CHAPTER 4

IN SITU SURVIVAL OF *PHYLLOSTICTA CITRICARPA*

ABSTRACT

Conidia of the citrus black spot (CBS) anamorph, *Phyllosticta citricarpa*, did not infect unwounded harvested packhouse-treated citrus fruit at 0.5 and 25 °C in artificial and natural inoculation studies. A low percentage infection of wounded fruit was evident at 25 °C, but not at 0.5 °C. Conidial adhesion occurred more readily on the synthetic polyethylene micro-wax layer applied to fruit than on the natural waxy surface layer. Cobalt irradiation of up to 400 Gy did not eliminate inoculum of *P. citricarpa* on CBS-infected fruit. Packhouse procedures which consistently reduced the incidence of *P. citricarpa* in CBS lesions included warm water treatment (43-47 °C for three minutes), chemical tank (imazalil sulphate, guazatine and 2,4-D), and a combination of chlorine, high-pressure spraying, warm water, the above chemicals, and polyethylene waxing. Conidial viability was reduced to zero by keeping CBS-infected fruit for three weeks at 25 °C, as well as by exposure of the fruit to chlorine, warm water, the above chemicals or all treatments. Fungicides in the triazole group, although not registered for postharvest use in citrus, substantially reduced the mycelial inoculum present in CBS lesions. Conidia of the pathogen were inactivated by various fungicides not registered for postharvest treatment of citrus, one notable exception being the systemic plant-defence activator, acibenzolar-*s*-methyl.

INTRODUCTION

Phyllosticta citricarpa (McAlp.) Van der Aa is the anamorph of the citrus black spot (CBS) pathogen, *Guignardia citricarpa* Kiely (Sutton & Watterston, 1966). Although the asexual phase does not play a significant role in the CBS disease cycle (Kiely, 1948; Kotzé, 1963; McOnie, 1964), the pathogen occurs in this form on fruit, where it may develop further during storage or shipment (McOnie, 1964; Kotzé, 1981). Evidence nevertheless indicates

that the pycnidial stage poses no threat as a source of infection, except when conidia from out-of-season or late-hanging fruit are washed onto young susceptible fruit below (Darnell-Smith, 1918; Kiely, 1948; Wager, 1949; Kotzé, 1963, 1981).

Citrus is produced in South Africa mainly as an export commodity. Most overseas markets, despite applying stringent phytosanitary standards, have thus far accepted CBS-infected fruit. In recent years, however, European Union countries have expressed concern that the CBS pathogen from South Africa may become established in their orchards, and hence adopted legislation which prevents the introduction of fruit infected with CBS to European countries free of the disease. This obviously necessitates a reappraisal of the potential of *P. citricarpa* as a source of infection, particularly regarding its fate on fruit after picking.

In this report evidence is presented on the infectivity of *P. citricarpa* in artificial and natural postharvest inoculation studies, and on the effect of radurisation and standard packhouse procedures on its viability. As the various experiments also facilitated monitoring of other citrus fruit pathogens, details in this regard are provided as well.

MATERIALS AND METHODS

Artificial inoculation of citrus fruit with *P. citricarpa*. A benomyl-resistant isolate of *P. citricarpa* from Mpumalanga Province (PPRI 5027), South Africa, was cultured for 10-14 days at 22 °C under continuous fluorescent lighting and high humidity on a medium consisting of 24 g potato-dextrose agar (PDA) (Merck Biolab), 1 g yeast-extract (Difco), 1 g malt-extract (Difco) and 8 g agar (Merck Biolab) per litre of deionised water (hereafter referred to as PYMA). Conidia were harvested by aseptically transferring the pycnidial crust of each culture to a 500 ml Erlenmeyer flask containing 200 ml sterile deionised water (SDW), and agitating the flask for one hour on a slow-speed rotary shaker. The resultant conidial suspension was dispensed into sterile centrifuge tubes and centrifuged twice for five minutes at 5 000 rpm. Pellets of conidia were suspended in SDW and the conidial

concentration was adjusted to 10^7 ml^{-1} spores/conidia for inoculation purposes. A fresh conidial suspension was prepared for each inoculation date referred to below.

CBS-free Valencia oranges (*Citrus sinensis* (L.) Osbeck) were collected from Crocodile Valley packhouse, Nelspruit, South Africa. The fruit were packed into cartons, ready for export, and passed through a packhouse line consisting of a chlorine bath (100 ppm, pH 7, for three minutes), high-pressure descaler water spray (20-30 kPa), hot water bath (42 °C for three minutes), fungicide brushing with 1000 ppm imazalil sulphate, and polyethylene waxing. A total of 3 000 fruit were inoculated with *P. citricarpa* as set out in Table 1. To account for physiological changes in the fruit, inoculation took place over a three-week period at the beginning of each week, using 250 fruit for each temperature/inoculation procedure on each occasion. Inoculation was facilitated by arbitrarily delineating a circle, ca. 15 mm in diameter and ca. 3 mm deep, with silicone sealant on the surface of each fruit (Fig. 1A). Prior testing indicated that the sealant, once set, has no inhibitory effect on *P. citricarpa* (Fig. 1B). Wounding of fruit was accomplished by puncturing the peel 3 mm deep at two spots within each silicone circle with a self-designed two-pronged punch. Each circle, whether wounded or not, received 500 µl of conidial inoculum.

After inoculation, fruit were stored for three weeks at 0.5 or 25 °C at Outspan Citrus Centre in Nelspruit and then transported to the University of Pretoria, where they were kept at 25 °C for a further week to simulate overseas marketing conditions. All 250 fruit per treatment were inspected for visible symptoms caused by the CBS pathogen. The fruit selected for isolations were surface-disinfested for 3 min in 70 % ethanol. Three citrus peel sections (3mm^3) from within the silicone circle on each of 100 fruit from each treatment were aseptically dissected and plated on PYMA. Sections yielding *P. citricarpa* were recorded after incubation for seven days at 27 °C. Plates were also screened quantitatively for the presence of *Colletotrichum gloeosporioides* Penzig, *Penicillium digitatum* (Pers. ex St.-Am.) Sacc., *Penicillium italicum* Wehmer, *Rhizopus stolonifer* (Ehrenb. ex Link) Lind and *Trichoderma viride* Pers. ex Gray. A BMDP 4F data set was completed and the variables (temperature, inoculation procedure and time) tested by means of Chi-square for

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

relationship with the incidence of the organisms. With $df = 2$ and $P > 0.05$, Chi-square values lower than 3.84 indicated no statistical significance.

For electron microscopy, sections of citrus peel were cut from within the silicone circle on 20 fruit from each treatment and fixed for 24 hours in 6 % glutaraldehyde, followed by three 15 minute washes in 0.7 M phosphate buffer. Fixed samples were each dehydrated for 15 minutes in increasing concentrations (50, 70, 90 and 100 %) ethanol and dried in a Hitachi HCP-2 critical-point drier. After gold-plating in an Eiko IB 3 ion-coater, specimens were viewed in a Hitachi 450 scanning electron microscope.
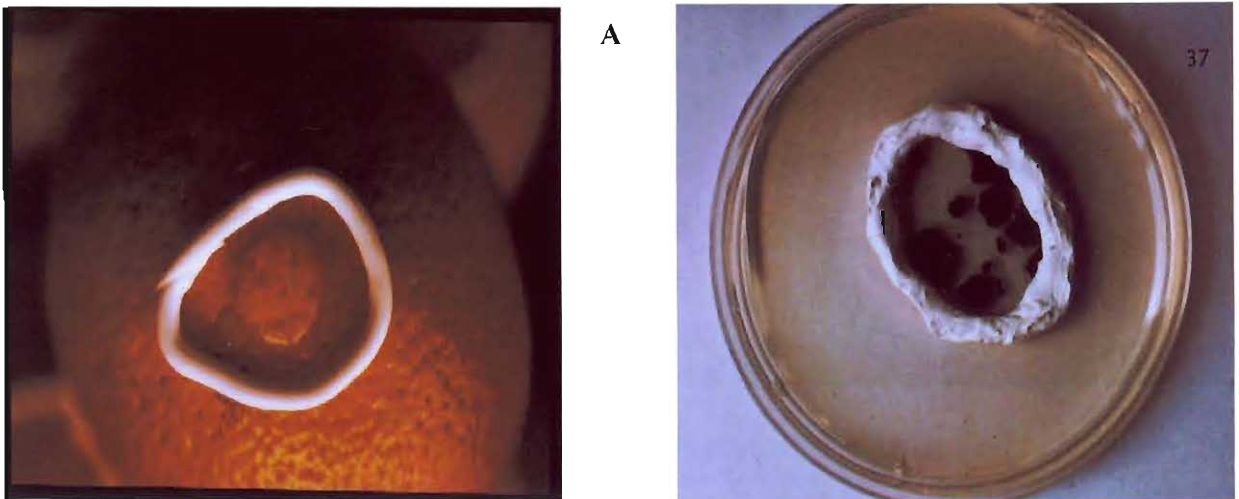

Figure 1: Silicone circle on citrus for retaining artificial inoculum of *Phyllosticta citricarpa* (A) and colonies of *Phyllosticta citricarpa* developing from a conidial inoculum within a silicone circle on nutrient medium, indicating the absence of toxicity of the sealant towards the fungus (B).

Natural infection of harvested fruit by *P. citricarpa*. Valencia fruit extensively covered with hard-spot CBS lesions full of mature pycnidia were collected at Malelane Estates, South Africa. Twenty-five to thirty of these heavily-infected fruit were placed on top of 100

CBS-free packhouse-processed Valencia fruit (Crocodile Valley packhouse, Nelspruit) in each of eight perforated plastic lug-boxes. Four lug-boxes were stored at 0.5 °C and the remaining four at 25 °C at the Outspan Citrus Centre, Nelspruit. One litre of sterile deionised water was gently poured twice a week over the fruit in each box to facilitate dissemination of conidia. After three weeks, the originally-symptomless fruit were inspected visually for symptoms of CBS. Fifty fruit were arbitrarily selected from each box and surface-disinfested for three minutes in 70 % ethanol. Five 5 mm^3 pieces were aseptically cut from each fruit, concentrating particularly on suspected CBS-lesions, and plated on PYMA. Pieces yielding *P. citricarpa* and other citrus fruit pathogens were recorded after incubation for seven days at 27 °C.

Radurisation of CBS-infected fruit. CBS-infected Valencia fruit obtained from Malelane Estates and treated at the Crocodile Valley packhouse as described earlier, were exposed to cobalt irradiation of 100, 200, 300 and 400 Gy at High Energy Processing Cape in Cape Town, using 100 fruit per dosage. Following irradiation, all the fruit were incubated for one week at 25 °C at high humidity, after which isolations were made from 50 fruit of each treatment as described under natural infection.

Effect of packhouse procedures on the viability of *P. citricarpa*. Two experiments were conducted, one in 1997 with Valencia fruit (Vorster Estates, Mahela Farm) severely infected with a benomyl-resistant strain of *P. citricarpa*, and one in 1998 with Navel fruit from Letaba Estates severely infected with a benomyl-sensitive strain of the pathogen. In both instances, fruit were subjected to one of the following packhouse procedures: chlorine bath (100 ppm active chlorine, pH 7, in 1997 and 15 ppm chlorine dioxide in 1998); high-pressure spraying (20-35 kPa); warm water bath (43-47 °C for three minutes); chemical tank (550 ppm imazalil sulphate, Fungazil 75 %, Janssen Pharmaceutica; 1 000 ppm guazatine, Deccotine 20 % SL, Rhône-Poulenc Agrichem; 500 ppm 2,4-D sodium salt, Deccomone 25 % SL, Rhône-Poulenc Agrichem); polyethylene wax application (Citrashine, Dormas); all the above processes; none of the processes. The number of fruit per treatment was 300 in 1997 and 200 in 1998. After treatment, 100 fruit from each process were kept for three weeks at 4.5, 10 or 25 °C (1997), and at 4.5 or 25 °C (1998). At the end of the three weeks,

50 fruit from each treatment at each temperature were arbitrarily selected and isolations made from hard spot lesions as previously described.

Viability of *P. citricarpa* conidia in the lesions was also determined on five additional fruit per treatment. Three viable hard spot lesions (Chapter 2) on each fruit were marked with a permanent marker and a drop of spore germination medium (Chapter 3) was pipetted onto each spot. Release of conidia was observed under a stereo-microscope, a process lasting between 30 seconds and 30 minutes to take place. When sufficient conidia were released into the germination medium (ca. 50 % of the pycnidia present in a CBS lesion), they were harvested with a sterile 0.5 mm diameter capillary tube and streaked onto a PYMA plate. It was not possible to quantify the conidia harvested with the capillary tube because of the small sample volume. However, an estimate of the conidial concentration present in the capillary tube would be between 10^2 and 10^5 conidia ml^{-1}. Plates were incubated for 14 days at 22 °C under fluorescent lighting and high humidity, and the number of *P. citricarpa* colonies that developed was recorded.

Survival of *P. citricarpa* on citrus fruit treated with unregistered fungicides. Valencia fruit from Malelane Estates containing viable CBS hard spots were incubated for one week at 25 °C under fluorescent lighting to promote formation of red freckle spots. *P. citricarpa* from these fruit tested positive for benomyl resistance. A batch of 150 fruit was dipped for one minute into one of the following aqueous fungicide suspensions: 350 ml 100 l^{-1} difenoconazole (Score 25 % EC, Ciba-Geigy); 30 ml 100 l^{-1} tebuconazole (Folicur 25 % EW, Bayer); 150 ml 100 l^{-1} triforine (Denarin 19 % EC, Shell); 250 ml 100 l^{-1} propamocarb-HCl (Previcur N 72.2 % SC, Schering); 50 ml 100 l^{-1} procymidone (Sumisclex 25 % SC, Agrihold); 20 g 100 l^{-1} kresoxim-methyl (Stroby 50 % WG, BASF); 40 ml 100 l^{-1} azoxystrobin (Ortiva 25 % EC, Zeneca Agrochemicals) and 12 g 100 l^{-1} acibenzolar-*s*-methyl (Bion 25 % WG, Novartis) The control batch was dipped in water. After treatment, fruit were kept at 25°C in humid conditions under fluorescent lighting. The incidence of *P. citricarpa, C. gloeosporioides* and *P. digitatum* was determined after 3 weeks, and viability of conidia of the CBS pathogen assessed weekly for three weeks as described before, using 50 fruit from each treatment for isolations and 10 fruit for conidial viability testing.

RESULTS

Artificial inoculation of citrus fruit with _P. citricarpa._ _P. citricarpa_ could not be isolated from wounded or unwounded fruit maintained at 0.5 °C and from unwounded fruit kept at 25 °C (Table 2). Wounded fruit kept at 25 °C infrequently produced CBS symptoms (Fig. 2) and, although viable spots sometimes developed (Fig. 3), low percentages (4-8 %) of the pathogen were recovered (Table 2). The Chi-square value of 16.81 ($P=0.0000$) for temperature and inoculation procedure indicates a significant effect of temperature and wounding on infection of fruit by _P. citricarpa_, while the value of 0.07 ($P=0.9652$) over time suggests that the physiological state of the harvested fruit had little or no effect on their susceptibility. With the exception of _T. viride_, the incidence of the other citrus fruit pathogens was related to temperature and, except for _C. gloeosporioides_, also to wounding.

Table 1: Schedule for inoculation of Valencia citrus fruit with _Phyllosticta citricarpa_ at different storage temperatures.

Post-packaging age of fruit at inoculation (weeks)	Wounding of skin	Inoculation procedure		
		Week 1	Week 2	Week 3
1	+	Inoculation (1day 25°C) 0.5°C	0.5°C	0.5°C
	-	Inoculation (1day 25°C) 0.5°C	0.5°C	0.5°C
	+	Inoculation 25°C	25°C	25°C
	-	Inoculation 25°C	25°C	25°C
2	+	0.5°C	Inoculation(1day 25°C) 0.5°C	0.5°C
	-	0.5°C	Inoculation (1day 25°C) 0.5°C	0.5°C
	+	0.5°C	Inoculation 25°C	25°C
	-	0.5°C	Inoculation 25°C	25°C
3	+	0.5°C	0.5°C	Inoculation (1day 25°C) 0.5°C
	-	0.5°C	0.5°C	Inoculation (1day 25°C) 0.5°C
	+	0.5°C	0.5°C	Inoculation 25°C
	-	0.5°C	0.5°C	Inoculation 25°C

Table 2: Recovery of citrus pathogens from wounded and unwounded Valencia fruit artificially inoculated with *Phyllosticta citricarpa* and stored at different temperatures.

Pathogen	Storage temperature (°C)	Wounding of skin	Positive isolations from 100 fruit				Chi-square (Probability)[λ]
			Week 1	Week 2	Week 3	Total	
	0.5	-	0	0	0	0	
	25	-	0	0	0	0	a) 16.81 ($P = 0.0000$)
Phyllosticta		Total	0	0	0	0	b) 16.81 ($P = 0.0000$)
citricarpa	0.5	+	0	0	0	0	c) 0.07 ($P = 0.9652$)
	25	+	7	8	4	19	
		Total	7	8	4	19	
	0.5	-	4	20	0	151	
	25	-	36	93	22	24	a) 406.32 ($P = 0.0000$)
Penicillium		Total	40	113	22	175	b) 40.14 ($P = 0.0000$)
digitatum	0.5	+	14	19	12	45	c) 20 49 ($P = 0.0000$)
	25	+	87	97	29	213	
		Total	101	116	41	258	
	0.5	-	8	2	4	14	
	25	-	7	70	22	99	a) 206.79 ($P = 0.0000$)
Penicillium		Total	15	72	26	113	b) 15.03 ($P = 0.0001$)
italicum	0.5	+	5	5	16	26	c) 11.07 ($P = 0.0040$)
	25	+	29	65	44	138	
		Total	34	70	60	164	
	0.5	-	0	0	0	0	
	25	-	49	23	27	99	a) 297.64 ($P = 0.0000$)
Rhizopus		Total	49	23	27	99	b) 15.98 ($P = 0.0001$)
stolonifer	0.5	+	3	9	0	12	c) 2.56 ($P = 0.2778$)
	25	+	54	40	43	137	
		Total	57	49	43	149	
	0.5	-	0	0	0	0	
	25	-	0	4	0	4	a) 1.38 ($P = 0.2407$)
Trichoderma		Total	0	4	0	4	b) 0.34 ($P = 0.5603$)
viride	0.5	+	0	0	1	1	c) 0.59 ($P = 0.7429$)
	25	+	0	1	0	1	
		Total	0	1	1	1	

Table 2: **(Continued)**

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Pathogen	Storage temperature (°C)	Wounding of skin	Positive isolations from 100 fruit				Chi-square (Probability)[x]
			Week 1	Week 2	Week 3	Total	
	0.5	-	22	17	42	81	
	25	-	21	33	28	82	a) 5.69 ($P = 0.0171$)
Colleto-		Total	**43**	**50**	**70**	**163**	b) 2.53 ($P = 0.116$)
trichum	0.5	+	25	16	11	52	c) 18.05 ($P = 0.0001$)
gloeosporioi-	25	+	17	43	27	87	
des		Total	**42**	**59**	**38**	**139**	

[x] A BMDP4F data set was completed and the variables (temperature [a], inoculation procedure [b] and time [c]) tested by means of Chi-square for relationship with the organisms. With $df = 2$ and $P = 0.05$, Chi-square values lower than 3.84 do not differ significantly.

A
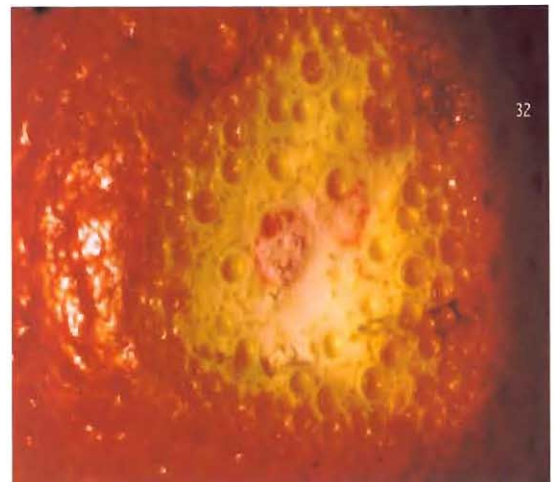
B

Figure 2: Typical early CBS symptoms on wounded citrus fruit artificially inoculated with *Phyllosticta citricarpa* and stored at 25 °C (A) and transverse section through the lesions in "A" showing red coloration of the albedo typical of infection by *Phyllosticta citricarpa* in the early stages of tissue colonisation (B).
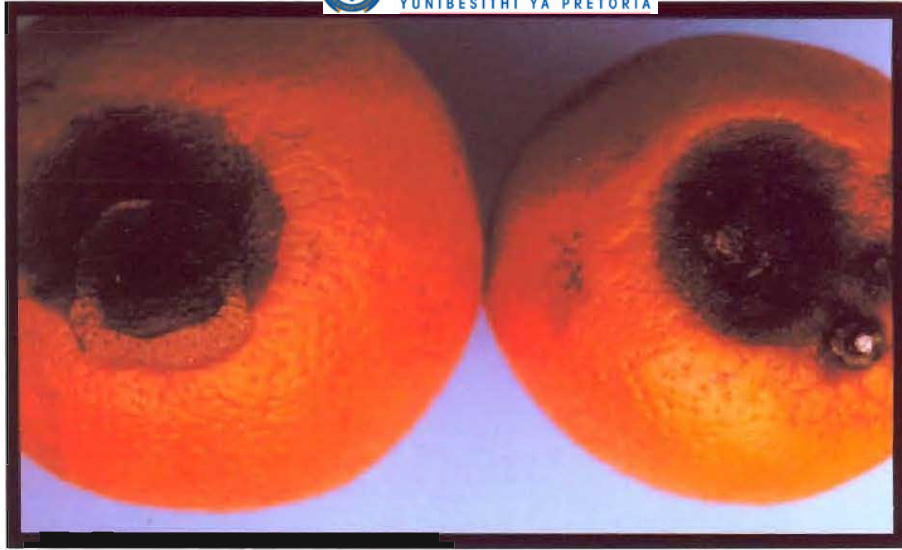
Figure 3: Development of virulent CBS spots on wounded citrus fruit kept for four weeks at 25 °C after artificial inoculation with *Phyllosticta citricarpa*.

Electron microscopy showed conidia of *P. citricarpa* to adhere to the synthetic micro-wax layer (Fig. 4 A and B) rather than to the natural wax layer on the rind (Fig. 5), thus indicating a preference of the conidia for hydrophobic synthetic wax surfaces. The conidia germinated sporadically in the inoculated area within the silicone circle on wounded fruit kept at 25 °C. Appresoria sometimes were produced directly after germination, without a visible germ-tube between the spore and appressorium (Fig. 6 A, B and C). No mycelial growth occurred on the fruit surface after germination, suggesting that the imazalil-containing wax layer prevented further development of the fungus.

Natural infection of harvested fruit by *P. citricarpa*. No CBS symptoms developed within three weeks at 0.5 or 25 °C on any of the disease-free fruit stacked underneath infected fruit in lug-boxes at Outspan Citrus Centre, Nelspruit, neither could *P. citricarpa* be isolated from them (Fig. 7). All five the other citrus fruit pathogens, particularly *C. gloeosporioides*, *P. italicum* and *P. digitatum*, were isolated from the receiving fruit stored at 25 °C. The latter three species were also the only ones recovered from fruit at 0.5 °C.
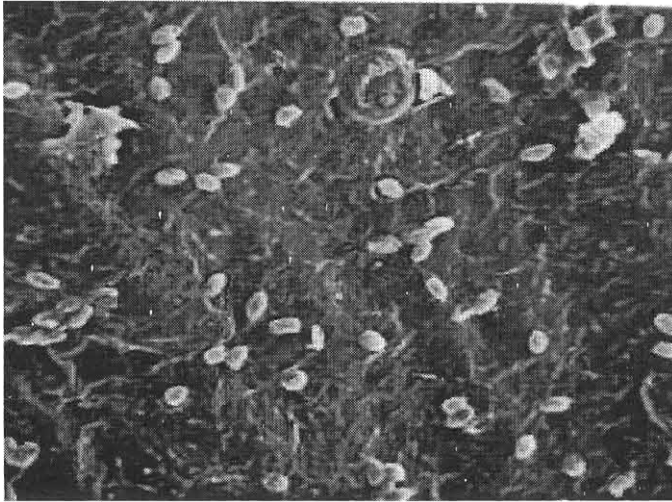
Figure 4: Adhesion of *Phyllosticta citricarpa* conidia to the synthetic polyethylene micro-wax layer on the surface of citrus fruit.
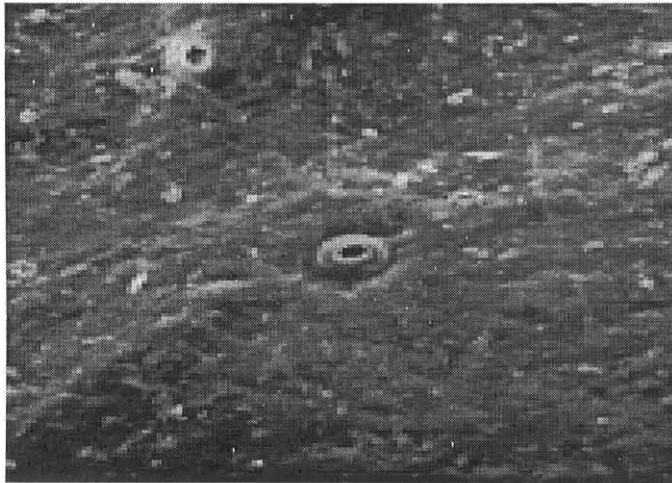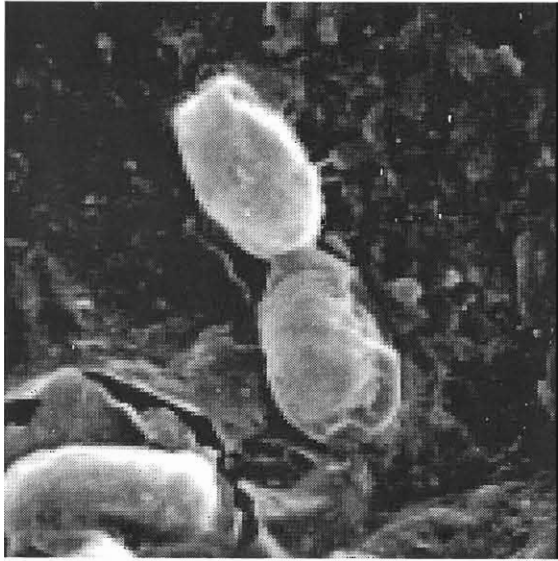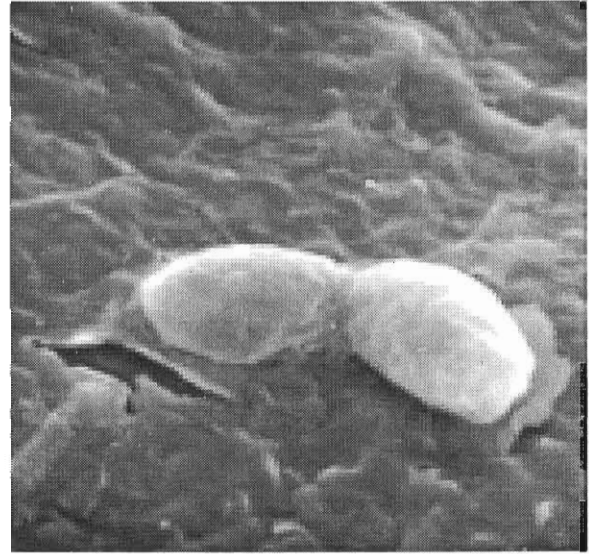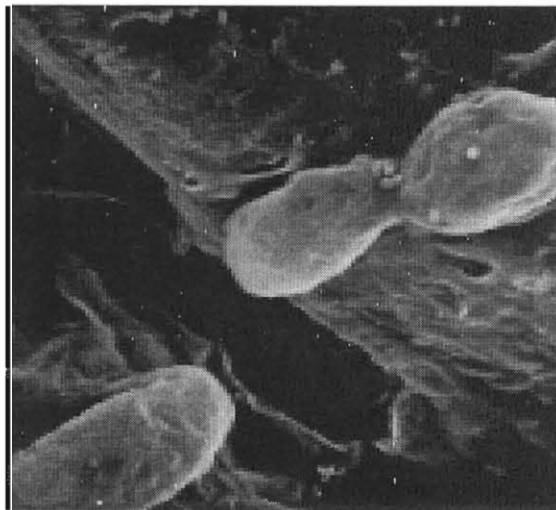

Figure 5: *Phyllosticta citricarpa* conidia on artificially-inoculated Valencia fruit peel not coated with a synthetic wax layer. Note that virtually no adhesion occurred in the inoculated area.

Figure 6: Germinated conidia of *Phyllosticta citricarpa* on the surface of a wounded citrus fruit stored at 25°C, with no visible germ tube between the spore and appressorium. Note attachment to synthetic wax layer (A, B & C).
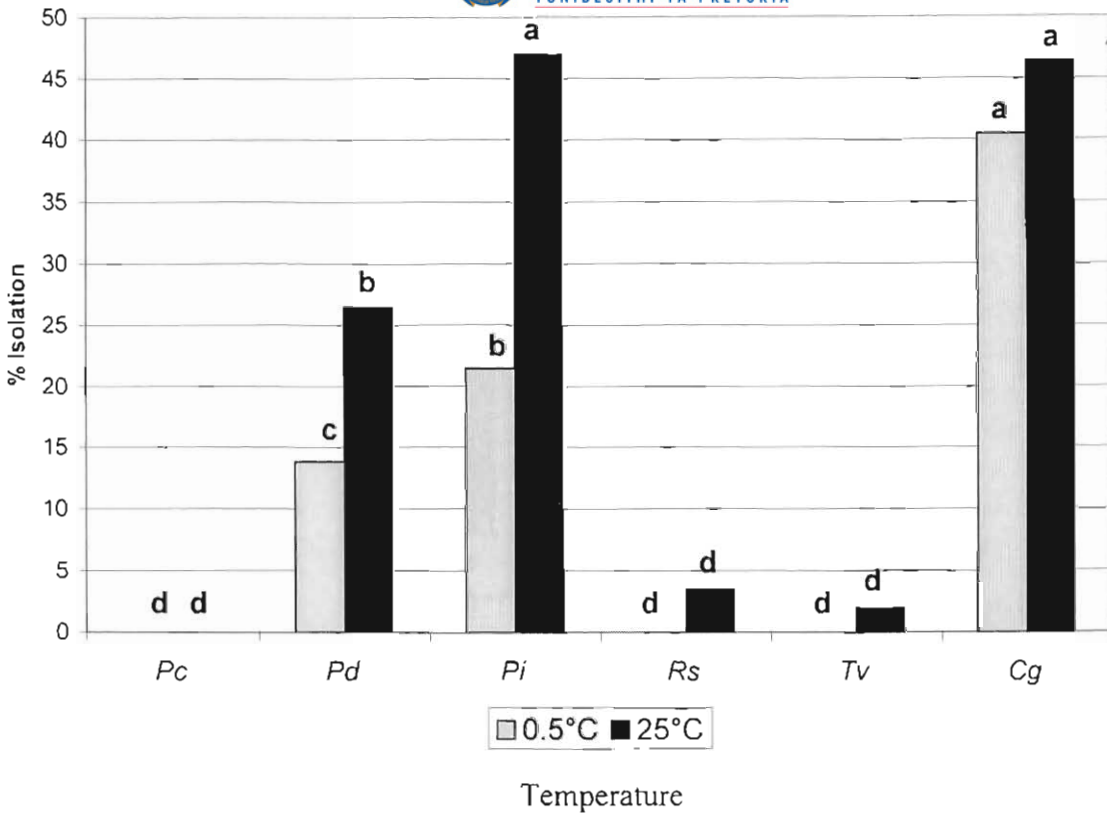
Figure 7: Isolation frequency of citrus pathogens (*Pc=Phyllosticta citricarpa; Pd=Penicillium digitatum; Pi=Penicillium italicum; Rs=Rhizopus stolonifer; Tv=Trichoderma viride; Cg=Colletotrichum gloeosporioides*) from originally-healthy citrus fruit exposed for three weeks at 0.5 °C and 25 °C, respectively, to inoculum of *Phyllosticta citricarpa* from CBS-infected fruit, and stored an extra week at 25 °C.

Bars (based on the means of isolations made from 200 fruit) followed by the same letter do not differ significantly, ($P = 0.05$) according to Duncan's multiple range test.

Radurisation of CBS-infected fruit. The incidence of *P. citricarpa* declined from 97.3% in non-irradiated fruit to 70.5% in fruit exposed to cobalt-irradiation of 400 Gy (Fig. 8). During subsequent incubation, new red freckle spot lesions formed on all fruit, irrespective of irradiation intensity, thus indicating the existence of viable inoculum on the fruit.
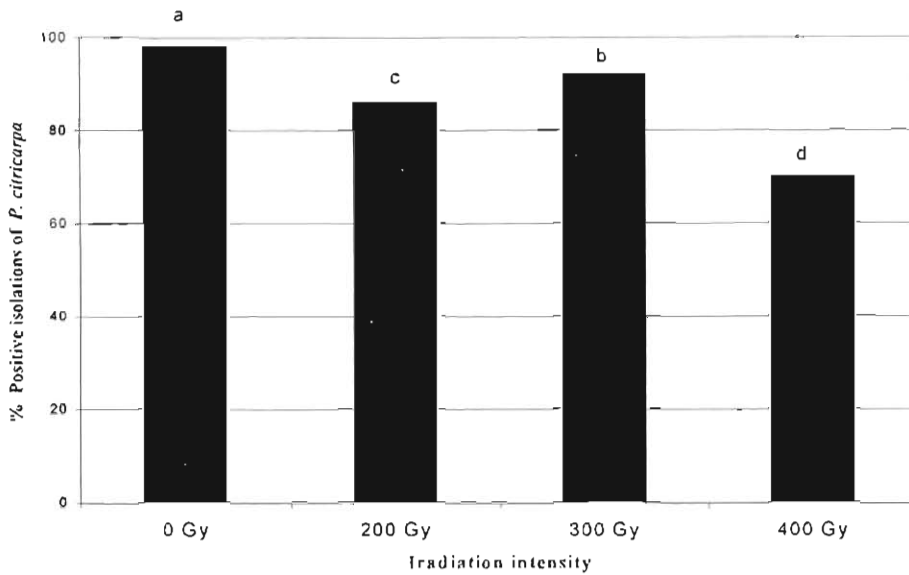
Figure 8: Isolation frequency of *Phyllosticta citricarpa* from black spot lesions on Valencia oranges exposed to different intensities of cobalt irradiation. Bars (based on the means of isolations made from 50 fruit) followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Effect of packhouse procedures on the viability of *P. citricarpa*. *P. citricarpa* could readily be isolated from CBS lesions on fruit kept at 4.5, 10 and 25 °C, but conidia of the pathogen apparently lost their viability at the higher temperature (Tables 3 & 4). Packhouse treatments which in both seasons significantly reduced the incidence of *P. citricarpa* in lesions included the warm water bath, chemical tank, waxing (at 25 °C only), and all treatments combined. However, none of the treatments eliminated the organism completely, the maximum reduction in its recovery being 86 % after exposure of fruit to all treatments in the processing line and storage at 4.5 °C. Conidial viability nevertheless was consistently reduced to zero by chlorine, the warm water bath, chemical tank, and combination of treatments.

The various treatments also affected other citrus fruit pathogens. Treatments which consistently reduced the numbers of one or more species to zero were chlorine (*R. stolonifer* at 4.5 °C and *T. viride* at 25 °C), high-pressure spraying (*P. digitatum* at 4.5 °C), warm water bath (*R. stolonifer* at 4.5 °C), chemical tank (*P. digitatum* at 25 °C, *P. italicum* at 4.5 °C, *R. stolonifer* at 25 °C, and *T. viride* at 4.5 and 25 °C), waxing (*T. viride* at 25 °C), and

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

the combination of all treatments (*P. digitatum* at 4.5 °C, *P. italicum* at 25 °C, *R. stolonifer* at 25 °C and *T. viride* at 25 °C). *C. gloeosporioides* seemed less sensitive to treatment, and in the 1997 season actually showed an increase in incidence in most of the treatments.

Table 3: Isolation frequency of citrus pathogens from CBS-infected Valencia fruit subjected to various packhouse procedures in 1997.

Pathogen	Temperature	Positive isolations from 100 fruit[x]						
		Control[y]	Chlorine[y]	Spray[y]	Warm water[y]	Chemicals[y]	Wax[y]	All[y]
Phyllosticta citricarpa	4.5°C	90 a +	93.3 a -	86.7 a +	76.7 b -	53.3 c -	80 ab	12.3 d-
	10°C	96.7 a +	86.7 ab -	83.3 b +	86.7 ab-	60 c -	80 b -	31.35 d-
	25°C	93.3 a -	93.3 a -	96.6 a -	70 b -	66.7 b -	56.7 c -	18.7 d-
Penicillium digitatum	4.5°C	6.7 a	0 a	0 a	6.7 a	0 a	0 a	0 a
	10°C	13.3 b	0 c	6.7 c	3.3 c	0 c	26.7 a	0 c
	25°C	60 a	40 b	13.3 c	0 d	0 d	43.3 b	2.0 d
Penicillium italicum	4.5°C	60 a	43.3 b	33.3 c	20 d	0 e	26.7 cd	3.0 e
	10°C	50 a	40 ab	13.3 c	16.7 c	6.7 d	33.3 b	0 d
	25°C	40 b	33.3 bc	60 a	40 b	0 d	30 c	0 d
Rhizopus stolonifer	4.5°C	40 a	0 c	13.3 b	0 c	40 a	50 a	5.7 c
	10°C	46.7 a	0 c	20 b	0 c	0 c	26.7 b	0 c
	25°C	26.7 a	0 b	0 b	20 a	0 b	0 b	0 b
Trichoderma viride	4.5°C	0 a	0 a	0 a	0 a	0 a	0 a	0 a
	10°C	13.3 a	6.7 b	0 b	0 b	0 b	0 b	0 b
	25°C	26.7 a	0.b	0 b	0 b	0 b	0 b	0 b
Colletotrichum gloeosporioides	4.5°C	6.7 d	33.3 b	40 a	36.7 ab	0 d	46.7 a	14.2 c
	10°C	20 d	46.7 b	90 a	36.7 bc	26.7 cd	26.7 cd	10 7 d
	25°C	20 c	56.7 a	36.7 b	53.3 a	20 c	50 a	27.4 bc

[x] Chlorine = 100 ppm active chlorine; Spray = high pressure spray; Warm water = water at 43-47 °C for 3min; Chemicals = imazalil sulphate + guazatine + 2.4 D; Wax = polyethylene wax application; All = Chlorine + Spray + Warm water + Chemicals + Wax.

[y] Values in rows (based on 50 replicates) followed by the same letter do not differ ($P = 0.05$) according to Fisher's LSD test.

+ = Germination of *P. citricarpa* conidia, harvested from viable hard spot lesions on medium.

- = No germination of *P. citricarpa* conidia, harvested from viable hard spot lesions on medium.

Table 4:   Isolation frequency of citrus pathogens from CBS-infected Valencia fruit subjected to various packhouse procedures in 1998.

| Pathogen | Temperature | Positive isolations from 100 fruit[x] | | | | | | |
		Control[y]	Chlorine[y]	Spray[y]	Warm water[x]	Chemicals[y]	Wax[y]	All[y]
Phyllosticta	4.5°C	85.2 a +	90.2 a -	82.3 a +	71.3 b -	45.6 c -	84.3 a +	25.2 d -
citricarpa	25°C	88.3 a -	82.6 a -	65.2 bc -	58.3 c -	28.2 d -	72.7 b -	16.3 c -
Penicillium	4.5°C	15.0 a	5.5 cb	0 c	8.4 b	5.2 cb	4.5 cb	0 c
digitatum	25°C	46.1 a	5.0 c	36.7 a	15.8 b	0 c	25.3 b	0 c
Penicillium	4.5°C	53.6 a	18.3 b	43.0 a	15.0 b	0 c	20 b	6.3 c
italicum	25°C	36.3 a	5.7 c	15.3 b	10.3 b	2.0 c	18.3 b	0 c
Rhizopus	4.5°C	23.7 a	0 b	6.3 b	0 b	0 b	15.3 a	0 b
stolonifer	25°C	0 d	17.9 b	8 0 c	0 d	0 d	28.5 a	0 d
Trichoderma	4.5°C	40.2 a	5.3 bc	1.2 c	8.0 b	0 c	3.5 bc	1.0 c
viride	25°C	10.7 a	0.0 b	6.3 b	2.4 b	0 b	0 b	0 b
Colleto-	4.5°C	15.3 c	23.3 b	27.5 ab	22 0 b	9.0 c	34 7 a	10.3 c
trichum gloeosporioides	25°C	32.0 b	48.7 a	34.0 b	34.6 b	17.6 c	20.0 c	8.7 d

[x] Chlorine = 10 ppm chlorine dioxide; Spray = high pressure spray; Warm water = water at 43-47 °C for 3 min; Chemicals = imazalil sulphate + guazatine + 2.4 D; Wax = polyethylene wax application; All = Chlorine + Spray + Warm water + Chemicals + Wax.

[y] Values in rows (based on 50 replicates) followed by the same letter do not differ ($P = 0.05$) according to Fisher's LSD test.

+ = Germination of *P. citricarpa* conidia, harvested from viable hard spot lesions, on medium.

- = No germination of *P. citricarpa* conidia, harvested from viable hard spot lesions, on medium.

Survival of *P. citricarpa* on citrus fruit treated with fungicides not registered for postharvest use on citrus. Conidia of *P. citricarpa* present on the control fruit lost their viability after the first week, whereas all fungicides, except acibenzolar-s-methyl, reduced conidial viability to zero within the first week (Table 5). Fungicide treatment also had a suppressive effect on mycelial inoculum of *P citricarpa*, with difenoconazole providing the greatest reduction in isolation frequency.

Table 5:　　Isolation frequency of *Phyllosticta citricarpa*, *Penicillium digitatum* and *Colletotrichum gloeosporiodes* from citrus fruit treated with fungicides not registered for postharvest use of citrus.

Pathogens	Mean percentage positive isolations after 3 weeks [x]								
	Control	Difenoconazole	Triforine	Tebuconazole	Propamocarb-HCl	Kresoxim-methyl	Azoxystrobin	Acibenzolar-s-methyl	Procymidone
P.citricarpa	66.7 a	4.8 c	27.0 b	33.3 b	22.2 b	23.8 b	20.8 b	30.2 b	31.8 b
Time Harvested	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3	W1 W2 W3
Viable conidia	+ - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - -	- - -
P. digitatum	46.0 b	14.3 d	15.9 d	28.6 c	42.9 b	39.7 b	49.2 b	36.5 cb	69.8 a
C. gloeosporioides	41.3 b	69.8 a	49.2b	68.3 a	55.6 ab	60.3 a	58.7 a	65.1 a	47.6 b

[x] Values in rows (based on 50 replications) followed by the same letter do not differ significantly

(P = 0.05) according to Fisher's LSD test.

w1 = week 1; w2 = week 2; w3 = week 3

+ = Viable *P. citricarpa* conidia

- = No viable *P. citricarpa* conidia.

DISCUSSION

Koch's postulates were essentially satisfied by re-isolating *P. citricarpa* from artificially-inoculated wounded citrus fruit stored at 25 °C. However, the fact that no infection occurred in unwounded fruit at 0.5 and 25 °C after artificial or natural infection, or in artificially-inoculated wounded fruit at 0.5 °C, indicates that infection of unblemished harvested citrus fruit by conidia of *P. citricarpa* is highly unlikely, particularly at low temperatures. Even the apparent infection of wounded fruit probably can be ascribed to the extremely high inoculum pressure of ca. 10^5 conidia mm^{-2} within the confines of the silicone circle and the stimulation of conidial germination provided by sap exuding from the wounded fruit. The

constatation by Wager (1949) that conidia of *P. citricarpa* can infect citrus fruit only until about three months after petal drop is therefore strongly supported. Results of the natural inoculation experiment furthermore corroborates the view of Wager (1953) that *P. citricarpa* is incapable of spreading from CBS fruit to clean fruit originating from healthy orchards. What is interesting though, was the tendency for *P. citricarpa* conidia to adhere to the synthetic polyethylene micro-wax layer on fruit rather than to the natural waxy surface of the peel. Affinity for hydrophobic surfaces has previously been reported for *Phyllosticta ampelicida* (Engleman) Van der Aa (Kuo & Hoch, 1996) and could, in the case of *P. citricarpa*, imply that waxing of fruit actually promotes adherence, and hence dissemination, of conidia. Fungicides like imazalil retained in the wax nevertheless appear to prevent propagation of the pathogen.

Although none of the packhouse treatments effectively eliminated *P. citricarpa* from CBS-infected fruit, most of the processes rendered the conidia non-viable. Indeed, conidial viability could be reduced to zero just by storing fruit at 25 °C. Results presented in this study indicate that low temperatures (0.5 – 10 °C) may prolong the life span of conidia present on CBS infected fruit. In terms of overall efficacy it was particularly the combined treatment that resulted in the greatest reduction in propagule numbers of the pathogen. This hurdle technology, which is practised in most modern citrus packhouses, therefore contributes significantly to decontamination of infected fruit, but does not free them of inoculum. However the form in which the pathogen obviously remains after treatment, viz. mycelium instead of conidia, can hardly be regarded as infective (McOnie, 1967). Where available facilities do not provide a full component of treatments, a warm water bath or chemical tank should suffice for restraining *P. citricarpa*, whereas chlorine dipping seems sufficient for killing conidia produced by the fungus.

Considering the failure of cobalt irradiation to eliminate *P. citricarpa* mycelium from citrus fruit, submitting fruit to radurisation merely for rendering them free of a dubious source of CBS inoculum, seems futile. Most of the unregistered fungicides evaluated in this study suppressed conidial viability of *P. citricarpa* but, considering the rapid mortality of conidia on fruit processed according to standard packhouse procedures, utilisation of the fungicides

for this purpose probably would be a waste. Nevertheless, particularly difenoconzole showed great potential in reducing mycelial inoculum of the pathogen on infected fruit. This was not unexpected since triazole fungicides, including difenoconazole, are known for their efficacy against CBS in the field at high rates (Schutte, 1995). With other fungicides, such a correlation was not evident. For instance, the biotically-derived compounds, kresoxim-methyl and azoxystrobin, were comparatively ineffective as postharvest treatment, despite giving excellent preharvest control of CBS (Schutte, 1995). Acibenzolar-*s*-methyl, which induces systemic resistance in plants, similarly failed to provide notable control in the packhouse, probably because it acts preventatively rather than curatively (Technical information, Novartis Ltd.).

Although this study primarily concerned *P. citricarpa*, the response of other pathogens to the various treatments also warrant brief deliberation. The aggravating effect of wounding on infection by *P. digitatum*, *P. italicum*, *R. stolonifer* and *T. viride* (Eckert, 1978; Brown, 1988; Smilanick, 1995) was confirmed. Of particular interest, however, was the apparent independence of wounding for *C. gloeosporioides* to infect, and its relative insensitivity to packhouse treatments directed at superficial fruit colonists. This is primarily ascribed to the endophytic occurrence of *C. gloeosporioides* in citrus (Timmer *et al.*, 1998). Reduction of the natural epiphytic microflora by the packhouse procedures in 1997 probably created an environment on or near the fruit surface conducive to colonisation by endophytically-residing propagules of *C. gloeosporioides* where it proliferated in the absence of competition. Whether it contributed to the suppression of *P. citricarpa* in this study is unclear, but Kotzé (1963) previously reported a negative correlation between the isolation frequencies of *P. citricarpa* and *C. gloeosporioides*. A study of the association between these two organisms could therefore provide valuable information regarding the effect of *C. gloeosporioides* on the pre- and post harvest destiny of the CBS pathogen.

REFERENCES:

Brown, G.E. 1988. Post harvest decays of Florida citrus. *The Citrus Industry* 6:31-39.

Darnell-Smith, G.P. 1918. An account of "black spot" disease in citrus fruit in New South Wales. *Proceedings of the Linnean of Society New South Wales* 72:251-291.

Eckert, J.W. 1978. Post-harvest diseases of citrus fruit. *Outlook on Agriculture* 9:225-232. Department of Plant Pathology, University of California, Riverside, California 92521, USA.

Kiely, T.B. 1948. Preliminary studies on *Guignardia citricarpa*: the ascigerous stage of *Phoma citricarpa* McAlp and its relation to black spot of citrus. *Proceedings of the Linnean Society of New South Wales* 93:249-292.

Kotzé, J.M. 1963. Studies on the black spot disease of citrus caused by *Guignardia citricarpa* Kiely , with particular reference to its epiphytology and control at Letaba. D.Sc (Agric) Thesis, University of Pretoria, Pretoria.

Kotzé, J.M. 1981. Epidemiology and control of citrus black spot in South Africa. *Plant Disease* 65:945-950.

Kuo, H.C. & Hoch, R. 1996. Germination of *Phyllosticta ampelicida* pycnidiospores - Prerequisite of adhesion to substratum and the relationship of substratum wettability. *Fungal Genetics and Biology.* 20: 18-29.

McOnie, K.C. 1964. Control of citrus black spot disease. A comparison of the relative merits of copper fungicide and maneb for the control of black spot disease on the Valencia orange. *South African Citrus Journal* 361:5,7,9, & 11.

McOnie, K.C. 1967. Germination and infection of citrus by ascospores of *Guignardia citricarpa* in relation to control of black spot. *Phytopathology* 57:743-746.

Schutte, G.C. 1995 Evaluation of control strategies for citrus black spot in South Africa. PhD Thesis, University of Pretoria, Pretoria.

Smilanick, J.L. 1995. Evaluation of heated solutions of sulphur dioxide , ethanol and hydrogen peroxide to control post harvest green mold of lemons. *Plant Disease* 19:742-747.

Sutton, B.C. & Watterston, J.M. 1966. Descriptions of pathogenic fungi and bacteria. *Guignardia citricarpa* No. 85. Commonwealth Mycological Institute, Kew, Surrey.

Timmer, L.W., Brown, G.E. & Zitko, S. E. 1998. The role of *Colletotrichum* spp. in postharvest anthracnose of citrus and survival of *C. acutatum* on fruit. *Plant Disease* 82:415-418.

Wager, V.A. 1949. The occurrence of the black spot fungus in the citrus areas of South Africa. *Farming in South Africa*. August: 367-369.