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.

3.6 References

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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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