap for the entire evaluation period of October to February for the 2001/2002, 2002/2003 and 2003/2004 seasons. Some fungal spores frequently observed on the disks include *Alternaria*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Penicillium* and *Stemphylium* species. A large number of unknown elongated to subglobose hyaline to dematiaceous spores and numerous pollen grains were observed, without any attempt to identify them. Apart from the spores and pollen grains, numerous miscellaneous particles, consisting of mainly dust particles, were also observed.

Leaf litter treatment applied during the 2001/2002 season resulted in significantly less CBS in the mulched rows than the non-mulched rows (Table 7.1). CBS index was 87.5% lower in the mulched rows compared to the non-mulched. Both treatments resulted in lower CBS than the control trees. The layer of wheat mulch remained sufficiently intact to cover the soil effectively and about a 5 cm thick mulch layer remained at the end of the growing season (Fig. 7.3)

The mean minimum and maximum temperatures per month during September to March were very similar for the four years, with mean minimum and maximum temperature of 12.7 to 13.4°C and 20.8 to 23.6°C, respectively (Fig. 7.4). Season 2003/2004 had slightly higher mean monthly maximum temperature of 26.9°C for December 2003 and 26.2°C for January 2004, compared to the second highest of 24.7°C for February 2005. A bigger difference was observed for rainfall between seasons. Total rainfall during September to March was recorded as 877.2, 597.4, 516.6, 492.5 and 160.6 mm, respectively, for the 2003/2004, 2000/2001, 2001/2002, 2002/2003 and 2004/2005 seasons.

No significant differences in results were obtained between years for a specific treatment repeated in the same orchard (Table 7.1). No significant differences were evident between mulching and no mulching for the 2002 to 2005 seasons, and both were significantly lower than the control for each year (Table 7.1). Leaf litter treatments reduced CBS incidence by 95.9% to 97.2% compared to the control for the 2002 to 2005 seasons.

No significant differences were evident when percentage infected fruit per tree were compared between fruit borne within the canopy and on the outside, as well as for fruit on top, middle or bottom part of tree (Table 7.2). The same observations were evident when percentage infected fruit per tree were compared for the aspectual distribution within a tree, with no significant differences in CBS occurrence for northern, eastern, southern or western part of the tree (Table 7.3). No CBS was present on chemically sprayed fruit in the adjacent orchard blocks at the time of assessment (results not shown).

CBS infection occurred mostly in the same trees for the 2002 to 2005 seasons. In rows receiving wheat mulch 46.7% of the trees bearing symptomatic fruit were infected each year during the three-year period, whereas 91.7% of the trees were infected each year in rows were leaf litter was removed (results not shown).

7.5 Discussion

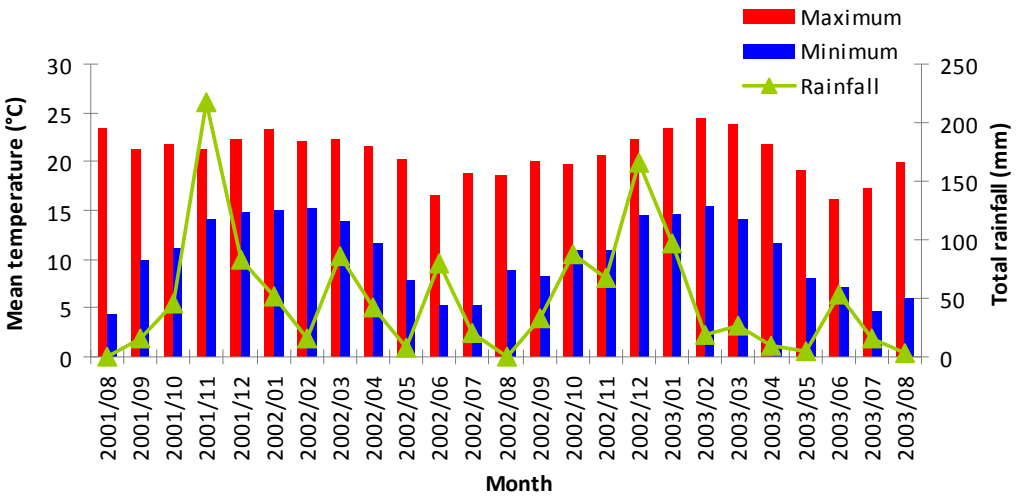
This study confirmed that sanitation practices, such as leaf litter removal and mulching of leaf litter with wheat straw can decrease the primary inoculum of CBS and contribute to better management of the disease in a commercial orchard. Leaf litter removal or mulching can provide an alternative to chemical control and improve control in an integrated approach. Regardless of the prevailing climatic conditions each year, the number of infected fruit at harvest was on average reduced by 89% and 96% by leaf removal and mulching, respectively, compared to the control.

The mean minimum and maximum temperatures per month during September to March were very similar for the four years. In contrast, differences in rainfall between seasons were observed. Total rainfall during September to March was similar for 2000/2001 to 2002/2003 seasons, while 2003/2004 season received about 40% more rain than the previous seasons. In contrast, season 2004/2005 was the driest, receiving about 80% less rain than the previous season.

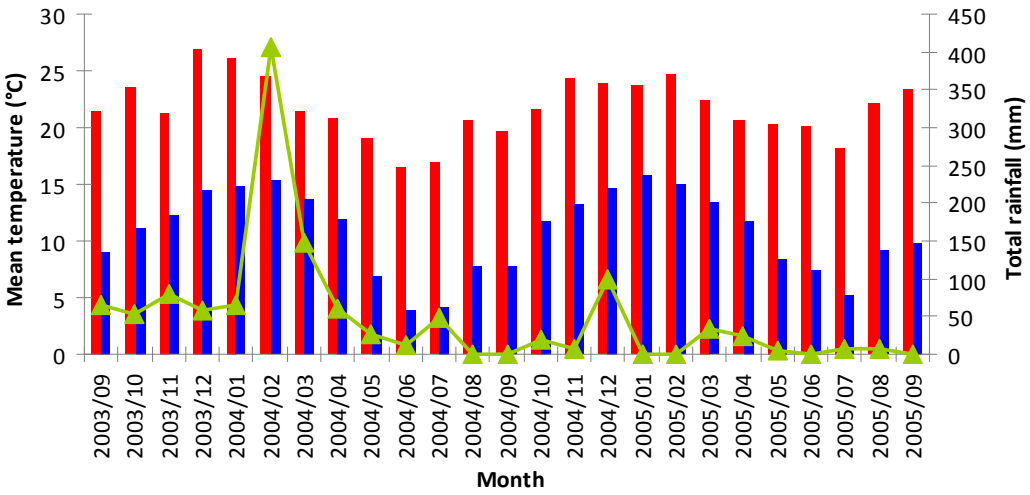
CBS-symptomatic fruit can be as high as 60% or more at harvest in orchards where no control measures were applied (Sutton & Waterson, 1966; Brodrick, 1969). In this study up to 40.8% of fruit in control rows were infected, indicating that the disease pressure in the area was severe and serious economic losses could occur without CBS control. In a similar study, no control measures resulted in up to 40.9% of fruit infected to some extent with CBS, whereas mulching with buffalo grass increased clean fruit at time of harvest by 21% compared to no treatment (Schutte & Kotzé, 1997). The higher level of control

Figure 7.2. Quest volumetric spore trap used to capture spores during October to February.

Figure 7.3. Layer of wheat straw mulch on the orchard floor at the end of the season (March).



A



B

Figure 7.4. Prevailing climatic conditions in Burgersford area, Mpumalanga, A: August 2001 to August 2003 and B: September 2003 to September 2005.

Table 7.1. Incidence and severity of citrus black spot in a commercial Valencia orange orchard where leaf litter was either left undisturbed (control), removed or removed together with mulching with wheat straw[a]

Parameter	Control	Non-mulched	Mulched
2001/2002			
Infected trees (%)	100 a	87.2 b	23.3 c
Infected fruit (%)	52.6 a	12.3 b	2.3 c
CBS-index	16.9 a	4.8 b	0.6 c
2002/2003			
Infected trees (%)	100 a	15.0 b	17.9 b
Infected fruit (%)	40.8 a	1.7 b	1.4 b
CBS-index	11.5 a	0.4 b	0.4 b
2003/2004			
Infected trees (%)	100 a	13.8 b	12.8 b
Infected fruit (%)	38.2 a	1.5 b	1.2 b
CBS-index	10.7 a	0.4 b	0.3 b
2004/2005			
Infected trees (%)	100 a	12.5 b	9.0 b
Infected fruit (%)	33.5 a	1.0 b	1.4 b
CBS-index	9.8 a	0.3 b	0.4 b
Mean			
Infected trees (%)	100 a	32.1 b	15.8 b
Infected fruit (%)	41.3 a	4.1 b	1.6 b
CBS-index	12.2 a	1.5 b	0.4 b

[a]Values are the mean of 48 fruit per tree with 12 to 80 trees per treatment; Values followed by the same letter in a row do not differ significantly according to Student's *t*-least significant difference ($P \leq 0.05$)

Table 7.2. Vertical and horizontal distribution of citrus black spot-infected fruit in trees in a commercial Valencia orange orchard from which leaf litter was either left undisturbed, removed or removed together with mulching with wheat straw

Treatment	Percentage infected fruit per tree[a]				
	Vertical distribution			Horizontal distribution	
	Top	Middle	Bottom	Internal	Peripheral
2001/2002					
Control	17.7 a A	17.2 a A	17.7 a A	25.9 a A	26.7 a A
Non-mulched	4.8 a B	4.8 a B	5.5 a B	9.7 a B	5.4 a B
Mulched	0.4 b B	6.0 a B	3.4 B	5.2 a B	4.6 a B
2002/2003					
Control	13.9 a A	12.7 a A	14.2 a A	20.3 a A	20.5 a A
Non-mulched	5.0 a B	4.5 a B	2.1 b B	6.3 a B	5.4 a B
Mulched	3.0 a B	2.5 a B	2.5 a B	4.5 a B	3.6 a B
2003/2004					
Control	12.7 a A	12.7 a A	12.8 a A	17.2 A	21.0 A
Non-mulched	4.0 a B	4.0 a B	3.2 a B	5.3 a B	5.9 a B
Mulched	2.9 B	5.0 a B	1.3 b B	5.2 a B	4.0 a B
2004/2005					
Control	11.5 a A	10.4 a A	11.5 a A	17.9 a A	15.6 a A
Non-mulched	4.0 B	1.5 B	2.1 B	4.5 a B	3.0 a B
Mulched	4.0 a B	3.5 a B	3.8 a B	6.7 a B	4.6 a B
Mean					
Control	14.0 a A	13.3 a A	14.1 a A	20.3 a A	21.0 a A
Non-mulched	4.5 a B	3.7 a B	3.2 a B	6.4 a B	4.9 a B
Mulched	2.6 a B	4.3 a B	2.8 a B	5.4 a B	4.2 a B

[a]Values are the mean of 16 fruit (vertical) or 24 fruit (horizontal) per tree with 12 to 80 trees per treatment; Values followed by the same letter in a row within vertical or horizontal distribution (lower case) or in a column within a season (upper case) do not differ significantly according to Student's *t*-least significant difference ($P \leq 0.05$).

Table 7.3. Aspectual distribution of citrus black spot-infected fruit in trees in a commercial Valencia orange orchard from which leaf litter was either left undisturbed, removed or removed together with mulching with wheat straw

Treatment	Percentage infected fruit per tree[a]			
	North	East	South	West
2001/2002				
Control	16.5 a	12.5 a	11.6 a	12.0 a
Non-mulched	4.4 a	3.3 a	2.9 a	4.2 a
Mulched	3.1 a	1.5 a	2.4 a	2.8 a
2002/2003				
Control	14.2 a	10.1 a	7.1 a	9.4 a
Non-mulched	5.0 a	2.4 a	1.4 a	2.8 a
Mulched	4.0 a	1.2 a	0.6 a	2.2 a
2003/2004				
Control	12.7 a	8.9 a	8.5 a	8.2 a
Non-mulched	4.9 a	2.5 a	1.5 a	2.3 a
Mulched	3.8 a	2.1 a	0.8 a	2.5 a
2004/2005				
Control	10.2 a	7.6 a	7.3 a	8.3 a
Non-mulched	3.8 a	1.1 a	0.8 a	1.9 a
Mulched	4.8 a	2.1 a	2.1 a	2.3 a
Mean				
Control	13.4 a	9.8 a	8.6 a	9.5 a
Non-mulched	4.5 a	2.3 a	1.7 a	2.8 a
Mulched	3.9 a	1.7 a	1.5 a	2.5 a

[a]Values are the mean of 12 fruit per tree with 12 to 80 trees per treatment; Values followed by the same letter in a row do not differ significantly according to Student's *t*-least significant difference ($P \leq 0.05$).

obtained in this study was mainly due to persistent leaf litter removal in the specific orchard for three years before commencement of treatments. Although the study of Schutte & Kotzé (1997) was only over two years, the authors noted a decrease of disease occurrence due to mulching from the first to the second year.

The persistent removal of leaf litter from the entire estate (46 blocks on about 215 ha combined) since 1998 has dramatically reduced the ascospore inoculum within the orchard and no ascospores of *G. citricarpa* could be detected on the discs of the volumetric spore trap for the full evaluation periods of October to February for the 2001/2002, 2002/2003 and 2003/2004 seasons. The spore trap was not operational for the last season, as the solar panel, generating energy for the trap, was stolen in the winter of 2004. A shortcoming of this study is that no data on ascospore levels within the orchard were collected before commencement of leaf litter removal and that no spores were trapped in the control block.

The source of inoculum, other than ascospores, in this particular orchard was investigated after no ascospores were captured during two consecutive seasons. No infected fruit remained on the trees after harvest (July), and very few dead twigs or branches were found which could harbour pycnidiospores. It is unlikely that pycnidiospores were the source of inoculum, as distribution of infected fruit in the trees, as well as spots on the fruit were random, indicating air-borne inoculum rather than water-borne inoculum as source for infection. It is possible that the low level of ascospores present in the orhard was not effectively captured with the spore trap.

A similar reduction in ascospore levels were obtained in a study on black rot of grape, caused by *G. bidwellii*, when overwintering mummified berries were covered with wheat straw (Becker & Person, 1993). The severity of black rot on clusters was significantly reduced by up to 62% when mummified berries were removed compared to control and number of ascospores released was often reduced to undetectable levels (Becker & Person, 1993). A significant reduction in *V. inaequalis* inoculum and scab symptoms on apple was achieved with various leaf litter management strategies, including shredding of leaf litter, application of urea and/or biocontrol products to leaf litter, leaf sweeping with leaf ploughing within rows (Sutton *et al*., 2000; Vincent *et al*., 2004; Holb *et al*., 2006; Gomez *et al*., 2007).

Studies on *G. bidwellii* and *V. inaequalis* indicated that the correct timing of mulching and/or leaf litter removal is of utmost importance and is linked to the epidemiology of the

disease. Similar to chemical control programmes, sanitation practices aiming at reducing/eliminating the inoculum in overwintering leaf litter has to be applied before the onset of the critical infection period. In SA the critical fruit infection period is from October to January when newly set fruit are highly susceptible and infective mature ascospores are released at the commencement of the rainy season (Kotzé, 1981). These high levels of CBS control obtained through sanitation in this study can be ascribed to thorough leaf litter removal or confinement of the inoculum prior to the onset of the critical infection period.

The type of mulching material is important as the decay rate of material with a high carbon/nitrogen ratio immobilises nitrogen and can result in a temporary nitrogen shortage (Casale *et al*., 1995). Wheat has a carbon/nitrogen ratio of about 100 and ratios above 100 are considered to be too high and unsuitable for use as mulch (Handreck & Black, 1994). Since citrus trees normally produce nearly 80% of their roots in the top 50 cm of soil (Cahoon *et al*., 1956), the effect of decomposing wheat mulch on citrus growth and soil nutrient levels should be evaluated in future studies.

This type of sanitation practices would most likely be applied in organic orchards as the cost for manual removal of leaf litter and even mulching is very high. Leaf removal in the specific orchard near Burgersfort was terminated after the 2004/2005 season mainly due to labour cost implications. Manual removal of leaf litter in the 215 ha estate was also very difficult to complete within such a short time (August to beginning of October). Nevertheless, reduction in CBS can be achieved by persistent and entire removal, inactivation or immobilisation of overwintering inoculum residing in infected leaf litter on the orchard floor.

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CHAPTER 8

General discussion

Citrus black spot (CBS), caused by *Guignardia citricarpa* Kiely, gained prominence as an economical important disease of citrus in the 1990's due to the phytosanitary risk associated with infected plant material. Restrictive trade barriers have been introduced to more effectively regulate the movement of citrus fruit from CBS-infected production regions to CBS-free countries in the Mediterranean and European Union (EU), as well as in Chile, Japan, United States of America and New Zealand (European Union, 1998; Paul *et al*., 2005; Everett & Rees-George, 2006; Lemon & McNally, 2010). A whole consignment of fruit may be rejected, if, during inspection at packinghouses or ports, one spot on one fruit within the consignment is found (European Union, 1992; Bonants *et al*., 2003). Consequently, CBS has a great impact on global citrus trade and is of great concern to affected growers.

The disease originated in south east Asia (Smith *et al*., 1997) and has spread world-wide to summer rainfall production areas mainly through infected, but symptomless nursery trees (Kiely, 1949; Wager, 1952; Calavan, 1960; Kotzé, 1981). The main source of inoculum of CBS in infected orchards is ascospores produced only on leaf litter (Kiely, 1948; Kotzé, 1981). Pycnidiospores, when present on symptomatic fruit or twigs within a citrus tree, may also be a source of inoculum. Symptomatic CBS fruit may contain pycnidia with viable pycnidiospores and are regarded by the EU as a source of inoculum for CBS-free areas, therefore justifying their phytosanitary regulations. Furthermore, infected symptomless fruit may develop symptoms during transport or storage, increasing the possibility of symptomatic fruit reaching European markets.

Symptomatic CBS fruit or peel lying on the ground underneath citrus trees is not considered by us to be a source of inoculum. Only pycnidiospores are produced on symptomatic fruit and these spores have a relative short viability period. The current study clearly demonstrated that pycnidiospores of *G. citricarpa* from various sources failed to infect mature detached green leaves or leaf litter under controlled and field conditions. This is the first report on artificial inoculation of leaf litter with pycnidiospores of *G. citricarpa*. Symptomatic fruit or peel lying on the ground underneath citrus trees therefore cannot lead to infection and colonisation of freshly detached leaves and leaf litter by *G. citricarpa* and do not contribute to the production of subsequent inoculum in an orchard.

Therefore, commercial fruit are not considered to be a high risk for introduction of the pathogen into areas free of CBS.

Since *G. citricarpa* cannot infect freshly detached citrus leaves or leaf litter as shown in the current study, leaves have to be infected by the pathogen while still on the tree. Therefore, the inoculum produced on the leaf litter, thus depends on the level of infection of young leaves while attached to the tree (Kiely, 1948; Wager, 1952; Kotzé, 1963; McOnie, 1964c; Whiteside, 1967). Infected young citrus leaves forms a vital part in the survival of the pathogen and the period of susceptibility of citrus leaves to *G. citricarpa* was investigated in the current study.

The current study provided the first scientifically-founded data, substantiated by molecular identification of the pathogen, on the duration of the susceptibility to CBS of newly emerging citrus leaves monitored over time. The study indicated that the susceptibility period of citrus leaves to infection by the black spot pathogen was up to 10 months, considerable longer than previously perceived. Citrus trees can produce more than one new leaf flush per year. This implies that some part of the leaves on a citrus tree will be susceptible to *G. citricarpa* throughout the year. This, together with the long susceptibility period of newly formed leaves, makes chemical control of leaf infections unpractical. Therefore, apart from protecting susceptible fruit, control should also focus on reducing inoculum in the orchard.

Although sanitation practices in citrus orchards whereby infected late hanging fruit are removed before the new crop sets and pruning of dead and possible infected twigs are widely practiced within SA, no sanitation practices are currently employed to reduce inoculum from leaf litter (Kotzé, 1981). The current study showed that leaf litter removal or mulching can provide an alternative to chemical control. Sanitation through leaf litter management can also improve control in an integrated approach. Environmental and human health concerns have led to increasing restrictions on the use of chemical fungicides and greater focus on alternative non-chemical control measures that can contribute to disease control and resistance management in an integrated approach (Janisiewicz & Korsten, 2002).

Regardless of the prevailing climatic conditions each year, control achieved with litter management in the current study resulted in control equal to that achieved with the industry standard for fungicides (Schutte *et al*., 2003). This study confirmed the findings of Schutte & Kotzé (1997) that sanitation practices, such as leaf litter removal and

mulching of leaf litter with wheat straw can decrease the primary inoculum of CBS and contribute to better management of the disease in a commercial orchard. This type of sanitation practices would most likely be applied in organic orchards as the cost for manual removal of leaf litter and even mulching is very high. Leaf removal in the orchard used for this study, was terminated mainly due to labour cost implications. Also, manual removal of leaf litter in the 215 ha estate was very difficult to complete in less than three months (August to beginning of October). Nevertheless, reduction in CBS can be achieved by persistent and entire removal, inactivation or immobilisation of overwintering inoculum residing in infected leaf litter on the orchard floor.

Once *G. citricarpa* has infected young citrus leaves, the pathogen usually remains latent as a small knot of mycelium directly under the cuticula (McOnie, 1967), until leaf drop and senescence (Kiely, 1948; Kotzé, 1981). After leaf drop, the pathogen is able to grow saprophytically and produce spores on the dead leaves within 40 to 180 days, depending on the temperature and frequency of wetting (Kotzé, 1981). The rate and severity of spore production, especially ascospores, on newly formed leaf litter in an orchard will provide valuable information on availability of inoculum and potential infection events.

The Kotzé Inoculum Monitor (KIM) was successfully applied to capture ascospores of *G. citricarpa* from naturally formed citrus leaf litter. Ascospores were captured from leaf litter collected during October to March each year with peak ascospore availability between December to February. Ascospore production was seasonal with most spores captured from leaf litter collected between October and February each year and no spores collected during the winter months. This seasonal production and maturation of spores has been reported for *G. citricarpa* as well as numerous other fungi (Pady, 1957; Kotzé, 1963; McOnie, 1964a, b; Chatterjee & Hargreave, 1974; Smith, 1996; Guerin *et al*., 2001; Rossi *et al*., 2001; Swart & Kotzé, 2007). The peak ascospore production recorded in the current study also corresponds to the period of reported fruit susceptibility in SA (Kotzé, 1981), starting from flowering (September to October) up to five months later (February to March).

The study using the newly developed KIM provided supporting information on ascospore maturity not previously accessible with the field-based volumetric sucking-type spore traps, such as the Hirst and Burkard versions. The KIM has the advantage over field-based spore traps of providing information on the presence of mature, ready to be dispersed, ascospores on leaf litter before a natural spore release event. Another advantage of the KIM is that variations in external factors such as temperature, water

(dew/rain) and wind are eliminated, making data from different samples from the same orchard over time or from different orchards more comparable. Inoculum densities between orchards can be compared and the potential CBS risk can be assigned to these orchards, which in turn will contribute to improved management of the disease.

Various citrus production regions in South Africa have officially been declared free of CBS and include some of the regions in the Northern Cape, Free State, North West and all the citrus producing regions within the south-western Western Cape Province (European Union, 1998; Mabiletsa, 2003; APHIS, 2009, Shea, 2010). To verify and maintain the pest-free status of a production region, extensive monitoring work is required. In the absence of symptomatic fruit or sporulating fruiting bodies of *G. citricarpa* on leaf litter, detection techniques relies on isolations and DNA amplification with species-specific primers from symptomless plant tissue. Generally, detection of the pathogen from symptomless fruit or leaf material has a low success rate due to the restricted growth of the pathogen in latently infected tissue. An artificial leaf wilting method was optimised in the current study to provide an alternative detection method for *G. citricarpa* from symptomless leaves.

Alternate wetting and drying of leaf litter and variation in temperature have been reported to provide optimal conditions for spore formation and maturation (Kiely, 1948; Kotzé, 1981). Kiely (1948) described an artificial wetting and drying technique to induce sporulation of the CBS pathogen on freshly detached mature green leaves. However, few researchers have applied the technique with success (Wager, 1952; Kotzé, 1963; McOnie, 1964c, 1967; Whiteside, 1967). Most attempts to replicate the artificial leaf wilting described by Kiely in the current study failed. Results achieved were too variable and the method was found as not suitable for application in routine surveys.

After several adaptations from the original method described by Kiely (1948), formation of visual fungal fruiting structures on treated leaves developed after six to 14 days, in the current study. This is significantly faster than in the field under natural conditions or reported from Kiely's wilting treatment. Furthermore, detection of the pathogen was improved considerably when combining the artificial leaf wilting with polymerase chain reaction (PCR) with species-specific DNA primers compared to PCR results of untreated green leaves or treated leaves without PCR. The artificial leaf wilting technique was very reliable, fast and effective in enhancing growth and sporulation of the CBS pathogen in latently infected citrus leaves. The wilting treatment in combination with PCR can be used to monitor citrus nurseries and orchards throughout the year, especially for CBS-free

orchards to verify and maintain its pest-free status. Larger samples rather than smaller ones should be used due to natural variation in level of infection in leaves. This is the only detection method not dependant on season for sample collection and can greatly enhance the detection of the CBS pathogen throughout the year.

Some of the outcomes of this study have been included in a pest risk assessment (PRA) on CBS that have been presented to the EU. This study supports the PRA of South Africa stating that the risk associated with fruit for introduction of *G. citricarpa* is low. It is also my opinion that the current EU phytosanitary regulations pertaining to *G. citricarpa* on fresh citrus fruit imported into the EU are without adequate technical justification and are unnecessarily restrictive and disruptive to trade relevant to risk. In accordance with the International Plant Protection Convention principles of technical justification and minimal impact, failure to overturn current phytosanitary regulations pertaining to *G. citricarpa* on fresh citrus fruit imports would constitute an unjustified technical barrier to trade. It is recommended that the current EU phytosanitary regulations pertaining to *G. citricarpa* in association with fresh fruit should be re-evaluated.

Some aspects of the pathogen-host interactions require further clarification and future work should focus on:

- asco- and pycnidiospore production on leaf litter, and possibilities to reduce or inhibit especially ascospore production on the leaf litter without the need for labour intensive removal or confinement of the leaf litter.
- re-evaluation of leaf and fruit inoculations in the field as greenhouse studies may not be representative of field conditions.
- refined optimal as well as extreme conditions for infection of susceptible host material.
- survival of *G. citricarpa* in latently infected citrus plants. Can the pathogen be eradicated from an infected tree and to what extent does the pathogen move within citrus tissue?
- interaction between *G. citricarpa* and *G. mangiferae* isolates in the same host tissue. Do these two fungi compete for space and nutrients, and does *G. mangiferae* influence *G. citricarpa* in any way with disease expression?
- the underlining mechanisms of symptom development and the conditions required for formation of different symptom types on fruit.
- improved detection methods that can distinguish between *G. citricarpa* and *P. citriasiana*, the causal agent of citrus tan spot.

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Appendix A. Description and operation of the Kotzé Inoculum Monitor

The Kotzé Inoculum Monitor (KIM) (Fig. A.1) operates through an electric evacuator motor, which requires a power source of 220/250 volt AC. The evacuator motor extracts air from the low-pressure chamber, resulting in the air being drawn via the plant material contained in a circular plastic grid into the funnel-shaped hopper. Velocity of the spore-laden air increases considerably as it is sucked through the gradually tapering ducting and eventually a jet of air passes through the orifice at the base of the ducting. At a distance of 3 mm away from the exit of the orifice, and in a plane perpendicular to the jet of accelerated air, the air strikes the surface of an adhesive-coated standard microscope slide or a Petri dish (65 mm diam.) containing nutrient medium, causing the spores to adhere to the sticky surface.

The microscope slide or Petri dish is mounted on a carrier frame (Fig. A.2), which, over a period of two hours, moves linearly through a distance of 30 mm. This movement is achieved by means of a clockwork device and switch combination. The purpose of this movement is to deposit spores over an extended area that could be related to the actual time of spore release.

Winding of the clockwork automatically switches on the evacuator motor and starts the two-hour operating cycle. On completion of the cycle, the evacuator motor switches off as the clockwork fully unwinds with an audible click. The construction of the air-duct, which connects the hopper to the low-pressure chamber, is designed so that water dripping from the plant material is drained away from the system without affecting the functioning thereof. The complete stainless steel construction of the KIM protects the operating process from the effects of static electricity.

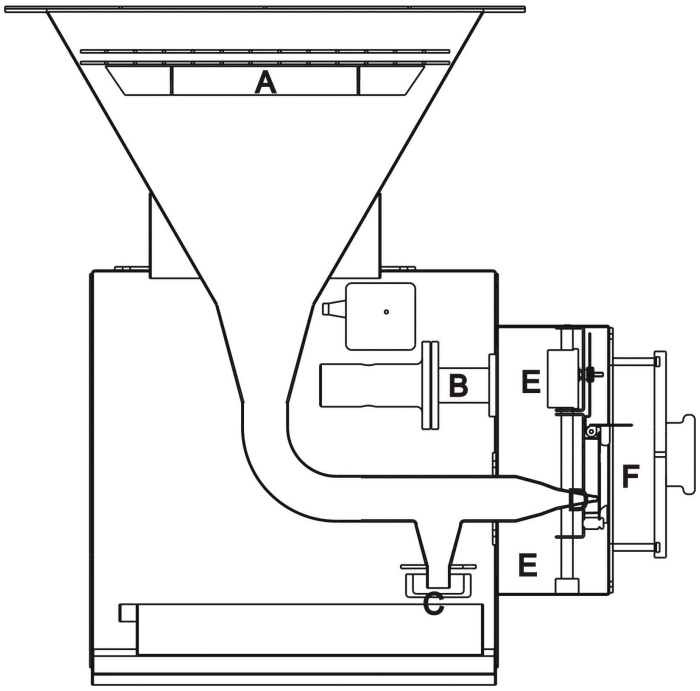

Figure A.1. Diagrammatic cross-section of the Kotzé Inoculum Monitor unit. A: grid support for plant material in hopper; B: evacuator motor unit; C: water trap with overflow water tray; D: air orifice; E: low-pressure chamber; F: carrier for Petri dish or microscope slide.

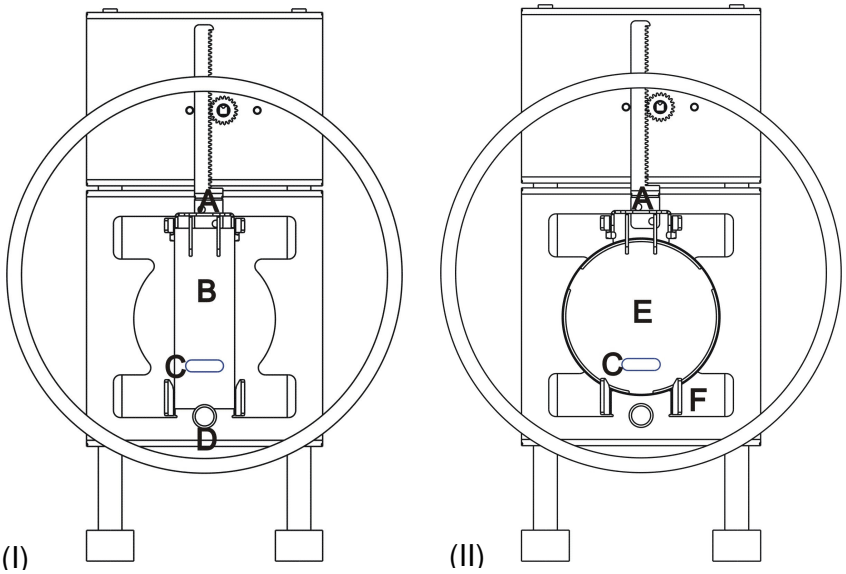

Figure A.2. Diagrammatic cross-section of the front view of the low-pressure chamber to illustrate position of microscope slide (I) and Petri dish (II). A: gravity catch; B: microscope slide; C: orifice (behind the slide or dish); D: slide support pin; E: Petri dish; F: carrier bracket.

Appendix B. Protocol to artificially wilt green citrus leaves to improve detection of *Guignardia citricarpa* in latently infected leaves

1. Picked mature green leaves randomly from all four sides of the tree, with about 20 leaves per tree and from at least 20 trees per orchard block.
2. Keep detached leaves cool and process within six hours.
3. Wash leaves in running tap water to remove dirt and drain to remove excess water.
4. Air dry leaves for 12 hours out of direct sunlight OR air dry leaves for two to four hours in direct sunlight.
5. Soak air-dried leaves in tap water for 30 minutes, drain to remove excess water and place in a 20 µm thick clear plastic bag. Use 20 to 50 leaves per bag, depending on size of leaves and bag.
6. Closed bag, including as much as possible air within the bag, and place bag with leaves in an incubator at 42 ℃ for 6 h.
7. After 6 h, remove the bag from the incubator and mixed leaves by shaking the bag.
8. Open the bag to allow leaves to air dry and incubate under florescent and near-UV light for 18 h.
9. Repeat steps 5 to 8 for at least 21 days or until ample fructification of *Guignardia* is visible on the leaf surface.

Note: It is important to monitor the moisture within the bag closely, since no fungal fruiting structures will develop if the leaves are to dry and the leaves will rot if it is too wet. Unfortunately the correct moisture levels are only known through experience. Leaves have to air-dry completely on a daily basis to limit the growth of other fungi such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.