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.

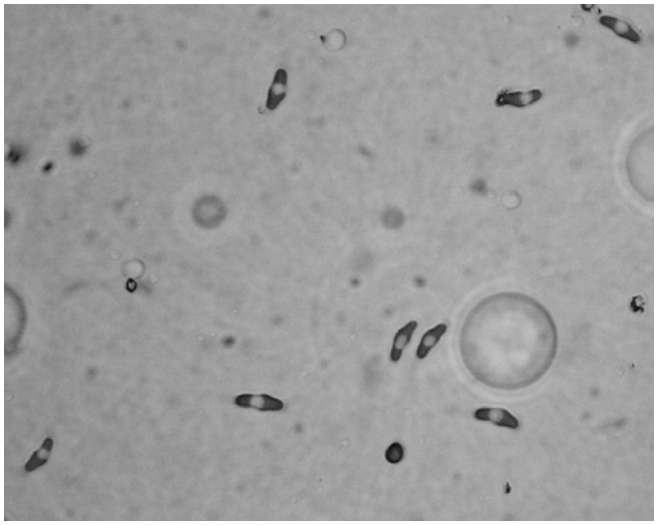

Figure 5.2. Ascospores of ***Guignardia citricarpa*** collected from Eureka lemon leaf litter on silicone-coated microscope slide with the Kotzé Inoculum Monitor.

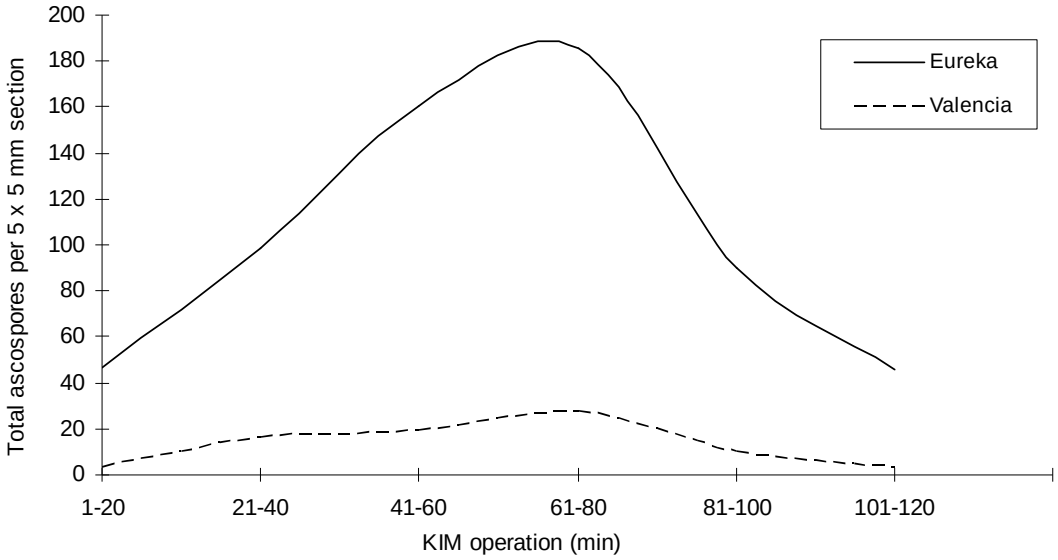

Figure 5.3. ***Guignardia citricarpa*** ascospores collected with the Kotzé Inoculum Monitor (KIM) from Eureka lemon and Valencia orange leaf litter. Total ascospore counts were done along the centre transect row (30 x 5 mm) in 25 mm^2 sections of a microscope slide coated with silicon. Each 25 mm^2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

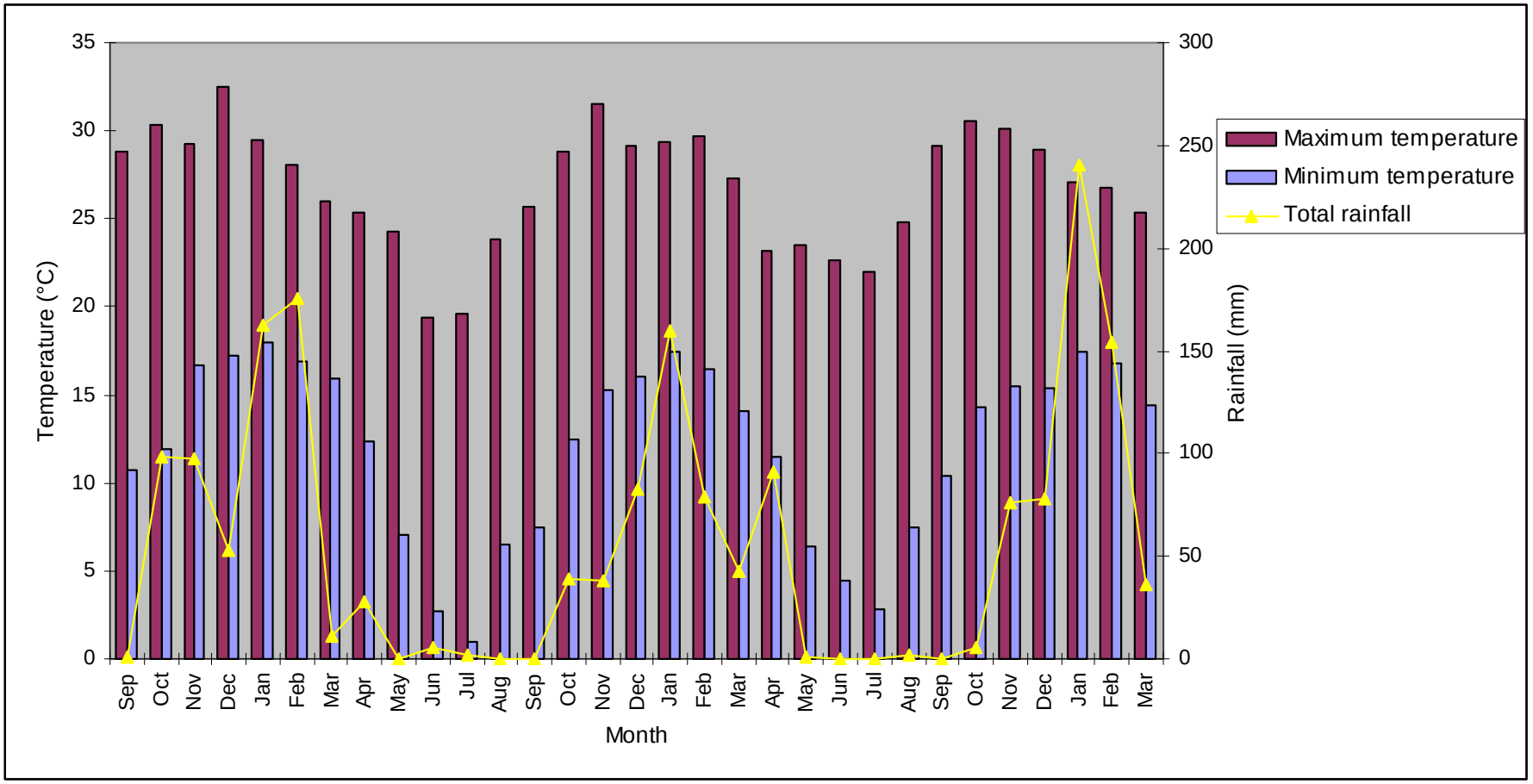

Figure 5.4. Mean maximum and minimum temperature and total rainfall per month recorded in Mooinooi in North West Province during September 2003 to March 2006. Data obtained from the South African Weather Service.

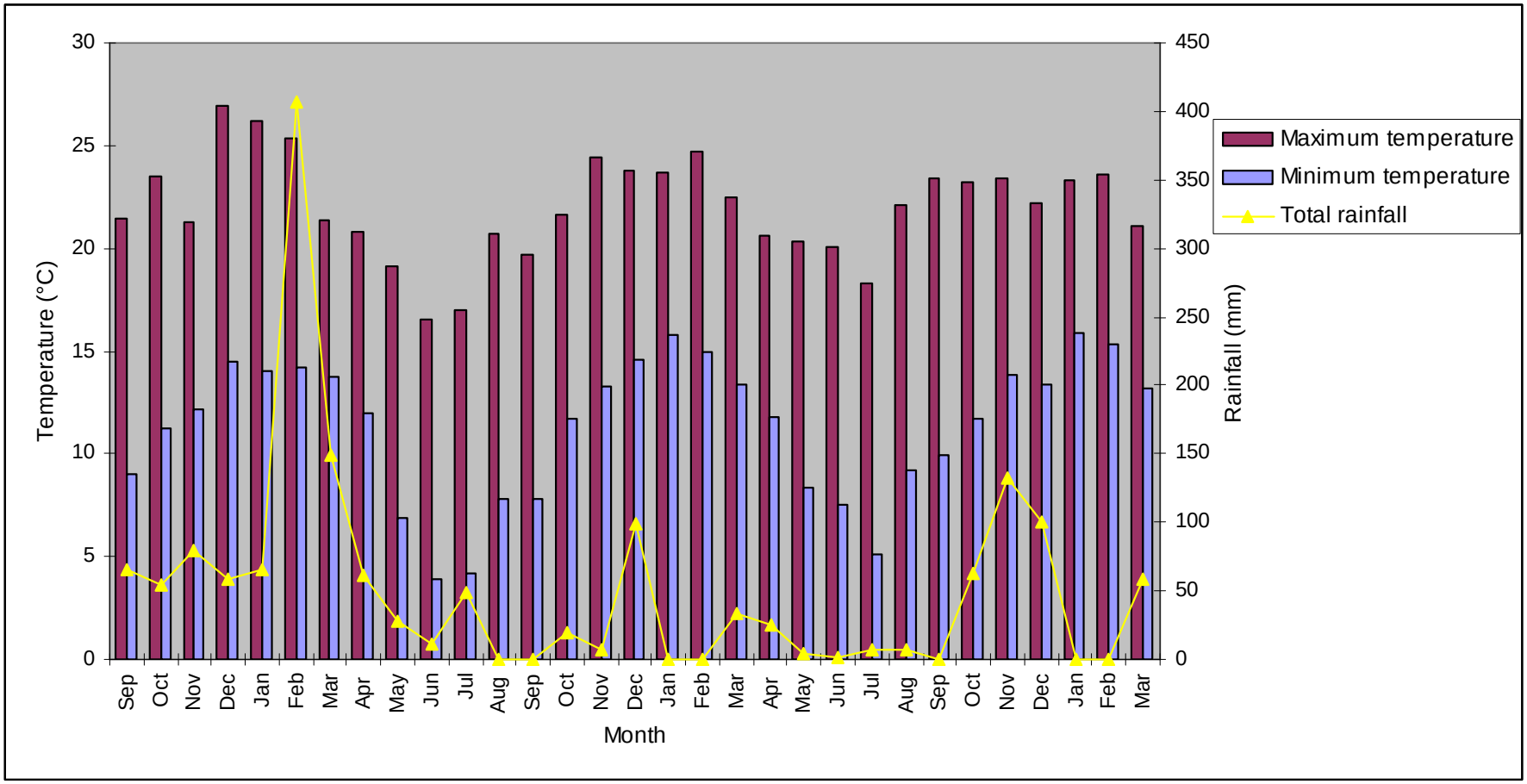

Figure 5.5. Mean maximum and minimum temperature and total rainfall per month recorded in Burgersford in Mpumalanga Province during September 2003 to March 2006. Data obtained from the South African Weather Service.

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.

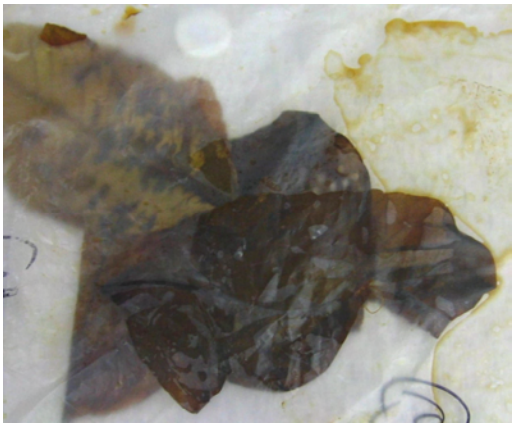
A  B 

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.

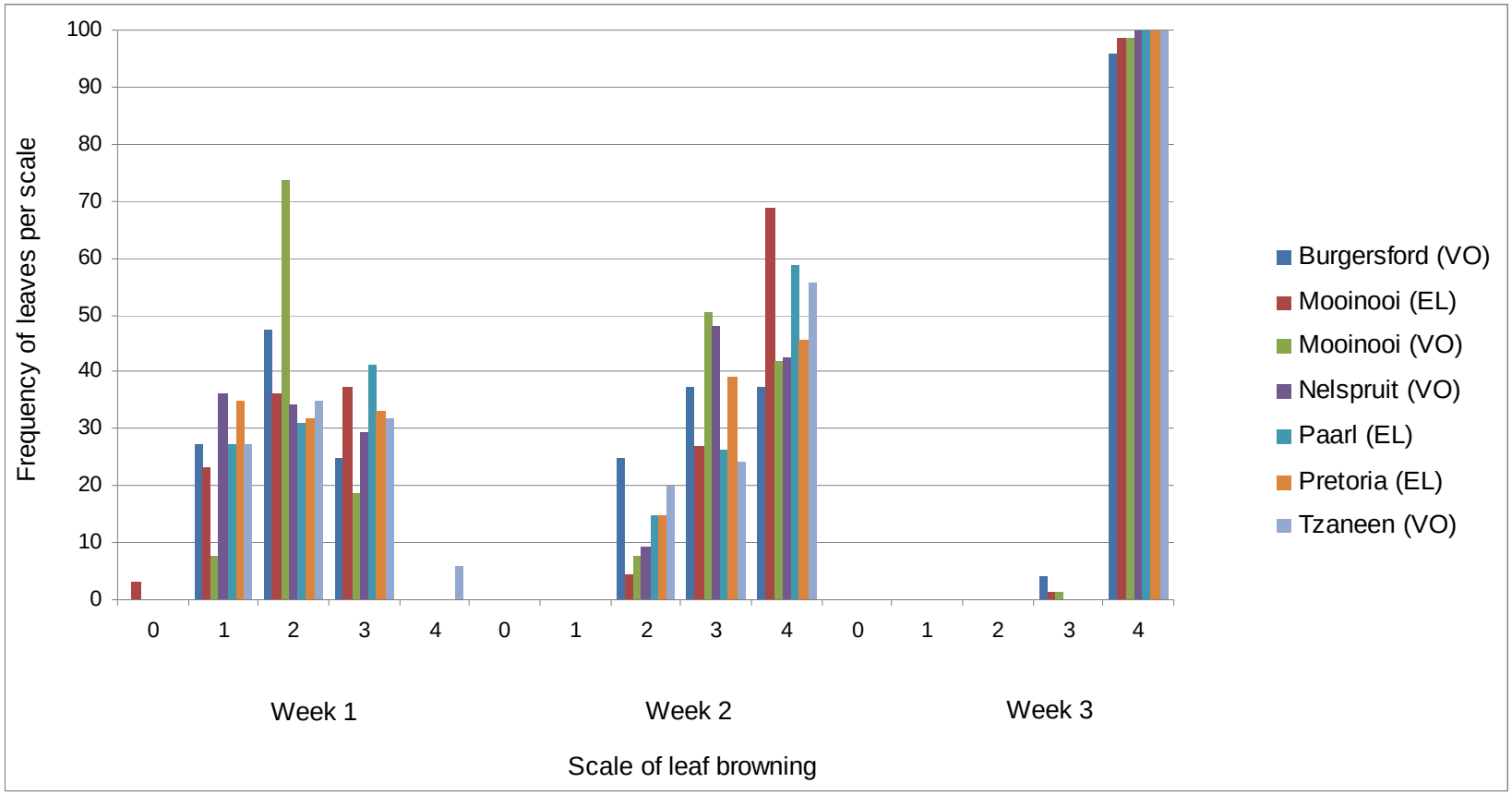
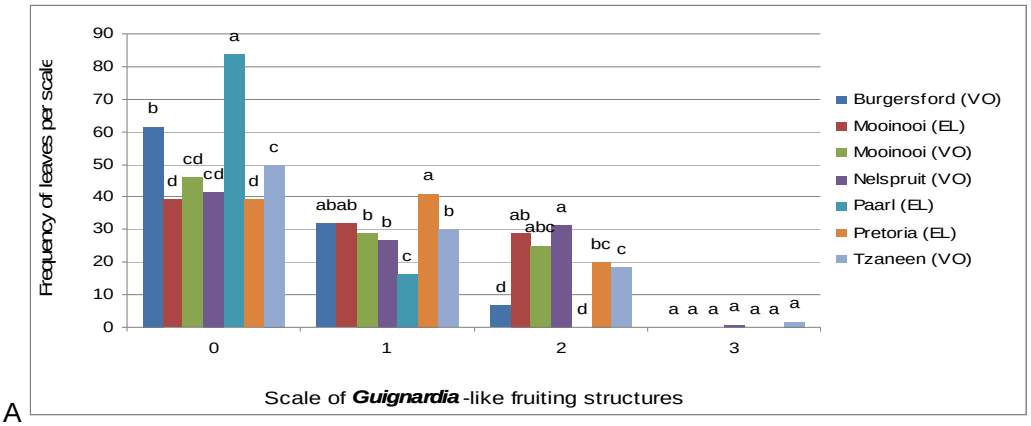
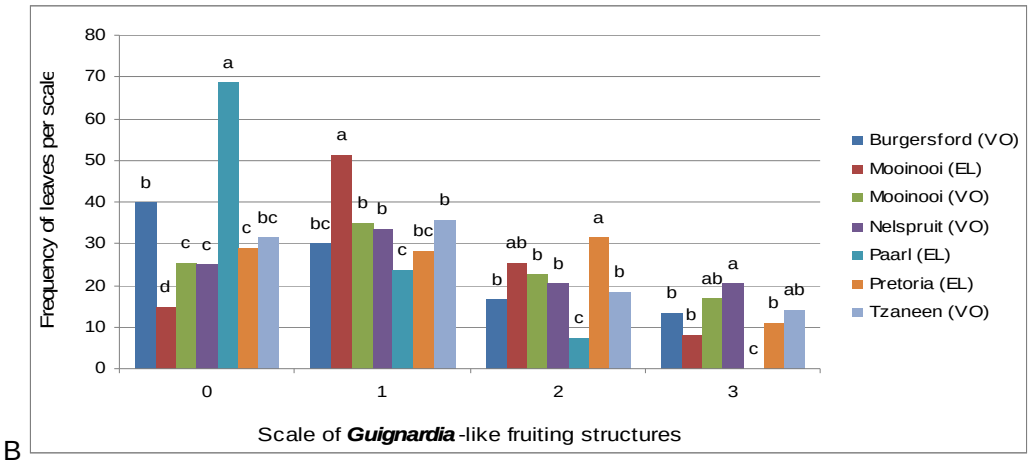


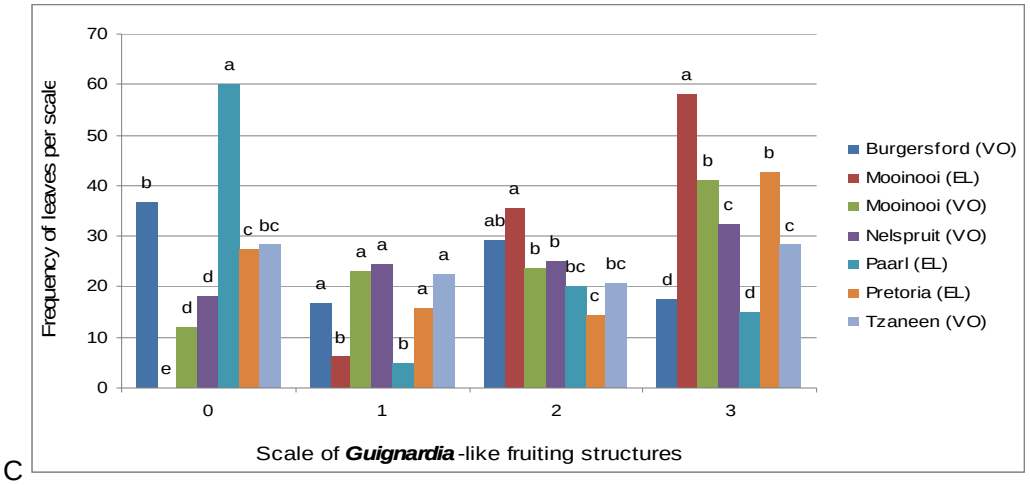
Figure 6.4. Leaf browning of treated mature green Eureka lemon (EL) and Valencia orange (VO) leaves expressed as frequency of leaves in each rating scale (0 to 4), 7, 14 and 21 days after wilting treatment commenced using 20 µm thick clear plastic bags. Leaf browning was scored on a five point ordinal 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.

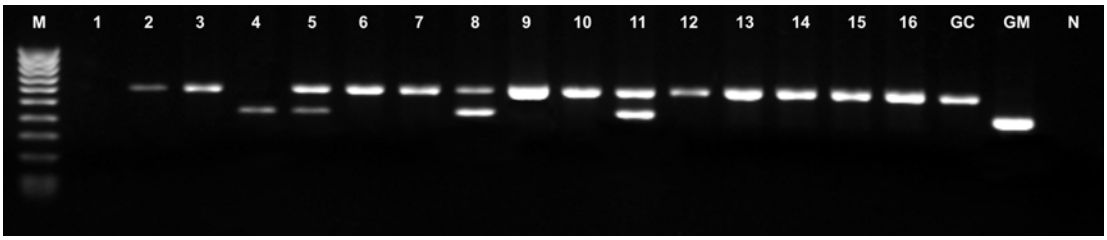


Figure 6.6. PCR amplicons of ***Guignardia citricarpa*** and ***Guignardia mangiferae*** from DNA extracted from symptomless citrus leaves. 1 to 7 = green, un-wilted leaves, 1 = Valencia Orange (VO) from Burgersford, 2 = VO from Nelspruit, 3 = VO from Tzaneen, 4 = Eureka lemon (EL) from Paarl, 5 = VO 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.

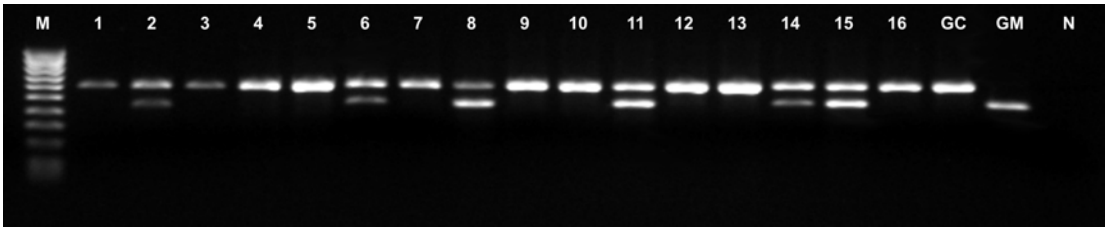


Figure 6.7. PCR amplicons of ***Guignardia citricarpa*** and ***Guignardia mangiferae*** from DNA extracted from symptomless artificially wilted citrus leaves. 1 to 6 = leaves wilted for seven consecutive days, 1 = Valencia Orange (VO) from Nelspruit, 2 = VO from Burgersford, 3 = VO from Tzaneen, 4 = 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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