SURVIVAL OF *PHYLLOSTICTA CITRICARPA*, ANAMORPH OF THE CITRUS BLACK SPOT PATHOGEN

by

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CHAPTER 1

GENERAL INTRODUCTION

Occurrence and significance of citrus black spot. Citrus black spot (CBS) causes substantial economic losses in all major citrus-producing countries subject to summer-rainfall (Kotzé, 1981). Countries in which CBS occurs include Argentina, Brazil, Peru, Venezuela, Uruguay, summer-rainfall regions of South Africa, Nigeria, Uganda, Kenya, Zimbabwe, Swaziland, Mocambique, India, China, Hong Kong, Taiwan, Japan, Philippines and coastal regions of Australia (Kiely, 1948; Sutton & Waterston, 1966; Kotzé, 1981; Whiteside *et al.*, 1988; Schutte *et al.*, 1996). The disease is absent in Mediterranean regions subject to winter-rainfall, e.g. Israel, Italy, Spain, Greece, Cyprus, Chile, and California in the USA (Kotzè, 1981; Schutte *et al.*, 1996).

Citrus orchards in the northern, eastern and central Transvaal, Natal, and lemon orchards in the eastern Cape represent approximately 50% of the total citrus plantings in South-Africa (Schutte, 1995). These areas must continuously be protected against CBS with registered fungicides. The cost of control measures of CBS in South Africa during the 1995 season amounted to between R11 million and R16.5 million (Schutte, 1995). In the 1997 season, South Africa spent in the region of R30 - 50 million on fungicides alone (mainly mancozeb and benomyl) for preharvest control of CBS (G.C. Schutte, personal communication). This estimate excluded the indirect losses due to CBS, viz spray cost (labour, tractor and spraying equipment maintenance, etc.) and rejection of exportable fruit due to the development of CBS in transit (packhouse processing and packing material costs, etc.).

Causal organism. McAlpine (1899) assigned the imperfect state of the CBS pathogen to the genus *Phoma* and described it as a new species, *Phoma citricarpa* McAlp. Sutton & Watterson (1966) reclassified the imperfect state in 1966 as *Phyllostictina citricarpa*

(McAlp.) Petrak, and Van der Aa (1973) changed it to *Phyllosticta citricarpa* (McAlp.) Van der Aa. The latter classification was confirmed on basis of the conidial appendages by Punithaingam & Woodhams (1982) and is currently still accepted.

Pycnidia of *P.citricarpa* may occasionally be found in lesions on green leaves and dead twigs attached to the tree (McOnie, 1964). Appreciable numbers of conidia can develop on old fruit lesions (hard spots), whereas numerous conidia are present on fallen decaying citrus leaves (Kiely, 1948; Kotzé 1963). Conidia are exuded in a sticky gelatinous mass in the presence of moisture and depend on running water for dispersal (Darnell-Smith, 1918; Kiely 1948; McOnie, 1965). The condia are borne in tandem in large numbers on short conidiophores in the pycnidium and are released upon contact of the conidioma with water (Darnell-Smith, 1918; Kiely, 1948). The dimensions of the conidia are in the order of 8.0–10.5 μm x 5.5–7.0 μm (Sutton & Watterson, 1966). Spermatia, measuring 7.5 x 1.6 μm, are sometimes present (Darnell-Smith, 1918). Lesions on mature fruit (hard spots) can contain one to more than 50 pycnidia.

The parasitic relationship between *P.citricarpa* and citrus was studied by Darnell-Smith (1918), Kiely (1948), Wager (1949) and Kotzé (1963). Since then little new information has been gathered in this regard. There is particularly a lack of basic knowledge concerning conidium attachment, germination and appressorium formation by the fungus.

Conidia of *P. citricarpa* are not airborne and do not play an important role in the epidemiology of CBS in orchards (Kiely, 1948; McOnie, 1964; Kotzé 1981). They only contribute to the epidemiology of CBS if infected out-of-season fruit remaining on the trees (Kiely, 1948; Wager, 1949; McOnie, 1964; Kotzé, 1981). Conidia may cause infection on young susceptible fruit and leaves when splashed from out-of-season fruit by rain (Wager, 1953). Fruit are only susceptible during the first few months after fruitset, whereafter they become resistant to infection (Wager, 1953; Kotzé, 1981).

Ascospores produced in ascomata (perithecia) by the teleomorphic state of the pathogen are the primary source of inoculum (McOnie, 1964; Kotzé, 1981). Perithecia are found on dead leaves on the orchard floor (Kiely, 1948; Wager, 1949; Kotzé 1963; McOnie, 1964; Kotzé,

1981). Spermatia referred to by Darnell-Smith (1918) as X-spores are present on fallen decaying citrus leaves and can also be produced in culture. There is strong evidence that spermagonia produce functional male gametes, entitled to be called spermatia. Their appearance always precedes perithecium formation on decaying citrus leaves and they therefore have a distinct sexual function (Kiely, 1948). This supports the view that, although pycnidia mature on dead leaves several weeks before perithecia are produced, detectable infection in orchards does not occur before ascospores are available (McOnie, 1964). To date, ascospores could not be retrieved from fruit lesions (Kiely, 1948; Kotzé, 1963; McOnie, 1965; Kotzé, 1981).

Ascospores on dead leaves are forcibly discharged into air currents after rainy spells and are disseminated by wind (Kotzé, 1981). Infection of citrus fruit by ascospores takes place early in the season in the presence of moisture when the spores germinate and produce appressoria. A thin infection peg penetrates the cuticle and expands, forming a small mat of mycelium between the cuticle and the epidermis (McOnie, 1967). This is referred to as latent infection and ends when the fruit becomes mature. Infection depends on prevailing environmental conditions and the stage of fruit development (Brodrick, 1969). Light and temperature play an important role in lesion development on the fruit, and hence on the development of pycnidia and conidia. Black spot symptoms and sporulation occur optimally on citrus fruit and flavedo pieces exposed to light at 27 °C (Brodrick & Rabie, 1970).

The host. Except for sour orange (*Citrus aurantium* L.) and its hybrids, all commercially grown citrus varieties are susceptible to CBS (Kotzé, 1981). Lemons are particularly prone to the disease, although heavy losses may also occur on Valencia and Navel oranges (*C. sinensis* (L.) Osbeck) as well as in grapefruit (*C. paradisi* Mart.) (Kiely, 1948; Kotzé, 1981). The flavourless glucoside, hesperidin, ($C_{28}H_{34}O_{15}$) occurs abundantly in oranges and other citrus varieties, especially in young fruit (Hendrickson & Kesterson,1961) and can readily be isolated from chopped citrus peel by methanol extraction. Heperidin was found to be the best carbon source for supporting growth of *P. citricarpa* (Frean,1964). All citrus fruit furthermore produce oil which is contained in numerous oil sacs in the rind. Oil yield of citrus fruit at different developmental stages was directly correlated with the increase in fruit surface area up to maturity. Oil can be separated into two basic chemical groups, the terpenes and the

terpenoids (Bernard, 1961). The main terpene in citrus oil is d-limonene ($C_{10}H_{16}$), which comprises 90% of the total constituents in the oil (Braverman, 1949). Brodrick (1971) showed that d-limonene as such is toxic to fungi and that it inhibits growth of *P. citricarpa*.

Export conditions. Export procedures have changed appreciably since the first detailed description of CBS in 1899. It now takes up to a week from harvesting to pack fruit for the export market. Degreening of fruit is an additional time factor, extending handling of fruit with 2-4 days (A Du Pisanie, personal communication). The packhouse procedures ($Ca(OCl)_2$ or chlorine dioxide dump trough, high pressure spray, warm water bath, fungicide dip tank, and wax application) minimise the risk of decay. The time it takes for citrus fruit to reach the harbour depends on several factors, including the type of transport available. For instance, fruit packed at Letaba Estates are transported by rail and reach Durban harbour within 2-3 days (A Du Pisanie, personal communication). When the consignment reaches the harbour, the fruit are kept under cool conditions. Loading it onto the ship can take another day during which the cold chain is broken. It takes another 20-30 days on the ship under refrigeration (specific temperatures for specific citrus cultivars) to reach the destination and another week before the fruit is sold. Although Wager (1949) reported that conidia of *P. citricarpa* on CBS-infected fruit have a short lifespan, the fate of these conidia during processing and transit has not been investigated.

Objectives of the study.

- To determine the viability of conidia and mycelium of the CBS pathogen on citrus fruit.
- To categorise the different types of CBS lesions according to appearance and risk as source of infection.
- To determine the time needed for pycnidia to develop in newly-formed lesions and for the production of conidia in the pycnidia.
- *In vitro* studies concentrating on the physical and chemical conditions influencing conidial germination, and on the efficacy of existing packhouse procedures and alternative fungicides to suppress germination.
- *In vivo* experiments determining the possibility for CBS fruit to cross-contaminate clean (symptomless) packhouse-treated fruit.
- Evaluating existing packhouse treatments *in vivo* for their effect on *P. citricarpa* mycelium

and conidia present in the lesions. The entire process from harvesting in the orchard to reaching the final destination on foreign markets (incubation time) will be simulated in the lab. This will be done to determine if conidia from CBS fruit are still viable.

- Determination of the effect of cobalt irradiation on the viability of CBS propagules.

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CHAPTER 2

DEVELOPMENT AND VIABILITY OF BLACK SPOT LESIONS ON CITRUS FRUIT

ABSTRACT

Citrus black spot (CBS) lesion types occurring commonly on fruit were indexed into four groups, viz. lesion type A (hard spots or shot-hole spots), B (false melanose, speckled blotch or inky spots), C (freckle spots) and D (virulent, spreading or galloping spots). This indexing accommodated the inclusion of atypical CBS lesions occurring on fruit into one of these classes. Freckle spot was the first symptom to develop on harvested naturally-infected symptomless Valencia fruit after 5 - 7 days of incubation at 27 °C, high humidity and 24 h fluorescent lighting. Pycnidia formed in these lesions contained viable conidia after 9 – 13 days of incubation. Mycelium in the margins of viable hard spot lesions survived packhouse processing while conidia present in the lesions were rendered sterile by processing and also rapidly lost viability on unprocessed fruit under optimal storage conditions. CBS symptoms could not be induced in healthy mature intact Valencia leaves by artificial inoculation with *P. citricarpa* conidia. Conidia of *Phyllosticta citricarpa* germinated abundantly in citrus juice extracts (Valencia orange, Eureka lemon and Texas Star Ruby grapefruit) while germination in distilled water, 0.5 g l^{-1} sucrose and citrus peel washings was negligible.

INTRODUCTION

Guignardia species, including their *Phyllosticta* and *Leptodothiorella* anamorphic states, comprise an economically important group of about 40 plant pathogenic species (Hawksworth & David, 1989). Among them, *Guignardia citricarpa* Kiely (anamorph: *Phyllosticta citricarpa* (McAlp.) Van der Aa), the causal agent of citrus black spot

(CBS), is a pathogen of major concern to citrus-growing in South Africa (Doidge, 1919; Wager, 1949; Kotzé, 1963; McOnie, 1964; Brodrick, 1969).

CBS is characterised by lesions which develop on maturing fruit of lemon, sweet orange, grapefruit and tangerine citrus varieties (McOnie, 1964). Various types of lesions can be distinguished during development of the disease, e.g. speckled blotch, freckle spot, hard spot and virulent spot (Kiely, 1948; Calavan, 1960; Kotzé,1963). There is, however, no general consensus regarding this terminology. For instance, hard spot is also known as shot-hole or type "A" spot, virulent spot as spreading or type "D" spot, freckle spot as type "B" spot and speckled blotch as false melanose or type "C" lesions (Kiely, 1948; Brodrick, 1969; Garrán, 1996). Furthermore, CBS lesions often cannot be classified into a specific category (S. Kamburov, personal communication). Also, although the *G. citricarpa* teleomorph does not occur in any of the lesion types on citrus fruit (McOnie, 1964; Kotzè, 1981), it is not clear to what extent each type contributes towards the production of viable conidial inoculum. A need thus exists for reassessing the lesion classification scheme, particularly with regard to viability of the various spots.

The purpose of this study is to propose a modified classification system for CBS fruit symptomology, present evidence on pycnidial development in various lesion types, to elucidate the effect of packhouse processing on conidial and mycelial viability in these lesions, investigate the susceptibility of citrus leaves to conidial infection and, to additionally, describe a medium for assessing the viability of *P.citricarpa* conidia.

MATERIALS AND METHODS

Lesion development on citrus peel. Valencia fruit exposed to a high inoculum of *G. citricarpa* ascospores in the orchard, but not showing visible CBS symptoms, were harvested in the 1997 season at Malelane Estates. The fruit did not receive any postharvest treatment except for surface-sanitising in 70 % ethanol for 5 minutes. Fifty fruit were placed in each of three growth chambers (Conviron) with 24 h exposure to full-

strength fluorescent light at 27 °C under high humidity. Lesion development was monitored and samples were prepared for electron microscopy at different developmental stages of the disease. Citrus peel blocks (ca. 5 mm^3) containing the dissected lesions were fixed for 24 h in 6 % glutaraldehyde. The samples were then washed three times for 15 min in 0.7 M phosphate buffer, dehydrated for 15 min in ascending percentages (50 %, 70 %, 90 %, 100 %) absolute ethanol, and dried in a critical-point dryer (Hitachi HCP-2). After goldplating in an Eiko IB3 ion coater, specimens were viewed in a Hitachi 400 scanning electron microscope.

Viability of different types of hard spot CBS lesions. Valencia oranges exhibiting typical hard spot CBS symptoms were collected in July 1997 at Malelane Estates and either left untreated or treated at the Crocodile Valley packhouse near Nelspruit. The packhouse process comprised a high-pressure descaler water spray (20 – 25 kPa), hot water bath (42 °C for 2 minutes), brushing with imazalil sulphate (1000 ppm Fungazil 75 % SP, Janssen Pharmaceutica) and waxing.

Hard spot lesions on the treated and untreated fruit were categorised into four classes, viz (a) red margin hard spots (RMH), (b) brown margin hard spots (BMH), (c) dark brown margin hard spots (DBMH) and (d) black margin hard spots (BLMH) (see Figs 9-12). Three lesions of a particular hard spot lesion category were selected per fruit and demarcated with a permanent marker. Twenty treated and 20 untreated fruit of each hard spot category were incubated at 27 °C under high humidity and continuous fluorescent lighting.

Five treated and untreated fruit from each hard spot category were removed from the incubator after 5, 14 and 21 days respectively. Each of the three selected hard spot lesions on each fruit was covered with a drop of spore germination medium (Chapter 3). When 50% or more of the pycnidia (3 to 200) present in the lesion had released their contents into the droplet, the conidia were harvested with a sterile 0.5 mm diameter glass capillary tube and streaked onto a single Petri dish containing the medium (described in Chapter 3). Concommitantly, a citrus peel block (ca. 3 mm^3) was dissected aseptically

from the lesion margin of each selected hard spot and plated onto medium in a separate Petri dish. Petri dishes were incubated for 10 days at 22 °C under high humidity and 24 h fluorescent lighting, and inspected visually for *P. citricarpa* colonies.

Evaluation of media for sustaining germination of *P. citricarpa* conidia. Germinability of conidia of *P. citricarpa* isolate from Natal (PPRI 5350) was determined as described in Chapter 3 in sterile distilled water (SDW) supplemented with the following substances (rates are per litre of SDW): (a) 15 g blended mature Valencia orange peel (flavedo and albedo), (b) 0.5 g sucrose, (c) 5 g citric acid (d) 20 ml mature Valencia orange juice, (e) 20 ml Eureka lemon juice, (f) 20 ml Star Ruby grapefruit juice, and (g) citrus peel washings from three mature Valencia oranges agitated for 8 hours in a litre SDW on a slow-speed rotary shaker. The various suspensions and solutions were filtered through Whatman no1 paper and autoclaved for 20 min at 120 °C. Unamended SDW served as control. Conidial concentration was 1 x 10^7 ml^{-1}, three replicates were used, and germination was recorded after 48 h.

Categorising of CBS symptoms. Symptoms on citrus fruit from various localities in South Africa were photographed to obtain a representative photo-gallery of the different symptoms occurring on fruit. Variations on the commonly-described symptoms were noted and the lesion types were categorised into main groups.

***P. citricarpa* leaf inoculation.** Ten 5-year-old greenhouse-reared Valencia orange on Swingle citrumelo citus trees were pruned, removing all existing leaves, and allowed to flush. Mature CBS-free leaves were selected in the new flush, washed with SDW, and inoculated with a conidial suspension of *P. citricarpa* isolate from Natal (PPRI 5350). Inoculation was accomplished by sticking a 15-mm diameter rubber ring with Vaseline® to the abaxial or adaxial surface of respectively five leaves on each tree, and depositing 500 µl of a 1 x 10^7 ml^{-1} conidial suspension of the *P. citricarpa* isolate in each of the rings. Control leaves received 500 µl SDW per rubber ring adaxially on five leaves per tree. After inoculation, each leaf was covered for one week with a small plastic bag to maintain a humid environment.

Inoculated leaves were harvested and inspected for CBS symptoms after four weeks. Isolations were made from the inoculated leaf areas on potato-dextrose agar (Merck, Biolab), and plant tissue sections were processed and observed under the electron microscope as described earlier.

RESULTS

Lesion development on citrus peel. The first lesions on mature CBS-infected Valencia fruit appeared as freckle spots after 5 – 7 days incubation at 27 °C with high humidity and constant fluorescent lighting. Development of these small, red, sunken dots on the citrus rind was restricted, with no notable tissue colonisation beyond the infection loci (Fig. 1). Small numbers of conidia were present in some of the pycnidia after 9 – 13 days (Figs. 2 & 3). At 20 days, lesions that developed under optimal conditions each contained more than one centrally-positioned pycnidium (Fig. 4). However, these pycnidia were at different stages of maturity, indicating that they would not release their conidia synchronously. In mature pycnidia present at 20 days, myriads of conidia were closely packed in a gelatinous mass (Figs. 5 & 6). The gelatinous substance and cap-like appendages on each conidium (Fig. 7) facilitated attachment of the conidia to the substrate.

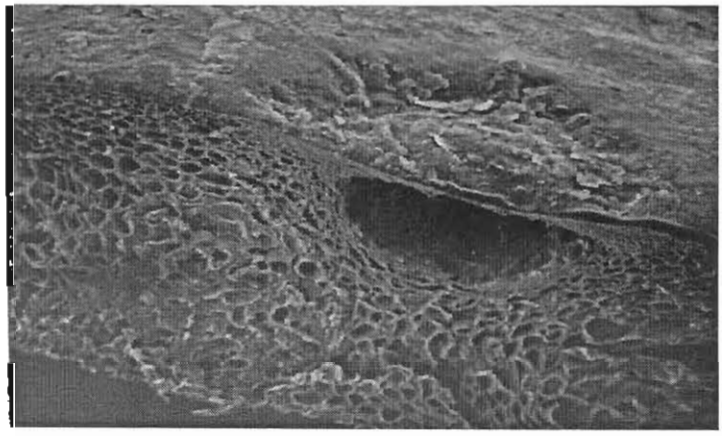

Figure 1: Section through a freckle spot lesion caused by *Phyllosticta citricarpa* on a Valencia orange after 7 days under optimal conditions for disease development.

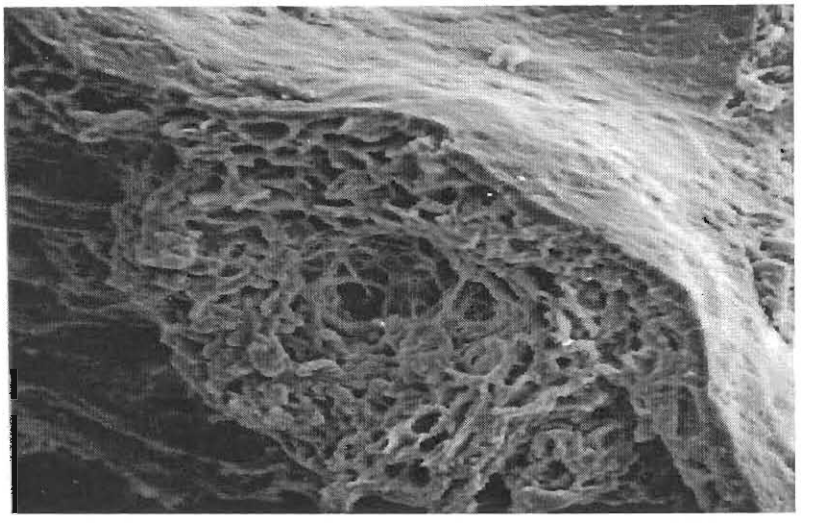

Figure 2: Primordial pycnidia of *Phyllosticta citricarpa* developing in a freckle spot lesion on Valencia citrus peel after 9 days under optimal conditions for disease development.

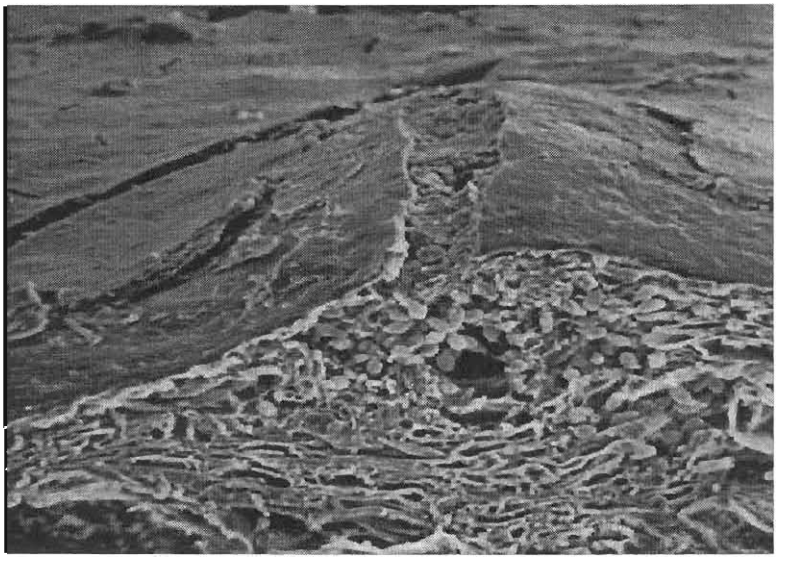

Figure 3: Conidia of *Phyllosticta citricarpa* in the first pycnidium formed in a freckle spot lesion after 13 days under optimal conditions for disease development.

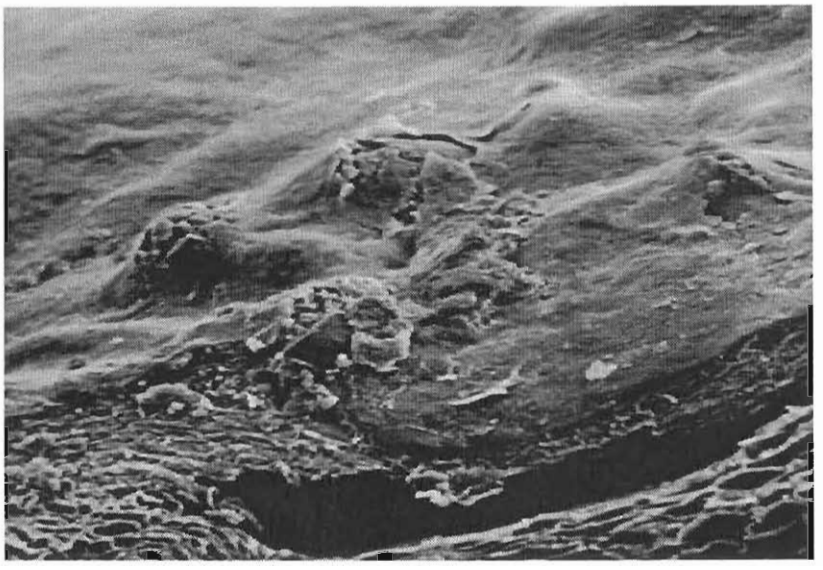

Figure 4: Pycnidia of *Phyllosticta citricarpa* at different stages of maturity after 20 days under optimal conditions for disease development.

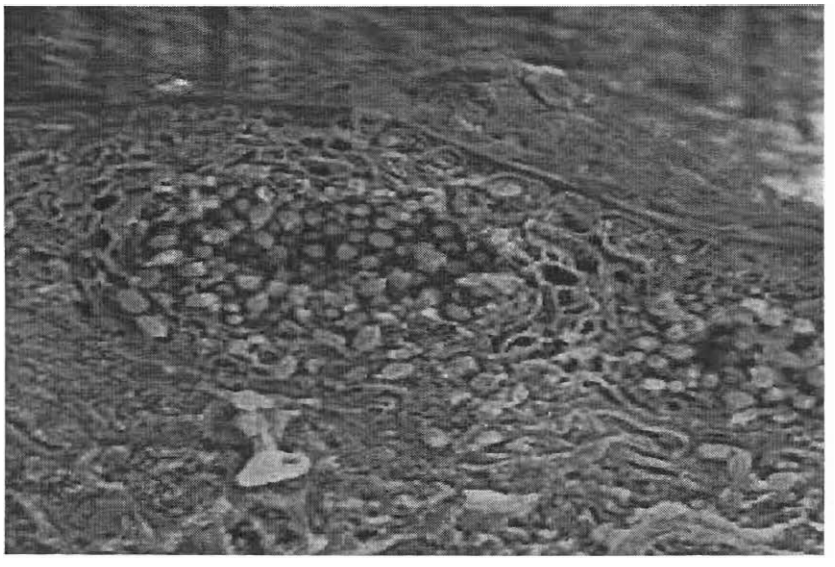

Figure 5: Fully-developed pycnidium of *Phyllosticta citricarpa* filled with mature viable conidia in a freckle spot lesion after 20 days under optimal conditions for disease development.

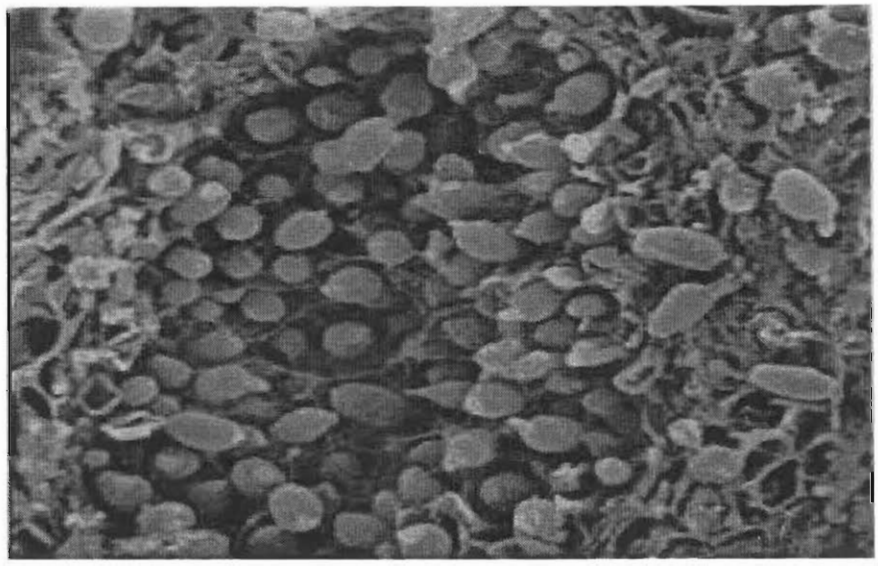

Figure 6: Enlargement of Fig. 5 showing numerous viable conidia of *Phyllosticta citricarpa* imbedded in a gelatinous mass ready for dispersal.

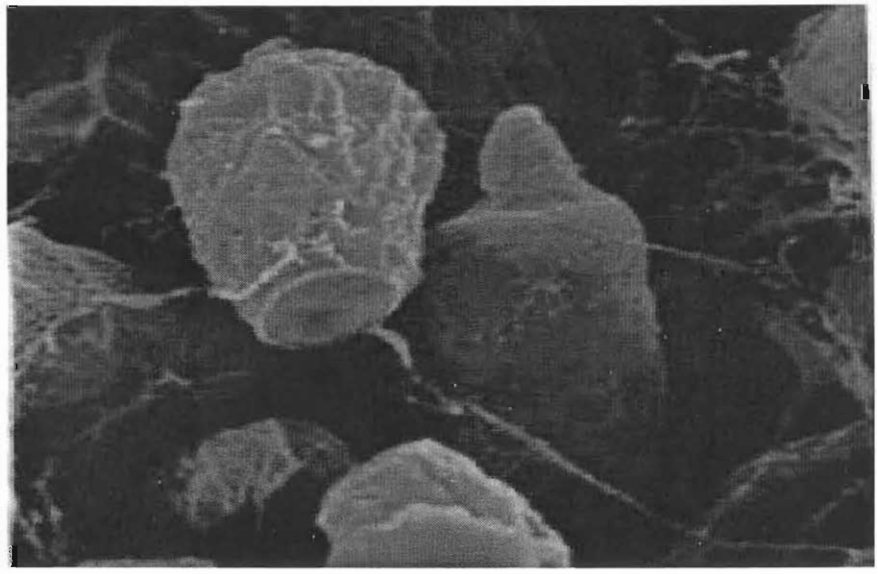

Figure 7: Viable conidia of *Phyllosticta citricarpa* present in a pycnidium on fruit incubated for 20 days. Note the gelatinous cap-like appendage on each conidium which is used for attachment.

Viability of different types of hard spot CBS lesions. At day 5, only RMH-type hard spot lesions on unprocessed fruit yielded an appreciable percentage of viable *P. citricarpa* conidia (Table 1). However, the incidence of viable conidia progressively declined to zero at day 21. Viability of conidia in BMH lesions on unprocessed fruit was about 73% less than in RMH lesions at day 5 and also of a transient nature. Very few conidia from DBMH lesions germinated when plated out, and none at all from BLMH lesions. Packhouse processing drastically reduced conidial viability, with RMH lesions being the only type yielding some viable conidia after processing, albeit only temporarily.

Table 1: Viability of *Phyllosticta citricarpa* conidia in different categories of CBS hard spot lesions on Valencia orange fruit subjected or not subjected to packhouse treatments.

Hard spot Category[x]	%Viable conidia on untreated fruit[y]			%Viable conidia on treated fruit[y]		
	5d	14d	21d	5d	14d	21d
RMH	43.3 a	8.3 b	0c	1.4 c	0 c	0 c
BMH	10.6 a	1.2 b	0 b	0 b	0 b	0 b
DBMH	0.3 a	0 a	0 a	0 a	0 a	0 a
BLMH	0 a	0 a	0 a	0 a	0 a	0 a

[x] RMH = red margin hard spots; BMH = brown margin hard spots; DBMH = dark brown margin hard spots; BLMH = black margin hard spots.

[y] Means in a row (based on 15 replicates) followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

A similar tendency was evident with mycelial viability, although packhouse processing had a less pronounced effect on survival (Table 2). BLMH lesions nevertheless contained no viable mycelium, whether fruit were treated or not.

Table 2: Viability of *Phyllosticta citricarpa* mycelium in different categories of CBS hard spot lesions on Valencia orange fruit subjected or not subjected to packhouse processing.

Hard spot category[x]	% Viable mycelium on untreated fruit[y]			% Viable on treated fruit[y]		
	5d	14d	21d	5d	14d	21d
RMH	98.2 a	90.4 a	87.2 a	67.2 b	54.6 b	58.3 b
BMH	83.3 a	74.8 b	63.2 b	17.3 c	11.3 cd	8.7 d
DBMH	8.6 a	5.9 a	4.3 a	1.2 b	0.4 b	1.2 b
BLMH	0 a	0 a	0 a	0 a	0 a	0 a

[x] RMH = red margin hard spots; BMH = brown margin hard spots; DBMH = dark brown margin hard spots; BLMH = black margin hard spots.

[y] Means in a row (15 replicates) followed by the same letter do not differ significantly (P= 0.05) according to Duncan's multiple range test.

Evaluation of media for sustaining germination of *P. citricarpa* conidia. Best germination of *P. citricarpa* conidia occurred in SDW supplemented with Eureka lemon or with Valencia orange juice, although Star Ruby grapefruit juice also supported good germination (Table 3). Stimulation of germination was evident in the citric acid solution, but considerable variation existed between replicates. Compared to the juice extracts, germination in the Valencia orange peel suspension was low, but nevertheless higher than in Valencia peel washings, sucrose solution, and SDW.

Table 3: Germination rate of *Phyllosticta citricarpa* conidia in sterile distilled water (SDW) supplemented with various substances.

Substance (per litre SDW)	PH	% Germination[x]
Sterile distilled water	7.25	0.2 c
15 g mature Valencia orange peel	4.62	27.8 d
0.5 g sucrose	6.92	0.3 c
5 g citric acid	4.00[y]	33.4 bd
20ml mature Valencia orange juice	4.34	63.9 a
20 ml mature Eureka lemon juice	3.84	69.2 a
20 ml mature Star Ruby grapefruit juice	3.93	44.0 b
Valencia peel washings	6.52	0.8 c

[x] Means in a column (based on 3 replicates) do not differ significantly (P = 0.05) according to Duncan's multiple range test.

[y] pH adjusted with 1 N NaOH.

Categorising of CBS symptoms. Four main types of CBS lesions could be distinguished, viz. Type A (hard spot or shot-hole spot) (Figs. 8-12), Type B (false melanose or speckled blotch) (Figs. 13 & 15), Type C (freckle spot) (Figs. 15-17), and Type D (virulent spot, spreading spot or galloping spot) (Figs. 18 & 19).

Type A symptoms (Fig. 8) are commonly present and may have a green halo on mature fruit or a yellow halo on green fruit. The symptom can be distinguished from other lesion types by a parchment-like, grey-white to light-brown depressed centre in which pycnidia may or may not be present. The rind of these lesions is slightly raised and vary in colour from light red to pitch black. Variations within lesion type A include type A1, (RMH) with a red margin (Fig. 9), type A2 (BMH) with a brown margin (Fig. 10), type A3 (DBMH) with a black margin and no halo (Fig. 11), and type A4 (BLMH) with a black margin (Fig. 12).

Type B lesions (Fig. 13) are an early-season expression of infection by the CBS pathogen and appear within months after fruit has reached the resistant stage towards infection. These lesions are small, circular, slightly raised, dark brown to inky black, surrounded by tiny black specks, and do not produce pycnidia. With time they may develop into type A lesions, whereas type C lesions can appear amongs them on mature fruit. Type B_1 lesions (mud-cake blotches) (Fig. 14) differ from classical false melanose or speckled blotch in being larger in diameter and having an abrasive texture. The centres of these lesions are cracked, giving them a mud-cake like appearance. They also do not contain pycnidia.

Type C lesions (Fig. 15) are red to dark red, sunken, and usually appear on mature fruit after the colour has changed from green to orange. Individual spots sometimes coalesce into type D lesion (Fig. 16). One or several (Fig. 17) pycnidia may be present in the lesions, but the grey-white craters characteristic of type A lesions are absent.

Type D symptoms (Fig. 18) mainly develop late in the season on fully mature fruit, even after harvesting (Fig. 19). The necrotic, sunken lesions are brown to brick red at the periphery, irregular, confluent and spread rapidly. Numerous black pycnidia may be present in collapsed tissue in the centre of old type D lesions. However, pycnidia and conidia are absent in newly-formed virulent spots.

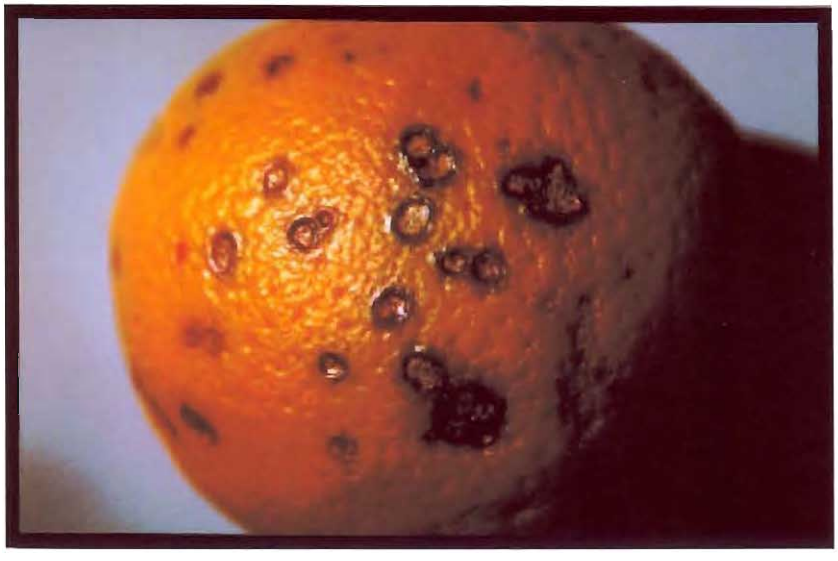

Figure 8: Citrus black spot lesion type A , hard spot or shot-hole spot.

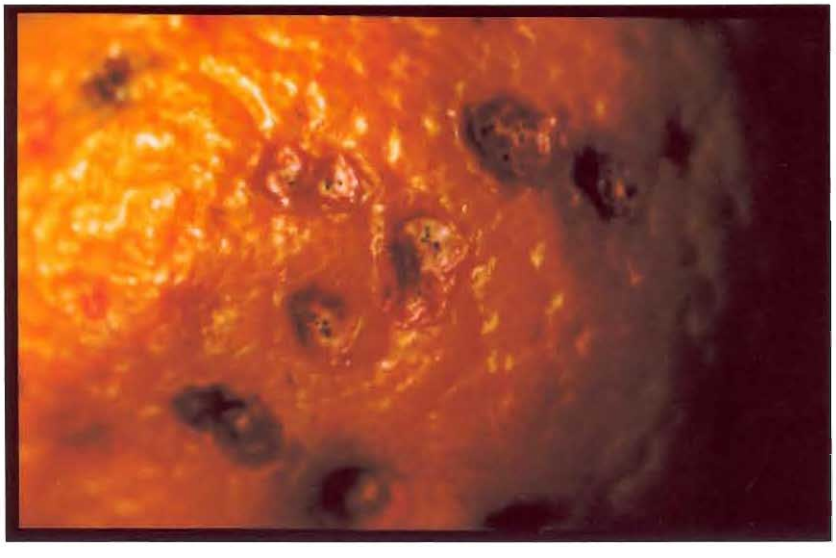

Figure 9: Citrus black spot lesion type A1, red margin hard spot (RMH).

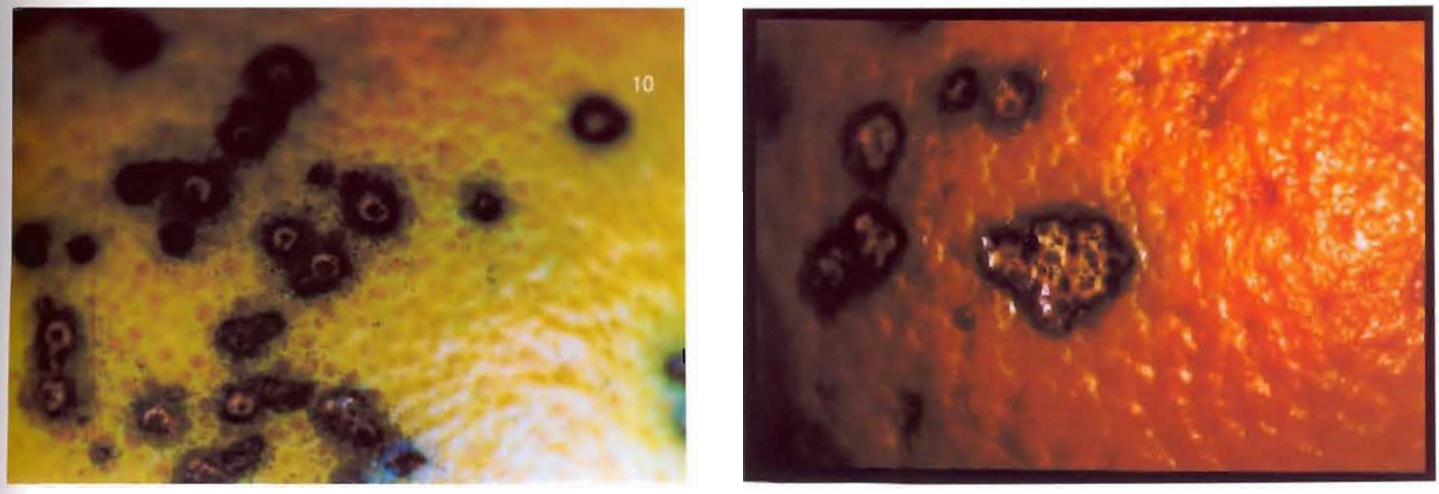

Figure 10: Citrus black spot lesion type A2, brown margin hard spot (BMH).

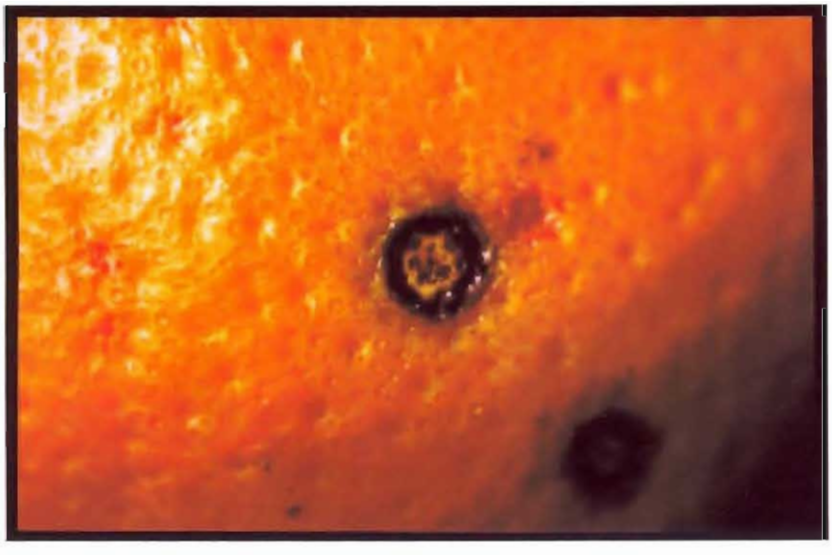

Figure 11: Citrus black spot lesion type A3, dark brown margin hard spot with no halo present (DBMH).

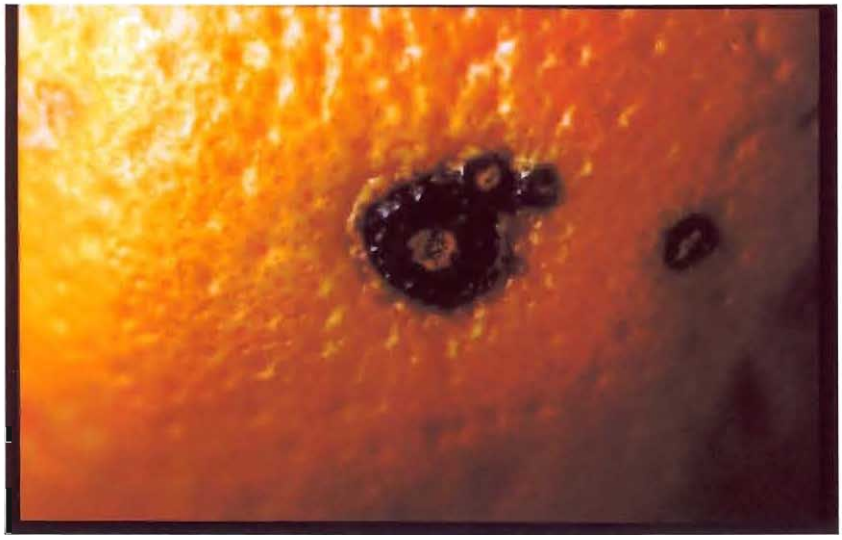

Figure 12: Citrus black spot lesion type A4, black margin hard spot (BLMH).

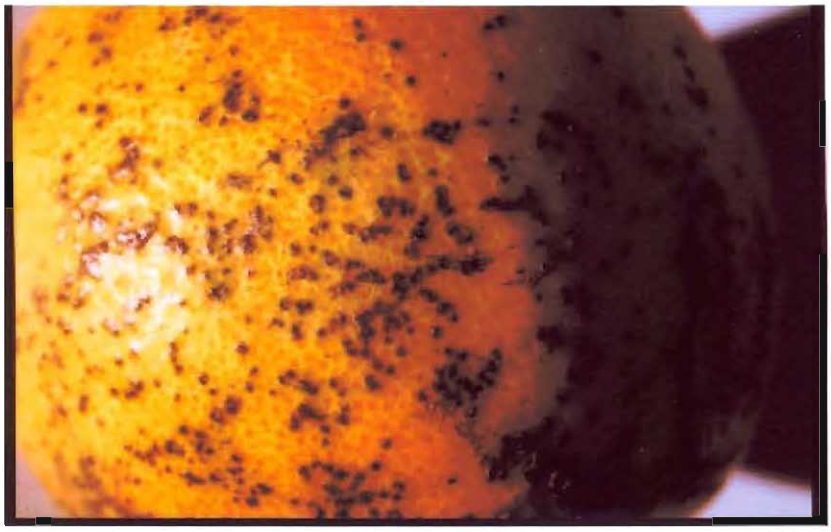

Figure 13: Citrus black spot lesion type B, false melanose or speckled blotch

Figure 14: Citrus black spot lesion type B2, mud-cake blotches.

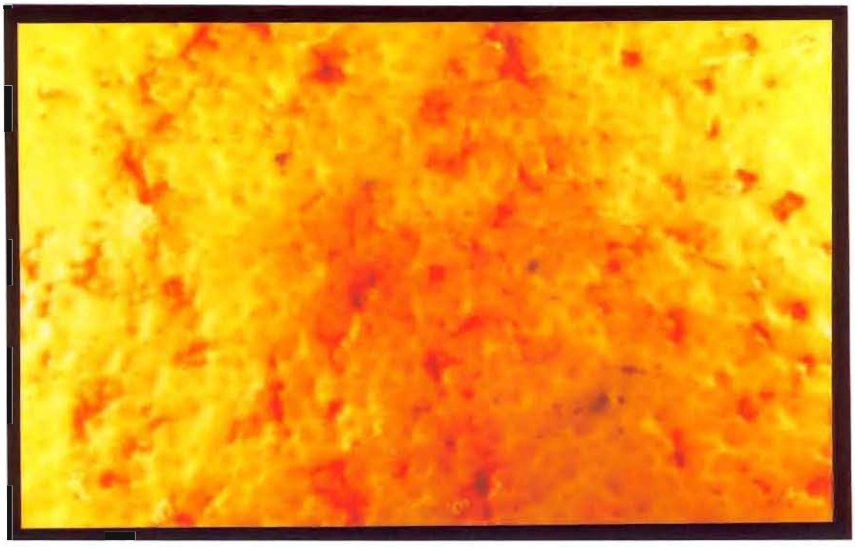

Figure 15: Citrus black spot lesion type C, freckle spot.

Figure 16: Coalescing type C freckle spots forming type D virulent spots.

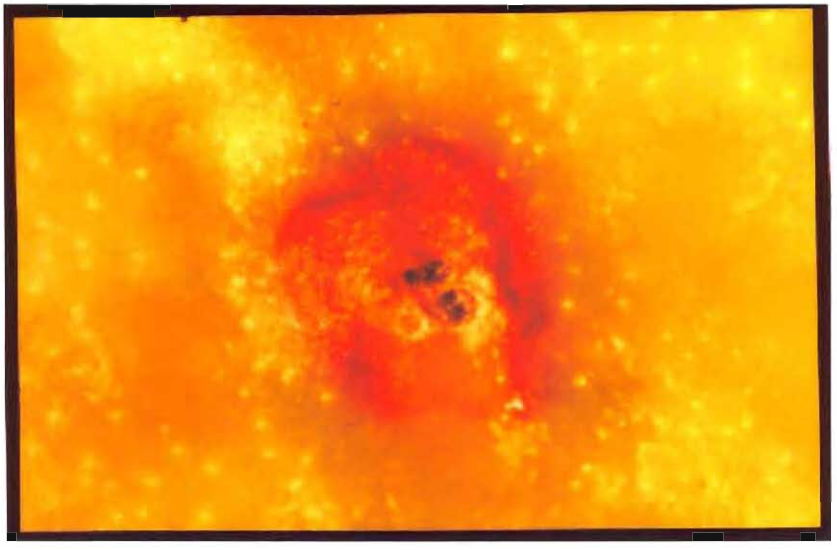

Figure 17: Citrus black spot lesion type C freckle spot with several pycnidia in the centre.

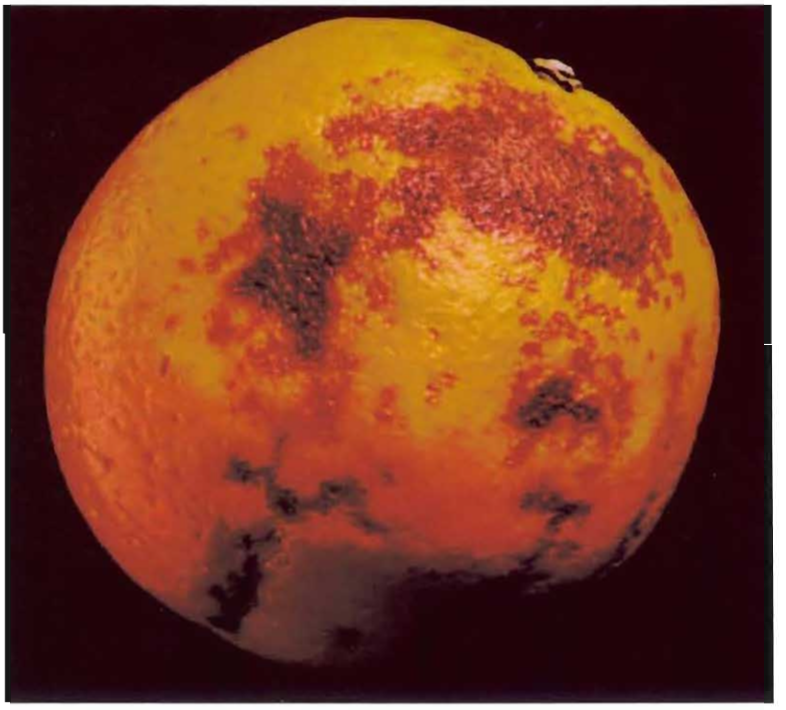

Figure 18: Citrus black spot lesion type D, virulent, spreading or galloping spot.

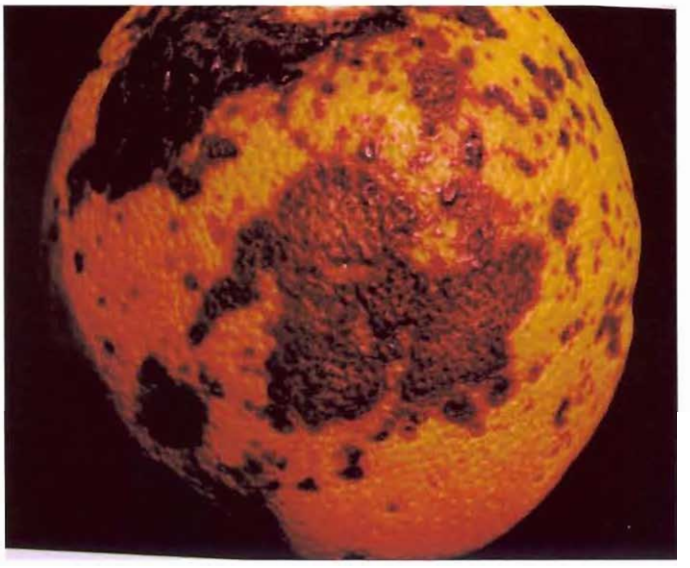

Figure 19: Virulent spot which developed after post harvesting.

***P.citricarpa* leaf inoculations.** *P. citricarpa* could not be re-isolated from any of the artificially inoculated mature Valencia leaves (Table 4). *C. gloeosporoides*, on the other hand, developed from about 40% of the inoculated and uninoculated leaf discs plated out. Electron microscopy indicated poor adhesion of *P. citricarpa* to the intact natural surface of leaves, but good adherence was observed in wounds and cracks (Fig. 20), which typically contained mucous clusters of conidia (Fig. 21). However, no conidial germination was evident after 4 weeks

Table 4: Isolation frequency of *Phyllosticta citricarpa* and *Colletotrichum gloeosporioides* from intact Valencia citrus leaves artificially inoculated or not inoculated with a conidial suspension of the CBS pathogen.

Tree no	Inoculation	% *P. citricarpa*[x]	% *C. gloeosporioides*[x]
1	Abaxial	0 a	34.2 b
	Adaxial	0 a	59.3 a
	Control	0 a	54.5 a
2	Abaxial	0 a	13.2 c
	Adaxial	0 a	66.8 a
	Control	0 a	43.2 b
3	Abaxial	0 a	25.3 b
	Adaxial	0 a	51.4 a
	Control	0 a	57.6 a
4	Abaxial	0 a	38.6 a
	Adaxial	0 a	10.3 b
	Control	0 a	12.5 b
5	Abaxial	0 a	66.6 a
	Adaxial	0 a	74.4 a
	Control	0 a	23.5 b
6	Abaxial	0 a	16.0 c
	Adaxial	0 a	62.4 a
	Control	0 a	42.7 b
7	Abaxial	0 a	8.5 b
	Adaxial	0 a	21.1 a
	Control	0 a	24.5 a
8	Abaxial	0 a	63.4 a
	Adaxial	0 a	62.7 a
	Control	0 a	50.5 b
9	Abaxial	0 a	39.5 a
	Adaxial	0 a	46.2 a
	Control	0 a	18.5 b
10	Abaxial	0 a	27.3 b
	Adaxial	0 a	56.4 a
	Control	0 a	58.3 a
Mean	Abaxial	0 a	33.3 a
	Adaxial	0 a	44.4 a
	Control	0 a	42.5 a

[x] Mean of 15 isolations from 5 leaves on each of 10 trees; values in columns within trees followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

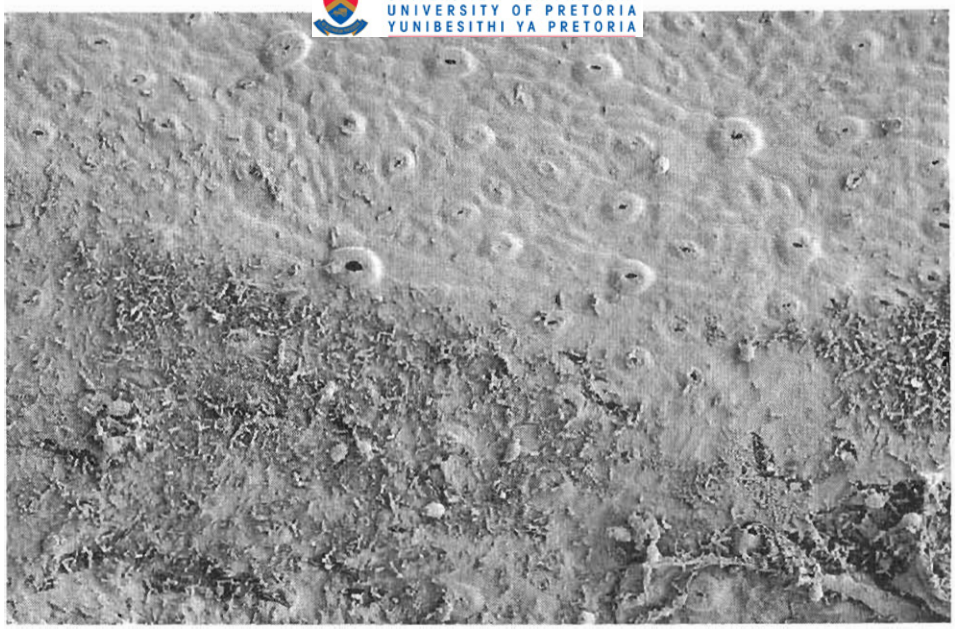

Figure 20: Poor adhesion of *Phyllosticta citricarpa* conidia to the intact natural leaf surface of abaxially-inoculated Valencia leaves (top of picture), and good adhesion in wounds and cracks

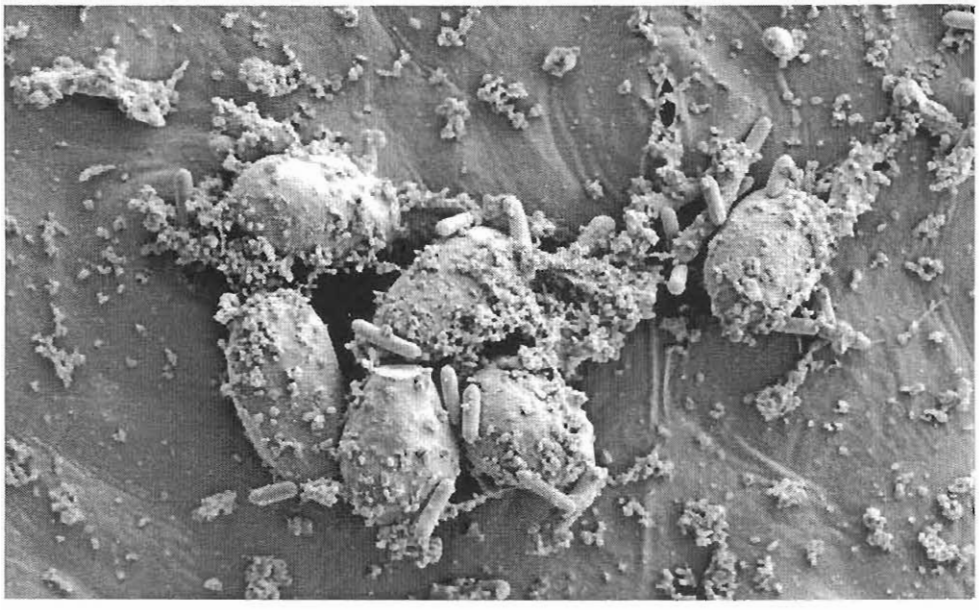

Figure 21: *Phyllosticta citricarpa* conidia in a wound on a Valencia leaf.

DISCUSSION

Brodrick & Rabie (1970) showed that light and temperature promote CBS symptom development. In the present investigation, CBS lesions developed within 5 – 7 days on symptomless, naturally-infected Valencia oranges at a storage temperature of 27 °C and 24 h fluorescent lighting. Lesions appearing first, were freckle spots and upon further incubation they developed into virulent spots. Pycnidia were produced after 9 – 13 days of incubation under optimal conditions. Viable conidia could be discerned in these pycnidia the moment they changed colour from whitish translucent to brownish black, i.e. from day 13 onwards. No conidia were present in primordial pycnidia sampled after 10 days.

Brodrick & Rabie (1970) observed significantly slower CBS lesion development at 20 °C than at 27 °C, whereas Wager (1948) found that lesion development at 4.5 °C was delayed to such an extent that practically no further increase occurred. The recommended export temperature for citrus varies between 10 °C and 11 °C (Venter & Cook, 1998). It is thus clear that under export conditions, lesion development and conidium production would be delayed. Furthermore, latently-infected fruit do not have the potential to produce new viable *P. citricarpa* conidia within the time allowed for marketing.

Valencia oranges receiving no postharvest treatment contained higher percentages of viable mycelium and conidia in the different hard spot lesions than fruit subjected to standard packhouse treatments. The combination of packhouse treatments and incubation time reduced the conidial inoculum present on fruit to zero. This is in accordance with findings in Chapter 4. It is clear from the higher percentage mycelium surviving the packhouse treatments that a brush-on application of imazalil sulphate is less effective than an imazalil, 2,4-D and guazatine dip treatment. Findings by Kiely (1948) and Darnell-Smith (1918) were confirmed that negligible *P. citricarpa* conidial germination occurs in distilled water. Germination in citrus peel extracts was not comparable to

germination in juice extracts. This might have been the result of toxicity of citrus peel extracts to *P. citricarpa* (Brodrick, 1971).

Grouping of the four basic CBS symptoms present on citrus fruit into lesion types A, B, C and D not only consolidated the various existing classification systems, but facilitated allocation of atypical CBS symptoms to a specific type (Table 5). The only lesion type capable of producing a significant amount of viable conidia was Type A1, red margin hard spot. Conidial inoculum produced by freckle and virulent spots can be regarded as insignificant compared to viable hard spots. Type A, "hard spots" are conspicuous blemishes on harvested fruit and are actively culled in the packhouse. It is furthermore unlikely for Type A lesions to develop in transit (Wager, 1948) and the chances of new viable conidia reaching CBS-free recipient countries, therefore remote. Lesion Type B holds no threat to importing countries. It is an early season expression of CBS containing no pycnidia or conidia and is also actively culled in the packhouse. Thus, the only viable CBS propagules potentially capable of reaching their end-destination via citrus fruit are viable mycelium in the margin of Type C and D lesions. Only under extreme conditions will it be possible to spread through viable mycelium on fruit to intact citrus leaves. This infection phase, together with a summer-rainfall climate, are essential for the continuation of the CBS disease cycle which will ultimately lead to a CBS epidemic (Kotzé, 1981).

Table 5: Comparison of proposed and existing citrus black spot lesion descriptions.

Proposed CBS lesion classification on fruit	Existing CBS lesion classification on fruit	Reference	Description of lesion type	Viable conidia and mycelium after packhouse processing
Type A	Hard spot (synonym: shot-hole spot)	Darnell-Smith, 1918; Kiely,1948; Calavan,1960; Kotzé, 1963, 1981; Brodrick, 1969; Garrán, 1996	▪ Circular crater-like lesions often surrounded by a green or yellow halo. ▪ Rims of the crater are slightly raised with a red to pitch black colour. ▪ Parchment-like centres are grey-white to light brown and may or may not contain numerous pycnidia.	Viable mycelium with the ability to produce viable conidia.
Type B	Speckled blotch (synonyms: false melanose; inky spot)	Kotzé, 1963; McOnie, 1965; Brodrick, 1969; Garrán, 1996.	▪ An early season expression of CBS. ▪ Lesions are small, circular and slightly raised. ▪ Lesions are dark brown to inky black, surrounded by tiny black specks and have an abrasive texture.	Viable mycelium. No conidia are found in this lesion type.
Type C	Freckle spot	Kiely,1948; Wager, 1948; Calavan,1960; Kotzé, 1963, 1981; Brodrick, 1969	▪ Small red to brick red sunken, irregular to circular lesions, which develop on mature fruit after the colour has changed from green to orange. ▪ No grey-white crater center is present. ▪ Pycnidia can develop in the lesions but never in the same quantities as in Type A lesions.	Viable mycelium in the margins of lesions.
Type D	Virulent spot (synonyms: spreading or galloping spot)	Darnell-Smith, 1918; Wager, 1948 Kiely,1948; Calavan,1960; Kotzé, 1963, 1981; Brodrick, 1969; Garrán, 1996	▪ Develop late in the season on fruit which is fully matured. ▪ Lesions are sunken and brown to brick red at the periphery, irregular, confluent and spread rapidly. ▪ Pycnidia may develop in the centers of these lesions upon incubation.	Viable mycelium. This lesion type frequently develops in transit and thus can contain viable conidia

Reisolation of *P. citricarpa* from artificially-inoculated intact mature Valencia leaves was unsuccessful and Koch's postulates could not be satisfied. Wager (1953) also failed to establish infection in intact citrus leaves by spraying a *P. citricarpa* conidial suspension onto healthy trees during the few months following petal drop. He proposed that spells of wet weather are necessary for the conidia to germinate and penetrate young fruit or leaves. The reluctance of *P. citricarpa* conidia to adhere to natural leaf surfaces observed

here corroborates the findings in Chapter 4 regarding conidial adhesion to the natural waxy layer present on fruit. However, more detailed investigations are necessary to determine if the age and state of citrus leaves could determine susceptibility towards infection by *P. citricarpa* conidia infection.

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CHAPTER 3

IN VITRO EFFECTS OF FUNGICIDES, DISINFECTANTS AND PHYSICAL CONDITIONS ON CONIDIAL VIABILITY OF THE CITRUS BLACK SPOT PATHOGEN, *PHYLLOSTICTA CITRICARPA*

ABSTRACT

Seven *Phyllosticta citricarpa* isolates from different regions of the world were screened *in vitro* for sensitivity towards postharvest fungicides and disinfectants registered for use in South African citrus packhouses. A method was developed which facilitated conidial germination and appressorium formation as criteria for evaluating fungistatic activity. Guazatine and *o*-phenylphenol (sodium salt) inhibited conidial germination and appressorium formation completely at registered packhouse concentrations. Prochloraz, thiabendazole, and two emulsion formulations of imazalil, viz. Chloramizol and Sanazil, inhibited germination by 99.93 %, 99.13 %, 99.13 % and 98.66 % respectively. Imazalil sulphate was less effective, with 21.19 % of the conidia exposed to the compound germinating. Chlorine dioxide inhibited conidial germination more effectively and at lower concentrations than calcium hypochlorite. Based on their response to the various fungicides, the *P. citricarpa* isolates could be separated into three distinct groups, viz highly-sensitive, moderately-sensitive and sensitive. Optimal germination of conidia occurred at 22 °C and was not affected by light. Optimal pH for germination in a citric acid pH-range varied between 4.0 and 4.2.

INTRODUCTION

Citrus black spot (CBS), incited by *Guignardia citricarpa* Kiely (anamorph *Phyllostictina citricarpa* (McAlp.) Van der Aa), causes severe economic losses in all major citrus producing countries subject to summer-rainfall, e.g. Argentina, Uruguay, Brazil, South

Africa, Zimbabwe, Swaziland, Mocambique, India, China, Hong Kong, Taiwan, Japan and Australia (Sutton & Waterston, 1966; Kotzé, 1981; Schutte *et al.*, 1996a). The disease is absent in Mediterranean regions (winter-rainfall) (Schutte *et al.*, 1996a).

All commercial citrus cultivars are susceptible to CBS (Kotzé, 1981). Infection occurs through ascospores produced on dead, infected fallen leaves, but lesions on fruit contain only pycnidia bearing conidia (McOnie, 1964; Kotzé, 1981). Although conidia can be a significant source of inoculum when washed from out-of-season or late-bearing fruit onto young susceptible fruit below (Kotzé, 1981), they are not considered a potential source of infection, particularly on fruit exported to Mediterranean countries where rainfall occurs mainly in winter (Darnell-Smith, 1918; Kiely, 1948; Wager, 1949; Kotzé, 1981). Despite this assurance, European Union (EU) countries such as Spain, Italy, Greece and Cyprus have recently expressed concern that CBS from the Southern Hemisphere may infect their citrus crops. Directions have consequently been implemented by the EU to prevent the import of CBS-infected fruit by member countries that are free of the disease.

The above legislation obviously could have serious consequences for the South African citrus industry. Notwithstanding extensive preharvest spray programmes with fungicides such as copper, dithiocarbamates, benzimidazoles (Kellerman & Kotzé, 1977; De Wet, 1987) and strobilurines (Schutte *et al.*, 1996b), the incidence of CBS in South Africa are very low (S. Kamburov, personal communication). However, compared to other citrus diseases, one infected fruit could result in the rejection of an entire consignment. Measures therefore have to be taken to ensure that CBS-affected fruit remain non-infective. A feasible option for such a purpose is to render infections on fruit non-viable by chemical treatment.

Fungicides registered for postharvest use on citrus in South Africa include benomyl, guazatine, imazalil, imazalil sulphate, *o*-phenylphenol (sodium salt), prochloraz and thiabendazole (Krause *et al.*, 1996). Another group of compounds with extensive postharvest application are the various chlorine formulations. Although not classified as fungicides, they effectively reduce inoculum levels of decay pathogens (Brown &

Wardowski, 1984). Considering the activity of the above chemicals against various fungal species and their compatibility with citrus, at least some of them have the potential to inhibit the CBS-pathogen on citrus fruit. This chapter reports on their *in vitro* efficacy in this regard and also provides information on the effect of environmental factors on conidial germination by *P. citricarpa*.

MATERIALS AND METHODS

Agrochemicals. The following fungicides were evaluated: guazatine (Decotine 20 % SL; Rhône-Polenc Agrichem; registered rate 5 $\mu l\ ml^{-1}$), imazalil (Chloramisol 80 % EC; Janssen Pharmaceutica, and Sanazil 80 % EC; Sanachem; registered rate 1.32 $\mu l\ ml^{-1}$), imazalil sulphate (Fungazil 75 % SP; Janssen Pharmaceutica; registered rate 1.36 mg ml^{-1}), *o*-phenylphenol (sodium salt) (SOPP 32 % SL; Thor Chemicals; registered rate 4.8 $\mu l\ ml^{-1}$), prochloraz (Omega 45 % EC; AgrEvo; registered rate 3.3 $\mu l\ ml^{-1}$) and thiabendazole (Tecto Flowable Fungicide 45 % SC; Logos Pharmaceuticals; registered rate 4.5 $\mu l\ ml^{-1}$). Fungicides were screened at their respective registered rates (RR), as well as at quarter (¼RR), half (½RR) and double (2RR) these dosages. Aqueous stock solutions of the compounds were prepared in sterile deionised water and maintained in a refrigerator at 4.5°C.

The chlorine compounds evaluated comprised calcium hypochlorite from HTH and chlorine dioxide from BTS Products & Services. They were tested at registered rates of 100 ppm and 10 ppm respectively, as well as at ¼RR and ½RR. Solutions were freshly prepared for each screening and the pH of calcium hypochlorite was adjusted to 7 for optimal efficacy.

***P. citricarpa* isolates.** Isolates of the CBS pathogen were kindly supplied by the National Collection of Fungi, Plant Protection Reasearch Institute (PPRI), Pretoria, and included acquisitions from Taiwan (ATCC 32757), Hong Kong (PPRI 1565), Brazil (IBS 2/94), Argentina (PPRI 5365), India (IMI 304799), Malelane, South Africa (PPRI 5351) and Eshowe, South Africa (PPRI 5350).

Conidial germination studies. The isolates of *P.* citricarpa were cultured on a medium consisting of 24 g potato-dextrose agar (Merck Biolab), 1 g yeast-extract (Difco), 1 g malt-extract (Difco) and 8 g agar (Merck Biolab) per litre deionised water. Three replicate cultures of each isolate were incubated under continuous fluorescent lighting at 22 °C and RH > 85 %. Conidia were harvested after incubation for 10 to 14 days by aseptically removing the crust of each culture with a scalpel and placing it into a 250 ml Erlenmeyer flask containing 30 ml sterile deionised water. Spores were dislodged by rotary shaking for 1 hour and the resultant conidial suspension was dispensed into 1.5 ml Eppendorf tubes. Tubes were centrifuged twice for 10 minutes at 5 000 rpm, with the pellet being resuspended in sterile deinonised water after the first centrifugation. The spore concentration was adjusted to 1-3 x 10^6 ml^{-1} with deionised water.

A germination medium was prepared consisting of 20 ml Valencia orange juice made up to 1 litre with deionised water. The suspension was filtered through Whatman No. 1 paper and autoclaved at 121°C for 20 minutes. It had a final pH of 4.34.

Microwell plates (196FW/Lids AEC-Amersham (Pty) Ltd.) with a pit volume of 400 µl were used for observing conidial germination and appressorium formation. Each pit received 150 µl of the germination medium, 50 µl of fungicide or chlorine dilution and 50 µl of the conidium suspension. Control wells each received 50 µl sterile deionised water instead of fungicides or chlorine. Five replicate wells were used for each chemical/concentration/isolate combination. The total number of spores, germinated spores and appressoria (only for fungicides) in a 400 x magnified field in each well were counted after 6, 12, 24, 48 and 96 hours under an inverse light microscope.

Environmental factors. The environmental factors concerned were temperature, light and pH. Conidial suspensions of *P. citricarpa* PPRI 5350 Taiwan (ATCC 32757), Hong Kong (PPRI 1565), Brazil (IBS 2/94), Argentina (PPRI 5365), India (IMI 304799), Malelane, South Africa (PPRI 5351) and Eshowe, South Africa (PPRI 5350) were prepared as above. For temperature studies, the microwell plates with germination medium were adapted for 24 hours to 0.5, 4.5, 10, 15, 22, 27 and 40 °C, before adding 50µl of conidial suspension of each *P. citricarpa* isolate to each of three wells at each temperature. Plates were incubated for a further 48 hours under continuous fluorescent lighting at the respective temperatures, after which germination was recorded as described previously. Plates at 0.5 and 4.5 °C were incubated for 7 days before being assessed. The entire experiment was repeated with plates incubated in total darkness.

The effect of pH on conidial germination was determined in a citric acid pH-range, adjusted with 1 N NaOH to 3.2, 3.4, 3.8, 4.0, 4.2, 4.4, 4.6 and 4.8. Conidial suspensions of the above four *P. citricarpa* isolates were added to 50 ml of the various citric acid solutions in glass beakers. A 200 µl aliqout from each isolate/solution was aseptically transferred to each of three wells in a microwell plate. Plates were incubated for 48 hours at 22 °C and the number of germinated conidia recorded.

RESULTS

Conidia of the different *P. citricarpa* isolates germinated readily in the Valencia orange juice medium. A single germ-tube emerged randomly from each conidium, particularly in the area in contact with a solid surface. Germ-tube length varied considerably and in some instances boxing glove-like appressoria formed directly after germination (Fig. 1).

Effect of agrochemicals on conidial viability. All the fungicides significantly impeded conidial germination and appressorium formation at RR and ½RR (Table 1). Except for imazalil sulphate, the compounds also reduced conidial viability at ¼RR, with guazatine and SOPP providing the same inhibition at ¼RR as at RR. The latter two compounds were furthermore the only ones suppressing conidial and appressorium formation

completely at RR. Doubling the RR improved fungicidal efficacy only in the case of imazalil sulphate.

Based on germination response, the fungicides could be classified as moderately effective (imazalil sulphate), effective (thiabendazole, and imazalil as Sanazil and Chloramizol), highly effective (SOPP, guazatine and prochloraz) (Table 2). Similarly, the various *P. citricarpa* clustered as moderately sensitive (IBS 2/94 and IMI 304799 from Brazil and India respectively), sensitive (PPRI 5351 and PPRI 5350 from Malelane and Eshowe, South Africa respectively), and highly sensitive (PPRI 5356, PPRI 1565 and ATCC 32757 from Argentina, Hong Kong and Taiwan respectively) (Table 3).

At RR, calcium hypochlorite and chlorine dioxide inhibited conidial germination completely (Table 4). Both formulations also significantly suppressed germination at ¼RR and ½RR, but only chlorine dioxide provided the same inhibition at ½RR as at RR.

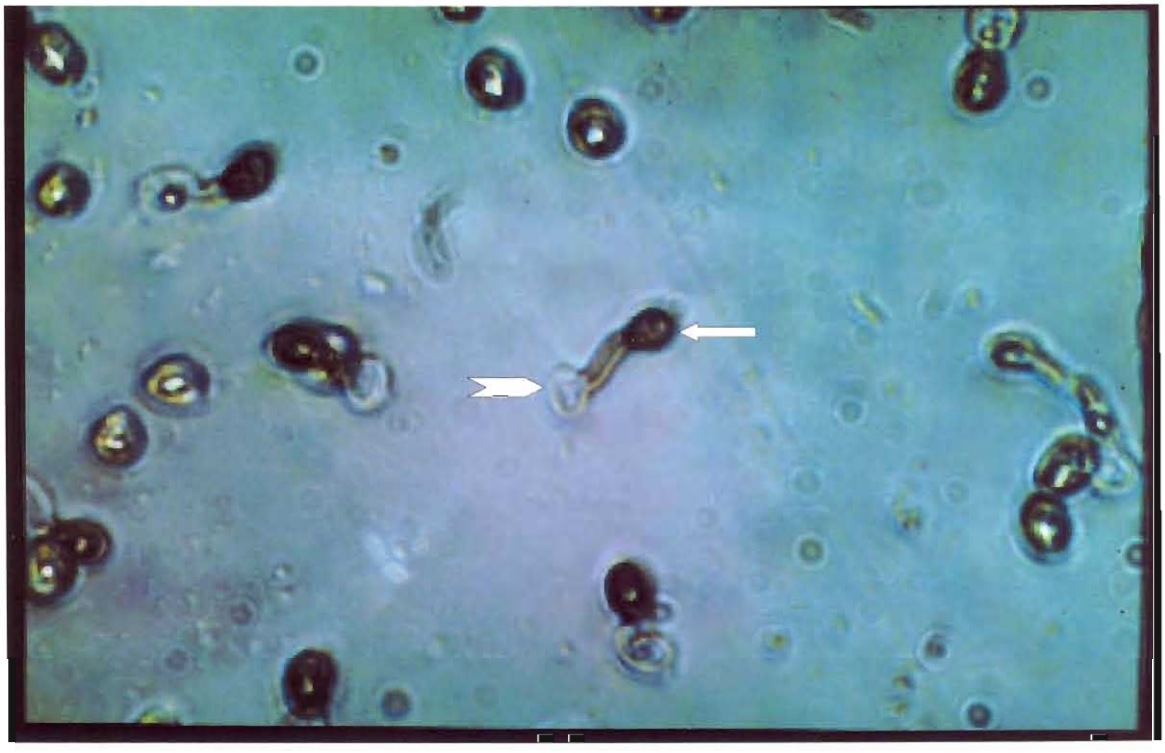

Figure 1: Inverse light microscopy of germinating *P.citricarpa* conidia (⇨) Appressoria (⇦) are dark brown to black with a boxing glove appearance.

Table 1: *In vitro* effect of registered post harvest fungicides in South Africa on conidial germination and appressorium formation by *Phyllosticta citricarpa*.

Fungicide	Fungicide concentration ($\mu l\ ml^{-1}$)	% Conidial germination [y]	% Appressorium formation [y]
Imazalil sulphate	0 a [x]	68.8 a	55.2 a
	0.34	51.8 a	47.9 a
	0.68	40.8 b	34.7 b
	1.36 [z]	21.2 c	20.8 c
	2.72	2.03 d	0.8 d
Imazalil (Sanazil)	0	69.45 a	56.9 a
	0.33	28.1 b	19.1 b
	0.66	5.5 c	2.5 c
	1.32 [z]	1.4 d	0.5 d
	2.64	0.07 d	0 d
Imazalil (Chloramisol)	0	68.0 a	55.6 a
	0.33	9.9 b	6.3 b
	0.66	3.5 c	3.1 b
	1.32 [z]	0.4 d	0.3 c
	2.64	0.07 d	0 c
Thiabendazole	0	64.8 a	56.7 a
	1.13	10.3 b	7.6 b
	2.25	2.9 c	1.6 c
	4.5 [z]	0.9 d	0.5 c
	9	0 d	0 c
Prochloraz	0	70.9 a	58.3 a
	0.83	4.5 b	2.0 b
	1.65	1.5 c	0.7 c
	3.3 [z]	0.07 c	0 c
	6.6	0 c	0 c
Guazatine	0	62.45 a	50.3 a
	1.25	1.12 b	0.6 b
	2.5	0.29 b	0 b
	5.0 [z]	0 b	0 b
	10	0 b	0 b
SOPP	0	68.27 a	54.0 a
	1.2	0.10 b	0 b
	2.4	0 b	0 b
	4.8 [z]	0 b	0 b
	9.6	0 b	0 b

[x] Concentration in $mg\ ml^{-1}$

[y] Mean of 5 replicates of each of 7 *P. citricarpa* isolates evaluated over 5 time intervals at each fungicide concentration; values in columns within fungicides followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's studentised range test.

[z] Recommended application rate.

Table 2: *In vitro* efficacy of different registered post harvest fungicides in South Africa for suppressing conidial germination and appressorium formation by *Phyllosticta citricarpa.*

Fungicide	% Conidial germination[x]	% Appressorium formation[x]	Fungicide grouping
Imazalil sulphate	28.98 a	26.05 a	Moderately effective
Imazalil (Sanazil)	8.73 b	5.53 a	Effective
Imazalil (Chloramisol)	3.48 b	2.43 b	Effective
Thiabendazole	3.52 c	2.44 c	Effective
Prochloraz	1.50 d	0.67 d	Highly effective
Guazatine	0.35 d	0.15 d	Highly effective
Sodium *o*-phenyl phenate	0.025 d	0.00 d	Highly effective

[x] Mean of 5 replicates of each of 7 *P. citricarpa* isolates evaluated over 5 time intervals at 5 concentrations of each fungicide; values in columns followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's studentised range test.

Table 3: *In vitro* sensitivity of different *Phyllosticta citricarpa* isolates to registered post harvest fungicides in South Africa.

Isolate	% Conidial germination[x]	% Appresorium formation[x]	Isolate grouping
Brazil	12.29a	8.96 a	Moderately sensitive
India	9.48 b	8.86 a	Moderately sensitive
Malelane	6.45 c	5.54 b	Sensitive
Eshowe Natal	4.38 d	3.43 c	Sensitive
Argentina	2.87 e	2.18 d	Highly sensitive
Hong Kong	2.30 e	1.98 d	Highly sensitive
Taiwan	2.17 e	1.57 d	Highly sensitive

[x] Mean of 5 replicates of each of 7 fungicides evaluated over 5 time intervals at 5 concentrations of each fungicide; values in columns followed by the same letter do not differ significantly (P=0.05) according to Tukey's studentised range test.

Table 4: *In vitro* effect of chlorine compounds on conidial germination by different *Phyllosticta citricarpa* isolates.

Isolate	Concentration in ppm		%Germination	
	ClO_2	$Ca(OCl)_2$	ClO_2 [x]	$Ca(OCl)_2$ [x]
Brazil	0	0	68.4 a[a]	65.3 a
	2.5	25	1.2 b	10.0 b
	5	50	0.04 b	1.9 c
	10 [y]	100 [y]	0 b	0 c
India	0	0	61.2 a	55.8 a
	2.5	25	1.8 b	12.9 b
	5	50	0 b	2.6 b
	10 [y]	100 [y]	0 b	0 b
Malelane (South Africa)	0	0	48.4 a	60.7 a
	2.5	25	0.8 b	10.1 b
	5	50	0 b	3.4 c
	10 [y]	100 [y]	0 b	0 d
Eshowe Natal (South Africa)	0	0	56.5 a	54.9 a
	2.5	25	3.1 b	10.2 b
	5	50	0.08 c	2.4 c
	10 [y]	100 [y]	0 c	0 c

[x] Mean of five replicates for each chlorine concentration at each of 5 time intervals; values in columns followed by the same letter do not differ (P = 0.05) according to Tukey's studentised range test.

[y] Recommended application rate.

Effect of environmental factors on conidial germination. Optimal conidial germination and appressorium formation by *P. citricarpa* isolates from Brazil, India, Malelane and Eshowe South Africa occurred at 22 °C, regardless of whether the cultures were incubated in light or in darkness (Fig. 2; Table 5). No germination was evident at 0.5, 4.5 and 40 °C and only a few conidia produced germ tubes at 35 °C. Germination at 10, 15 and 27 °C was also significantly less than at 22 °C when incubation took place in light. Optimal pH for conidial germination and appressorium formation by the four *P. citricarpa* isolates in citric acid solution was between 4.0 and 4.2, whereas maximum germination rate of the isolates varied between 27 % (IBS 2/94 from Brazil) and 49 % (PPRI 5350 from Eshowe South Africa) (Fig. 3; Table 6).

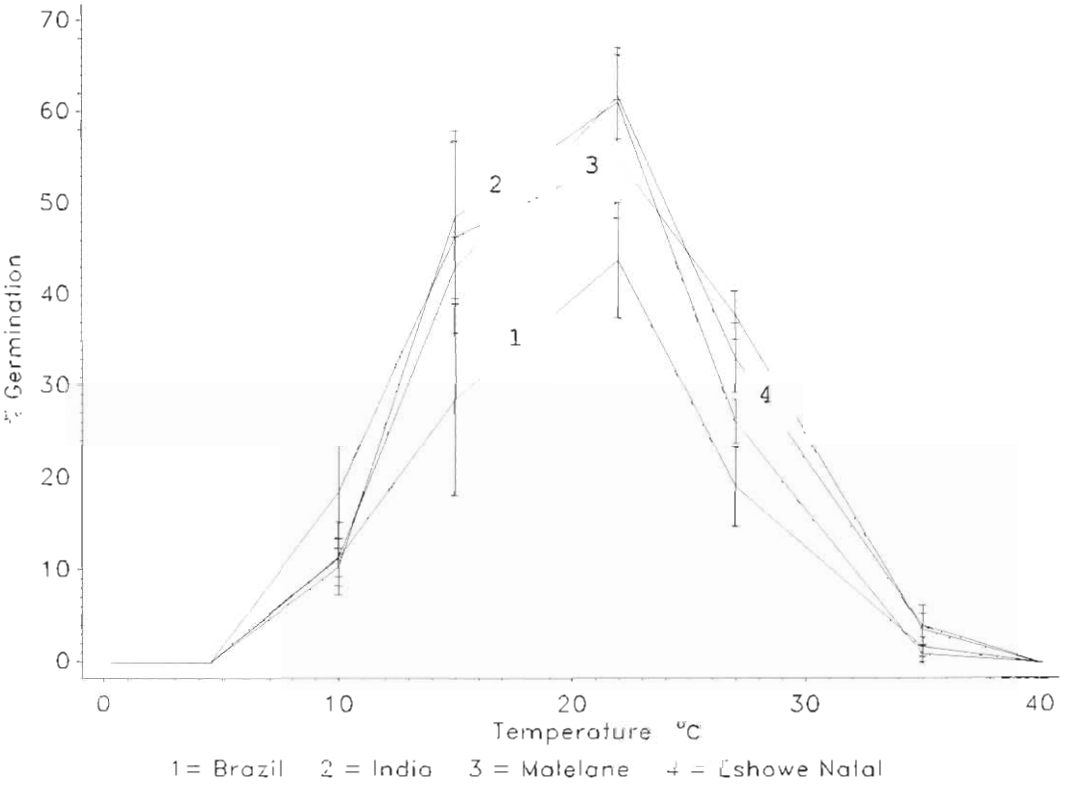

Figure 2 *In vitro* effect of temperature on conidial germination by 4 *Phyllosticta citricarpa* isolates. Evaluation points in each line represents the mean of 6 observations at 48 h.

Table 5: *In vitro* conidial germination and appressorium formation rate of *Phyllosticta citricarpa* at different temperatures in the presence and absence of light.

Temperature	% Conidial germination in light[x]	% Conidial germination in darkness[x]	% Appressorium formation in light[x]	% Appressorium formation in darkness[x]
0.5°C	0 e	0 d	0 d	0 d
4.5°C	0 e	0 d	0 d	0 d
10°C	12.9 d	10.5 c	6.0 c	8.9 c
15°C	41.6 b	45.3 a	30.5 b	32.7 b
22°C	55.3 a	48.9 a	46.7 a	43.7 a
27°C	29.0 c	27.6 b	25.4 b	25.5 b
35°C	2.5 e	1.9 d	1.7 d	0.6 d
40°C	0 e	0 d	0 d	0 d

[x] Mean of 6 observations at 48 hours on each of 4 *P. citricarpa* isolates; values in columns followed by the same letter do not differ significantly (P=0.05) according to Tukey's studentied range test.

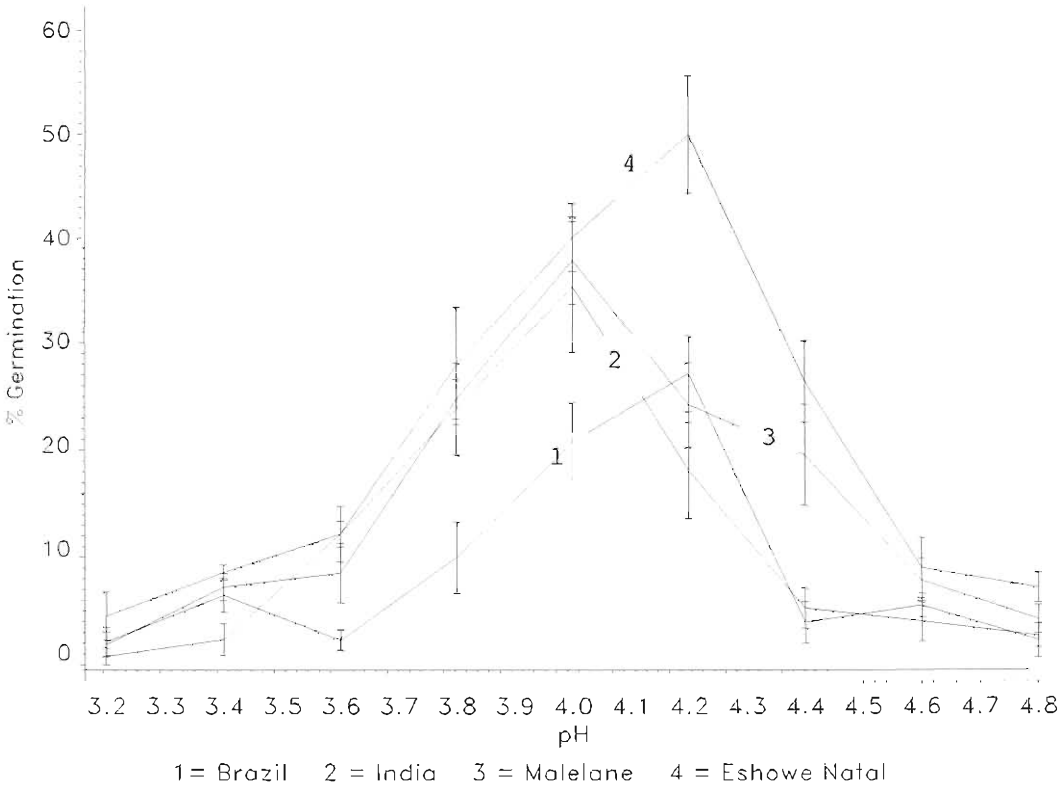

Figure 3 *In vitro* effect of pH on conidial germination by four *Phyllosticta citricarpa* isolates. Evaluation points in each line represent the mean of 6 observations at 48 h.

Table 6: *In vitro* conidial germination and appressorium formation rate of *Phyllosticta citricarpa* in citric acid at different pH values.

pH	% Conidial germination[x]	% Appressorium formation[x]
3.2	2.3 d	1.7 c
3.4	6.2 d	3.1 c
3.6	8.8 cd	5.4 c
3.8	21.6 b	13.3 b
4.0	33.5 a	20.4 a
4.2	29.8 ab	24.9 a
4.4	13.8 c	10.4 b
4.6	6.60 d	4.1 c
4.8	4.08 d	3.1 c

[x] Mean of 6 observations at 48 hours on each of 4 *P. citricarpa* isolates; values in columns followed by the same letter do not differ ($P = 0.05$) according to Tukey's studentised range test.

DISCUSSION

The Valencia orange juice medium used in this study generally supported conidial germination and appressorium formation by *P. citricarpa* more prolifically than the citric acid solution, despite having a pH 1.7 to 3.4 times higher than that for optimal germination in the latter solution. This endorses the belief of Darnell-Smith (1918) and Kiely (1948) that *P. citricarpa* conidia require an external stimulus to germinate, and that this stimulus is provided by a nutritional factor, as emerged from Chapter 2. It furthermore indicates that the spore environment in which the various experiments of the present investigation were conducted, readily allowed germination of viable conidia.

All the fungicides evaluated here suppressed conidial germination by *P. citricarpa* and could potentially be used for reducing inoculum of the pathogen on citrus fruit. However, due to the existence of complicating factors, expectations created by *in vitro* results are seldom realised in practice. For instance, pycnidia in CBS hard spot lesions differ in maturity and therefore do not release their conidia synchronously (Darnell-Smith, 1918; Kiely, 1948), thus implying that timing and scheduling of fungicide application are crucial. Even when mature, not all conidia are released from the pycnidia when in contact with water (or fungicide suspension), and conidia not exposed to the fungicide at the time of application obviously would escape its effect. Furthermore, propagules of *P. citricarpa* not only consist of conidia, but also of mycelium, which may survive fungicide treatment and give rise to new lesions in which fresh pycnidia could eventually be produced (Wager, 1948).

Considering the above reservations and the peculiarity of the situation at hand, it is evident that only fungicides providing total *in vitro* control of *P. citricarpa* could be considered for commercial eradication of CBS inoculum from citrus fruit. The two fungicides which completely inhibited conidial germination by *P. citricarpa* in the laboratory, were guazatine and SOPP. The latter compound has such a broad mode of

action that it can actually be used as a disinfectant in packhouses (Smithwick & Kaplan, 1984). However, its sensitivity to pH and phytotoxicity towards citrus fruit (Smithwick & Kaplan, 1984) preclude its general use. Guazatine, although comparatively non-toxic to plants and animals, acts as a diffusion barrier on the plasma membrane of cells (Dekker & Georgepoulos, 1982), and is therefore fungistatic rather than fungicidal. The only other fungicide which was classified as highly effective, viz. prochloraz, has the same limitation as guazatine. Being a sterol biosynthesis inhibitor, it also acts fungistatiallly (Dekker & Georgepoulos, 1982). Prochloraz is furthermore not acceptable to some European markets while guazatine residues are prohibited by Eastern markets (Venter & Cook, 1998). Concerning their spectrum of activity, and hence their universality of application, guazatine is known to control sour rot, blue and green mould and *Alternaria* black rot effectively. SOPP controls blue and green mould to a lesser extent than guazatine, but also has activity against *Diplodia* stem-end rot, *Alternaria* black rot and sour rot. Prochloraz is effective against all the above postharvest pathogens (Venter & Cook, 1998).

Contrary to the above fungicides, chlorine has a cidal action and is accepted by virtually all overseas markets (Brown & Wardowski, 1984). Of the two compounds evaluated, chlorine dioxide proved to be the most effective and can also be applied at lower rates. The better performance of chlorine dioxide probably is due to its more stable formulation and lower reactivity with organic material (Brown & Wardowski, 1984; Lesar, 1997). Whether these characteristics would enable the compound to eradicate *P. citricarpa* from citrus fruit *in vivo*, remains to be seen.

Although quantitative differences were evident in the response of the different *P. citricarpa* isolates to the various chemical treatments, the isolates did not differ qualitatively in reaction. The same phenomenon prevailed with the physical requirements for germination of the isolates, and the tendencies observed can therefore be regarded as representative of the species. The optimal temperature of 22 °C for conidial germination by *P. citricarpa* corresponds with that for conidium formation by the organism *in vitro* (Brodrick & Rabie,1970), but is lower than the 27 °C reported by Brodrick (1969) and

Brodrick & Rabie (1970) as conducive to mycelial growth, spermatium production and symptom development. Unlike conidium production and symptom development (Brodrick & Rabie,1970), conidial germination by *P. citricarpa* did not respond positively to light. While light could thus induce sporulation of the CBS pathogen on citrus fruit, even when in transit, it would not enhance germination of the conidia. Of particular importance is the fact that conidia of *P. citricarpa* germinated optimally at pH 4.0-4.2, and hardly at all at pH 4.8. As the pH of orange, grapefruit and even lemon peel exceeds 5 (Sinclair, 1984), germination of conidia on or within the peel seems unlikely.

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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

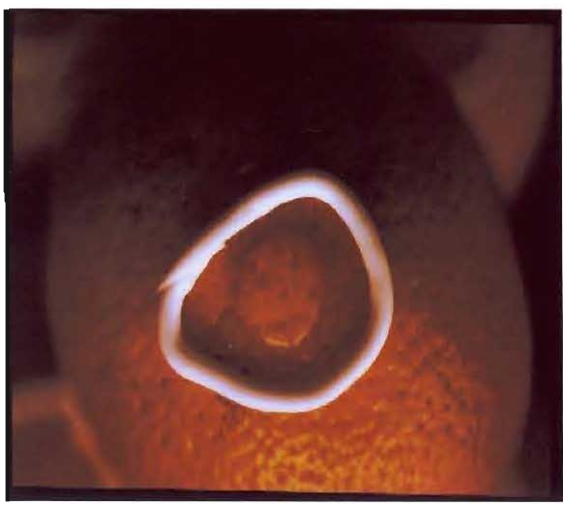
concentration was adjusted to 10^7 ml^{-1} spores/conidia for inoculation purposes. A fresh conidial suspension was prepared for each inoculation date referred to below.

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

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.



A

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.

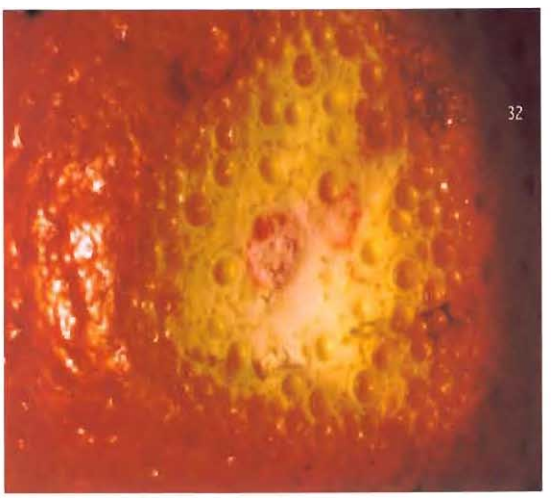
Pathogen	Storage temperature (°C)	Wounding of skin	Positive isolations from 100 fruit: Week 1	Week 2	Week 3	Total	Chi-square (Probability)[λ]
	0.5	-	0	0	0	0	
	25	-	0	0	0	0	a) 16.81 ($P = 0.0000$)
Phyllosticta		Total	**0**	**0**	**0**	**0**	b) 16.81 ($P = 0.0000$)
citricarpa	0.5	+	0	0	0	0	c) 0.07 ($P = 0.9652$)
	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)

Pathogen	Storage temperature (°C)	Wounding of skin	Positive isolations from 100 fruit Week 1	Week 2	Week 3	Total	Chi-square (Probability)[x]
	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).

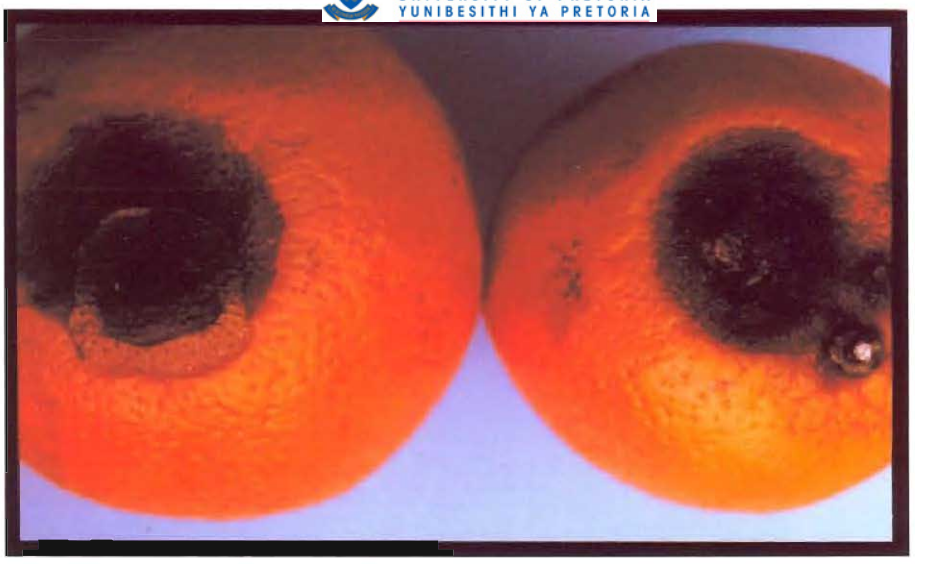


Figure 3: Development of virulent CBS spots on wounded citrus fruit kept for four weeks at 25 °C after artificial inoculation with *Phyllosticta citricarpa*.

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.

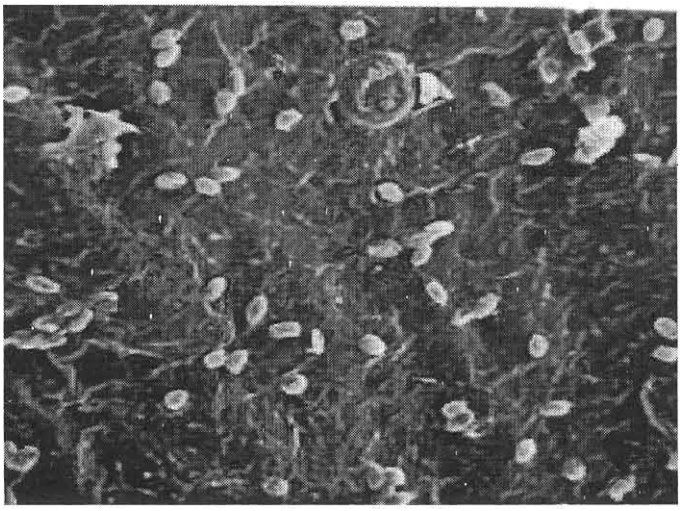


Figure 4: Adhesion of *Phyllosticta citricarpa* conidia to the synthetic polyethylene micro-wax layer on the surface of citrus fruit.

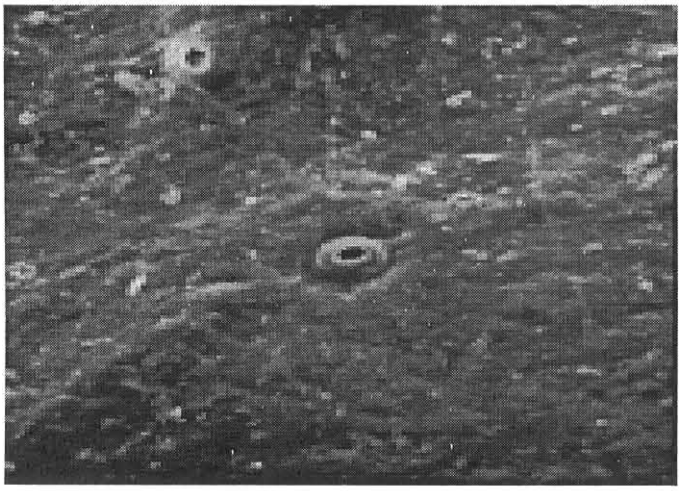


Figure 5: *Phyllosticta citricarpa* conidia on artificially-inoculated Valencia fruit peel not coated with a synthetic wax layer. Note that virtually no adhesion occurred in the inoculated area.

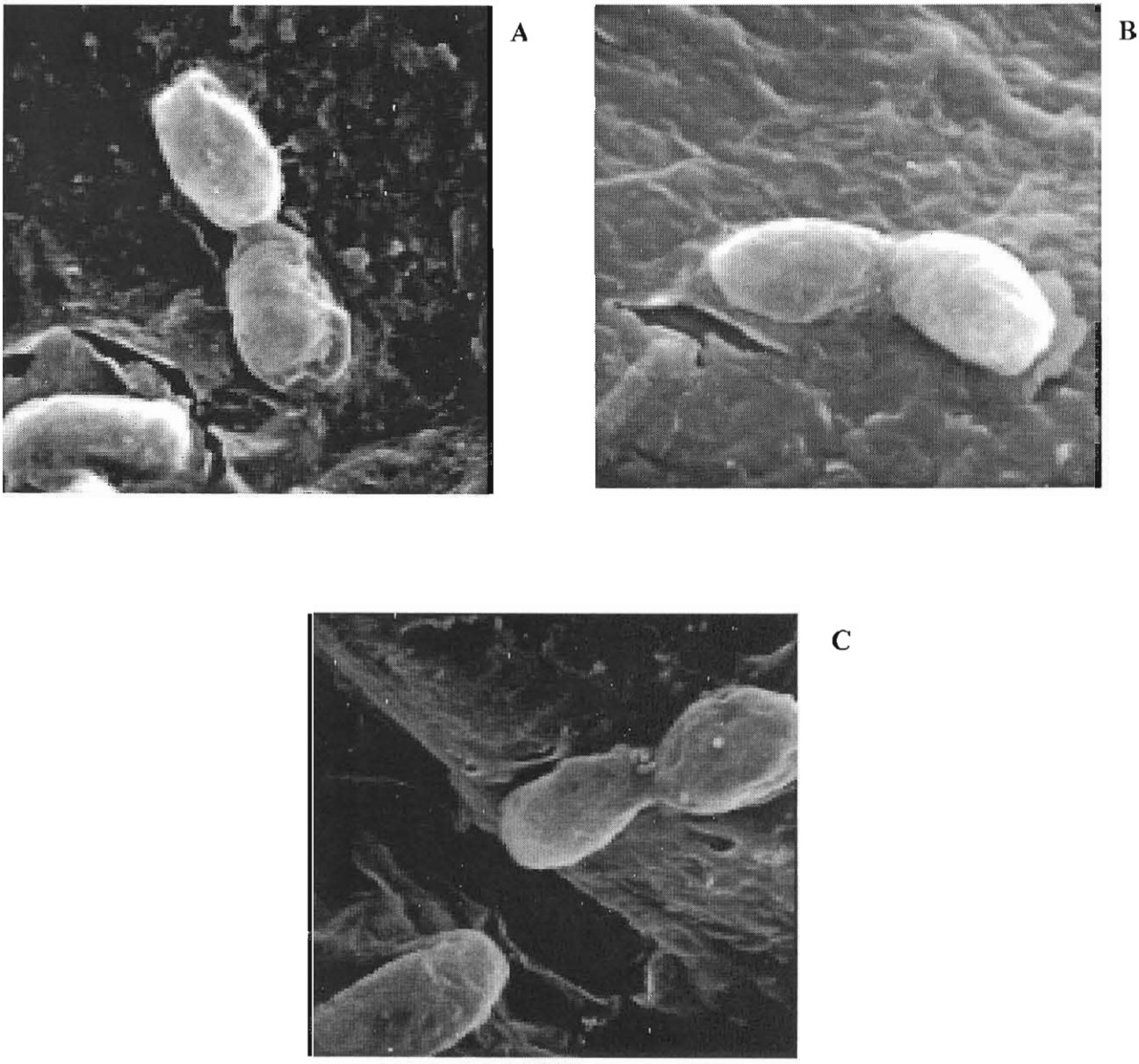


Figure 6: Germinated conidia of *Phyllosticta citricarpa* on the surface of a wounded citrus fruit stored at 25°C, with no visible germ tube between the spore and appressorium. Note attachment to synthetic wax layer (A, B & C).

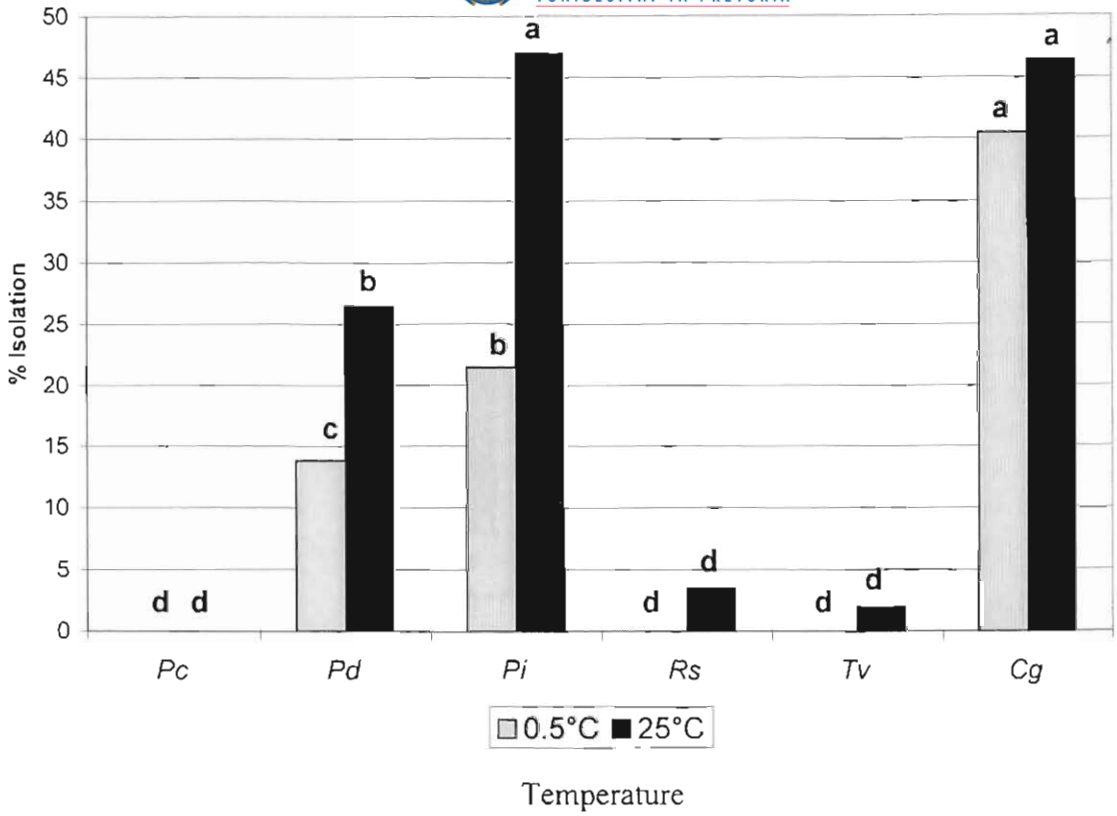


Figure 7: Isolation frequency of citrus pathogens (*Pc=Phyllosticta citricarpa; Pd=Penicillium digitatum; Pi=Penicillium italicum; Rs=Rhizopus stolonifer; Tv=Trichoderma viride; Cg=Colletotrichum gloeosporioides*) from originally-healthy citrus fruit exposed for three weeks at 0.5 °C and 25 °C, respectively, 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.

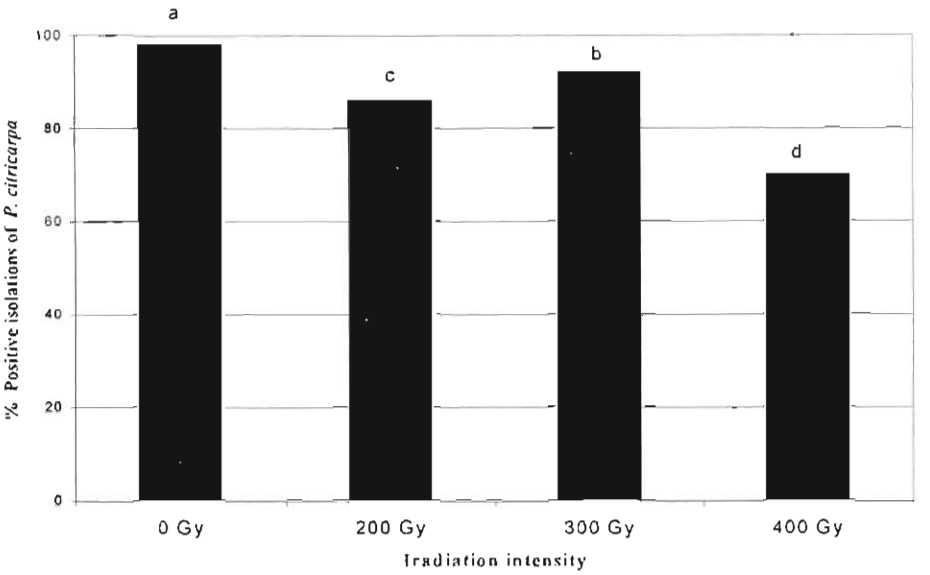


Figure 8: Isolation frequency of *Phyllosticta citricarpa* from black spot lesions on Valencia oranges exposed to different intensities of cobalt irradiation. Bars (based on the means of isolations made from 50 fruit) followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's 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

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.

		Positive isolations from 100 fruit[x]						
Pathogen	Temperature	Control[y]	Chlorine[y]	Spray[y]	Warm water[x]	Chemicals[x]	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
Colleto-trichum gloeosporioi-des	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.

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

	Mean percentage positive isolations after 3 weeks [x]								
Pathogens	Control	Difenoconazole	Triforine	Tebucconazole	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.

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CHAPTER 5

GENERAL DISCUSSION

The possible spread of citrus black spot from South Africa to Mediterranean countries was investigated. Existing evidence indicates that citrus fruit are susceptible to infection by ascospores form the teleomorphic state, *Guignardia citricarpa* Kiely, for 3-4 months after petal drop. Thereafter the pathogen lies dormant until maturity sets in (Wager, 1953; Kotzè, 1963; McOnie, 1964). Spells of wet weather are necessary for ascospores to germinate and infect the young fruit and leaves. Data on the prerequisites for *Phyllosticta citricarpa* (McAlp.) Van der Aa conidia to incite disease in citrus fruit or leaves are vague and inconclusive.

CBS lesions first appeared on naturally infected symptomless Valencia fruit after 5-7 days of incubation under optimal conditions (27 °C, high humidity and continious fluorescent lighting). Pycnidia containing viable conidia were visible after 9-13 days under optimal conditions. Unlike CBS lesion development and condium and spermatium production in culture, (Brodrick & Rabie, 1970) light had no effect on condial germination by *P. citricarpa*, while the optimal germination temperature, 22 °C, corresponded with the optimal temperature for conidium production in culture. During the 30-40 days it takes for shipment and marketing of fruit conditions for CBS lesion development are suboptimal and it is doubtful if new lesions with viable conidia could develop in this period. Wager (1948) showed that lesion development at 4.4 °C was significantly slower than at 29.4 °C. According to him, *P. citricarpa* conidia do not appear to have a long live-span but the present results showed a tendency for conidia to stay longer viable at cooler storage temperatures (4.5 °C and 11 °C). Artificial inoculation with *P. citricarpa* conidia proved unsuccessful at 0.5 °C on wounded and unwounded fruit.

A combination of packhouse treatments and storage of fruit eliminated *P. citricarpa* conidial inoculum on CBS fruit, although mycelium in the periphery of viable lesions survived. Fruit are exported under cool (10 – 11 °C) conditions (Venter & Cook, 1998). There is thus little opportunity for pycnidia with viable conidia to develop from surviving mycelium on packhouse-processed fruit during shipment and marketing.

Existing packhouse procedures and the fungicides utilised in South Africa, with the exception of imazalil sulphate, reduced viable mycelium and eliminated conidia of *P. citricarpa* from fruit. Combinations of factors are employed in packhouses to reduce decay (hurdle technology) and it is therefore unlikely that imazalil sulphate will be applied as the only postharvest treatment in a packhouse, and the inability of the compound to control conidial germination is no reason for concern. Chorine in the receiving bins is sufficient for reducing the *P. citricarpa* condial inoculum on fruit. The warm water bath and subsequent postharvest chemical treatment will eliminate the remaining condial inoculum and reduce viable mycelium significantly. The only CBS lesion type capable of producing viable conidia in quantities sufficient to cause infection were red margin hard spots (Chapter 2, Fig.9). Mycelium in the periphery of lesions showing red active growth (freckle spots, virulent spots and red margin hard spots), unlike conidial inoculum present in these lesions, can survive the packhouse treatments.

It was further demonstrated in artificial and natural inoculation studies that *P. citricarpa* conidia could not infect healthy mature packhouse-treated oranges. This is in accordance with findings of Wager (1953) that infected CBS fruit cannot transfer the disease to healthy mature oranges. A low percentage infection occurred on wounded fruit artificially inoculated with *P. citricarpa* conidia and stored for 4 weeks at 25°C and high humidity. These conditions do not occur under export conditions and there is therefore little chance of cross-contamination between infected and clean fruit.

Even though remote possibility exists for *P. citricarpa* conidia on CBS-infected fruit to infect wounded citrus fruit during shipment, the requirements for the onset of a CBS epidemic is not met. The disease must spread from the rind of imported infected fruit to

intact citrus leaves in recipient countries free of the disease. This step is crucial because the teleomorphic state, *G. citricarpa*, develops only on pre-infected decaying citrus leaves on the orchard floor (Kiely, 1948; McOnie,1964; Kotzé, 1981). The presence of a summer rainfall climate is a further prerequisite for the onset of a CBS epidemic (Kotzé, 1981; Schutte, 1996). Mature intact Valencia leaves could not be inoculated through artificial inoculation with *P. citricarpa* conidia (Chapter 2). According to Wager (1948), young citrus leaves are susceptible to infection by *P. citricarpa* conidia, but this statement is contradicted in findings where he had sprayed leaves with viable *P. citricarpa* conidial suspensions during the first few months after petal drop without achieving infection of mature or young leaves (Wager, 1953).

It is undeniable that in the past 40 years citrus fruit infected with CBS were exported to European countries where the disease is absent. During this time no transference of the disease occurred. Results made available through this study demonstrated that for the CBS pathogen to spread from infected fruit to orchards where the disease is absent, is highly unlikely if not impossible.

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SURVIVAL OF *PHYLLOSTICTA CITRICARPA*, ANAMORPH OF THE CITRUS BLACK SPOT PATOGEN

by

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RESUMÉ

Citrus black spot (CBS) is caused by *Guignardia citricarpa,* a member of the *Mycosphaerellaceae* in the ascomycete order, *Dothideales. G. citricarpa* infects primarily by ascospores produced on detached senescent leaves, but exists on citrus fruit in the anamorpic or conidial state, *Phyllosticta citricarpa*. Although conidia of *P. citricarpa* are regarded as of little or no significance in the epidemiology of CBS, concern has recently been expressed that infected fruit could serve as source of infection. This report describes the production of inoculum by *P citricarpa* and provides evidence on the survival of conidia and mycelium of the pathogen.

CBS lesions commonly occurring on citrus fruit were indexed into four distinct categories, viz. lesion type A (hard spot or shot-hole spot), B (false melanose, speckled blotch or inky spot), C (freckle spot), and D (virulent, spreading or galloping spot). This

indexing system allowed the allocation of atypical CBS lesions present on fruit to a particular class. Type C lesions developed first on naturally-infected Valencia fruit, appearing within 5-7 days at 27 °C, high humidity and continuous fluorescent lighting. Pycnidia produced in these lesions after 9-13 days already contained viable conidia. Conidia present in type A lesions remained viable for 14 days on unprocessed fruit stored under optimal conditions, but succumbed to standard packhouse treatments. Mycelium of *P. citricarpa* in the periphery of type A lesions survived packhouse processing.

Conidia of *P. citricarpa* did not infect unwounded 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 no infection occurred at 0.5 °C. Conidia adhered more readily to the synthetic polyethylene micro-wax layer on fruit than to the natural waxy surface layer. CBS could not be induced in healthy mature Valencia leaves inoculated with conidia of *P. citricarpa*. In the laboratory, conidia germinated optimally at 22 °C, with no germination evident at 0.5 °C and 40 °C. Optimal pH for germination in a citric acid pH-range was 4.0-4.2, the minimum 3.2, and the maximum 4.8. Light had no effect on germination rate.

Seven *P. citricarpa* isolates from different regions of the world were screened *in vitro* for sensitivity towards fungicides and disinfectants registered for use in South African citrus packhouses. Guazatine and *o*-phenylphenol (sodium salt) inhibited conidial germination and appressorium formation completely at recommended rates. Prochloraz, thiabendazole, and two emulsion formulations of imazalil, viz. Chloramizol and Sanazil, reduced germination by more than 98 %. Imazalil sulphate was less effective, with 21 % of the conidia exposed to the compound remaining viable. Chlorine dioxide inhibited conidial germination more effectively, and at lower concentrations, than calcium hypochlorite.

Packhouse procedures which consistently reduced the incidence of *P. citricarpa* in CBS lesions *in vivo* 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 viablility was reduced to zero by keeping CBS-infected fruit for three weeks at 25 °C, as well as by exposing the fruit to chlorine, warm water, the above chemicals, or all treatments combined. Various fungicides not registered for use on harvested citrus fruit inactivated conidia of *P. citricarpa*, one notable exception being the plant resistance-activating substance, acibenzolar-*s*-methyl. Difenoconazole, although not registered on citrus, substantially reduced mycelial inoulum present in CBS lesions. Cobalt irradiation of up to 400 Gy did not eliminate inoculum of *P. citricarpa* on infected fruit.

DIE OORLEWING VAN *PHYLLOSTICTA CITRICARPA*, ANAMORF VAN DIE SITRUS SWARTVLEK PATOGEEN

deur

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OPSOMMING

Sitrus swartvlek (SSV) word veroorsaak deur *Guignardia citricarpa*, 'n lid van die *Mycosphaerellaceae* in die askomiseete-orde, *Dothideales*. *G. citricarpa* infekteer hoofsaaklik deur askospore wat geproduseer word op sitrusblare wat van die boom afgeval het. Die patogeen kom voor op sitrusvrugte in die anamorf- of konidiale fase, *P. citricarpa*. Alhoewel konidium van *P. citricarpa* beskou word as onbelangrik in die epidemiologie van SSV, is kommer onlangs uitgespreek dat geïnfekteerde vrugte kan dien as bron van infeksie. Hierdie verslag beskryf die produksie van inokulum deur *P. citricarpa* en voorsien inligting oor die oorlewing van konidiums en miselium van die patogeen.

SSV letsels wat algemeen voorkom op sitrusvrugte is opgedeel in vier duidelik-onderskeibare kategorieë, nl. letseltipe A (hardevlek of haelgatvlek), tipe B (vals

melanose, spikkelvlek of inkvlek), C (sproetvlek) en D (virulente- of spreiende vlek). Hierdie indekseringsisteem laat die plasing van atipiese SSV letsels op vrugte in een van die onderskeie katogorieë toe. Tipe C letsels het eerste ontwikkel op natuurlik-geïnfekteerde Valencia vrugte na inkubasie van 5-7 dae by 27 °C met 'n hoë humiditeit en deurlopende fluoresserende beligting. Piknidiums wat na 9-13 dae in hierdie letsels geproduseer is het reeds kiembare konidiums bevat. Konidiums teenwoordig in tipe A letsels het vir 14 dae lewensvatbaar gebly vir onbehandelde sitrusvrugte wat gestoor is onder optimale toestande, maar is totaal uitgewis deur standaard pakhuisbehandelings. Miselium in die perifirie van tipe A letsels het die pakhuisbehandeling oorleef.

Konidium van *P. citricarpa* kon nie onbeskadigde pakhuisbehandelde sitrusvrugte by 0.5 en 25 °C infekteer in kunsmatige en natuurlike infeksiestudies nie. 'n Lae persentasie infeksie het voorgekom in verwonde vrugte gestoor by 25 °C, maar geen infeksie is waargeneem by dieselfde vrugte gestoor by 0.5 °C nie. Konidiums het meer geredelik geheg aan die sintetiese poliëtileen mikro-wakslaag op sitrusvrugte as op die natuurlike wakslaag. SSV kon nie geïnduseer word in gesonde, volwasse Valencia blare geïnokuleer met *P. citricarpa* konidiums nie. In die laboratorium het *P. citricarpa* konidiums optimaal ontkiem by 22 °C en geen waarneembare ontkieming het plaasgevind by 0.5 en 40 °C nie. Die optimale pH grense vir ontkieming in 'n sitroensuur pH-reeks was 4.0-4.2, met 'n minimum en maksimum pH van 3.2 en 4.8, onderskeidelik. Lig het geen effek op die ontkiemingstempo van *P. citricarpa* konidiums gehad nie.

Sewe *P. citricarpa* isolate van verskillende dele van die wêreld is *in vitro* getoets vir sensitiwiteit teenoor swamdoders en ontsmettingsmiddels geregistreer vir gebruik in Suid Afrikaanse sitruspakhuise. Guasatien en *o*-fenielfenol (natriumsout) het ontkieming en appressoriumvorming van konidium geheel en al geïnhibeer by die aanbevole konsentrasies. Prochloras, tiabendasool en twee emulsie-formulasies van imasalil nl. Chloramizol en Sanazil het ontkieming met 98 % geïnhibeer. Imasalil sulfaat was minder doeltreffend, met 'n oorlewingsyfer van 21 %. Chloordioksied het konidiumontkieming meer doeltreffend en teen laer konsentrasies geïnhibeer as kalsiumhipochloriet.

Pakhuisbehandelings wat die voorkoms van *P. citricarpa* in SSV letsels *in vivo* konsekwent verminder het, sluit in: warm water behandeling (43-47 °C vir 3 minute), chemiese dompelbad (imasalil sulfaat, guasatien en 2,4-D) en 'n kombinasie van chloor, hoë-druk spuit, warm water, bogenoemde chemikalieë en poliëtilieen polering van sitrusvrugte. *P. citricarpa* konidiums is totaal uitgewis deur SSV-geïnfekteerde sitrusvrugte te stoor vir 3 weke by 25 °C na behandelings met chloor, warm water, bogenoemde chemikalieë, of 'n kombinasie van al die behandelings. Verskeie swamdoders wat nie geregistreer is vir na-oesgebruik op sitrusvrugte nie, het *P. citricarpa* konidiums gedood. 'n Uitsondering was die plantbestandheidsaktiveerder, asibensolar-*s*-metiel. Difenoconasool wat ook nie geregistreer is vir na-oes sitrusbehandeling nie, het die miseliuminokulum in SSV letsels aansienlik verminder. Kobaltbestraling van tot 400 Gy het nie die *P. citricarpa* inokulum in besmette sitrusvrugte uitgeskakel nie.