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

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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 &

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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.

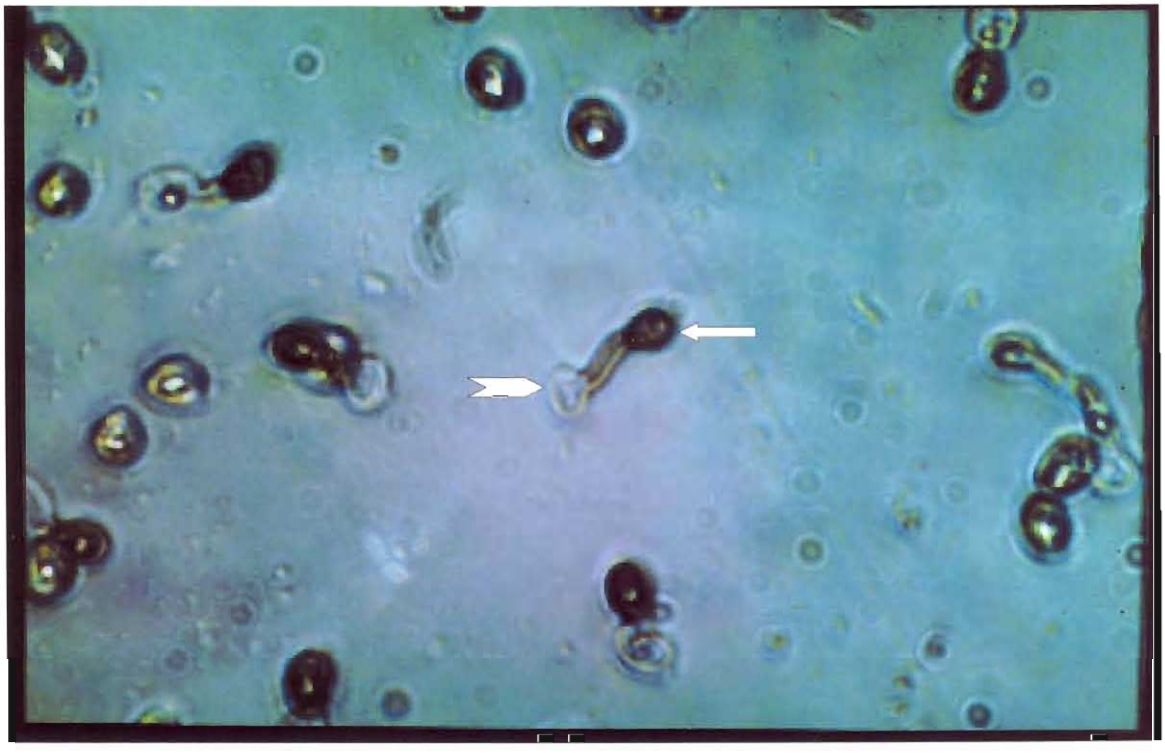

Figure 1: Inverse light microscopy of germinating *P.citricarpa* conidia (⇨) Appressoria (⇦) are dark brown to black with a boxing glove appearance.

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.

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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).

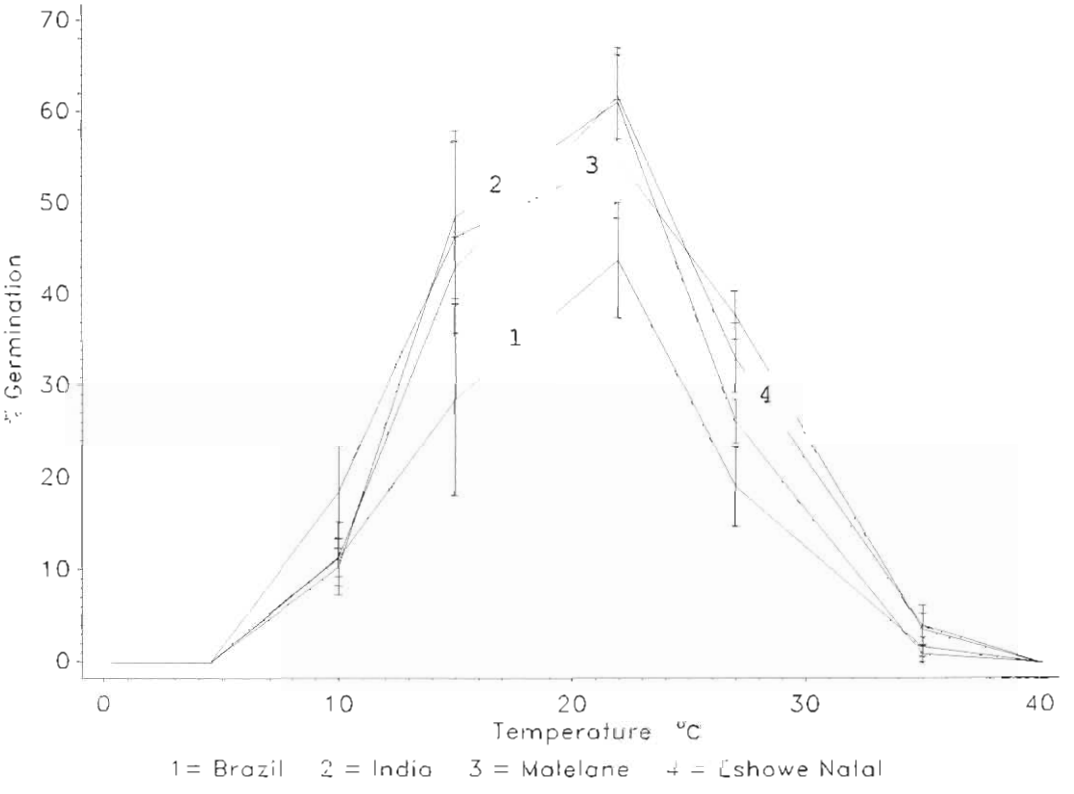

Figure 2 *In vitro* effect of temperature on conidial germination by 4 *Phyllosticta citricarpa* isolates. Evaluation points in each line represents the mean of 6 observations at 48 h.

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.

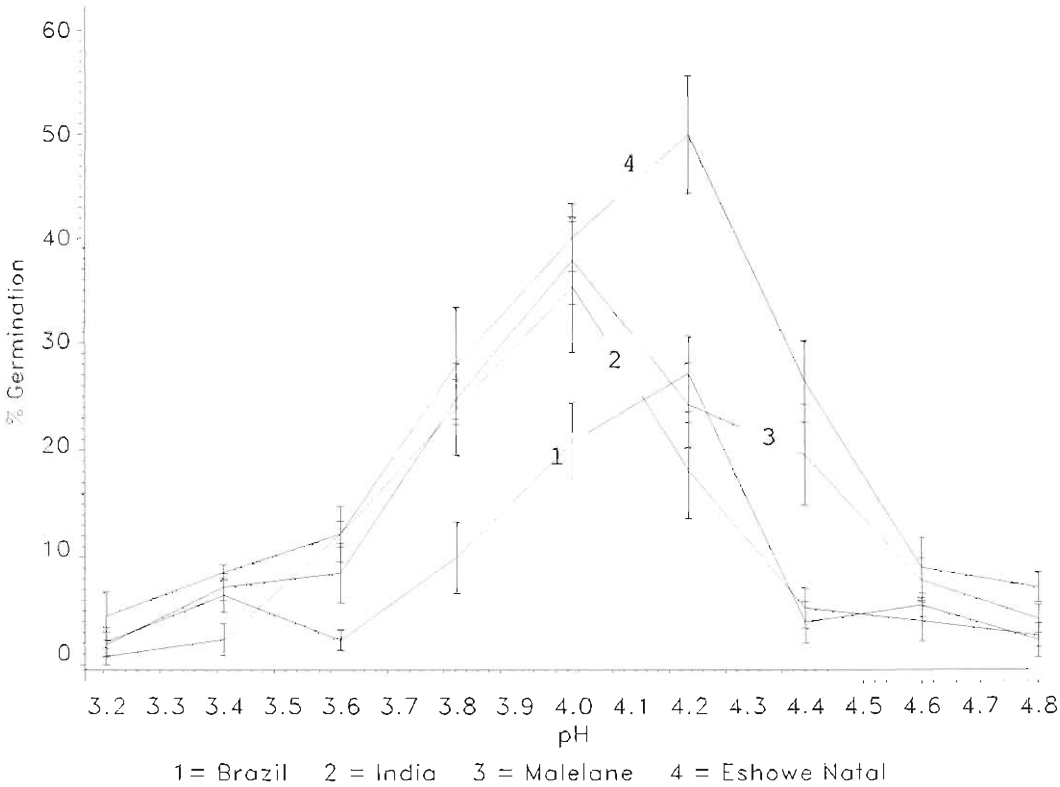

Figure 3 *In vitro* effect of pH on conidial germination by four *Phyllosticta citricarpa* isolates. Evaluation points in each line represent the mean of 6 observations at 48 h.

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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