STUDIES ON THE EXPRESSION OF RESISTANCE OF *COFFEA* SELECTIONS TO *HEMILEIA VASTATRIX*

by

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ABSTRACT

Physiological races of *Hemileia vastatrix* in southern Africa were identified. Prevalent races were I (2.2%), II (88.9%) and III (2.2%). Six samples could not be identified. Twelve biotypes of race II were distinguished. In some cases, the biotypes only occurred in specific regions.

It was established, using fluorescence microscopy, that, in some cases, the percentages of germinated urediospores that did not form appressoria, appressorium formation over stomata, and aborted appressoria, were significantly different between susceptible and resistant selections of the host, and non-hosts. The sequence of events leading to successful infection was investigated using scanning electron microscopy (SEM). When a stoma is encountered by a germ tube tip a uniquely shaped appressorium forms over one end of the stomatal slit. A distinct appressorial foot is wedged within the stomatal vestibule. In coffee, a torpedo-shaped substomatal vesicle initial (SSVI) develops bilaterally from the apex of the infection wedge, while in bean, the infection wedge protrudes into the substomatal chamber. The substomatal vesicle (SSV), at 48 hours post inoculation (hpi) is anchor-shaped. Haustorial mother cells are formed on stubby primary infection hyphae which curve back onto subsidiary cells. No differences in appearance of these structures were noted between resistant and susceptible coffee selections. A much-branched mycelium ramifies through the intercellular spaces of the mesophyll cells 96hpi. In bean, the SSV began to collapse 48hpi. Bayfidan® only slightly suppressed fungal development on the leaf surface. However, within susceptible tissue, this systemic fungicide had an effect on the

morphology of the fungus. Extracellular material accumulated on the SSVI and the SSV. The SSV appeared swollen, and disruptions in the vesicle wall was noted.

The discovery of teliospores on locally infected trees led to a SEM study on their structure, development and germination.

Infection structure formation on the leaf surface, latent period, reaction score and urediosorus concentration differed between susceptible coffee leaves of different ages. Generally, mature leaves are more susceptible than very young or old leaves.

A range of fungicides, mainly systemics, were tested in the field on naturally infected coffee trees. Various epidemiological and climatic factors influence rust development in the field. The role of these factors at the fungicide site and in commercial coffee-growing regions of southern Africa was evaluated.

PREFACE

The experimental work described in this thesis was carried out in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg, under the supervision of Professor F.H.J. Rijkenberg.

All chapters have been prepared as for journal submission, and therefore some repetition was unavoidable.

There are a few "cited by" references in this thesis. These articles were unobtainable due to their limited circulation.

I hereby declare that these studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

T.A. COUTINHO

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FRONTISPIECE

The praises of coffee are sung in the Coffee Cantata "Schweigt stille, plaudet nicht ..." BMV 211, written (1732-35) by J.S. Bach who used the words of Picander (Christian Friedrich Henrici):

Ei! wie schmeckt der Coffee süsse, lieblicher als
tausend Küsse, milder als Muskatenwein
Coffee, Coffee musse ich haben: und wenn jemand
mich will laben, ach so schenkt mir Coffee ein!

which translates roughly into English as:

Oh! how sweet is the taste of coffee, choicer than
a thousand kisses, milder than muscatel wine
Coffee, Coffee must I have: and if anyone
wishes to comfort me, pour me out some coffee.

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GENERAL INTRODUCTION

Hemileia vastatrix (Berk. et Br.) is the causal organism of leaf rust on the genus *Coffea* L. (Javed, 1984). This plant disease was one of the earliest to be studied scientifically (Ward, 1882), and, due to its major impact on the world's coffee industry, it is still the subject of detailed biological investigations.

Penetration is confined to the abaxial leaf surface and results in the formation of colonies of up to 15mm in diameter. Slight chlorosis of the adaxial surface is observed. The fungus grows in a radial manner so that mature urediospores are found in the centre of the affected areas and young spores are produced in marginal surface tissue. The urediosori are bright yellow to orange in colour. The tissue in the centre of the affected area becomes necrotic with increasing age. The economic damage stems from the extensive, premature defoliation of the host (Waller, 1982). Urediosori are readily attacked by *Verticillium lecanii* (Zimm.) Viegas. This hyperparasite was present in all rust samples collected by the present author, regardless of province, region or site.

The disease is not only of economic importance, but it has a profound social effect in large areas of the world. Since harvesting of the crop is done manually and the amount of hand-labour required is determined by the yield, producing a healthy crop is of paramount importance. In the areas from Panama to Mexico a 20% infection has been estimated to result in the reduction of labour by as much as 31 million men/day (Muyshondt, 1971, cited by Schieber, 1972).

After oil, coffee is the most important commodity in international trade. It is produced in approximately 45 countries (Schieber & Zentmyer, 1984). *H. vastatrix* can infect all the species of *Coffea* (Waller, 1982), of which three are of economic importance, namely *C. arabica* L., *C. canephora* Pierre ex Froehner and *C. liberica* Bull ex Hiern (Carvalho *et al.*, 1969). They make up 90%, 9% and 1% of the world's coffee, respectively (Javed, 1984). *C. arabica* and *C. canephora* are grown in the Republic of South Africa, Venda, Gazankulu,

Lebowa and KaNgwane. They make up 99.28% and 0.72% respectively of the total coffee grown in southern Africa (Anon., 1988).

C. arabica is a self-pollinating, allotetraploid species and is the only polyploid described in this genus. *C. canephora* (Robusta) and *C. liberica* are diploid, cross-pollinating species (Carvalho *et al.*, 1969). Arabica coffee is preferred over all other species because of its superior quality (Van der Vossen, 1985), while Robusta is known to have a better resistance to rust (Rodrigues *et al.*, 1975). *C. liberica* has been shown to have a high level of susceptibility (Van der Graaff, 1986). Since Robusta is imported in great quantities for the instant coffee market, there is a move to increase Robusta plantings in southern Africa (Anon., 1988). Besides this factor, other reasons for this move include the comparatively low production costs, the less intensive management required, and its relatively good resistance to diseases and pests (Anon., 1988).

The earliest report of *H. vastatrix* in southern Africa was from Natal in 1878 (Rayner, 1960; 1972). By 1904 it was reported to have devastated coffee-growing regions of the Transvaal also (Rayner, 1972). As a result of these infections, the coffee plantings were completely abandoned in both regions (Brodrick, 1971). Abandoned trees are still present and have been observed growing in the wild pathosystems of northern Natal (M.D. Laing, pers. comm.). In the 1930s, *H. vastatrix* was found in many of the warmer areas of southern Africa, parasitic on indigenous plants of the order Rubiaceae (Gyde, 1932). At this time, coffee trees still grew in small numbers, but it was found that, if the size of the plantation was increased, the fungus became established and spread very rapidly (Gyde, 1932). In the 1960s coffee was again planted in Natal and the disease was observed (Nyenhuis, 1967). In 1969 it was once again recorded in the Transvaal (Brodrick, 1971). Cultivars of *C. arabica* resistant to *H. vastatrix* are not grown commercially in southern Africa. Thus, coffee rust can still be a serious problem, especially when chemical control is not included in farming practices.

In order to determine the importance of coffee rust to the industry in southern

Africa, and to offer advice to growers on future plantings of coffee selections resistant to rust, a survey of the coffee-growing regions was done. This included collecting rust for race identification, and sampling seed or seedlings from selections, regarded by the farm managers concerned to be less susceptible, in an attempt to assess their susceptibility to the rust race/races present in southern Africa.

Although the infection process of *H. vastatrix* has been studied since the first observations by Ward (1882), certain aspects have still not been thoroughly investigated. Various researchers have reported on factors affecting this process (Montoya & Chaves, 1981; De Jong *et al.*, 1987), however, little work has been done on the morphology of pre- and post-penetration stages on the susceptible host. Similarly, there is little information on these processes on and within resistant coffee selections, non-host plants and fungicide-treated hosts. This study has attempted to address these deficiencies in our knowledge.

H. vastatrix occasionally produces teliospores which will germinate to produce a basidium and basidiospores (Gopalkrishnan, 1951). The role of basidiospores is obscure: they do not infect coffee or any other known host of *Hemileia* spp., and no alternate host of coffee rust has been found. In the literature, reports of their occurrence are relatively rare. An opportunity to study the morphology of this stage of the life cycle arose and a scanning electron microscopy study was undertaken.

Leaf age is known to influence the resistance of *Coffea* selections to *H. vastatrix*. Early reports indicated that younger leaves were more resistant to infection than older leaves (D'Oliveira, 1957, cited by Kushalappa & Eskes, 1989). The literature does not provide sufficient evidence to indicate at which stage or stages in the infection process this resistance mechanism comes into operation. Infection structure formation as well as subsequent sporulation on leaves of different ages were therefore investigated.

A field trial investigating the efficacy of chemicals to control coffee rust was

included in an attempt to advise growers on which fungicide provides the most efficient control. The timing of fungicide applications is known to be critical for the adequate control of coffee rust. In order to provide such information, knowledge of aspects of the epidemiology of this disease in southern Africa was required. In-depth questionnaires to farm managers, observations and recordings by the present author, as well as a thorough literature search provided an understanding of the epidemiological situation in this country.

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CHAPTER 1

IDENTIFICATION OF PHYSIOLOGICAL RACES OF *HEMILEIA VASTATRIX* IN SOUTHERN AFRICA

INTRODUCTION

In 1932, Mayne presented the first experimental evidence of physiologic specialization of *Hemileia vastatrix* Berk. & Br. while working on coffee in India. His findings necessitated a careful survey of the parasite's specialization throughout the world, and a search for new sources of resistance in *Coffea arabica* L. Dr. A.L.B. d'Oliveira initiated such a programme, and the Centro de Investigação das Ferrugens do Cafeeiro (CIFC) was established in Oeiras, Portugal in 1955 (Eskes, 1989). Today, 31 rust races have been identified (Bettencourt & Rodrigues, 1988). According to Rodrigues (1984), 938 rust isolates were obtained from 37 countries and the number of times each race was identified, expressed as a percentage of the total number of race cultures established by the CIFC, indicated that the prevalent races are race II (58.2%), race I (14.4%), race III (8.9%) and race XV (3.6%). The remaining races constitute about 15% of the total. The presence of Race II has been reported in 30 out of 33 different world areas where coffee rust is found, including southern Africa (Rodrigues *et al.*, 1975).

Host genotypes with similar resistance spectra have been classified at the CIFC into physiological groups or clones. The coffee differentials, routinely used in rust race identification, are considered as type varieties or clones representing these different resistance or physiological groups (Bettencourt & Noronha-Wagner, 1971; Rodrigues *et al.*, 1975; Lopes & Godinho, 1976; Eskes, 1989). The present list of differential hosts consists of clonal lines of five *Coffea arabica* L. selections, six tetraploid hybrids of *C. arabica* x *Coffea* spp., and six *Coffea* spp. selections (Bettencourt & Rodrigues, 1988).

The present survey was undertaken to identify local *H. vastatrix* races, and use

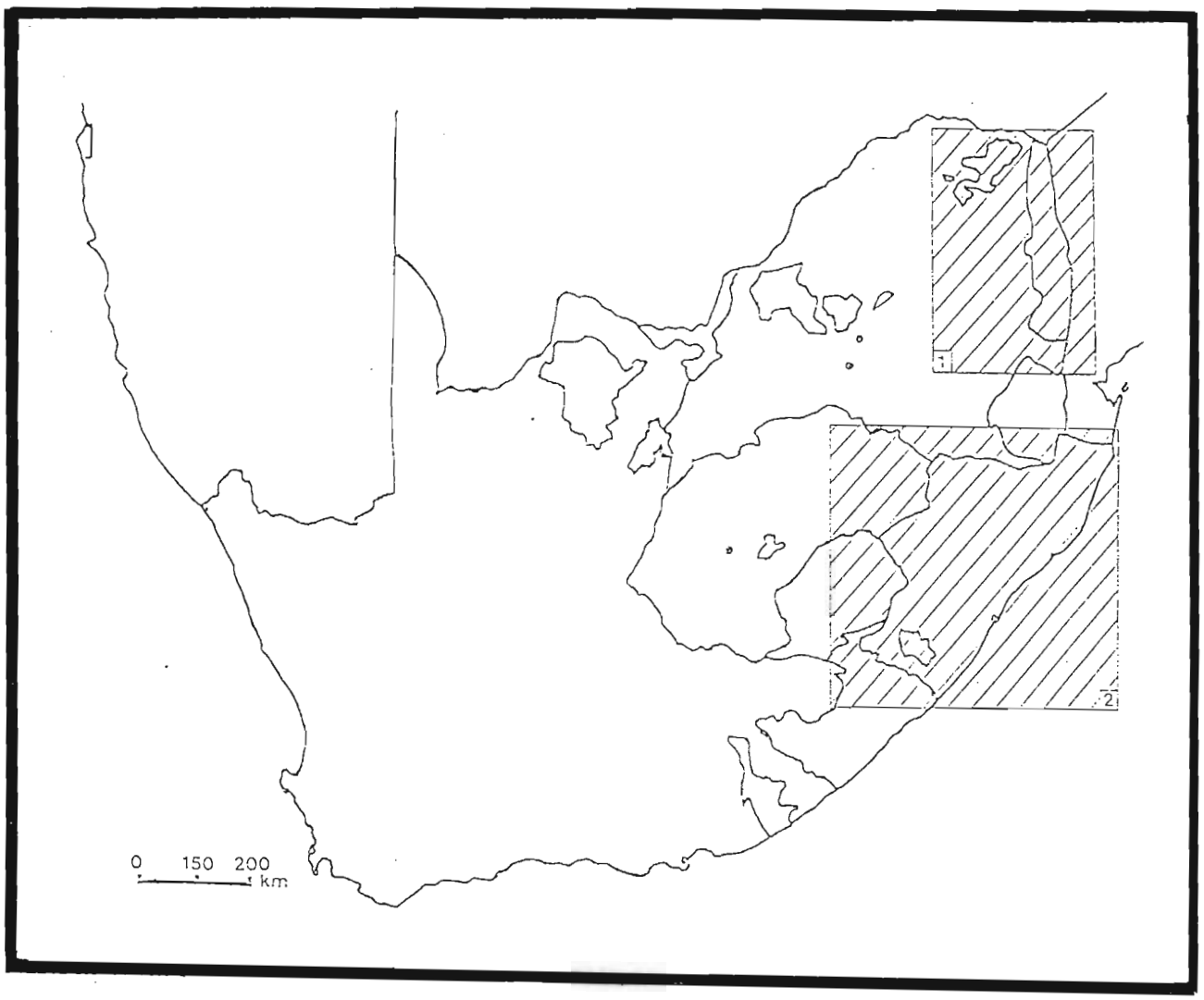
them to screen for the rust resistance of the coffee cultivars commonly grown in southern Africa. Coffee selections obtained from Malawi, Portugal and Colombia were also evaluated for resistance, following inoculation with the rust race/races identified in the present study.

MATERIALS AND METHODS

Rust collection. Rust urediospores were collected from different localities in both the Transvaal and Natal (**Figs.** 1, 2 and 3). Urediospores were stored in liquid nitrogen in gelatin capsules (size 00) placed in Nunc® tubes (1.8 ml). The samples were obtained from plantations of *C. canephora* L. and different *C. arabica* cultivars. The latter included SL cultivars 14, 28 and 34 (based on appearance these cultivars appeared to be indistinguishable), Caturra (red- and yellow-cherry varieties), and Catuai [hybrid between Mundo Nova and yellow Caturra (Anon., 1987)]. On one estate, *Gardenia thunbergia* L. was grown in close proximity to coffee plantings. Rust, similar in appearance to *H. vastatrix,* was noticed and collected.

Coffee selection sources. Seeds of a range of *Coffea* selections were obtained from the Tea Research Foundation of Central Africa, Malawi, the CIFC, Portugal, and CENICAFÉ, Colombia (**Table 1**). Cuttings of a range of differentials of *C. arabica* (**Table 2**) were also obtained from the CIFC. According to Dr. L. Rijo (pers. comm.), these differentials were adequate for the identification of the majority of coffee rust races. A few of the selections listed in **Table** 1 belong to physiological groups different from those used as differentials, and therefore assisted in the race identification.

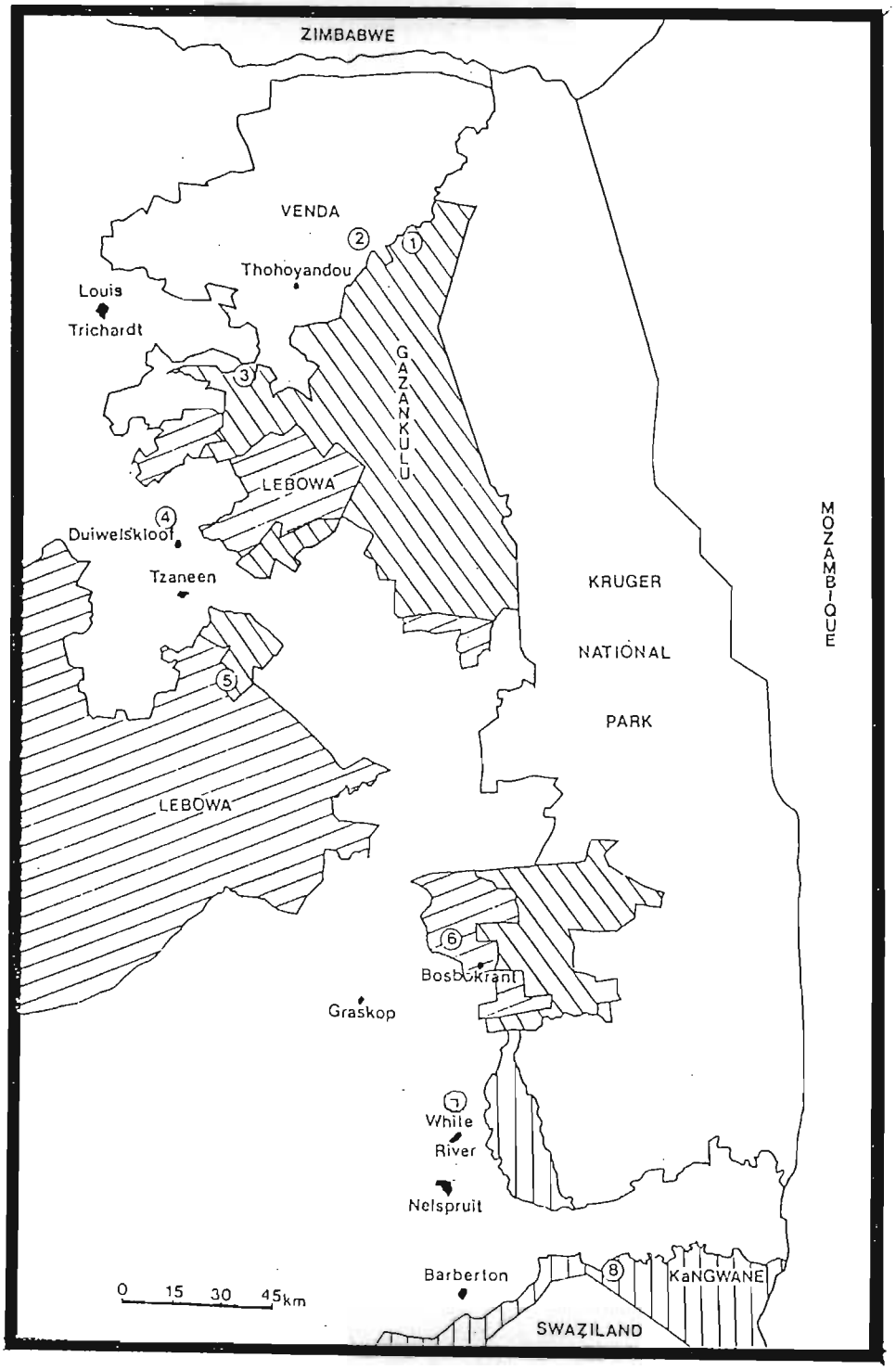
Planting conditions for coffee selections. Seeds, seedlings and cuttings were planted in pre-composted bark medium supplemented at monthly intervals with N:P:K fertilizer and copper chelate. They were grown under 50% shade at temperatures between 15 and 30°C.



1 Eastern Transvaal

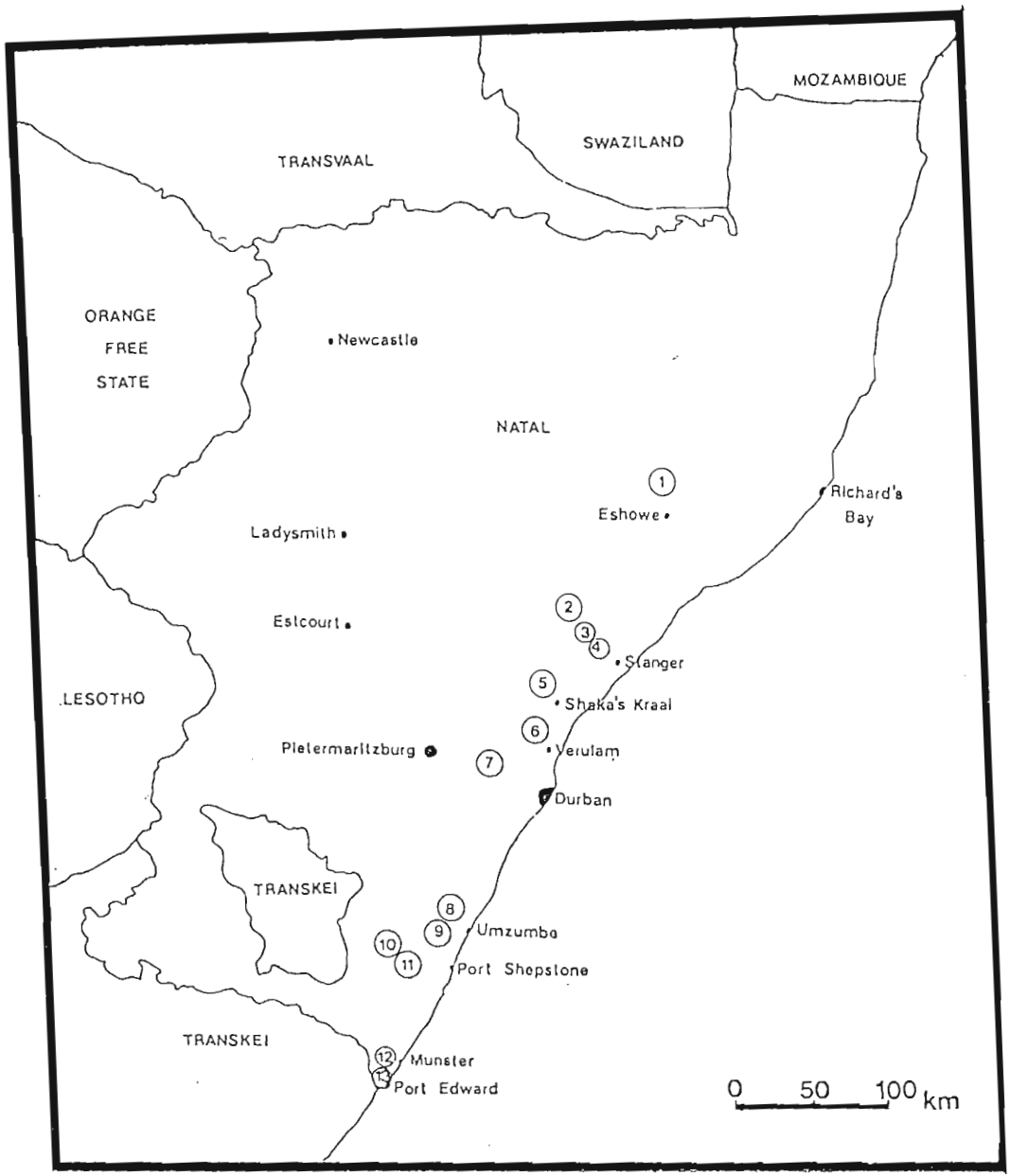
2 Natal

Fig. 1 Regions in southern Africa, depicted in more detail in Figs. 2 and 3, where coffee is commercially cultivated



1. MHINGA ESTATE
2. PHASWANA ESTATE
3. LA ROCHELLE ESTATE
4. WESTFALIA ESTATE
5. TOURS ESTATE
6. ZOEKNOG ESTATE
7. BURGERSHALL EXPT. FARM
8. SCHOEMANSDAL ESTATE

Fig. 2 Eastern Transvaal estates/plantations where coffee is cultivated



1. FARM ERICA
2. MAYFIELD ESTATE
3. ISLAND FARM
4. OCEAN LODGE ESTATE
5. SHAKA'S KRAAL FIELD STAT.
6. OAKFORD PRIORY
7. DRUMMOND FARM
8. OCEAN VIEW ESTATE
9. KILINDI ESTATE
10. THORNTON FARM
11. ECHO VALLEY FARM
12. BOULDER HILL ESTATE
13. BEAVER CREEK FARM

Fig. 3 Natal estates/plantations where coffee is cultivated

Table 1 Coffee selections, obtained from various countries, used to evaluate their resistance/susceptibility to *Hemileia vastatrix* race/races present in southern Africa

REF. CODE	SELECTION	PHYSIOLOGICAL GROUP/CLONE*	INSTITUTE AND COUNTRY OF ORIGIN OF COFFEE SELECTIONS
A	Catimor 1	A	Centro de Investigação das Ferrugens do Cafeeiro, Portugal
B	Catimor 4	A	Centro de Investigação das Ferrugens do Cafeeiro, Portugal
C	S. Agaro	J	Tea Research Foundation of Central Africa, Malawi
D	Geisha	C	Tea Research Foundation of Central Africa, Malawi
E	Caturra	E	Tea Research Foundation of Central Africa, Malawi
F	K7	?	Tea Research Foundation of Central Africa, Malawi
G	NC-169	?	Centro Nacional de Investigaciones de Café, Colombia
H	NP-547	?	Centro Nacional de Investigaciones de Café, Colombia
I	Coffea canephora	?**	Mhinga Estate, Transvaal
J	C. excelsia	?**	Burgershall Experimental Station, Transvaal

*Groups identified with the aid of a reference table in a research paper by Rodrigues *et al.* (1975)

**A specific selection of both *C. canephora* and *C. excelsia* are used as differentials (Rodrigues *et al.* 1975)

Table 2 Differential selections of *Coffea* spp. used to identify races of *Hemileia vastatrix* present in southern Africa

CIFC CODE	SELECTION	PHYSIOLOGICAL GROUP
87/1	Geisha	Group C
128/2	Dilla & Alghe	Group C
110/5	S.4 Agaro	Group J
33/1	S.288-23	Group G
32/1	DK 1/6 Kent's type	Group D
1343/269	Hybrido de Timor	Group R
134/4	S.12 Kaffa	Group I

Leaf disc preparation. Leaf discs have been used with success in determining resistance to coffee rust (Narasimhaswamy *et al.*, 1961; Eskes & Toma-Braghini, 1981) and identifying rust races (Ramachandran *et al.*, 1979). The standardized method described by Eskes (1982a), was used for disc preparation from third-leaf pairs of the *Coffea* selections. Leaf discs, 2cm in diameter, were punched out using a stainless-steel cork borer. The midvein and leaf margins were avoided. Discs were placed, adaxial surface down, on foam rubber saturated with tap water in glass trays (29 x 24 x 2.5cm).

Bulking of urediospores. A camel-hair brush (No. 1) was used to dust the abaxial surfaces of leaf discs of cv. Caturra lightly with urediospores collected from the various localities. Care was taken to prevent possible contamination between samples. Glass trays, containing these leaf discs, were transferred to a dew chamber, set at 20°C, and incubated in the dark for 24 h. Following this period, trays were covered with glass sheets and placed in a constant environment chamber (Conviron ®) set at 26°C with a 12 h photoperiod. Urediospores from a single colony of urediosori on the selected leaf disc, from each rust sample, were removed with a fine brush. The spores were dusted onto three freshly prepared leaf discs for each rust sample. The discs were incubated as described above. Again, following the formation of urediosori, urediospores were collected and the inoculation process repeated until sufficient spores had been accumulated in order to inoculate the differential leaf discs. During this process, spores from "single colony" isolations were stored in liquid nitrogen as described previously.

Race identification. Six leaf discs of each differential/selection were inoculated, as described above, with every rust sample collected and each rust sample was tested twice. Levels of resistance or susceptibility on the differentials were determined using the scale described by Eskes & Toma-Braghini (1981) (refer to **Table 3**). Once a reaction score was obtained for each differential the results were compared to **Table 4** (from Bettencourt, 1981, cited by Muller, 1984).

Evaluation of the susceptibility of imported *Coffea* selections to *H. vastatrix*. Six leaf discs from each *Coffea* selection (**Table 1**) were inoculated,

Table 3 A 0 - 9 scale developed by Eskes & Toma-Braghini (1981) to evaluate the reaction types of coffee leaf rust, *Hemileia vastatrix*

INDEX VALUE	CIFC CLASSIFICATION FOR INDIVIDUAL LESIONS	DESCRIPTION OF REACTION TYPES FOR LEAVES OR ENTIRE PLANTS
0	i	Immunity, no visible reaction.
1	fl-,t-	Minute chlorotic spots, often associated with small tumefactions.
2	fl,t,o	Larger chlorotic spots, often associated with tumefactions. No urediospore production.
3	fl,t,o,o+	A mixture of various sizes of chlorotic spots, including very large chlorotic areas. Fewer tumefactions. No urediospore production.
4	fl,t,o,1	A mixture of chlorotic spots of various sizes, with some urediospore formation on large chlorotic lesions*. Sporulation of less than 25% of all lesions. Few tumefactions may occur. Early necrosis of lesions is sometimes observed.
5	fl,t,o,-2	As in 4, but with more urediospore formation. Sporulation of less than 50% of all lesions.
6	fl,t,o,-3	As in 5, but with increased urediospore production. Sporulation of less than 75% of all lesions.
7	fl,t,o,-4	As in 6, but with abundant urediospore production. Sporulation of up to 95% of all lesions.
8	t, 2-4	A mixture of lesions with a varying degree of sporulation, sometimes associated with a few tumefactions.
9	4	Only lesions with abundant sporulation, without marked chlorosis at the lesion border.

* "Lesion" was understood to mean colony of urediosori

Resistant [R] = 1, 2 and 3; Moderately Resistant [MR] = 4 and 5; Moderately Susceptible [MS] = 6 and 7; Susceptible = 8 and 9

Table 4 Races of *Hemileia vastatrix*, selected differentials and coffee physiologic groups (after Bettencourt, 1981, cited by Muller, 1984)

PHYSIOLOGIC RACE OF HEMILEIA VASTATRIX	POSTULATED RACE GENOTYPE	HOST FACTORS OF RUST RESISTANCE						
		$S_H1,5$	S_H1	$S_H4,5$	$S_H3,5$	$S_H2,5$	S_H6	$S_H1,4$
		Coffea arabica and tetraploid interspecific hybrids (C. arabica x Coffea spp.)						
		87/1	128/2	110/5	33/1	32/1	1343/269	134/4
		COFFEE PHYSIOLOGIC GROUPS						
		C	α	J	G	D	R	I
I	v2,5	R	R	R	R	S	R	R
II	v5	R	R	R	R	R	R	R
III	v1,5	S	S	R	R	R	R	R
IV	v?	R	R	R	R	R	R	R
VI	v?	R	R	R	R	R	R	R
VII	v3,5	R	R	R	MS	R	R	R
VIII	v2,3,5	R	R	R	S	S	R	R
X	v1,4,5	S	S	MS	R	R	R	MS
XI	v?	R	R	R	R	R	R	R
XII	v1,2,3,5	S	S	R	S	S	R	R
XIII	v5,?	R	R	R	R	R	R	R
XIV	v2,3,4,5	R	R	S	S	S	R	R
XV	v4,5	R	R	S	R	R	R	R
XVI	v1,2,3,4,5	S	S	S	S	S	R	S
XVII	v1,2,5	S	S	R	R	R	R	R
XVIII	v?	R	R	R	R	R	R	R
XIX	v1,4,?	R	MS	R	R	R	R	MS
XX	v?	R	MS	R	R	R	R	MR
XXI	v?	R	R	R	R	R	R	R
XXII	v5,6	R	R	R	R	R	S	R
XXIII	v1,2,4,5	S	S	MS	R	S	R	MS
XXIV	v2,4,5	R	R	MS	R	S	R	R
XXV	v2,5,6	R	R	R	R	S	S	R
XXVI	v4,5,6	R	R	S	R	R	S	R
XXVII	v1,4,6,?	R	MS	R	R	R	MS	MS
XXVIII	v2,4,5,6	R	R	S	R	S	S	R
XXIX	v5,6,?	R	R	R	R	R	S	R
XXX	v5,?	R	R	R	R	R	R	R
XXXI	v2,5,6,?	R	R	R	R	S	S	R
XXXII	v6,?	R	R	R	R	R	MS	R

R = resistant reaction
S = susceptible reaction
MS = moderately susceptible
MR = moderately resistant

as described above, with each of the various rust samples, and the reaction types were assessed using the scale described by Eskes & Toma-Braghini (1981) (refer to **Table 3**). The experiment was repeated twice.

Latent period. The latent period was determined on cv. Caturra leaf discs except where the race/biotype was incapable of infecting this cultivar. In this case the latent period was determined on the selection which proved to be the most susceptible to the particular race/biotype. Six leaf discs were inoculated as described above and used as a single replication. The experiment was repeated twice. The latent period was taken as the time period from the beginning of incubation to the time at which 50 per cent of the first urediosori had appeared. Following their inoculation, discs were incubated as described above.

Evaluation of the susceptibility of *Coffea* selections, commonly grown in southern Africa, to *H. vastatrix*. Four *C. arabica* selections are commonly grown in southern Africa, namely, SL 28, Caturra, SL 34 and Catuai (listed in order of importance). *C. canephora* is grown on a few estates in relatively small numbers. Seed/seedling samples were selected on the basis of their reaction to *H. vastatrix* in the field and on their rust history. Of interest was the selection of SL 34 obtained from Drummond. The owner of this estate had previously grown large quantities of SL 34 down the South Coast of Natal in the 1960s, and due to a severe rust outbreak had decided to abandon the estate. He selected seed, which he later planted in Drummond, from one tree that showed no visible symptoms of rust. At the time of collecting the rust sample, during April, the rust severity on the Drummond estate was relatively low in comparison to what was observed on other estates. Twelve leaf discs of such locally grown selections were inoculated and the disease-reaction type assessed on two different occasions. The inoculation, incubation and disease assessment methods used are as those described above.

RESULTS

Race identification. All 18 rust samples collected in the Eastern Transvaal from the seven estates failed to infect any of the differentials (**Table 5**). From a comparison between **Tables 4** and **5**, it is evident that the race/s present in this area may be one or more of the following: Race II, IV, VI, XI, XIII, XVIII, XXI or XXX. The differentials obtained were not specific enough to allow further separation of these races. The sample collected from *Gardenia thunbergia* failed to infect any of the differentials indicating that it too may be one of the above races or another species of *Hemileia*. Of the 27 rust samples collected in Natal 25 were incapable of infecting the differentials (**Table 6**) and the situation as described above once again applies. Races I and III were identified on samples collected from the Shaka's Kraal field station and at Oakford Priory, repectively.

Evaluation of the susceptibility of imported *Coffea* selections to *H. vastatrix*. Following the inoculation of individual leaf discs of the different coffee selections with the various rust samples, it became evident that if a single race was present, biotypes existed (**Table 7**). Rodrigues *et al.* (1975), Muller (1984) and Eskes (1989) noted that only races II, XIII and/or XXX are capable of infecting cv. Caturra, a member of Physiological Group E. This indicates that all rust samples collected infecting this cultivar are either one or more of the above races. Races XIII and XXX are relatively rare, only occurring in the Phillipines and Timor, repectively; therefore, it is likely that besides races I and III, the other race present in southern Africa, is race II. The differences in the reaction scores recorded between isolates of this race, on the *Coffea* selections (**Table 7**), may be due to the presence of biotypes. Dr. C.J. Rodrigues Jr. (pers. comm.) confirmed this conclusion. The biotypes were given lower case letters and their occurrence in southern Africa recorded (**Fig. 4**). The race II**e** biotype was the most common followed by II**b**. Biotypes II**a**, **c**, **d**, **f** and **g** were confined to the Eastern Transvaal while, II**j**, **k**, and **m** only occurred in Natal. The latent period, even between biotypes, differed, and their separation was based purely on the reaction score recorded. Six of the 45 samples were incapable of infecting cv. Caturra (**Table 7**, reference code E). This/these race/s could not be distinguished. They may be

Table 5 Results of differential testing of races of *Hemileia vastatrix* collected from various sites and hosts in the Transvaal and independent homelands

COLLECTION SITE	HOST	DIFFERENTIAL SELECTIONS OF COFFEA SPP.*						
		87/1	128/2	110/5	33/1	32/1	1343/ 269	134/4
Mhinga Estate	SL 28 [1]***	0**	0	0	0	0	0	0
	SL 28 [2]	0	0	2	0	0	0	0
Phaswana Estate	SL 28	0	0	0	0	0	0	0
	Gardenia thunbergia	0	0	1	0	0	0	0
La Rochelle Estate	SL 28 [1]	0	0	0	0	0	0	0
	SL 28 [2]	0	0	0	0	0	0	0
	Robusta	0	0	0	0	0	0	0
Tours Estate	SL 28 [1]	0	0	0	0	0	0	0
	SL 28 [2]	0	0	0	0	0	0	0
Zoeknog Estate	SL 28	0	0	2	0	0	0	0
	Caturra	0	0	0	0	0	0	0
Burgershall Experimental Station	K7	0	0	0	0	0	0	0
	Catuai	0	0	0	0	0	0	0
	Caturra	0	0	2	0	0	0	0
	Robusta	0	0	2	0	0	0	0
	C. excelsia	0	0	0	0	0	0	0
Schoemansdal Estate	SL 28 [1]	0	0	0	0	0	0	0
	SL 28 [2]	0	0	0	0	0	0	0

* Differentials obtained from the CIFC, Oeiras, Portugal
** Mean reaction score (refer to **Table 3**) on twelve *Coffea* leaf discs
*** [1] - [2] different sites within a plantation

one or more of the following: Race IV, VI, XI, XVIII or XXI. By evaluating their reaction to the various *Coffea* selection (**Table 7**) three distinct "races" or possibly biotypes of one or more of the above, were evident and they were labelled **?1-3** in the present study. Although the rust sample collected from *G. thunbergia* failed to infect *C. arabica* cultivars or the *C. arabica* crosses it did infect *C. canephora* and to a lesser extent *C. exselsia* (**Table 7**).

Table 6 Results of differential testing of races of *Hemileia vastatrix* collected from various sites and hosts in Natal

COLLECTION SITE	HOST	DIFFERENTIAL SELECTIONS OF COFFEA SPP.***						
		87/1	128/2	110/5	33/1	32/1	1343/ 269	134/4
Ukulinga	Caturra	0**	0	0	0	0	0	0
Univeristy Farm	Unknown	0	0	0	0	0	0	0
Farm Erica	SL 34	0	0	0	0	0	0	0
	Caturra	0	0	0	0	0	0	0
	Robusta	0	0	2	0	0	0	0
	SL 28	0	0	0	0	0	0	0
Island Farm	Caturra [1]*	0	0	0	1	0	0	0
	Caturra [2]	0	0	0	0	0	0	0
	Caturra [3]	0	0	0	0	0	0	0
	Mundo Nova	0	0	0	2	0	0	0
Ocean Lodge Estate	SL 28 [1]	0	0	0	0	0	0	0
	SL 28 [2]	0	0	0	0	0	0	0
Shaka's Kraal Field Station	SL 28 [1]	0	0	0	2	1	0	1
	SL 28 [2]	0	0	0	0	0	0	2
	Caturra	0	0	0	0	8	0	2
Oakford Priory	Unknown	9	9	0	0	0	0	0
Drummond Farm	SL 34	0	0	0	2	1	0	1
Ocean View Estate	SL 28	0	0	0	2	1	0	0
	Caturra	0	0	0	0	0	0	0
Kilindi Estate	Catuai	0	0	0	0	0	0	0
	Caturra	0	0	0	0	0	0	0
	SL 28	0	0	0	0	0	0	0
Thornton Farm	SL 28	0	0	0	0	0	0	0
Echo Valley Farm	SL 28 [1]	0	0	0	0	0	0	0
	SL 28 [2]	0	0	0	0	0	0	0
Boulder Hill Estate	SL 28	0	0	0	0	0	0	0
Beaver Creek Farm	SL 28	0	0	0	0	0	0	2

* [1] - [2] = different sites within a plantation

** mean reaction score (refer to **Table 3**) on twelve *Coffea* leaf discs

*** Differentials obtained from the CIFC, Oeiras, Portugal

Table 7 Evaluation of selected *Coffea arabica* cultivars and *C. canephora* selections to races of *Hemileia vastatrix* present in southern Africa

ORIGIN OF RACE [ESTATE/FARM]	ORIGIN OF RUST SAMPLE [CV./SELECTION]	ASSESSMENT SCORE*										RACE	LATENT PERIOD (days)**
		A[a]	B[a]	C[a]	D[a]	E[a]	F[a]	G[a]	H[a]	I[a]	J[a]		
Mhinga	SL 28 [1]***	0	0	6	0	9	4	9	0	4	0	IIa	27
	SL 28 [2]	0	0	9	0	9	6	9	0	4	2	IIb	28
Phaswana	SL 28	0	0	6	0	9	6	9	3	4	0	IIb	21
	Gardenia thunbergia	0	0	0	0	0	0	0	0	9	4	****	28 (1)
La Rochelle	SL 28 [1]	0	0	9	0	9	0	9	0	0	0	IIa	27
	SL 28 [2]	0	0	6	0	9	9	9	0	9	1	IIc	20
	Robusta	0	0	0	0	9	0	9	0	9	0	IId	27
Tours	SL 28 [1]	0	0	3	3	9	0	9	0	0	0	IIe	27
	SL 28 [2]	0	0	3	0	9	4	9	0	0	0	IIe	27
Zoeknog	SL 28	0	0	6	3	9	4	9	0	9	0	IIf	25
	Caturra	0	0	3	0	9	0	9	0	9	0	IId	28
Burgershall	K7	0	0	9	0	9	9	9	3	0	0	IIb	28
	Catuai	0	0	0	0	9	0	9	0	0	0	IIe	25
	Caturra	0	0	6	4	9	6	9	2	4	1	IIb	20
	Robusta	0	0	6	3	9	4	9	0	9	0	IIg	21
	C. excelsia	0	0	0	0	9	0	9	0	0	9	IIh	28
Schoemansdal	SL 28 [1]	0	0	0	0	9	0	9	0	0	0	IIe	31
	SL 28 [2]	0	0	0	4	9	6	9	3	4	0	IIi	28
Ukulinga	Caturra	0	0	0	0	9	0	9	0	0	0	IIe	27
	Blue Mountain	0	0	0	0	9	0	9	0	0	0	IIe	27
Farm Erica	SL 34	0	0	0	0	9	0	9	0	0	0	IIe	27
	Caturra	0	2	0	1	9	7	9	3	0	0	IIi	26
	Robusta	2	0	0	0	0	9	0	0	0	0	?1	25 (1)
	SL 28	0	0	0	3	9	0	9	0	0	0	IIe	32
Island	Caturra [1]	3	0	0	1	9	5	9	2	0	0	IIi	26
	Caturra [2]	0	0	0	0	0	0	0	0	0	0	?2	32 (2)
	Caturra [3]	0	1	0	0	0	0	1	0	0	1	?2	32 (2)
	Mundo Nova	0	0	1	0	0	5	3	3	0	6	?3	32 (2)
Ocean Lodge	SL 28 [1]	0	0	0	0	0	0	0	0	0	0	?2	25 (3)
	SL 28 [2]	0	3	0	3	0	0	0	0	0	0	?2	34 (4)
Shaka's Kraal	SL 28 [1]	2	3	9	2	9	9	9	1	0	7	IIj	27
	SL 28 [2]	0	1	0	8	9	9	8	0	0	8	IIj	25
	Caturra	0	0	0	8	9	9	9	0	0	5	I	25
Oakford Priory	Unknown	0	0	9	0	9	3	9	0	9	2	III	20
Drummond	SL 34	1	0	0	2	9	0	9	0	0	0	IIe	32
Ocean View	SL 28	2	0	9	0	9	9	9	0	0	3	IIh	23
	Caturra	3	0	0	0	8	3	9	0	0	5	IIk	28
Kilindi	Catuai	0	0	7	0	9	0	0	0	0	0	IIe	23
	Caturra	0	0	0	0	9	1	9	0	0	0	IIm	32
	SL 28	0	0	3	0	9	9	9	0	0	8	IIh	23
Thornton	SL 28	0	0	0	0	9	1	9	1	0	7	IIk	28
Echo Valley	SL 28 [1]	0	0	0	0	9	0	9	0	0	0	IIe	34
	SL 28 [2]	0	0	0	0	8	0	9	1	0	0	IIe	28
Boulder Hill	SL 28	0	0	0	0	9	0	9	0	0	0	IIe	34
Beaver Creek	SL 28	0	0	0	0	9	0	9	0	0	0	IIe	32

a Refer to Reference code in Table 1

* Assessment scale of Eskes & Toma-Braghini (1981) used (refer to Table 3)

** Latent period determined on cv. Caturra [Malawi] or on (1) K7 [Burgershall], (2) Caturra [Kilindi Estate], (3) Catuai [Drummond] or (4) Catuai [Kilindi Estate],

*** Sites within a single plantation

**** Species of *Hemileia*

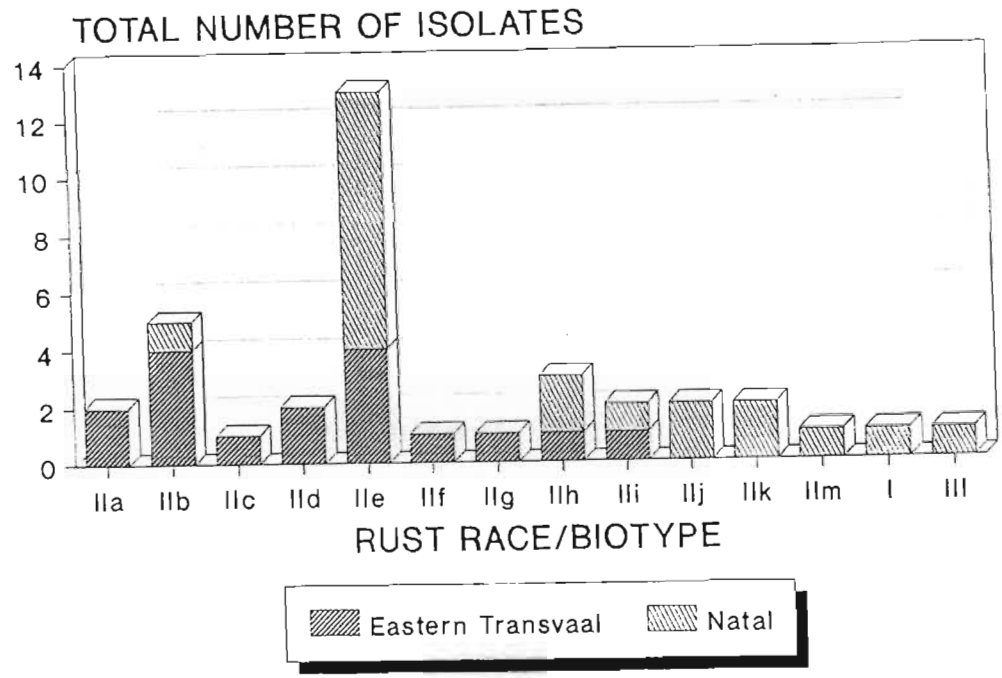


Fig. 4 Races and biotypes of *Hemileia vastatrix* in southern Africa

Latent period. The latent period, in some cases, differed between isolates of the same biotype (**Table 7**). For example, the latent period of biotype **IIe** ranges from 23 to 34 days depending on locality whence it was isolated.

Evaluation of the susceptibility of *Coffea* selections, grown in southern Africa, to *H. vastatrix*. Refer to **Table 8**. The highest recorded rating score on the *C. arabica* selection SL 28 was obtained following inoculation with race II biotypes **h** and **m**. Biotypes **IIj** and **k** were incapable of infecting this selection. Race III, biotypes **IIh** and **m** readily infected SL 34 whereas IIj, races **?1** and **?2** failed to infect this cultivar. Cultivar Caturra proved to be the most susceptible to race II biotypes **a** and **e**, and resistant to **?1**, **?2** and **?3**. Catuai was equally susceptible to Race II biotypes **a**, **c**, **d**, **e**, **h**, and **k**, while race **?3**, **IIj** and **m** failed to infect this selection. *C. canephora* selections from Schoemansdal and Island Estate were resistant to all tested races and biotypes of *H. vastatrix*. The remaining *Coffea* selections were only infected by specific race II biotypes.

Table 8 Disease assessment score recorded for selected *Coffea* selections commonly cultivated in the Eastern Transvaal and Natal against *H. vastatrix* races/biotypes present in southern Africa

COFFEE SELECTION	ORIGIN OF COFFEE SELECTION	RUST RACE/BIOTYPE																
		I	IIa	IIb	IIc	IId	IIe	IIf	IIg	IIh	IIi	IIj	IIk	IIm	?1	?2	?3	III
		ASSESSMENT SCORE *																
SL 28	Mhinga	0	9	8	8	8	9	8	8	9	5	0	0	9	2	4	0	9
	Phaswana	4	8	9	8	7	5	9	9	9	8	0	0	9	0	7	0	8
	La Rochelle	0	9	9	9	8	9	9	8	9	9	0	0	9	1	8	0	8
	Tours	0	9	9	9	9	9	8	9	9	9	0	0	9	0	7	0	9
	Zoeknog	9	9	9	9	9	9	9	7	9	9	0	0	9	0	8	0	9
	Schoemansdal	7	7	8	9	8	9	8	8	9	9	0	0	9	2	9	0	8
	Island Estate	9	9	8	8	9	9	9	9	8	9	0	0	9	1	9	0	9
	Kilindi	8	7	7	8	0	0	8	8	9	3	0	0	9	9	9	8	8
	Thornton	0	4	4	8	8	9	8	8	9	0	0	0	9	3	8	0	9
SL 34	Mayfield	8	8	0	7	7	9	6	7	9	9	0	8	9	0	0	4	9
	Drummond	9	0	9	0	0	5	0	0	9	4	0	0	9	0	0	8	9
Caturra	Zoeknog	8	9	9	9	9	9	9	9	9	9	0	0	9	0	0	0	8
	Island Estate	9	9	9	9	9	9	7	8	9	9	0	9	9	0	0	0	8
	Kilindi	0	9	0	8	8	9	0	5	8	4	8	0	8	0	0	0	9
Catuai	Drummond	9	9	0	9	9	9	9	9	9	9	0	9	0	5	0	0	9
	Kilindi	3	9	7	9	9	9	8	8	9	0	0	9	0	0	9	0	8
C. canephora	La Rochelle	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0
	Burgershall	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	4
	Schoemansdal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Island Estate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Echo Valley	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0

* Assessment scale of Eskes & Toma-Braghini (1981) used (refer to Table 3)

DISCUSSION

In order to establish the range of pathogen variation within a defined area, surveys have proved to be a reliable option. Physiological races of many rust fungi have been successfully identified using this method, for example, *P. coronata* Cda. in Canada (Chong & Kolmer, 1990). The ultimate aim of such work is to provide information of direct relevance to breeding for resistance (Wolfe & Schwarzbach, 1975) and/or in establishing a host gene deployment system (Roelfs, 1984).

Results from surveys can also be used to indicate in which region or regions a cultivar, resistant to a particular race prevalent in that region, can be grown. In coffee research, the identification of races and new sources of resistance has been a successful and an ongoing process since 1955 (Eskes, 1989). Researchers, particularly plant breeders, have exploited gene sources, and resistant material is now available to the coffee industry (Rodrigues, 1984).

Purification of rust races is usually achieved with single-pustule isolations (Green, 1971). Due to unique nature of urediospore formation of coffee rust, this method cannot be applied. Monosporic isolations have been attempted, but little success has been achieved. At the CIFC, infection success for two out of 300 monosporic cultures has been reported (Carneiro, 1980, cited by Eskes, 1989). Monosoric isolates have never been tried (Eskes, 1989). Single-colony isolations, used in this study, to obtain pure cultures was successful. Using a similar technique, Hoogstraten (1982) reported that from 28 single-colony isolates, obtained from inoculation with a 50:50 mixture of two races, 27 were pure.

Race identification. Dispite the fact that positive identification of all races could not be made, it is now clear that physiologic specialization of *H. vastatrix* does occur in southern Africa. According to Dr. C.J. Rodrigues, Jr. (pers. comm.) the assessment score used by the CIFC only characterizes differences in virulence between races and does not distinguish biotypes. An assessment score distinguishing between the heterogeneous reaction types frequently found in coffee, was developed by Eskes & Toma-Braghini (1981), and used successfully in the present investigation. This scale covers a large range of reaction types and slight differences recorded may be due to the physiological condition of the host, or the presence of a race or biotype (Dr. C.J. Rodrigues, Jr., pers. comm.).

Different isolates of the same rust race may have different levels of aggressiveness (Williams & Owen, 1975; Johnson & Taylor, 1976). Rodrigues (1984) noted differences in this parameter among isolates of races I, II and III of *H. vastatrix*. Silva *et al.* (1985) reported differences between the aggressiveness of two isolates of race III and the components of aggressiveness which they recognized

included percentage germination, percentage appressoria formed, growth rate, latent period, etc. They proposed that this variation is unrelated to resistant genes in the host. Thus there is a possibility that the observed differences between race II biotypes may be due to differences in aggressiveness.

The appearance of symptoms on *G. thunbergia* similar to those observed on *Coffea* spp. and the failure of urediospores to infect *C. arabica* (except *C. canephora* and to a limited extent *C. excelsia*) suggested that this fungus is either a race of *H. vastatrix* or a species of *Hemileia*. According to Eskes (1989), only two known races are incapable of infecting *C. arabica* and *C. arabica* crosses but infect *C. canephora* readily, namely, VI and XVIII. Dr. C.J. Rodrigues Jr. (pers. comm.) suggested that the isolate was possibly another species, *H. gardeniae-thunbergia*. However, further investigations are required before this can be verified.

Latent period. The variation in latent period within *H. vastatrix* race II biotypes (**Table 7**) on *Coffea* selections cannot be satisfactorily explained. The techniques used in this investigation were standardized; leaves removed from the trees were of similar age and physiological condition, and subsequent to inoculation were kept under controlled environmental conditions. Differences in the length of the latent period of rust races have been attributed to leaf age (Eskes & Toma-Braghini, 1982; Parlevliet, 1985; Pretorius *et al.*, 1988), and temperature (Pretorius *et al.*, 1988; Kushalappa, 1989). In the case of coffee, the latent period differs between leaves from branches which bear large quantities of fruit and those that bear little or no fruit (Eskes, 1982b). This factor together with a possible influence of urediospore density may partially explain the variation in the latent periods.

Variation in the reaction types of coffee commonly cultivated in southern Africa, and imported selections. Two selections of *C. arabica* show promise with regard to their susceptibility to leaf rust, namely, SL 34 and Catuai on the Drummond and Kilindi Estates, respectively. Both selections were however susceptible to certain races and biotypes. Generally, the selections of *C. arabica* available in southern Africa are highly susceptible to *H. vastatrix*. Despite the

fact that the most popular cultivar, SL 28, is susceptible to most rust races/biotypes, it has two other major cultural problems (Mr. J. Logan, Coffee Growers' Association of Zimbabwe, pers. comm.). Firstly, the tree grows to an extreme height making harvesting difficult, and, secondly, the berries ripen late in the year and therefore the plants are not allowed relief from the physiological stress of bearing prior to flowering. SL 28 has always been grown in southern Africa despite warnings by Department of Agriculture Officers concerning its susceptibility to rust (Nyenhuis, 1967). The cultivars Caturra and Catuai are semi-dwarf varieties; unfortunately they produce berries of a lower quality than the SL varieties (Anon., 1987). Intensive breeding of *C. canephora* is presently underway in an attempt to improve the plantings in this country. Robusta, as it is commonly known, is resistant to leaf rust. However, certain selections, particularly those under some form of stress, are susceptible to leaf rust (Coutinho, unpublished data). The rust samples collected from such material proved to be capable of infecting specific selections of *C. canephora* under controlled conditions (refer to **Tables 7** and **8**). Thus stress may not be an important factor in the susceptiblility of *C. caneohora* to leaf rust. Some *C. canephora* selections did, however, prove resistant to all isolates collected in this study (**Table 8**).

Considerable progress has been made over the past 30 years in the breeding of material resistant to *H. vastatrix*. At present, numerous hybrids with Hybrido de Timor (HDT) as one of the parents, constitute the most advanced resistant material available for the control of coffee rust (Varzea *et al.*, 1985). Catimor is an example of such material, and in this investigation proved resistant to all races and biotypes present in southern Africa. The durability of this resistance is questionable. A retrospective analysis showed that the resistance of the HDT common parent has not "broken down" in the preceding 40 years (Varzea *et al.*, 1985). However, there is a move towards breeding for horizontal resistance in *Coffea* spp. (Hoogstraten *et al.* 1982; Van der Graaff, 1986) and some progress has been made. At present, there are no plantings of resistant selections of *C. arabica* in southern Africa and there is an urgent need to introduce resistant cultivars to the industry.

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CHAPTER 2

APPRESSORIUM FORMATION BY *HEMILEIA VASTATRIX*

INTRODUCTION

Many fungal pathogens have developed specialised infection structures for host plant penetration. One type of such structures is termed the "appressorium", which can be simple, with very little difference in appearance from the germ tube, or thick-walled and separated from the germ tube by a septum (Staples & Macko, 1980). The term was introduced by Frank (1883; cited by Emmett & Parbery 1975), who believed the structure to be an adhesive disc. Defining "appressorium" in terms of morphology only, is regarded by Emmett & Parbery (1975) as inadequate and inaccurate, and, in their opinion, its definition must be qualified by stating that it gives rise to infection.

Germinating urediospores of rust fungi cannot usually achieve host penetration directly, and successful infection depends on the formation of an appressorium over a stoma. In the genus *Coffea*, the stomata are confined to the abaxial leaf surface. Thus, successful infection by *Hemileia vastatrix* Berk. & Br. can only occur on this surface (Chinnappa & Sreenivasan, 1968; Harr & Guggenheim, 1978). Studies by Harr & Guggenheim (1978) indicated that the appressorium of *H. vastatrix* is unusual in that a "vesicle" is present on its dorsal surface.

Considerable advances have been made recently in the understanding of the processes involved in infection site recognition (Staples *et al.*, 1985; Hoch & Staples, 1987; Mendgen *et al.*, 1988). The role of physical and chemical stimuli in these recognition mechanisms is well documented in the literature (Wynn, 1976; Grambow & Riedel, 1977; Staples *et al.*, 1983). In the *H. vastatrix-Coffea* interaction the factor, or factors, operating in leaf-surface recognition is/are unknown. In this study, the reaction of *H. vastatrix* to leaf surfaces was examined. A morphological study of appressorium formation on leaves of the host,

Coffea arabica L., and a non-host, *Phaseolus vulgaris* L. was conducted.

MATERIALS AND METHODS

Planting material. Seeds of *C. arabica* cv. Caturra were obtained from the Tea Research Foundation of Central Africa, Malawi. Following germination, seedlings were grown under 50% shade at temperatures between 15 and 30°C in pre-composted pine bark medium supplemented monthly with N:P:K fertilizer and copper chelate. Intact, detached leaves of *P. vulgaris* cv. Pinto 650 seedlings, grown by the Crop Improvement Research Unit, Pietermaritzburg, under greenhouse conditions (20 to 30°C), were also used in this investigation.

Leaf disc/intact detached leaf preparation. The standardized method, described by Eskes (1982), was used for leaf disc preparation. Third-leaf pairs of three-year-old *Coffea* seedlings were gently rinsed under a stream of tap water in order to remove soil debris. Leaf discs, 2cm in diameter, were punched out using a stainless-steel cork borer. The midvein and leaf margins were avoided. Six *Coffea* leaf discs and two intact detached leaflets of *P. vulgaris* were used for each replication and the experiment was repeated four times. Discs and leaves were placed, adaxial surface down, onto foam rubber saturated with tap water in glass trays (29 x 24 x 2.5cm) and inoculated as described below. To obviate morphogenetic effects induced by gravity, discs were initially attached to the underside of the lid of a glass tray with double-sided tape and inoculated. This practice was discontinued when it was found that the morphology of the appressorium on such material did not differ from those formed on discs with the abaxial surface uppermost.

Leaf disc and intact leaf inoculations. Urediospores of *H. vastatrix* were freshly collected from infected trees. They were lightly dusted onto the abaxial surface of *C. arabica* leaf discs and intact *P. vulgaris* leaflets with a camel-hair brush (No. 1). Glass trays were transferred to a dew chamber, set at 20°C and incubated in the dark for 24h. Following this period, trays were covered with glass

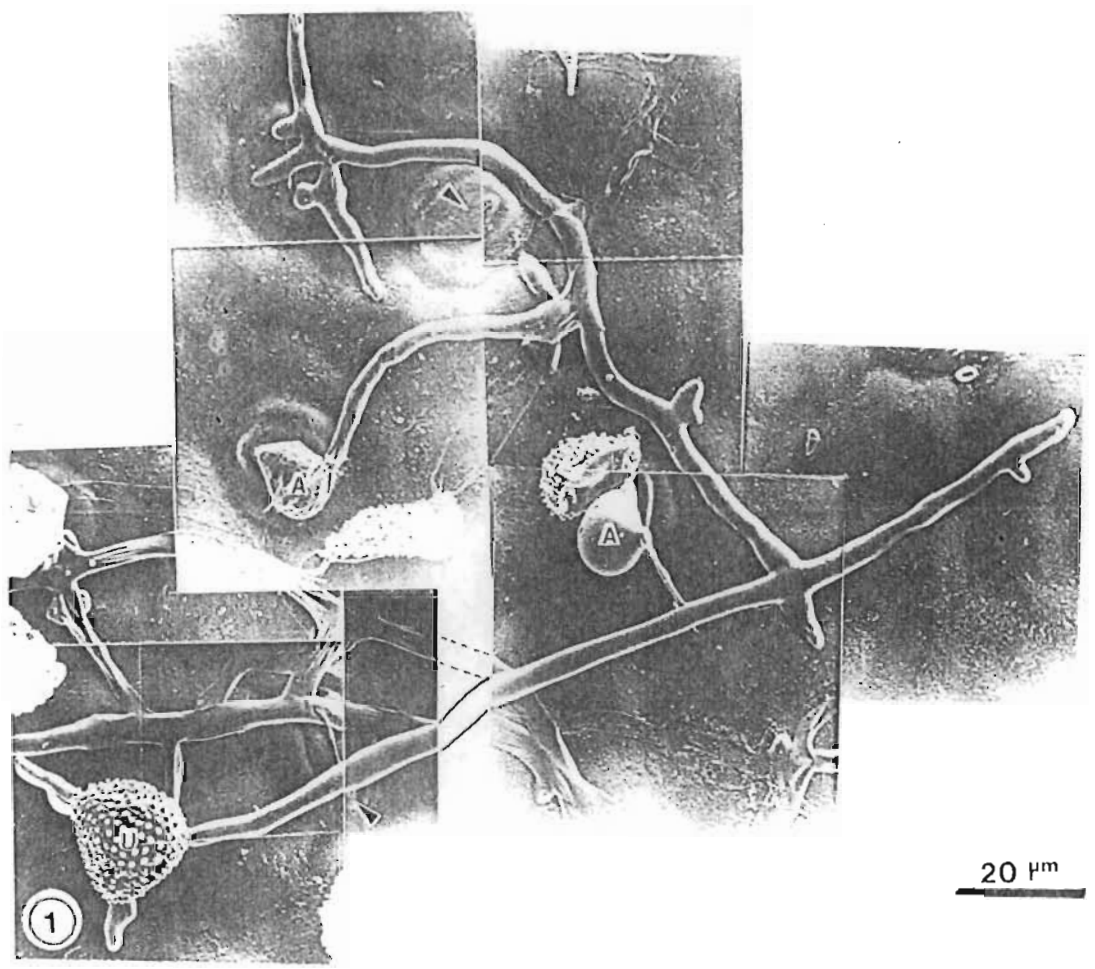
Plate 1

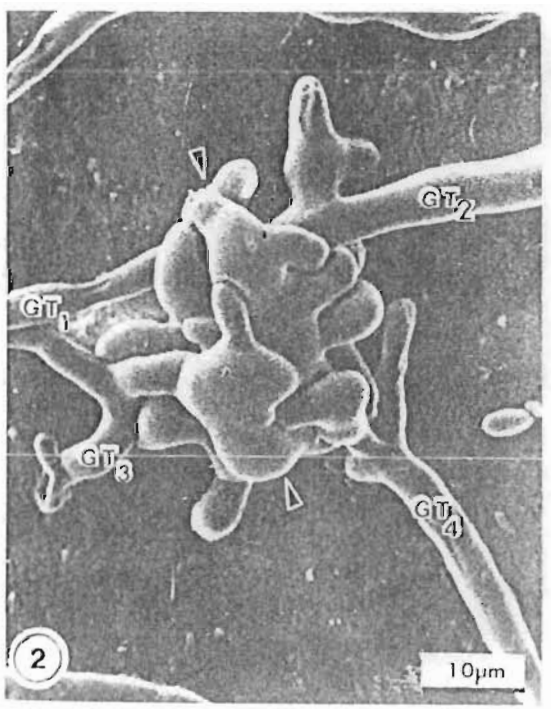
Germination of urediospores of *Hemileia vastatrix* on the leaf surface of *Coffea arabica*, and appressorium formation

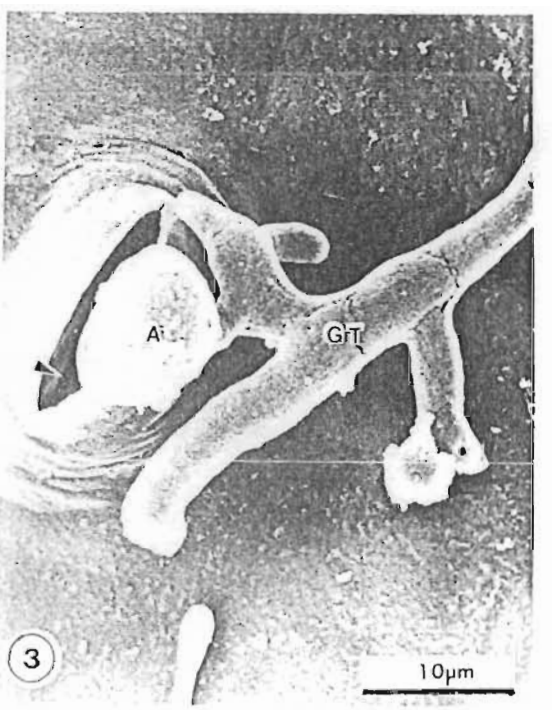
Fig. 1 Random extension of urediospore [U] germ tube 24hpi. Appressorium [A] has formed over a stoma. Note that the germ tube extends over a closed stoma and by-passes another [arrows]

Fig. 2 Entangled germ tubes [GT] from several urediospores with several appressorium-like structures [arrows] 24hpi

Fig. 3 Appressorium [A] at the raised lips of the stoma 8hpi. Note the inner stomatal pore at the base of the vestibule [arrow]


A
A
U
20 µm
1


GT2
GT1
GT3
GT4
10µm
2


Ai
GT
10µm
3

sheets and placed in a constant environment chamber (Conviron ®) at 26°C with a 12 h photoperiod.

Light microscopy. At 24 hours-post-inoculation (hpi) three pieces (ca. 5 x 5mm) of two of the inoculated *Coffea* leaf discs, and three pieces from two intact *P. vulgaris* leaflets, were used for this investigation. This material was fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer (pH = 6.8-7.2) overnight. The tissue was dehydrated through a graded water:ethanol:tertiary-butanol series and infiltrated with liquid paraffin and wax from Histosec ® pastilles as described by Jensen (1962). The embedded material was sectioned at 10 μm, using a rotary microtome and stained with safranin - fast green following the removal of the wax with xylene. Sections were viewed with a Zeiss photomicroscope and the stomatal complexes drawn to scale.

Scanning electron microscopy (SEM). Leaf material was sampled at 2, 4, 8 and 24hpi. One *Coffea* leaf disc and two pieces from each intact *P. vulgaris* leaflet were cut at each sample time and from each replication. Each leaf disc was cut into three pieces (ca. 5 x 5mm). Leaf samples were either viewed directly using an Emscope ® SP 2000 cryo-apparatus or fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer [pH = 6.8-7.2], rinsed in buffer, post-fixed in 2% osmium tetroxide and dehydrated in an ethanol series. The specimens were critical point dried (CPD) using a Hitachi ® HCP-2 with carbon dioxide as transition fluid, and mounted on metal stubs. Prior to coating, some metal stubs with mounted leaf specimens were gently pressed onto other stubs coated with double-sided tape. In so doing, the appressoria were stripped from the leaf surface, permitting examination of both dorsal and ventral surfaces. At 24hpi the infection structures from three leaf pieces were removed from the leaf surface with double-sided tape in a similar manner and viewed using cryo-SEM. The tissue was gold/palladium coated in a Polaron ® sputter coater. Infection structures were viewed with a Hitachi ® S-570 operating at either 5, 8 or 10 kV. Measurements of infection structures were made from SEM micrographs.

Plate 2

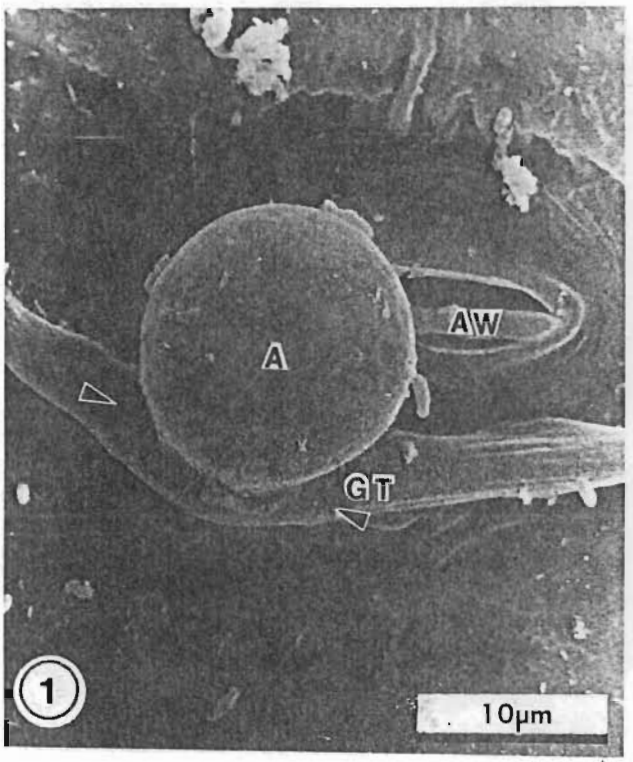
The appressorium of *Hemileia vastatrix* over the stoma of the host, *Coffea arabica*

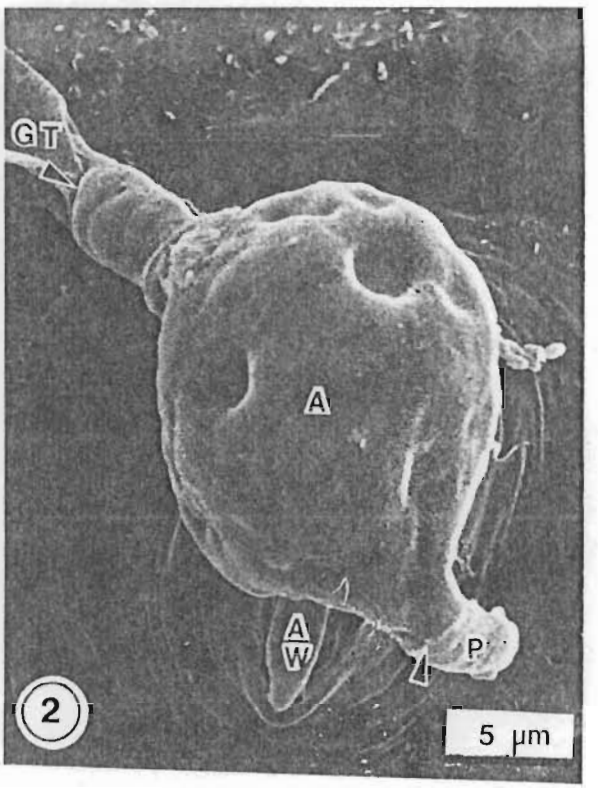
Fig. 1 Appressorium [A] on a germ tube [GT] branch 24 hpi. The appressorial wedge [AW] is within the stomatal vestibule

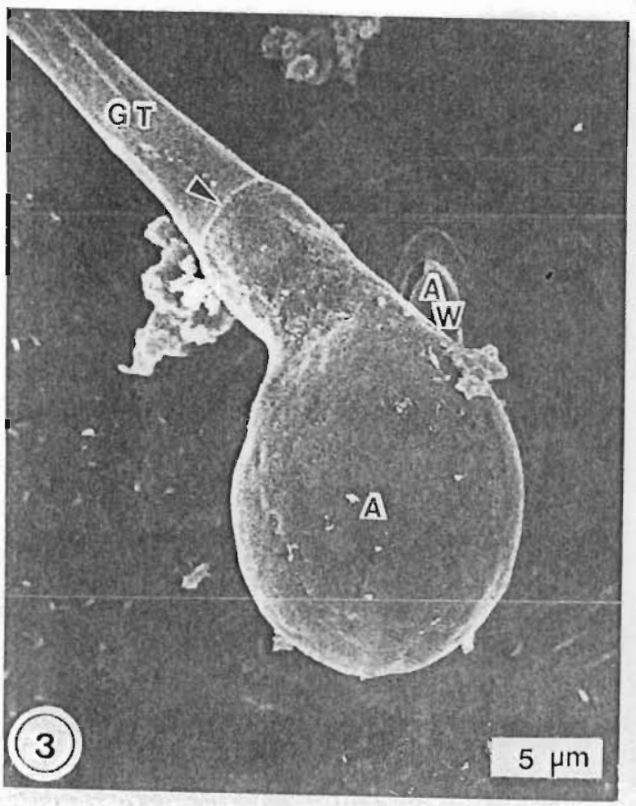
Fig. 2 Appressorium [A] terminally delimited from the germ tube [GT] by a septum [arrow] 24hpi. The appressorial wedge [AW] is again discernible. Opposite the collapsed germ tube a protruberance [P], cut off from the appressorium by a septum [arrow], is visible

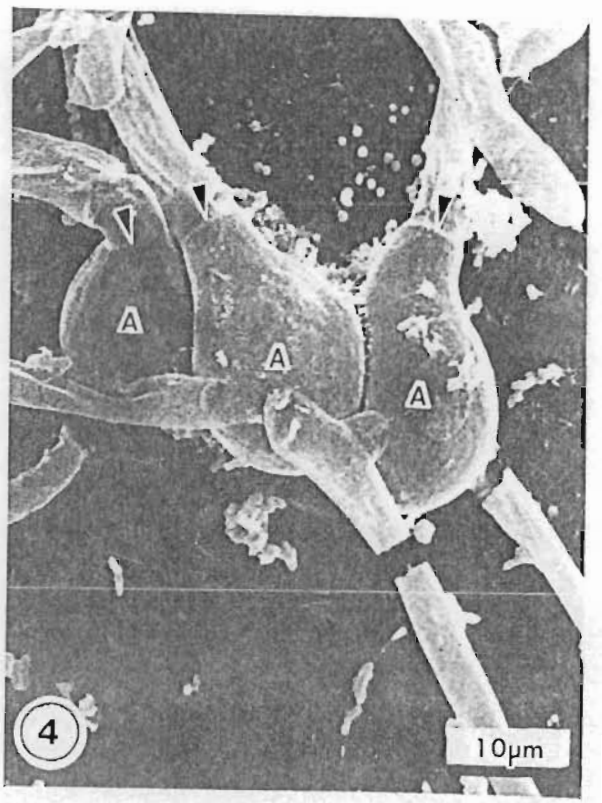
Fig. 3 Terminal appressorium [A] on the stoma 24hpi. The appressorial wedge [AW] is visible within the stomatal vestibule. A septum [arrow] cuts off the germ tube [GT] from the appressorium. No appressorial protruberance is visible

Fig. 4 Three appressoria [A] over a stoma 24hpi


A
AW
GT
1
10µm


GT
A
AW
P
2
5 µm


GT
AW
A
3
5 µm


A
A
A
4
10µm

RESULTS

Light microscopy. There are marked differences between the structure of stomata of *C. arabica* and *P. vulgaris* leaves (**Figs. 1 A** and **B**). In the former case, the subsidiary and guard cells are elevated above the epidermal cells; this is not the case in *P. vulgaris* leaves. In both *Coffea* and *P. vulgaris*, prominent stomatal lips are raised above the stomatal slit and measure approximately 4μm and 2μm (n = 10) in height, respectively. The vestibule is a small chamber which separates the stomatal lips and inner stomatal pore.

Germination. At germination (4hpi), germ tubes (dia. 4.20μm, n = 10) extrude through germ pores of the urediospore wall. As many as five germ tubes can be formed from a single urediospore, but only one elongates and extends randomly over the epidermis, in some cases past or over closed stomata [**Plate 1** Fig.1]. Exploratory branches form along its length. On a few occasions, particularly when the inoculum concentration was high, the germ tubes of several urediospores were seen to have become entangled causing a mass of appressorium-shaped structures [**Plate 1** Fig. 2]. Fungal morphology at germination was similar on host and non-host tissue. However, germ tubes were relatively short on *P. vulgaris* leaflets in comparison to those on *C. arabica* leaves.

Appressorium formation on host and non-host tissue. The formation of appressoria and their shape was similar on both host and non-host tissue. However, appressoria formed only sporadically on non-host tissue. They were first observed, in both cases, at 8 hpi.

When the raised subsidiary cell of coffee is encountered by the tip of either a side branch or the main axis of the germ tube, the tip swells and an appressorium is formed [**Plate 1** Fig. 3]. The near-spherical appressorium usually develops on one end of the stomatal slit. Following the formation of the appressorium, it is cut off from the germ tube by a septum which is formed approximately 6μm from the edge of the appressorial swelling. A wedge-shaped structure develops from the base of the appressorium into the vestibule of the stoma [**Plate 2** Fig. 1, 2 and 3; **Plate**

Plate 3

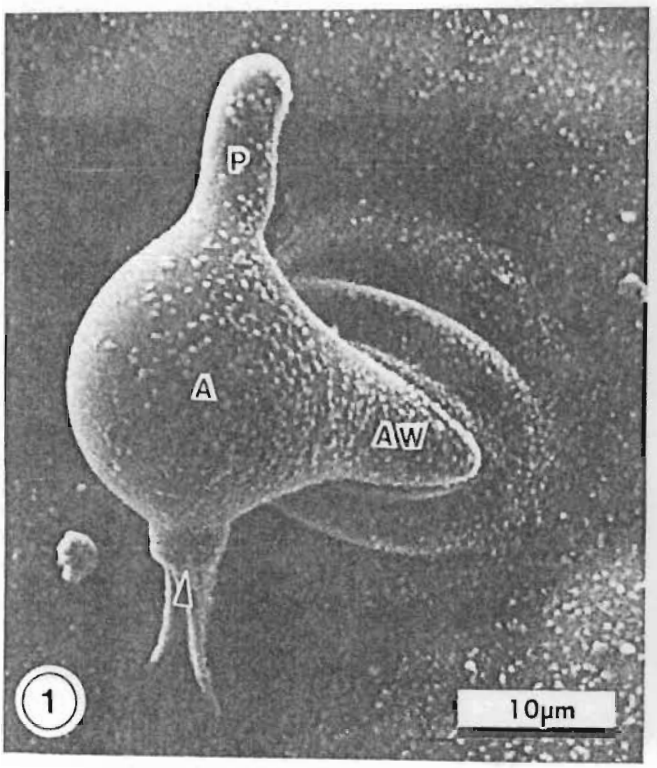
The upper and lower surfaces of appressoria of *Hemileia vastatrix* on, and removed from, the leaf surface of *Coffea arabica* 24hpi

Fig. 1 Cryo-scanning electron microscopy of the appressorium [A]. The appressorial wedge [AW] and the protruberance [P] opposite the collapsed germ tube, are visible [arrow]

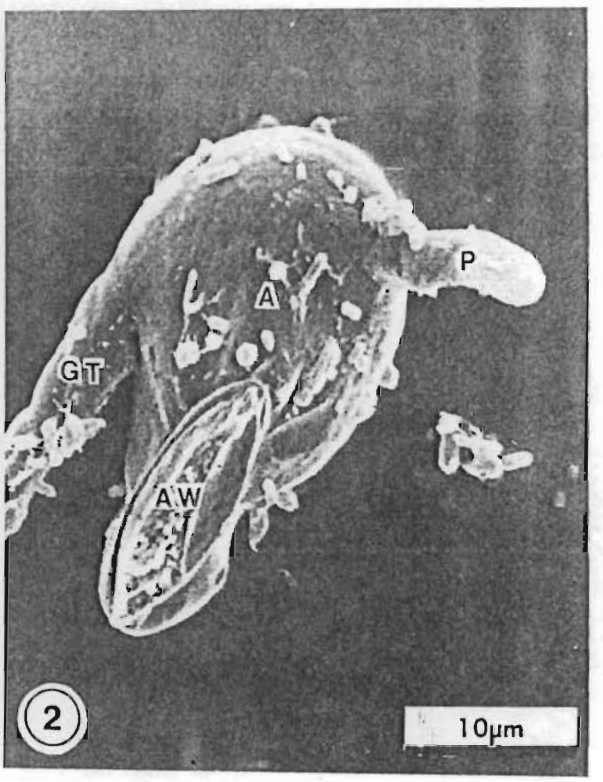
Fig. 2 The lower surface of the appressorium [A]. The appressorial wedge [AW] and the protruberance [P] are noticeable

Fig. 3 Appressorial wedge [AW] produced on the lower surface of the appressorium [A]. Note the appressorial wedge has tapered to form the infection wedge [white/black arrow], and the residue of some putatively adhesive material on the appressorium [black arrow]

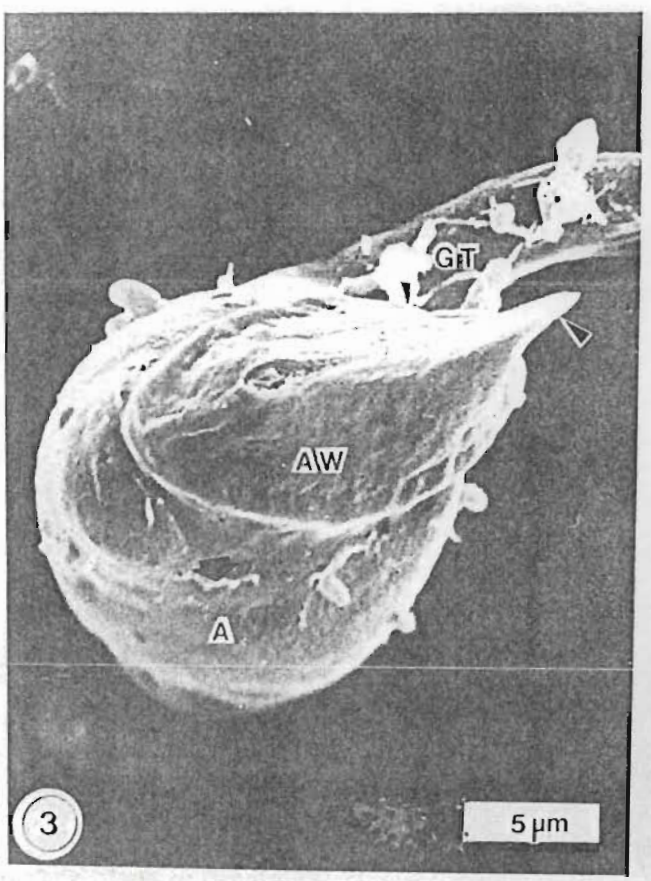
Fig. 4 A lateral view of an appressorium [A], appressorial wedge [AW] and infection wedge [arrow]



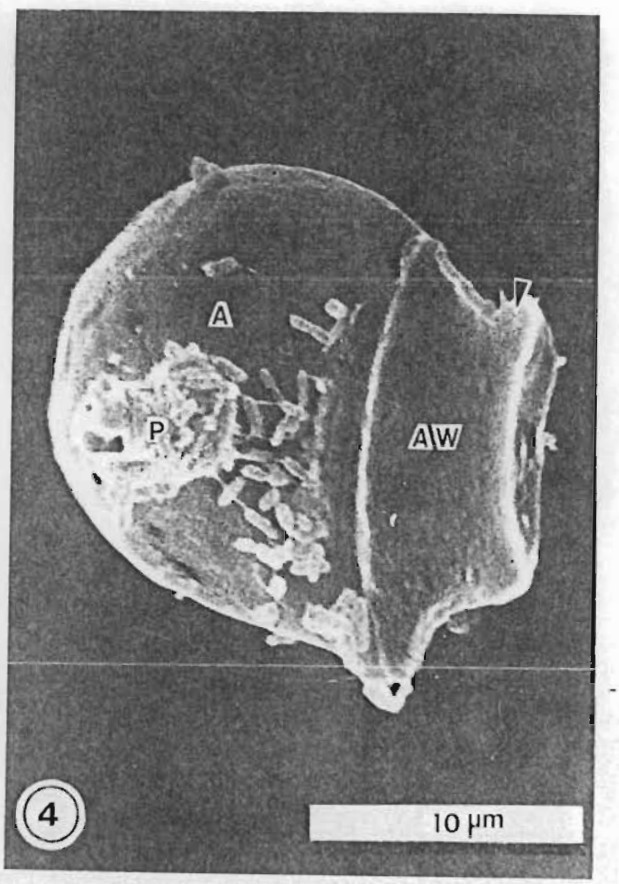
1



2



3



4

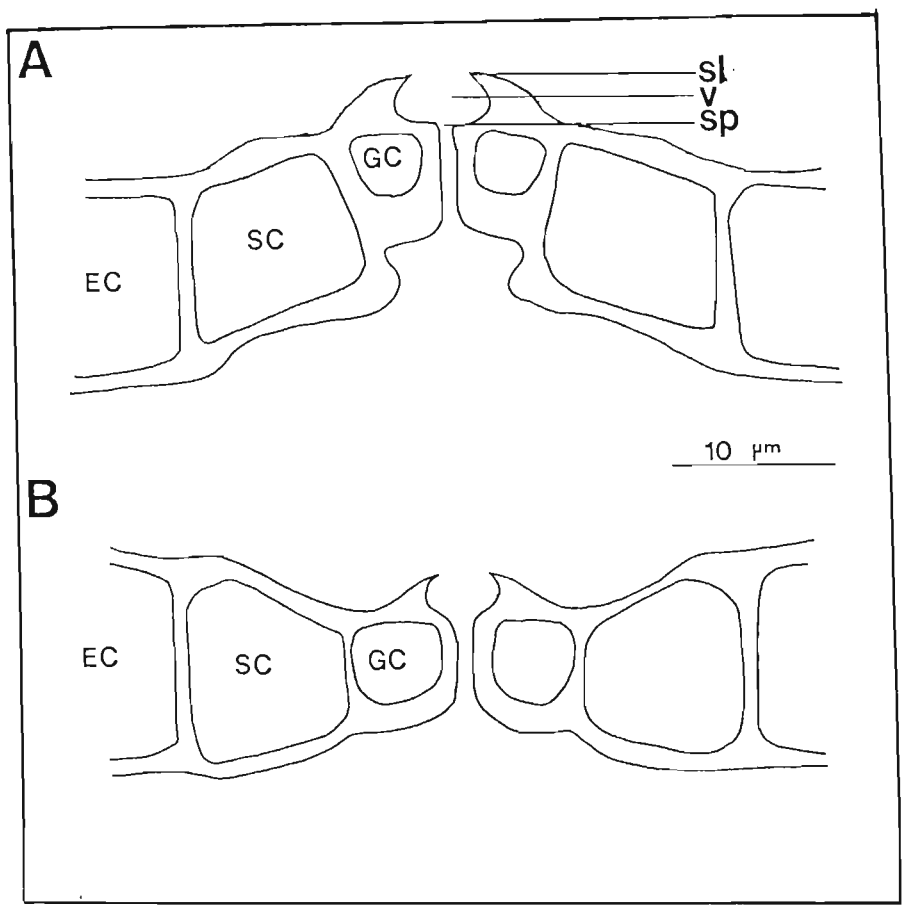


Fig. 1 Schematic representation of cross-sections through the stomata of *Coffea arabica* cv. Caturra [A] and *Phaseolus vulgaris* [B]. EC = epidermal cell; SC = subsidiary cell; GC = guard cell; sl = stomatal lip; v = vestibule; and sp = stomatal pore

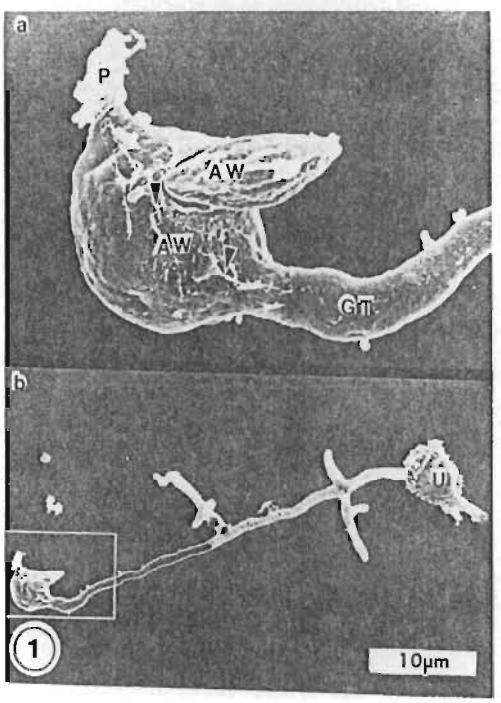
Fig. 1]. Although this wedge was not always seen using SEM when viewing the leaf surface, it was found that upon removing the appressorium with tape this wedge was consistently present [**Plate 3** Figs. 2, 3 and 4]. Upon reaching the stomatal pore the appressorial wedge tapers to form an infection wedge (comparable to the infection peg of other rust fungi) [**Plate 3** Figs. 3 and 4]. More than one appressorium was occasionally seen over a host stoma [**Plate 2** Fig. 4], but never on non-host stomata. A protruberance of between 9 - 10μm in length and 4μm in

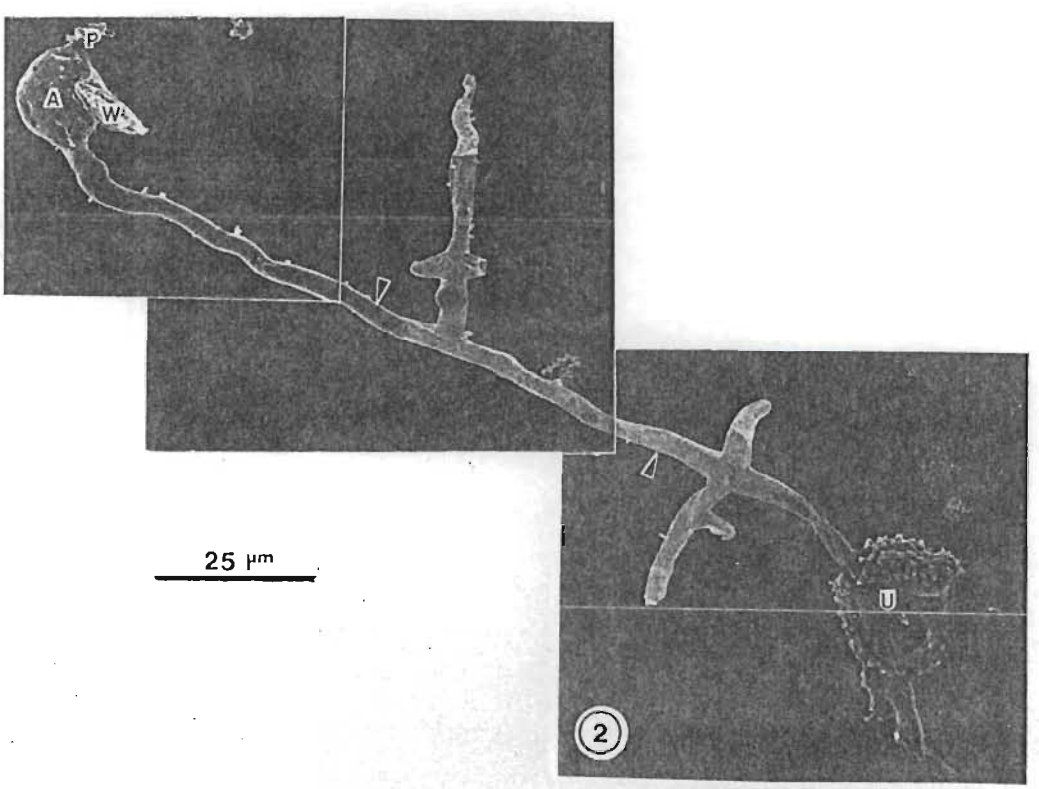
Plate 4

Lower surfaces of appressoria and germ tubes of *Hemileia vastatrix* removed from the leaf surface of *Coffea arabica* 24hpi

Fig. 1 An enlargement of the insert, Fig. 1b, in Fig. 1a depicts some remnants of putatively adhesive material [arrows] on the appressorium [A] but not on the germ tube [GT]

Fig. 2 An enlargement of Fig. 1b illustrates the absence of the putatively adhesive material from the urediospore [U] and germ tube [arrows]





25 μm

diameter is often found on the appressorium opposite the collapsed germ tube [**Plate 2** Fig. 2; **Plate 3** Fig. 1]. Appressoria without such a protuberance are also formed [**Plate 2** Fig. 3]. Remnants of this protrusion are seen on the surface of stripped appressoria [**Plate 3** Fig. 2].

The lower surfaces of the urediospore, germ tube and appressorium were viewed upon their removal from the host leaf surface with tape [**Plate 4**]. Remnants of putatively adhesive material are absent from the germ tube of both CPD- and cryo-SEM-prepared material [**Plate 4** Figs. 1 and 2]. However, remnants were observed on the appressorium of CPD leaf material [**Plate 4** Fig. 1]. Following the removal of the infection structures, the leaf surface was viewed with cryo-SEM and no trace of germ tube pathways could be detected.

The number of stomata on leaves of *C. arabica* cv. Caturra was counted with the aid of scanning electron microscopy. The mean number of stomata per mm^2 was found to be 117 (n = 10).

DISCUSSION

Germ tubes of rust fungi adhere closely to the cuticle of the host in order to respond to the topographical features essential for growth across leaf epidermal cells (Wynn, 1981). Directional germ tube extension increases the ability of the germ tube to locate a stoma more efficiently by reducing wandering (Lewis & Day, 1972). This process is well documented in the literature. *P. graminis* f.sp. *tritici* Erikss. & E. Henn. germ tubes become orientated perpendicularly to leaf venation thereby increasing their chance of locating stomata (Lewis & Day, 1972; Lennox & Rijkenberg, 1989). Gradients in pH at the leaf surface also influence the direction of germ tube growth of *Uromyces viciae-fabae* (Pers.) Schroet. (Edwards & Bowling, 1986).

H. vastatrix germ tubes appear to lack directional growth [**Plate 1** Fig. 1]. According to Wynn (1976), random growth may be due to the lack of close

adhesion between germ tubes and the leaf surface. This may explain the extensive exploratory branching of the germ tube of *H. vastatrix* in attempting to locate a stoma. In the *P. graminis* f.sp. *tritici*/*Triticum aestivum* L. interaction, appressorium formation has been found to be negatively correlated with the degree of branching (Broyles, 1955, cited by Emmett & Parbery, 1975). Niks (1990) observed a negative correlation between germ tube length of *P. hordei* Otth. on *Hordeum vulgare* L. and the chance of success in the establishment of a colony, since the formation of a long germ tube and exploratory branches decreases the amount of energy available to infect the host. The germ tubes of *H. vastatrix* are exceptionally long compared to those of other rust fungi, for example, *P. graminis* f.sp. *tritici* (Lennox & Rijkenberg, 1989). Ferreira (1988) reported that germ tubes of *U. transversalis* (Thüm.) Winter which failed to locate stomata often reached considerable length. The observation in the present study that germ tubes of *H. vastatrix* appear to randomly extend over the leaf surface is contrary to what would be expected, as there are a large number of stomata per square millimetre on *C. arabica* cv. Caturra leaves. Counts of stomata made in this study were slightly lower than the 167/mm^2 recorded by Franco (1939) for an unnamed *C. arabica* cultivar.

The presence of putative extracellular adhesive material at the interface between germ tubes and the waxy layer of the host, has been demonstrated on germ tubes of *Puccinia coronata* Corda (Onoe *et al.*, 1972, cited by Staples & Macko, 1980), *Uromyces appendiculatus* Pers. Unger (Epstein *et al.*, 1987). *Puccinia graminis* f.sp. *tritici* (Harder *et al.*, 1985) and *Puccinia recondita* Rob. & Desm. (C.A. Crookes, pers. comm.). However, the presence of this material has not been demonstrated consistently. According to Chaubal (1987), extracellular mucilage was only observed on germ tubes of *Puccinia sorghi* Schw. when a cationic detergent or a cationic stain was added to the fixation solutions used in electron microscopy. The absence of such material on the germ tubes of *H. vastatrix* [**Plate 4**], using cryo-SEM, indicates that the germ tube must utilize another method of remaining attached to the leaf surface. The method used by *H. vastatrix* may involve inter-molecular forces between germ tube wall components, and host wall. The observation, by the present author, that

the abaxial surfaces of *C. arabica* leaves lack wax crystals, fails to explain the absence of adhesive material on the *H. vastatrix* germ tube. Although the leaves of *Phaseolus vulgaris* also lack wax extrusions, the germ tubes of *U. appendiculatus* are reported to possess extracellular material that may be involved in binding germ tubes to an inductive surface (Epstein *et al.*, 1985; Epstein *et al.*, 1987). Wynn & Staples (1981) showed that when rust urediospores of *P. graminis* f.sp. *tritici*, *P. recondita*, *P. hordei* Otth. and *P. sorghi* Schw. were incubated on waxless leaves of their hosts, the germ tubes neither adhered to the surface nor to the stomata, and consequently, penetration did not occur.

Appressoria develop in response to stimuli, the nature of which differs in the different rust species (Wynn & Staples, 1981). In *P. recondita*, a topographical stimulus is sufficient to induce appressorium formation (Dickinson, 1970). In *C. arabica* the only morphological surface feature that distinguishes the stomata from the remainder of the leaf surface are the raised subsidiary and guard cells. These raised areas may well act as thigmotropic stimuli inducing the formation of *H. vastatrix* appressoria, although, not infrequently, germ tubes are seen to traverse stomatal slits. The stomatal guard cells as well as the ridges around the stomatal opening are thought to provide thigmotropic stimuli inducing the formation of appressoria of *U. appendiculatus* (Wynn, 1976). An appressorium apparently forms when the germ tube tip encounters a stoma. The stomatal lips of both *P. vulgaris* and *Coffea* are prominent (**Fig. 1**); it is therefore likely that the stimulus for appressorium formation in *H. vastatrix* is physical. Dimensions of the topographical features which determine both germ tube orientation and appressorium formation have previously been determined (Dickinson, 1949). Hoch *et al.* (1987) defined the exact size of the signal which induced appressorium formation of *U. appendiculatus*. This signal was found to be a ridge or groove in the substrate surface that raises or lowers the elevation by 0.5μm. Measurements of the diameter of the germ tube (4.20μm) of *H. vastatrix* correspond to the elevated height of the stomatal lips (4μm); and when the tip of a germ tube comes into contact with another germ tube, appressorium-like structures form [**Plate 1** Fig. 2]. This implies that ridges on the surface of

approximately 4μm may act as a stimulus inducing appressorium formation in *H. vastatrix*.

The method by which the germ tube perceives thigmotropic stimuli is not well understood. Staples & Hoch (1982) first suggested that the sensing mechanism involved elements of the cytoskeleton of germ tubes, facilitating reception and transmission of information about the surface of the host to the nucleus. As is the case with most actively growing fungi, apical vesicles are observed to be positioned in the tip of the germ tube and become dispersed during the early stages of appressorium formation (Staples & Macko, 1980). According to Staples *et al.* (1985), these apical vesicles become positioned in the germ tube at its interface with the leaf surface thus probably causing the swelling of the germ tube tip. According to Rijo & Sargeant (1984), vesicles are observed in large numbers throughout the germ tube of *H. vastatrix*, particularly near the plasmalemma and the apex of the tube. The fact that appressoria of this fungus are not consistently produced at the tip of a germ tube indicates that the process conditioning swelling of the appressorium in the germ tube of *H. vastatrix* is not solely associated with the tip, and that apical vesicles may possibly also arise in a non-apical position.

In *P. graminis* f.sp. *tritici*, a chemical, acrolein, produced by the urediospores, will induce appressorium formation (Macko *et al.*, 1978). A chemical stimulus induced by the host rather than by the rust might also be involved. Grambow & Riedel (1977), and Grambow & Grambow (1978), found that phenols extracted from the epicuticular wax and cell walls of *T. aestivum* leaves induced infection structures of *P. graminis* f.sp. *tritici* in conjunction with volatile substances from the leaf. The role of chemicals in appressorium induction by *H. vastatrix* were not investigated in the present study. However, according to Ablanque *et al.* (1988), quantitative and qualitative comparisons of the waxes of different *Coffea* selections showed differences between those susceptible and those resistant to *H. vastatrix*.

The morphology of the appressorial complex, the appressorium and wedge of *H. vastatrix*, is unique. The observations made during the present study confirm

those reported by Harr & Guggenheim (1978). They, however, regarded the adhering structure, which is termed the appressorial wedge in the present study, as the appressorium and called what is here referred to as the appressorium, a dorsal vesicle. To retain the nomenclature used for other rusts, the present author regards the initial swelling of the germ tube to be the appressorium. One can only speculate on the function of the appressorial wedge. *H. vastatrix* is only capable of infecting the abaxial leaf surface, therefore, the appressorial wedge may have an attachment role in the absence of adequate adhesive/binding forces. It is possible that, to guarantee penetration of the stomatal pore, the appressorium requires a firm hold and the wedge anchors the appressorium in the stomatal vestibule. If this is true then the structure here called an appressorium is unique when compared to appressoria in other rusts.

A protruberance on the appressorium on the side opposite to the germ tube [**Plate 2** Fig. 2] has been noticed previously by Kapooria & Mendgen (1985) in the *Uromyces fabae* (Pers.) de Bary/*Vicia faba* L. interaction. The appressoria of this fungus, similarly to those of *H. vastatrix*, are occasionally delimited by two septa, and the part of the germ tube confined by the septa enlarges forming the appressorium. They offered no explanation for its role. Observations imply that the sensing mechanism of *H. vastatrix* germ tubes is located at a distance from the germ tube tip and only once this region reaches the stimulus is an appressorium induced. According to Hoch & Staples (1987), "a germ tube frequently overgrows the signal by 5 - 20 μm before growth ceases". The patterns of appressorium development observed in this investigation were consistent on host and non-host tissue, suggesting that the observed morphology is genetically controlled.

Physical factors, such as temperature, water availability, and light intensity, may play a role in induction of, and variation in, the morphology of the appressoria of *H. vastatrix*. According to De Jong *et al.* (1987), appressorium formation of *H. vastatrix* occurred more rapidly at 13 to 16°C, temperatures lower than those required for germination. The shape of appressoria was affected by temperature; at temperatures beween 13 to 19°C they were torpedo-shaped or roundish, whereas at higher temperatures their shape was predominantly irregular in

appearance. In this study, appressoria were induced at a constant temperature of 20°C and no variation in appressorium shape could be found.

More than one appressorium over a stoma have been reported for other host-pathogen combinations (Niks, 1981; Falahati-Rastegar *et al.*, 1983; Lennox & Rijkenberg, 1989). In a study conducted by Torabi & Manners (1989) the authors proposed that the proportion of appressoria of *P. recondita* resulting in successful penetration was greater when two or more appressoria occurred over a stoma.

Recognition of stomata by germ tubes of rust fungi occurs with a high degree of precision (Heath, 1977). Despite the vast amount of information on leaf surface recognition, much is still unknown. In the present study, the involvement of contact stimuli in the induction of appressoria by *H. vastatrix* is presented.

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CHAPTER 3

DEVELOPMENT OF INFECTION STRUCTURES OF *HEMILEIA VASTATRIX* IN RESISTANT AND SUSCEPTIBLE SELECTIONS OF THE HOST, AND IN NON-HOSTS

INTRODUCTION

Many research papers have been published on the spread and control of *Hemileia vastatrix* Berk. & Br., but much less is known about the infection process, which was first described by Ward in 1882. The coffee rust urediospore germinates, forms an appressorium over a stoma, and gains entry into the substomatal cavity of the host (Chinnappa & Sreenivasan, 1968; Harr & Guggenheim, 1978; and **Chapter 2** of the present study). Details of early morphological development within the host are, however, vague and clarification is needed.

Scanning electron microscopy has become extremely useful in recent years as a tool in mycological research. Many previous studies on various host-pathogen interactions have, until recently, been confined to pre-infection features on the host's surface. However, the advent of an epidermal stripping technique, described by Hughes & Rijkenberg (1985) for *Puccinia sorghi* Schw. on *Zea mays* L., has made the investigation of early infection structure development within both host and non-host tissue possible. In recent years this technique has been applied to various other rust - host interactions, including those of *Uromyces transversalis* (Thüm.) Winter - *Gladiolus* L. (Ferreira & Rijkenberg, 1989) and *Puccinia graminis* f.sp. *tritici* Erikss. & E. Henn. - *Triticum aestivum* L. (Lennox & Rijkenberg, 1989).

The objectives of this study were to describe infection structure formation in resistant and susceptible selections of *Coffea* spp. and non-hosts, *Camellia sinensis* (L.) Kuntze and *Phaseolus vulgaris* L., with the aid of light,

fluorescence and scanning electron microscopy.

MATERIALS AND METHODS

Plant material. *Coffea arabica* cv. Caturra seed was obtained from the Tea Research Foundation of Central Africa, Malawi. This cultivar is susceptible to *H. vastatrix* (Anon., 1987). CENICAFÉ (Centro nacional de investigaciones de café), Colombia, supplied the lines NP-547 and NC-169, which are F6 progenies of a cross between *C. arabica* cv. Caturra and Hybrido de Timor (HDT). NP-547 gives a resistant, and NC-169 a susceptible, response when inoculated with *H. vastatrix* (G.A. Alvarado, pers. comm.). HDT is a population derived from a single tree, probably a spontaneous hybrid between *C. arabica* and *C. canephora* L., discovered around 1927 in East Timor (Gonçalves *et al.*, 1977, cited by Rodrigues, 1984). The majority of HDT selections are resistant to all known races of *H. vastatrix*.

Following germination, seedlings were grown under 50% shade at temperatures between 15 and 30°C in pre-composted pine bark medium supplemented monthly with N:P:K fertilizer and copper chelate. *Camellia sinensis* and *Phaseolus vulgaris* cv. Pinto seedlings were grown, under greenhouse conditions, by the Department of Horticultural Science, University of Natal, Pietermaritzburg and by the Crop Improvement Research Unit, Pietermaritzburg, respectively. Temperatures within the greenhouse ranged from 20 to 30°C.

Leaf disc and intact leaf preparation. The standardized method described by Eskes (1982), was used for disc preparation from the third-leaf-pairs of *Coffea* selections and *Camellia sinensis*. Intact, detached trifoliate leaves of *P. vulgaris* were used. Leaves were gently rinsed under a stream of tap water in order to remove soil debris. Leaf discs, 2cm in diameter, were punched out using a stainless-steel cork borer. The midvein and leaf margins were avoided. Discs and trifoliate leaves of *P. vulgaris* were placed adaxial surface down, onto foam rubber saturated with tap water, in glass trays (29 x 24 x 2.5cm).

Inoculations. Urediospores of *H. vastatrix* were freshly collected from infected trees. They were lightly dusted onto the abaxial surface of leaf discs and intact leaves with a camel-hair brush (No. 1). Second-leaf pairs (non-detached) of year-old seedlings of each *Coffea* selection were similarly inoculated. The glass trays with the inoculated discs and trifoliate bean leaves, and inoculated seedlings were initially placed in a dew chamber at 20°C for 24 h in darkness and later in a constant environment chamber (Conviron ®) at 26°C with a 12 h photoperiod.

Light microscopy. Six pieces (ca. 5 x 5mm) from two leaf discs of each of the inoculated *Coffea* selections, and six pieces (ca. 5 x 5mm) from two intact *P. vulgaris* leaves were used for this investigation. These specimens were sampled at 48 and 72 hours-post-inoculation (hpi) from two replications. The material was fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer (pH = 6.8-7.2) overnight. The tissue was dehydrated through a graded water:ethanol:tertiary-butanol series and infiltrated with liquid paraffin and wax from Histosec ® pastilles as described by Jensen (1962). The embedded material was sectioned at 10 μm, using a rotary microtome and stained with safranin - fast green following the removal of the wax with xylene. Sections were viewed with a Zeiss photomicroscope and the stomatal complexes of bean and coffee drawn to scale.

Fluorescence microscopy. The fluorochrome staining procedure described by Tiburzy *et al.* (1983) was used. The surfaces of leaves, sampled 24hpi, were examined using a Zeiss research microscope fitted with epifluorescence equipment (light source HBO 50; red suppression filter BG 38; exciter filter BP 390-440; chromatic beam splitter FT 460; barrier filter LP 475). Colour photographs were taken using Kodak ® Ektachrome 160 Professional 35 mm film.

Experiment 1: Three leaf discs of each resistant and susceptible *Coffea* selection were used in a single replication. The experiment was repeated three times. In the case of the *Coffea* seedlings, six leaf pieces (ca. 5 x 5mm) were cut from two attached leaves and used for a single replication. In this case, the experiment was repeated twice.

Experiment 2: Three leaf discs each of *Camellia sinesis* and *Coffea arabica* cv. Caturra and three pieces of a trifoliate leaf of *P. vulgaris* (5 x 5mm) were used in a replication. The experiment was repeated four times.

Scanning electron microscopy (SEM). Leaf specimens sampled at 24, 48, 72 and 96 hpi were fixed in 3% glutaraldehyde in 0.05M sodium cacodylate buffer [pH = 6.8-7.2], rinsed in buffer and post-fixed in 2% osmium tetroxide in the same buffer. The leaf pieces were dehydrated in an ethanol series and critical point dried using a Hitachi® HCP-2 with carbon dioxide as transition fluid and mounted on stubs. Immediately following critical point drying, the epidermal stripping technique, described by Hughes & Rijkenberg (1985), was employed. The technique had to be modified in the case of coffee, as the epidermis rarely strips from the underlying cells. The leaf specimen fractures between spongy mesophyll and palisade cells. Thus, the stub had to be pressed gently onto cellotape numerous times to remove mesophyll cell remnants. Despite numerous modifications to this technique, the epidermis of *Camellia sinensis* leaves did not strip from the underlying tissue, and as a result, SEM was not accomplished with this plant species. Tissue for SEM was gold/palladium coated in a Polaron® E5100 sputter coater. Infection structures were viewed with a Hitachi® S-570 operating at 5, 8 or 10 kV.

Sampling for SEM. Four discs of each selection, and four pieces (5x5mm) from attached *Coffea* and detached *P. vulgaris* leaves were used for a single replication. One disc/leaf piece was used at each sample time. The experiment was repeated nine times in the case of coffee leaf discs and three times in the case of attached coffee and detached bean leaves.

Data recording and processing. For fluorescence microscopy and SEM, the stage of infection structure formation by the fungus was classified and recorded. In the case of fluorescence microscopy the following three pre-infection stages were recorded: (1) germinated urediospores that did not form appressoria; (2) germinated urediospores that formed appressoria over stomata; and (3) germinated urediospores that formed appressoria not over stomata. In the case of Experiment 2 a fourth category was included, namely, germinated urediospores

that formed appressoria on a germ tube side branch. Percentage germination could not be determined as many ungerminated urediospores are washed off the leaf pieces during preparation. The whole leaf disc/piece from intact leaf was scanned and the infection structures, excluding those issuing from large clumps of urediospores, counted. The mean percentage of the total counts recorded on each leaf disc/piece of intact leaf, from each replicate, was calculated for all pre-infection stages. Differences between means were tested for significance using the ANOVA statistical test. Post-penetration categories, using SEM, included: (1) substomatal vesicle initials (SSVI) formed: this category comprised infection wedges with unproliferated swollen tips; (2) substomatal vesicles (SSV) formed: an SSV was considered to have formed once primary infection hypha formation was initiated; (3) aborted SSVIs and SSVs, that is, those observed at later sampling times (48hpi and later) which had failed to develop further; (4) SSVs with secondary infection hyphae, and (5) advanced infection stages (ramification of mycelium through the intercellular spaces).

RESULTS

Light microscopy. Infection structures were, unfortunately, rarely observed using this technique. However, there are marked differences between the structure of stomata of *Coffea* and *P. vulgaris* leaves (**Chapter 2** Fig. 1 **A** and **B**). In the latter case, the guard cells form an almost even surface with the surrounding epidermal cells in the substomatal chamber, whereas in coffee the guard cells in the substomatal chamber are considerably recessed with respect to the subsidiary cells. In both *Coffea* and *P. vulgaris*, prominent stomatal lips are present around the stomatal slit. The vestibule separates the outer and inner stomatal pores. Stomatal slit lengths, and guard and subsidiary cell heights of Caturra, NC-169, NP-547 and *P. vulgaris* are presented in **Table 1**. At the 0.05 level there were no significant differences between the three categories of host selections and the non-host.

Fluorescence microscopy. Urediospore germination and appressorium

Table 1 Measurements of stomatal features in *Coffea* selections and *P. vulgaris* (n = 10)

HOST/NON-HOST	STOMATAL SLIT LENGTH (μm)	GUARD CELL HEIGHT (μm)	SUBSIDIARY CELL HEIGHT (μm)
cv. Caturra	12	8	13
NC-169	15	7	12
NP-547	10	7	11
Bean	14	10	13

formation of *H. vastatrix* was found to be the same on susceptible and resistant host, and non-host leaf surfaces (**Appendix 1**). Internal infection structures of *H. vastatrix* were rarely observed in leaves. The number of germinated urediospores that did not form appressoria, or that successfully formed appressoria over stomata, or that resulted in aborted appressoria not over stomata, were in some cases, statistically significantly different between *Coffea* selections and non-hosts at LSD 0.05 values.

Experiment 1: Refer to **Table 2**. The recorded number of germinated urediospores that did not form appressoria, and those that successfully formed appressoria over stomata, was significantly different on all three *Coffea* selections. By comparing the values recorded for these two categories, it appears that the susceptible Caturra, and to a lesser extent NC-169, are more readily infected than the resistant NP-547. Also, the number of aborted appressoria was significantly higher on NP-547, than on either Caturra or NC-169. In general, the percentages of the three categories recorded between leaf discs of *Coffea* selections (**Table 2**) and attached leaves were similar (**Table 3**). Trends comparable with those discussed above, were still evident.

Experiment 2: Refer to **Table 4**. In the comparison between the non-hosts, *Camellia sinensis* and *P. vulgaris*, and the host, cv. Caturra, significantly more appressoria formed over host stomata. Aborted appressoria occurred more

Table 2 Percentages of recorded pre-infection stages of *H. vastatrix* on the leaf disc surfaces of susceptible and resistant selections of *Coffea*

CATEGORY	COFFEE SELECTIONS		
	CATURRA	NC-169	NP-547
% germinated urediospores that did not form appressoria*	16.03 a**	32.09 b	43.31 c
% appressoria formed over stomata*	73.42 a	58.63 b	43.89 c
% aborted appressoria not over stomata*	10.54 a	9.28 a	12.80 b

* Values calculated from the mean percentage obtained from three replicates (refer to **Appendix 1**)

** Letters indicate significant differences across a row according to the LSD 0.05 values

Table 3 Percentages of recorded pre-infection stages of *H. vastatrix* on the surfaces of attached leaves of susceptible and resistant selections of *Coffea*

CATEGORY	COFFEE SELECTIONS		
	CATURRA	NC-169	NP-547
% germinated urediospores that did not form appressoria*	11.00 a**	13.56 a	38.70 b
% appressoria formed over stomata*	80.30 a	72.20 a	49.75 b
% aborted appressoria not over stomata*	8.70 a	14.20 c	11.55 b

* Values calculated from the mean percentage obtained on two replicates (refer to **Appendix 1**)

** Letters indicate significant differences across a row according to the LSD 0.05 values

Table 4 Percentage of recorded pre-infection stages of *H. vastatrix* on leaf disc surfaces from host and non-host plants

CATEGORY	COFFEE SELECTIONS		
	CATURRA	TEA	BEAN
% germinated urediospores that did not form appressoria*	16.06 a**	10.52 b	28.12 c
% appressoria formed over stomata*	73.41 a	37.39 b	28.76 b
% aborted appressoria not over stomata*	10.54 a	52.09 c	43.12 b
% appressoria on a germ tube side branch*	55.70 a	40.96 b	15.98 c

* Values calculated from the mean percentage obtained from four replicates (refer to **Appendix 1**)

** Letters indicate significant differences across a row according to the LSD 0.05 values

frequently on the non-hosts than on the host. In the case of the non-hosts, significantly more appressoria formed apically than on a germ tube side branch.

SEM. The time scale involved in the infection process can be established from **Table 5**. In the case of the *Coffea* selections the following was noted: at 24hpi, SSVIs had formed in Caturra and NP-547, SSVs had already developed in NC-169 at this time; at 48hpi, SSVs had formed in all three selections; in Caturra, secondary infection hyphae were noticed at 48hpi, and at 72hpi intercellular spread through the tissue began. In the case of NC-169 and NP-547, secondary infection hyphae and intercellular spread were noted at 72hpi. In the case of *P. vulgaris*, at 24hpi the SSVs had already developed, and secondary infection hyphae were evident as early as 48hpi. It must be noted that the arrangement of primary and secondary hyphae occasionally makes their unequivocal identification impossible.

Table 5 Total counts of the stages of infection structure development of *H. vastatrix* within various *Coffea* selections and a non-host, *P. vulgaris*, using SEM

CATEGORY	HPI	COFFEE SELECTIONS			NON-HOST
		CATURRA	NC-169	NP-547	BEAN
% SSV initials formed	24	8	8	6	2
% aborted SSV initials	48	12	2	2	*
	72	2	4	2	*
% SSV formed	24	*	6	*	52
	48	18	4	4	53
	48				7 c
% aborted SSVs	72	*	4	6	8
	96	*	*	*	3
% SSVs with secondary infection hyphae	48	4	*	*	21
	72	*	16	6	27 c
	96	*	4	*	24 c
	96				2
% advanced infection	72	4	2	2	*
	96	6	4	2	*
Total number of infection structures observed at all sample times		54	54	28	199

* stage of fungal development not observed at the particular time under consideration

c collapsed

In the case of *P. vulgaris*, the entire epidermis was stripped from the underlying cells with relative ease. It was for this reason that the total number of infection propagules examined was much higher for *P. vulgaris* than for *Coffea* (**Table 5**).

Infection structure morphology on the leaf surface of host and non-host tissue. The appearance of infection structures on resistant and susceptible host tissue and on the non-hosts was similar. Unlike the abaxial leaf surfaces of *Coffea* and *P. vulgaris*, wax extrusions are present on *Camellia sinesis*

leaves. The leaf surface view of the stomata of *Camellia sinensis* is unique in that a prominent ridge in the form of a ring surrounds the complex.

Infection structure morphology within host tissue. Once the stomatal chamber is entered, a bulbous SSVI develops from the apex of the infection wedge (**Plate 1** Fig. 1). Occasionally, two SSVIs were observed protruding into the substomatal chamber (**Plate 1** Fig. 2). The initials extend in length and become torpedo-shaped (**Plate 1** Fig. 3). Occasionally, amorphous material adhered to the infection structures in cv. Caturra (**Plate 1** Fig. 3). In NP-547, the resistant selection, the infection wedge appears to extend into the substomatal chamber (**Plate 1** Fig. 1; **Plate 2** Fig. 1). This was not observed in either Caturra or NC-169 tissue. The SSVI soon bifurcates forming two short primary hyphae, the resultant structure appearing anchor-shaped, because the stubby primary hyphae curve back onto the subsidiary cells, where, by septum formation each cuts off a haustorial mother cell (HMC) (**Plate 2** Figs. 1, 2 and 3). In **Plate 1** Fig. 4, the HMC of the primary hypha appears to form on the mesophyll cells, which was not the usual situation. The SSVs are orientated at right angles to the stomatal opening. Secondary infection hyphae emerge directly on the surface of the SSV that, with reference to the leaf, is adaxial (**Plate 2** Fig. 1) and elongate until they reach the mesophyll cells. Once abutted onto these cells, a septum cuts off a terminal HMC. Further branching of the secondary hyphae occurs behind the HMC and a much branched mycelium ramifies through the intercellular spaces of the mesophyll cells (**Plate 2** Fig. 4). No differences in the appearance of these infection structures between resistant and susceptible hosts were noted.

Infection structure morhology within the non-host, *P. vulgaris*. The SSVI was similar to that observed in the host study. However, the infection wedge protruded through the stoma into the substomatal chamber (**Plate 3** Figs. 1-4). A bulbous structure was evident at the end opposite to the SSV and this structure did not develop further. After 48hpi, SSVs in various stages of collapse were observed (**Plate 4** Figures 1-4). Again, the SSVs were orientated at right angles to the stomatal opening. The HMC was the first structure to collapse, and this was followed by the remaining parts of the infection structure. By 96hpi most infection

Plate 1

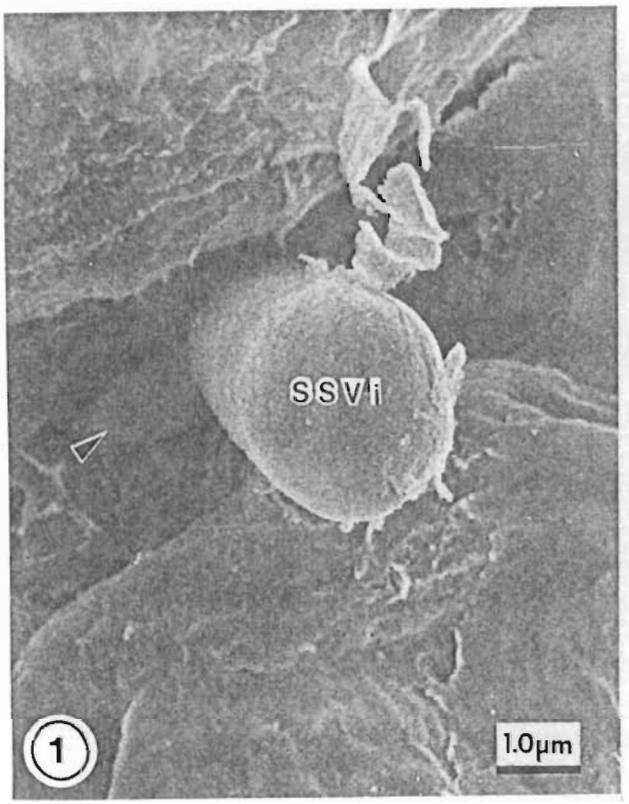
Infection structure development of *H. vastatrix* within host's substomatal chamber 24 and 48hpi

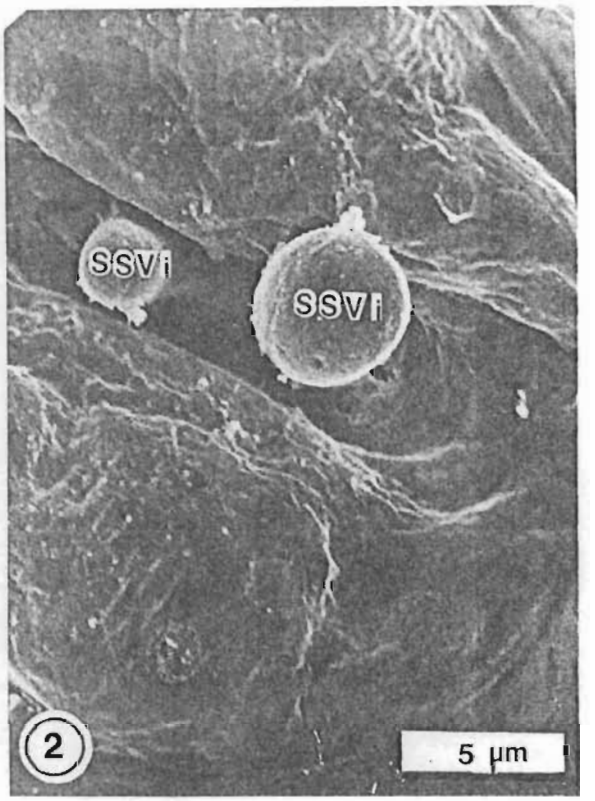
Fig. 1 A bulbous substomatal vesicle initial [SSVI] is visible within the substomatal chamber. Note the presence of the infection wedge (arrow) within the substomatal chamber

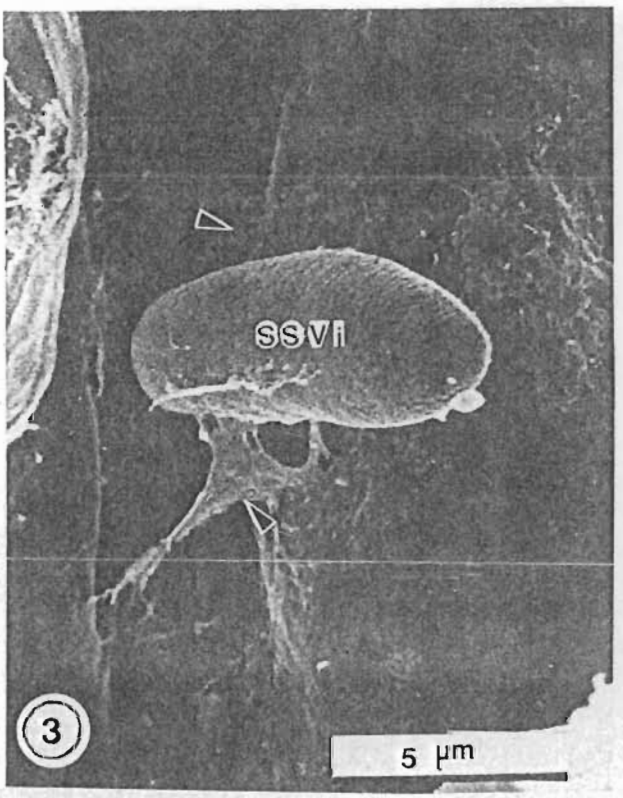
Fig. 2 Two substomatal vesicle initials [SSVI] on the same stoma

Fig. 3 Torpedo-shaped substomatal vesicle initial [SSVI]. The presence of amorphous material is noticeable [arrows]

Fig. 4 Bifurcate substomatal vesicle [SSV]. Note primary infection hyphae in contact with the mesophyll cells of host [arrows]


SSVi
1
1.0μm


SSVi
SSVi
2
5 μm


SSVi
3
5 μm

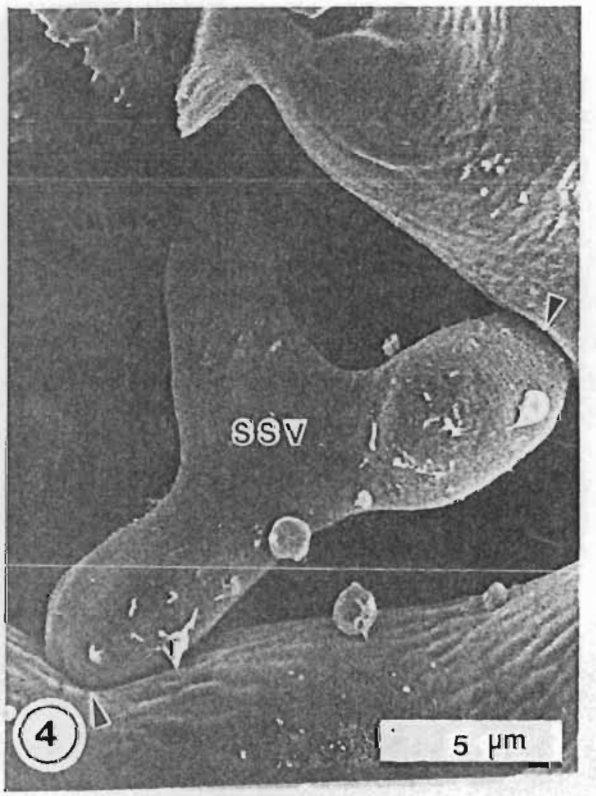

SSV
4
5 μm

Plate 2

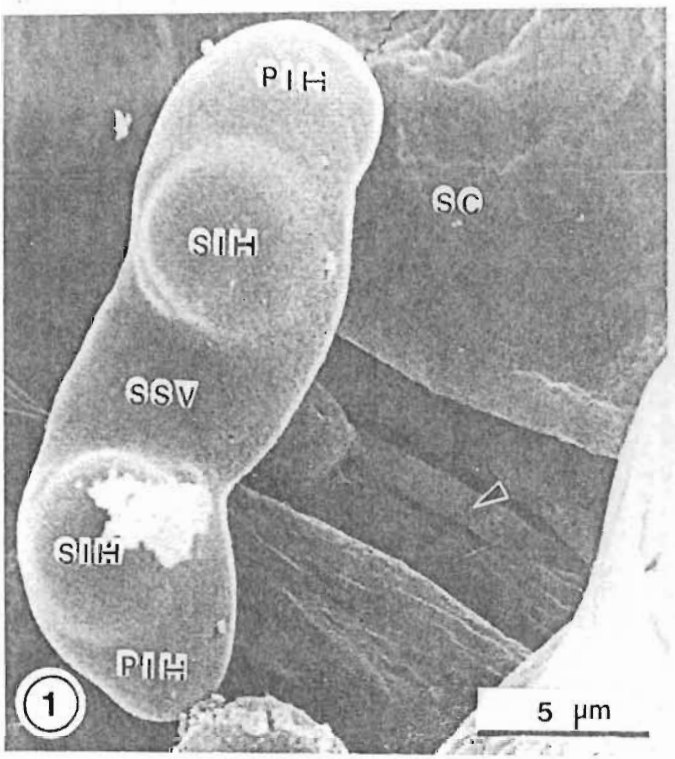
Infection structure development of *H. vastatrix* within host substomatal chamber

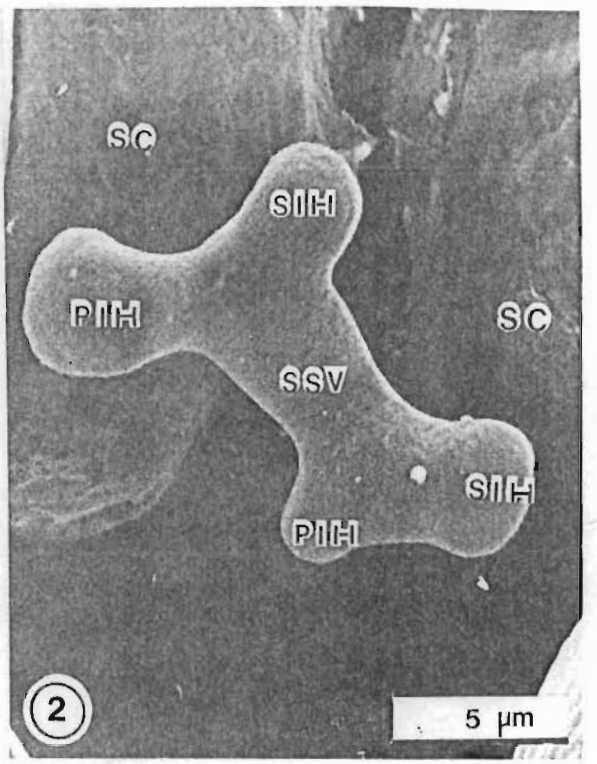
Fig. 1 The substomatal vesicle [SSV] orientated at right angles to the stomatal slit 48hpi. Note the appearance of the infection wedge which has protruded into the substomatal chamber of NP-547 [arrow]. The primary infection hyphae [PIH] are appressed to the subsidiary cells [SC] while the initials of the two secondary infection hyphae [SIH] are clearly evident on the SSV

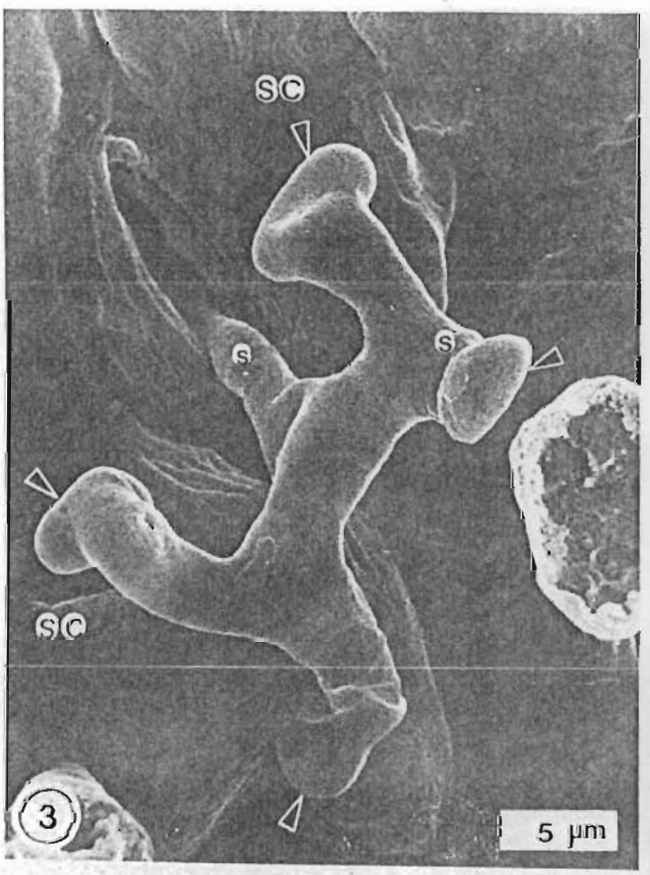
Fig. 2 The anchor-shaped SSV with primary and secondary infection hyphae 48hpi

Fig. 3 The haustorial mother cells [arrows] are appressed to the subsidiary cells [SC] and mesophyll cells of the host 72hpi. A septum [s] is seen both between the infection wedge and the SSV, and cutting off the HMC from the infection hypha [s]

Fig. 4 The fungus has numerous branched intercellular hyphae [arrows] 96hpi


PIH
SC
SIH
SSV
SIH
PIH
1
5 µm


SC
SIH
PIH
SC
SSV
SIH
PIH
2
5 µm


SC
S
S
SC
3
5 µm

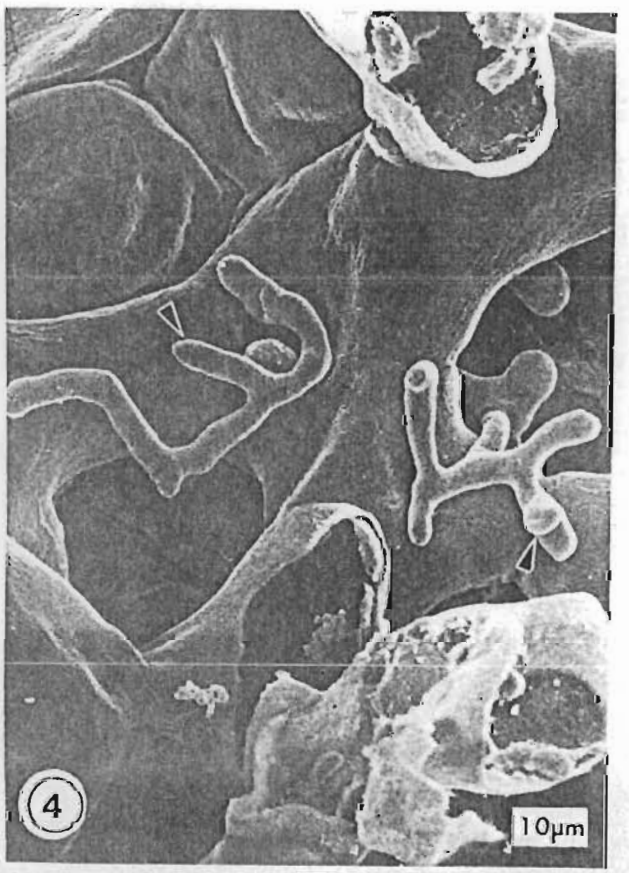

4
10µm

Plate 3

Infection structure development of *H. vastatrix* within the substomatal chamber of bean, a non-host, 24hpi

Fig. 1 The infection wedge [IP] protrudes into the substomatal chamber of bean. A bulb-shaped structure [BSS] is seen at one end of the infection wedge while at the other end a substomatal vesicle initial [SSVI] is visible

Fig. 2 A septum [arrow] is visible between the bifurcate substomatal vesicle [SSV] and the infection wedge

Fig. 3 A secondary infection hypha [SIH] has developed from the primary infection hypha [PIH] on the substomatal vesicle [SSV]

Fig. 4 Primary [PIH] and secondary [SIH] infection hyphae on the SSV. The arrangement of primary and secondary hyphae (for example, on right of this micrograph) occasionally makes their unequivocal identification impossible

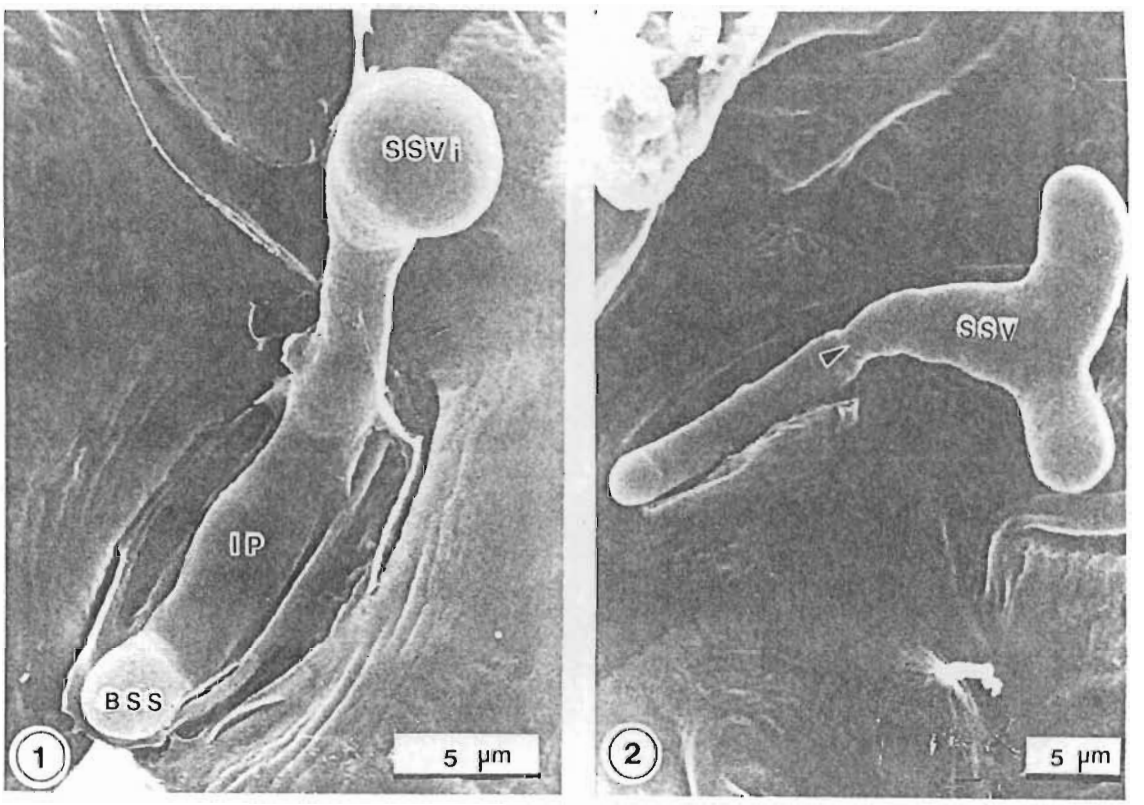

SSVi
IP
BSS
1
5 µm
SSV
2
5 µm
PIH
SGV
SIH
PIH
3
10µm
SIH
SIH
PIH
PIH
GC
4
5 µm

Plate 4

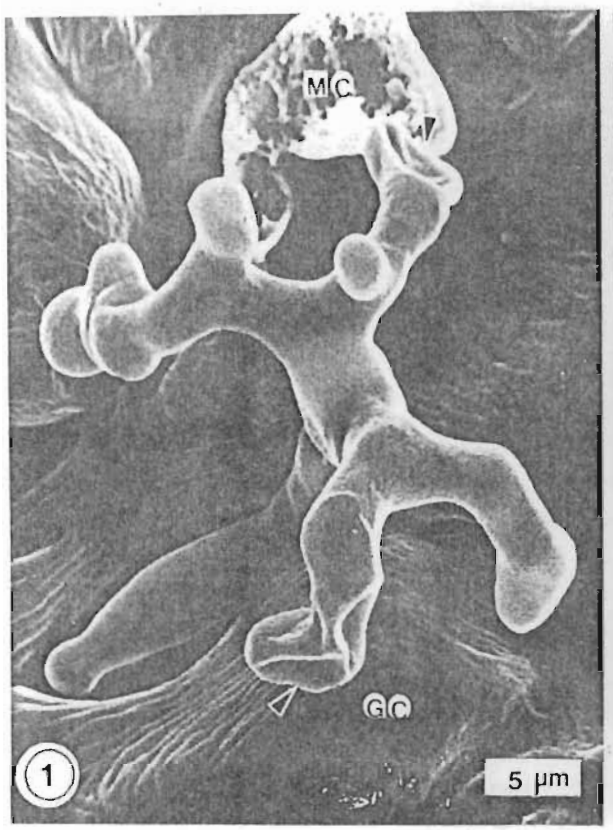
Infection structure development of *H. vastatrix* within the substomatal chamber of bean, the non-host

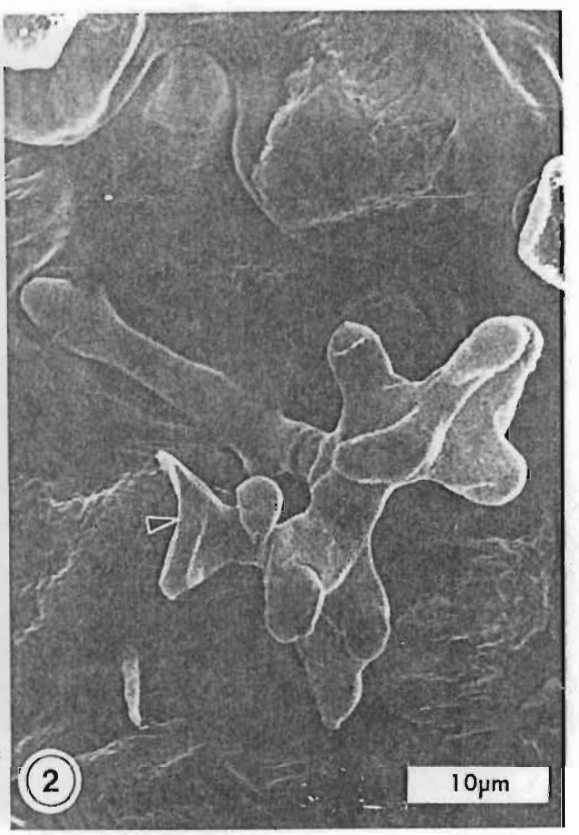
Fig. 1 Collapsed haustorial mother cells [arrows] in contact with the non-host's guard and mesophyll cells 48hpi

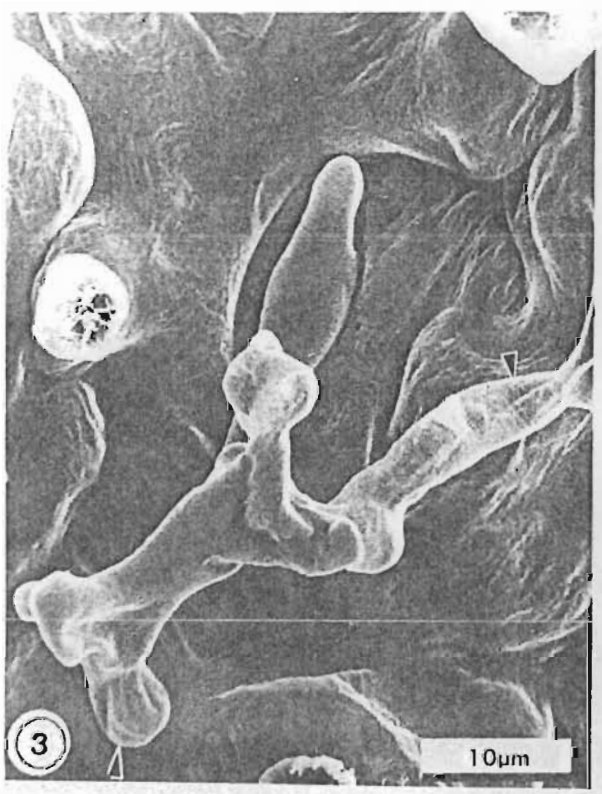
Fig. 2 Much-branched infection structure. The haustorium mother cell [arrow] has collapsed 48hpi. Note the septum between the wedge and the SSV

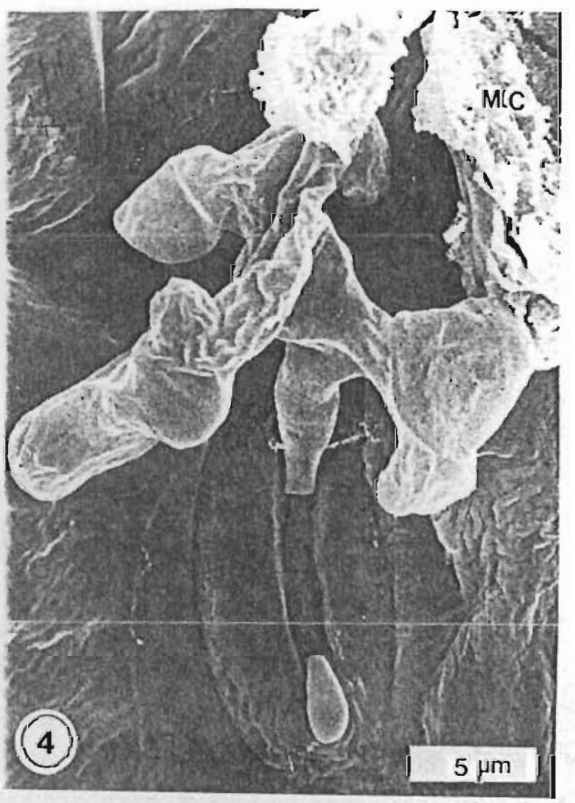
Fig. 3 Collapsed infection hyphae [arrow] 72hpi

Fig. 4 The entire infection structure collapsed by 72hpi


MC
GC
1
5 µm


2
10µm


3
10µm


MC
4
5 µm

structures had collapsed.

DISCUSSION

The present investigation on infection structure development of *H. vastatrix* revealed that the general pattern is similar to that observed for other rust fungi. During the infection process, the urediospore germ tube sequentially forms an appressorium, appressorial wedge (refer to **Chapter 2** of the present study), infection wedge, SSV and infection hyphae. The infection structures of *Hemileia vastatrix* do, however, have morphological features that are unique.

Fluorescence microscopy: Host study. According to Littlefield & Heath (1979) and Heath (1981), the development of rusts on the leaf surface is not a determinant in the resistance of host cultivars. However, in the case of other biotropic fungi, resistance to pre-infection structure formation can differ between the abaxial and adaxial leaf surfaces of a host. Amorphous sheets of epicuticular wax on the abaxial leaf surface of *Lolium* spp. prevented germinated conidia of *Erysiphe graminis* DC. from gaining access to features of the cuticular membrane which triggers normal infection structure development (Carver *et al.*, 1990). In the present study, the involvement of epicuticular wax on the *Coffea* leaves was not found to be responsible for the apparent pre-infection resistance expressed by the resistant selection. Significant differences, particularly in the percentages of germinated urediospores that did not form appressoria and appressoria successfully formed over stomata, were noted in this study between *Coffea* selections. This does indicate that conditions on the leaf surface of susceptible hosts are more conducive to pre-infection by *H. vastatrix* than those on the resistant selection (**Table 2** and **3**). However, Martins *et al.* (1985) found that the primary differences between resistant and susceptible *Coffea* selections were only evident after the penetration of the leaf surface by the rust fungus. There are few reports on reduced levels of appressorium formation by rust fungi in resistant hosts (Kochman & Brown, 1976; Russell, 1977). Rijo *et al.* (1982) with the aid of light microscopy, observed that changes in the morphology of guard and

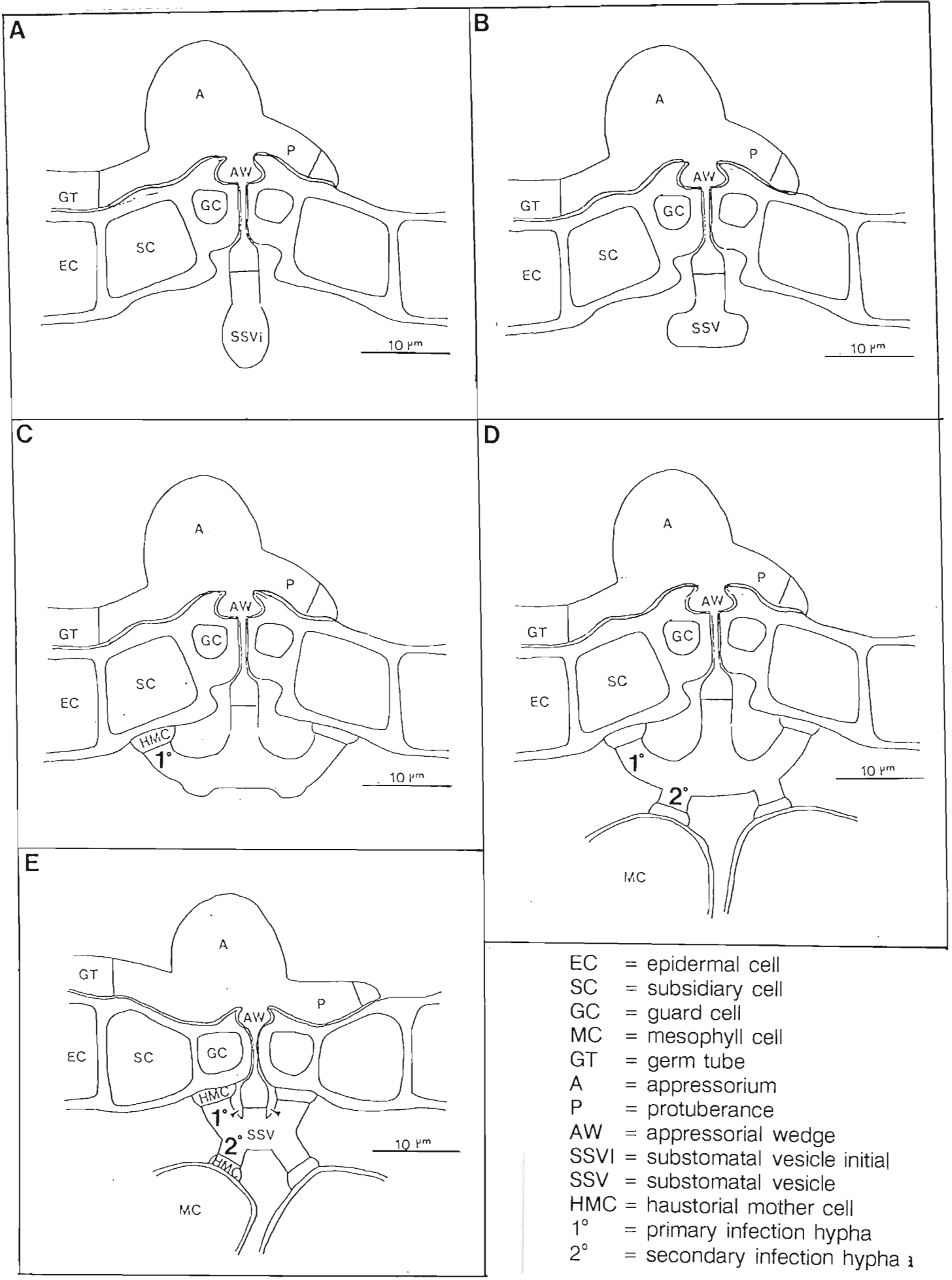
subsidiary cells of immune *C. arabica* selections following their inoculation with *H. vastatrix*. The cytoplasm of the affected cells was granular and they had "apparently thicker cell walls". These changes were induced without any apparent penetration by the fungus. It is therefore possible that similar changes occurred in NP-547 and these alterations in the stomatal complex may account for the failure of penetration and explain the relatively low numbers of infection structures observed in the resistant host (**Table 5**). This factor is unlikely to explain the lack of recognition by the germ tube and the failure of appressoria to form over stomata. Measurements of stomatal slit lengths failed to reveal a significant difference between selections (**Table 1**).

The time scale involved in the formation of infection structures of *H. vastatrix* in host tissue, as reported in the present study, is similar to that observed by Martins & Moraes (1985). In other rust/host combinations the first sampling was done 6hpi (Lennox & Rijkenberg, 1989). In the present study this was done 24hpi in accordance with the findings of De Jong *et al.* (1987) who recorded that, at this time, appressorium formation would be complete.

Fluorescence microscopy: non-host study. A number of factors have been implicated in non-host resistance. The surface characteristics of some plant species could account for non-recognition by the germ tubes of rust fungi. Such factors may include non-wettability of the leaf surface (Heath, 1977), lack of directional growth (Wynn, 1976), waxy or hairy leaf surfaces (Heath, 1974; 1977) and stomatal non-recognition (Wynn, 1976; Heath, 1977). It is evident that, on host leaves, the germ tubes of *H. vastatrix* lack directional growth and side branches are capable of developing appressoria. However, in the non-host, *P. vulgaris*, appressoria are generally formed terminally and in some cases on short germ tubes. Extensive exploration of the leaf surface in this case does not occur, and many appressoria abort. Thus, a surface feature on the bean leaf may act as a stimulus inducing appressorium formation by *H. vastatrix* almost immediately after germination. Wax extrusions and/or unique surface features may play a role in non-host resistance. *Camellia sinensis* leaves are waxy and together with the prominent ridge surrounding the stomatal complex may account for the low levels

of appressoria formed over stomata. This ridge appeared to induce appressoria, not over stomata, and probably is responsible for the high number of aborted appressoria on this plant species. Reduced percentages of appressoria over stomata, due to stomatal non-recognition, have been reported in the case of the inoculations of *T. aestivum* and *Avena sativa* L. with *U. appendiculatus* (Pers.) Unger (Wynn, 1976).

SEM study. The sequence of events in infection structure development of *H. vastatrix* within host and non-host tissue is summarized diagramatically in **Fig. 1**. In host tissue, the infection wedge is single, similar to that of *Uromyces transversalis* (Ferreira & Rijkenberg, 1989), but unlike the bifurcate infection peg of *Puccinia sorghi* (Hughes & Rijkenberg, 1985). The primary infection hyphae of *H. vastatrix* are formed in pairs, and their development is synchronous and at right angles to the stomatal slit (**Fig. 1 A** and **B**). According to Ferreira & Rijkenberg (1989), development of SSVs of *U. transversalis* is similarly orientated and they propose that this alignment has co-evolved with, or adapted to, substomatal chamber orientation. Instances of more than one apparently functional SSV occupying the same stoma have been reported previously for other rust fungi (Hughes & Rijkenberg, 1985; Lennox & Rijkenberg, 1990). Amorphous material on the SSVs occasionally deposited within the susceptible cultivar, Caturra, was not evident in the other susceptible selection, NC-169 nor in the resistant selection, NP-547. According to Onoe *et al.* (1987), similar material is formed in the association between *P. coronata* var. *avenae* Erikss. and susceptible tissue, and is thought to contribute to the establishment of susceptibility. Primary infection hyphae of *H. vastatrix* curve back onto the subsidiary cells, and HMCs are formed (**Fig. 1 C**). Similar to the situations pertaining in *P. sorghi* (Hughes & Rijkenberg, 1985), *U. transversalis* (Ferreira & Rijkenberg, 1989), and *P. graminis* f.sp. *tritici* (Lennox & Rijkenberg, 1989), secondary infection hyphae arise on the SSV side of the septum separating it from the HMC (**Fig. 1 C** and **D**). The time involved in the formation of the infection structures of *H. vastatrix*, within host tissue, is much longer than within any of the abovementioned rust/host interactions.



EC = epidermal cell
SC = subsidiary cell
GC = guard cell
MC = mesophyll cell
GT = germ tube
A = appressorium
P = protuberance
AW = appressorial wedge
SSVI = substomatal vesicle initial
SSV = substomatal vesicle
HMC = haustorial mother cell
1° = primary infection hypha
2° = secondary infection hypha

Fig. 1 Diagrammatic representation of infection structure development of *H. vastatrix* within *Coffea* [**A-D**] and *P. vulgaris* [E]

It would appear that the intra-tissue defence reaction fails to come into operation in the resistant *Coffea* selection, NP-547, within the 96hpi period examined. Light microscopy studies by Rijo (1972), Rijo & Rodrigues (1978) and fluorescence microscopy conducted by Martins *et al.* (1985), indicated that the differences between resistant and susceptible hosts of *H. vastatrix* start to appear 72hpi. They reported that in the incompatible combinations, fungal growth stopped in the pre-haustorium stage or sometimes after haustorium formation in the subsidiary cells. The morphology of the SSV is the same in both reaction types. The subsequent growth of the fungus is reduced in the resistant host, frequently limited to a single short hypha which senesces and apparently dies (Rodrigues *et al.*, 1982). According to Rijo & Rodrigues (1978) and Martins *et al.* (1985), in the resistant selections studied, all rust colonies stopped growing after 144hpi. The differences observed in the present study may be due to the particular resistant host examined or to the particular rust race.

In non-host tissue, the sequence of events in infection structure development is similar to that observed in host tissue, but with the following differences. The infection wedge and a bulb-shaped structure was consistently visible within the substomatal chamber (**Fig. 1 E**) of the non-host. The infection wedge was, however, also infrequently observed in the substomatal chamber of host tissue (NP-547). Differences in heights of guard and subsidiary cells (**Table 1**) could not adequately explain this feature in either host or non-host tissue. Collapse of the infection structures occurred in non-host after the development of HMCs from the secondary infection hyphae. This agrees with the reports by Heath (1974; 1977) that the defence reaction of rust fungi in non-host tissue usually started after the first haustorium was formed.

A criticism that can be levelled against the experiments presented in this Chapter is that differences in the expression of resistance may be different on leaf discs when compared to attached leaves. Mayama *et al.* (1975) reported an increase in susceptibility in detached incompatible wheat leaves floating in different solutions after inoculation. The normal incompatible reaction of attached wheat leaves to certain races of *Puccinia graminis* f.sp. *tritici* was changed and they became

highly susceptible. Eskes (1982) demonstrated that development of *H. vastatrix* was the same for leaf discs and attached leaves. However, Martins *et al.* (1986) noted an increase in susceptibility of *Coffea* to *H. vastatrix* in detached leaves when compared to attached leaves. In the present investigation, there were no demonstrable differences in the development of infection structures on the leaf surface and within tissue between leaf discs and attached leaves of resistant and susceptible selections.

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CHAPTER 4

THE EFFECTS OF THE FUNGICIDE, BAYFIDAN ®, ON INFECTION STRUCTURE FORMATION BY *HEMILEIA VASTATRIX* IN *COFFEA ARABICA* CV. CATURRA

INTRODUCTION

Triadimenol (ß-(4-chlorophenoxy-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol) is a systemic triazole fungicide introduced in 1977 (Frohberger, 1978). It was exclusively developed as a seed treatment for cereals [Baytan ®], and was later developed as a foliar fungicide [Bayfidan ®] (Kuck & Scheinpflug, 1986). Triadimenol is readily taken up by the plant roots and apoplastically translocated to transpiring leaves (Davidse & De Waard, 1984). This fungicide has both high levels of protectant, and curative action against *Hemileia vastatrix* Berk. & Br. (Anon., 1989). According to Dr. M. Deall (Bayer, pers. comm.), the fungicide has been further developed into a granular formulation, and combined with an insecticide, Disyston ®, for use in the coffee industry. It is sold under the trade name Repulse ® in Malawi, but has not as yet been released in southern Africa.

Triadimenol is a metabolite of triadimefon [Bayleton ®] and is considered to be the fungitoxic principle (Gasztonyi & Josepovits, 1979). The primary site of action of triadimenol is considered to be at the level of sterol biosynthesis at C-14 demethylation which, within the fungal metabolism, is inhibited (Buchenauer, 1978). Sterols, are required for growth and reproduction of eucaryotic organisms, and serve as architectural components of membranes (Siegel, 1981). In wheat, about 60% of the active ingredient of triadimefon, which penetrated the leaves, underwent the reduction to triadimenol within 48h at room temperature (Kuck, 1986, cited by Kuck & Scheinpflug, 1986). Similarly, Clark *et al.* (1978) found a 56% conversion of triadimefon to triadimenol in 5 days. The spectrum of fungicidal activity and application rates are, however, similar for both these fungicides (Kuck & Scheinpflug, 1986).

Previous research papers have dealt with the effect of systemic fungicides on the ultrastructure of rust fungi (Ferreira, 1988; Guggenheim *et al.*, 1989). Striking morphological abnormalities were produced in a number of fungal species by ergosterol biosynthesis inhibiting (EBI) fungicides; distortion of germ tubes, often with excessive branching, has been observed in *Monilinia fructicola* Wint. (Sisler *et al.*, 1983) and *Botrytis allii* Munn. (Richmond, 1984). Germination of fungal spores is known to be only incompletely suppressed by fungicides having this action (Kuck *et al.*, 1982). Consequently, a part of the fungal population usually completes colonization of the host plant, despite the presence of these fungicides.

The aim of this study was to determine at what stage, if at all, infection structure development of *Hemileia vastatrix* would be inhibited in a susceptible *Coffea arabica* L. cultivar, following the application of the granular formulation of triadimenol [Bayfidan®]. Fluorescence and scanning electron microscopy were used in this investigation.

MATERIALS AND METHODS

Planting conditions and material. *Coffea arabica* cv. Caturra seed was obtained from the Tea Research foundation of Central Africa, Malawi. Following germination, seedlings were grown in a greenhouse at temperatures between 15 and 30°C in pre-composted pine bark medium supplemented monthly with N:P:K fertilizer and copper chelate.

Application of fungicide. Six two-year old seedlings were treated monthly with Bayfidan® granules. The amount of fungicide used, 5g/seedling, was determined by taking into consideration the height of the seedlings as well as the container size. The recommended dose is 38g per cova (Dr. M. Deall, pers. comm.). Bayfidan® was applied by randomly scattering the granules over the soil surface. Six control seedlings were left untreated. The plants were kept under similar conditions as described above.

Leaf disc preparation. The method described by Eskes (1982) was used for disc preparation from the third-leaf pairs. Leaves were gently rinsed under a stream of tap water in order to remove soil debris. Leaf discs, 2cm in diameter, were punched out using a stainless-steel cork borer. The midvein and leaf margins were avoided. Discs were placed adaxial surface down, on to foam rubber saturated with tap water, in glass trays (29 x 24 x 2.5cm). Discs were cut from leaves 28 days after the second application, seven, 14 and 28 days after the third fungicide application; these times are referred to as A, B, C and D, respectively. The experiment was repeated three times and eight leaf discs were used in each replication.

Inoculations. Urediospores of *H. vastatrix* were freshly collected from infected trees. They were lightly dusted on to the abaxial surface of leaf discs with a camel-hair brush (No. 1). The glass trays were initially placed in a dew chamber at 20°C for 24 h in darkness, and later moved to a constant environment chamber (Conviron ®) at 26°C with a 12 h photoperiod.

Sampling. Two leaf discs were sampled at each sample time: 24, 48, 72 and 96 hours-post-inoculation (hpi). At 24hpi each leaf disc was cut in half and one half was prepared for fluorescence microscopy and the other for scanning electron microscopy.

Fluorescence microscopy. The fluorochrome staining procedure described by Tiburzy *et al.* (1983) was used. The leaf samples, 24hpi, were examined using a Zeiss research microscope fitted with epifluorescence equipment (light source HBO 50; red suppression filter BG 38; exciter filter BP 390-440; chromatic beam splitter FT 460; barrier filter LP 475). Colour photographs were taken using Kodak® Ektachrome 160 Professional 35 mm film.

Scanning electron microscopy (SEM). The infection structures formed on the leaf surface after/or by 24hpi were viewed with either cryo-microscopy, or prepared for SEM. Cryo-microscopy samples were prepared in an Emscope ® SP 2000 cryo-apparatus. Leaf specimens sampled at 24, 48, 72 and 96hpi were fixed

in 3% glutaraldehyde in 0.05M sodium cacodylate buffer [pH = 6.8-7.2], rinsed in buffer and post-fixed in 2% osmium tetroxide in the same buffer. The leaf pieces were dehydrated in an ethanol series, critical point dried using a Hitachi® HCP-2 with carbon dioxide as a transition fluid, and mounted on metal stubs. Immediately following critical point drying, an epidermal stripping technique, described by Hughes & Rijkenberg (1985), was employed. For coffee, this technique had to be modified, as the leaf epidermis most often fractures between the spongy mesophyll and palisade cell layers, and only rarely does the epidermis strip entirely from the mesophyll cells. To remove the adherent mesophyll cells, the stub with the exposed abaxial leaf surface was gently pressed onto cellotape numerous times. The tissue was gold/palladium coated in a Polaron® E5100 sputter coater. Infection structures were viewed with a Hitachi® S-570 operating at 5, 8 or 10 kV.

Data processing. Data obtained from the leaf exterior were processed separately from those of the leaf interior, since the stripping method precluded the stripping of the entire specimen. The stage of infection structure formation of *H. vastatrix* was classified and recorded. In the fluorescence microscopy study the following three pre-penetration stages were considered: (1) percentage of germinated urediospores that did not form appressoria; (2) percentage of germinated urediospores that formed appressoria over stomata; and (3) percentage of urediospores that formed appressoria not over stomata. The entire half of the leaf disc was scanned and the infection structures, excluding those issuing from large clumps of urediospores, counted. Percentage germination could not be determined as many ungerminated urediospores are washed off the leaf pieces during preparation. A mean percentage of the total counts recorded, from each replicate, was calculated for pre-infection stages. Differences between means were tested for significance using the ANOVA statistical test. Post-penetration counts using SEM considered: (1) number of substomatal vesicle initials (SSVI) formed: this category comprised infection wedges with unproliferated swollen tips; (2) number of substomatal vesicles (SSVs) formed: a SSV was considered to have formed once the primary infection hypha formation was initiated; (3) number of SSVs with secondary infection hyphae, and (4) number of advanced infections (ramification of mycelium through the intercellular spaces). Abnormalities in the

morphology of these structures were recorded.

RESULTS

Fluorescence microscopy. Refer to **Appendix 2** and **Table 1**. Although triadimenol had a statistically significant effect ($P < 0.05$) on the percentages of germinated urediospores that did not form appressoria and, appressoria formed over stomata, the effect was not great. In general, at B and D treatment times, the percentage appressoria formed over stomata was higher on the control than on the treated leaf discs. Fewer appressoria were formed over stomata at treatment times B and D. A significantly higher percentage appressoria aborted at time C in comparison to the other treatment times. No abnormal morphology of the urediospore, germ tube or appressoria was observed.

Table 1 Mean percentages of counts made of the stages of infection structure development of *H. vastatrix* on the leaf surface of cv. Caturra following the application of Bayfidan® at various time intervals

CATEGORY		DAYS AFTER THE SECOND*/THIRD** APPLICATION OF BAYFIDAN			
		28 days* [A]	7 days** [B]	14 days** [C]	28 days** [D]
		*****			
% germinated urediospores that did not form appressoria ****	TREATMENT	6.54 a A	21.98 b A	6.39 abc A	12.84 c A
	CONTROL***	15.89 ab B	20.82 ab A	17.25 ab AB	9.00 a B
% germinated urediospores that formed appressoria over stomata ****	TREATMENT	84.90 a A	73.32 b A	79.95 bc A	78.54 c A
	CONTROL***	75.77 ab B	73.49 ab B	72.79 ab AB	81.34 a B
% germinated urediospores that formed appressoria not over stomata ****	TREATMENT	8.56 ab A	4.70 a A	13.83 c A	8.62 ab AB
	CONTROL***	8.35 ab A	5.69 ab AB	9.96 b A	9.66 b B

*** Leaf discs cut from untreated seedlings

**** Values calculated from the mean percentage obtained from three replicates (refer to **Appendix 2**)

***** Lower and upper case letters indicate significant differences across a row and down a column, respectively, according to the LSD 0.05 values

SEM. The total counts of infection structures of *H. vastatrix* observed in cv. Caturra are presented in **Table 2**. There is a marked decrease in the number of structures seen with an extended fungicide treatment. The number of structures observed at time D is less than half the number observed at time A. Extracellular material accumulated on the infection structures at times A and B at 72hpi. This material was only noted on the SSVI observed at time D 24hpi. At time B advanced infection structures collapsed and occasionally hyphae with abnormal morphology were noted. At time D it would appear that further growth of the SSVs, after 48hpi was inhibited.

Table 2 Scanning electron microscopy total counts of early infection structure development of *Hemileia vastatrix* on *Coffea arabica* cv. Caturra after treatment with the fungicide, Bayfidan®

CATEGORY	HPI	DAYS AFTER SECOND*/ THIRD** APPLICATION OF BAYFIDAN				CONTROL
		28* [A]	7** [B]	14** [C]	28** [D]	
SSVI	24 24	23	13	17	9 4b	26
SSVs	24 48	3 13	1 12	*** 3	3b 5	26 2
SSVs + secondary infection hyphae	48 72 72	2 7 2b	4 2 4b	2 ***	*** ***	14 12
Advanced infection	72 96 96	*** 8 3b	4 3a 3c	12 14	*** ***	4 10
Total counts		61	46	48	21	94

HPI - hours post inoculation
a - collapsed
b - collection of extracellular material around the infection structure
c - abnormal hyphal structure
*** = no structures observed

Infection structure formation in triadimenol-treated tissue occurred as follows:- No abnormalities of pre-penetration structures were observed (**Plate 1** Fig. 1), confirming the results of the fluorescence microscopy study. A collection of extracellular material was occasionally observed on SSVIs 24hpi at time D (**Plate 1** Fig. 2). The SSV was sometimes similar in appearance to that observed in the control, that is, the fungicide appeared to have no effect on their morphology. However, on a number of occasions the SSV appeared swollen (**Plate 1** Fig. 3 and 4). Extracellular material was observed 72hpi (**Plate 1** Fig. 5; **Plate 2** Fig. 1), but it was not clear whether this originated from pathogen or host tissue. The appearance of the intercellular hyphae was abnormal, compared to the control, and hyphal branches were often swollen (**Plate 2** Figs. 2). Disruptions in the wall of the infection structures were also observed (**Plate 2** Fig. 4). Haustorial mother cell (HMC) collapse (**Plate 2** Fig. 3) was observed 96hpi, followed by collapse of the entire fungal structure.

DISCUSSION

Pre-penetration infection structure formation. One of the earliest opportunities for fungicides to interfere with the development of a pathogen is during germination and penetration of the host. Treatment of *C. arabica* cv. Caturra seedlings with triadimenol precluded appressorium formation as a target of attack. A weak effect on this process was observed in the present study. This observation confirmed reports that ergosterol inhibiting fungicides (EBI) have little effect on the infection process from germination to host penetration (Buchenauer, 1977; Kuck *et al.*, 1982; Paul, 1982; Zobrist *et al.*, 1982).

Post-penetration infection structure formation. Several workers have investigated the effects of fungicides on the fine structure of fungi as a step to elucidating their mode of action (Pring, 1984; Pring & Richmond, 1976). Scanning electron microscopy and fluorescence microscopy have also been successfully used to study the effects of fungicides on fungal morphology within host tissue (Ferreira, 1988; Kuck *et al.*, 1982; Paul, 1982).

Plate 1

Sequence of infection structure development of *H. vastatrix* on/within triadimenol treated *C. arabica* cv. Caturra leaves

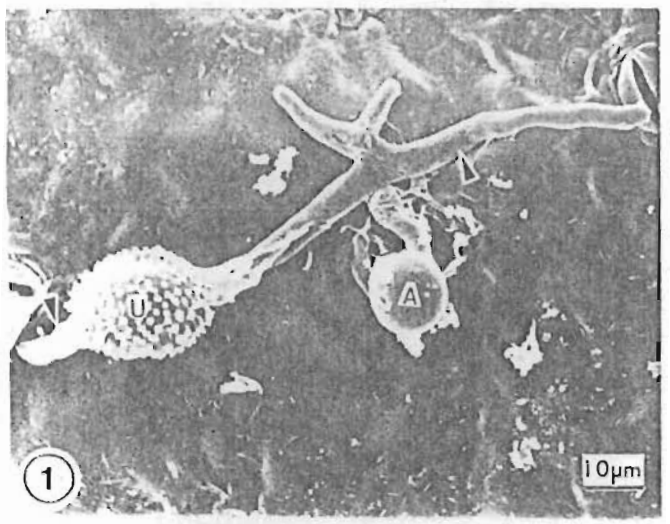
Fig. 1 Germinated urediospore (U) and single germ tube (arrow) on the leaf surface. An appressorium (A) has formed over a stoma 24hpi

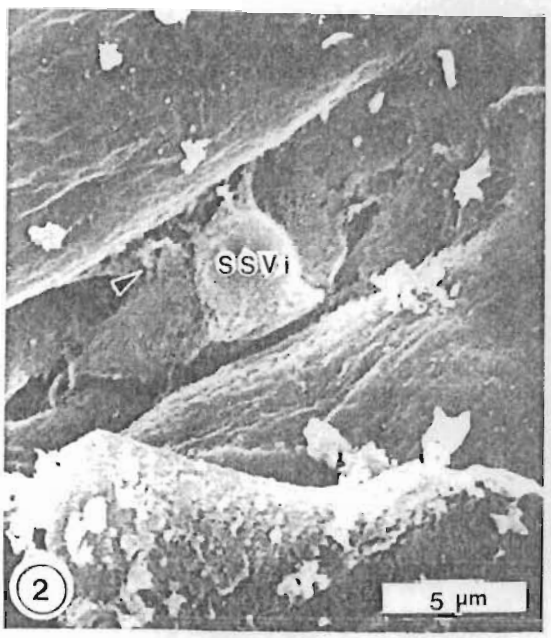
Fig. 2 Substomatal vesicle initial (SSVi) visible within the substomatal chamber. Note the appearance of extracellular material on this structure (arrow) 24hpi

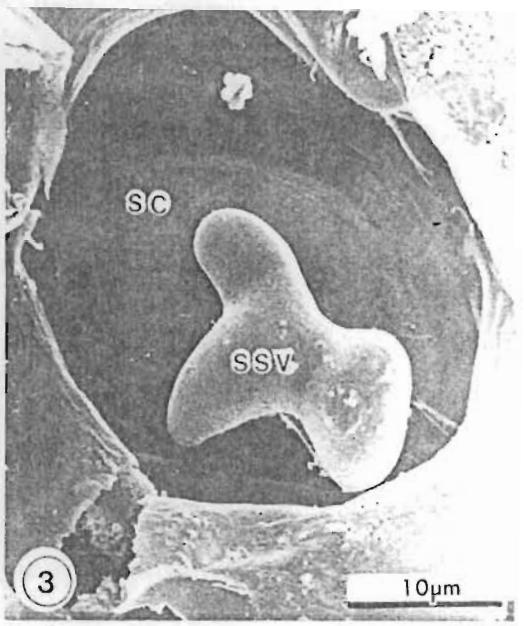
Fig. 3 Substomatal vesicle (SSV) appears swollen 96hpi

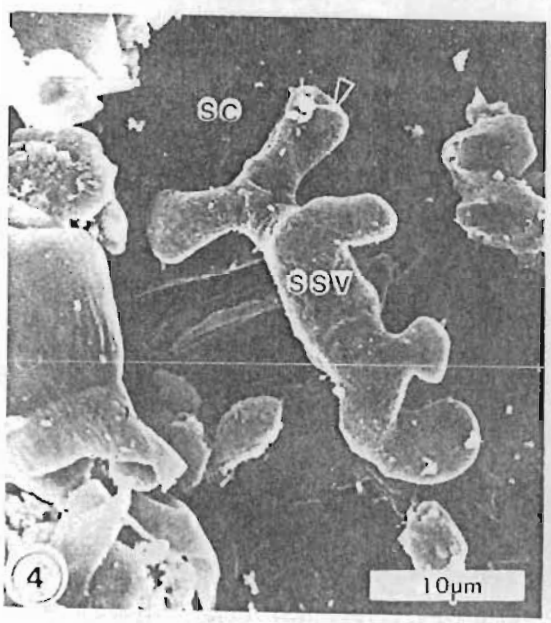
Fig. 4 Swollen SSV with infection hyphae and haustorial mother cells (arrows) 96hpi

Fig. 5 Extracellular material (arrows) seen on and surrounding the SSV 72hpi


U
A
1
10µm


SSVi
2
5 µm


SC
SSV
3
10µm


SC
SSV
4
10µm

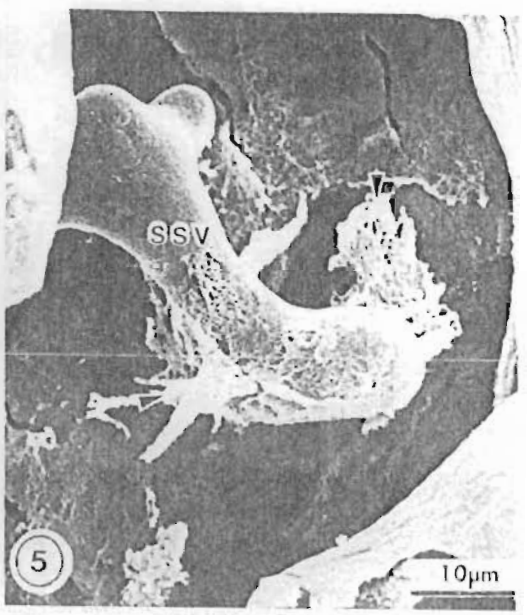

SSV
5
10µm

Plate 2

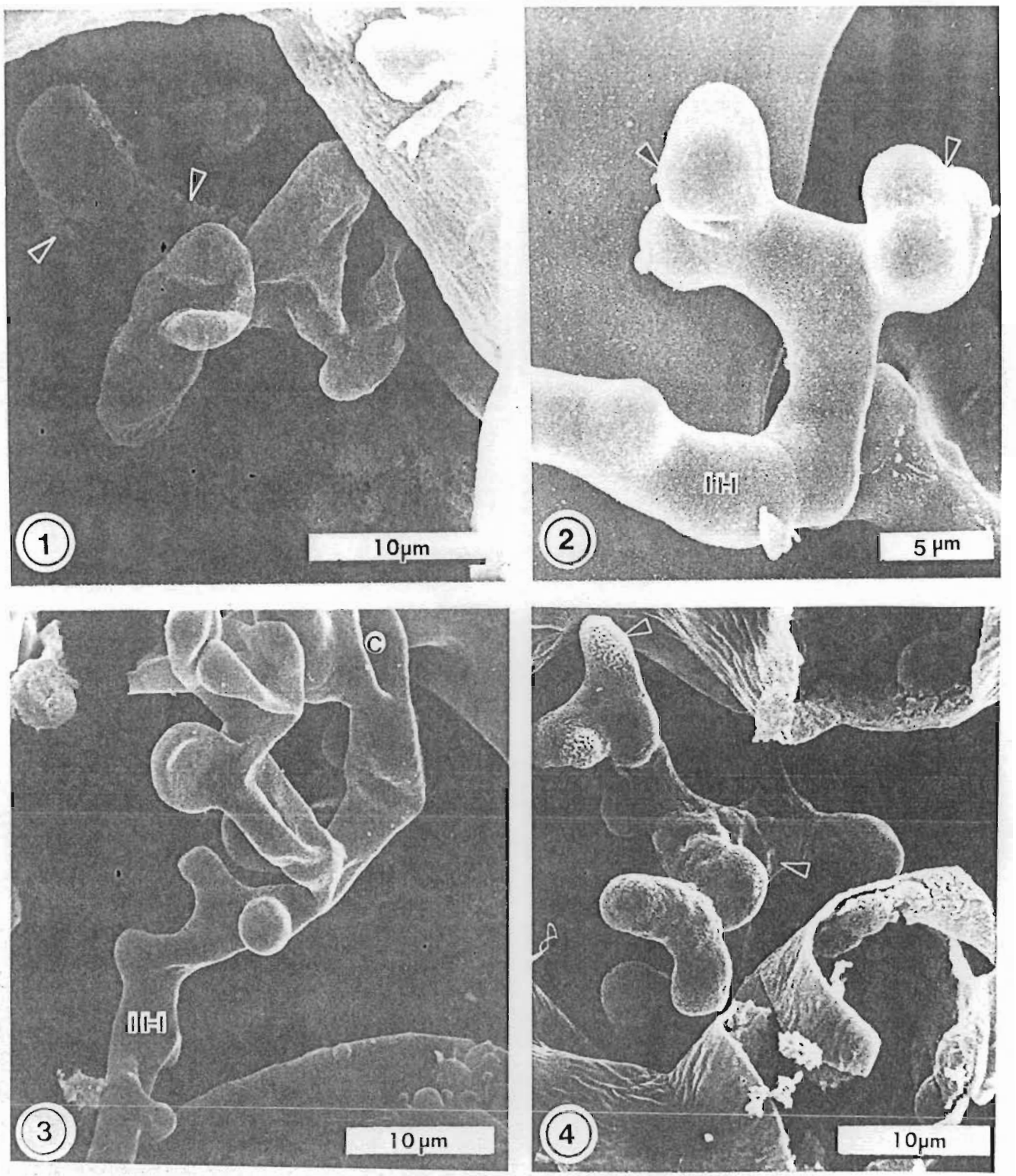
Abnormal infection structure morphology of *H. vastatrix* following the application of triadimenol 96hpi

Fig. 1 Extracellular material (arrows) seen surrounding collapsed infection hyphae and substomatal vesicle

Fig. 2 Tips of the intercellular hyphae showing abnormal morphology (arrows)

Fig. 3 Collapsed advanced infection structures

Fig. 4 Disruptions (arrows) in the walls of this infection structure are evident


1
10μm
2
5 μm
C
3
10μm
4
10μm

In the *H. vastatrix/Coffea* cv. Caturra interaction an effect of triadimenol on the morphology of SSVIs, SSVs, and later infection structures was noted with SEM. The collection of extracellular material on the infection structures may be due to the disruption of membrane permeability, which would subsequently result in plasmolysis. Permeability changes of fungal membranes in the presence of EBI fungicides have been recorded previously. Severe membrane damage is reported in the yeast, *Saccharomyces cerevisiae* Hansen., by econazole (Yamaguchi *et al.*, 1981, cited by Kato, 1986). The reasons for disruption of permeability is thought to be due to small changes in the precise molecular architecture of membranes (Kato, 1986). Richmond (1984) reported that triadimefon interferes with the molecular mechanism of morphogenesis responsible for the architectural structure of septa and walls of the fungus *Botrytis allii* Munn. This factor may account for the disruptions in the walls of the infection structures observed in the present study.

Occasionally the infection structures of *H. vastatrix* within treated tissue appeared swollen and this may be due to ultrastructural changes occurring within these structures. Extensive wall thickening of rust fungi was noted in *Triticum aestivum* L. and *Vicia faba* L. treated with triadimefon (Pring, 1984). Hippe & Grossmann (1982) made similar observations in the treatment of *Ustilago avenae* (Pers.) Rostr. sporidia with nuarimol and imazalil nitrate, both EBI fungicides.

According to Smolka & Wolf (1983), in the *Hordeum vulgare* L./*Erysiphe graminis* DC. complex, the most striking effect after treatment with triadimefon and triadimenol was the encasement of haustoria at 24 to 48hpi. *E. graminis* discontinued its growth after the production of the first haustoria. Similar observations have been made for other host/pathogen/fungicide combinations, for example, *Phaseolus vulgaris* L./*Uromyces phaseoli* (Pers.) Wint. treated with oxycarboxin (Pring & Richmond, 1976). However, in the case of the *Puccinia graminis* f.sp. *tritici* Eriks. & E. Henn./*T. aestivum* interaction following treatment with triadimenol and triadimefon the development of the first haustoria was not completely inhibited (Kuck *et al.*, 1982). Paul (1982) noted that

triadimefon reduced the number of SSVs and haustoria of *Puccinia recondita* Rob. ex Desm. within *T. aestivum* L. tissue. This fungus was totally inhibited at the stage of the formation of the first intercellular hyphae. Until transmission electron microscopy has been carried out with inoculated triadimenol-treated coffee tissue, no conclusions can be reached on the effects of this fungicide at the ultrastructural level.

In the present study the onset of fungal disruption by triadimenol occurred between 24 and 48hpi. This relatively late action of the fungicide may be due to the fact that in the initial stage of development the fungus still draws its reserves of ergosterol from the urediospore. By 72hpi this supply would be exhausted and fungal growth would cease. Kuck *et al.* (1982) made similar observations in the interaction between *T. aestivum/P. graminis* f.sp. *tritici* following treatment with triadimenol and triadimefon.

The excessive branching of intercellular hyphae, particularly at the hyphal tip, may be due to modifications in the activity of enzymes involved in wall synthesis as has been reported by Sisler & Ragsdale (1984). Excessive branching of the intercellular hyphae of *P. recondita* following treatment of cryproconazole was reported by Guggenheim *et al.* (1989).

It is evident from the present study that there appears to be no similarities between "natural" resistance conferred by the host genome (**Chapter 3**) and the "artificial" resistance conferred by the fungicide, at least in the time frame under consideration.

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CHAPTER 5

TELIOSPORES OF HEMILEIA VASTATRIX

INTRODUCTION

Hemileia vastatrix Berk. & Br. has three spore types, namely, urediospores, teliospores and basidiospores. As yet, the pycnial and aecial stages have not been found under either natural or controlled conditions (Gopalkrishnan, 1951). Urediospores are capable of infecting *Coffea* spp. and other members of the Rubiaceae (Frean, 1986). Ward (1882), Pole Evans (1909), Gyde (1932), Mayne (1936), Thirumalachar & Narasimhan (1947) and Gopalkrishnan (1951) were unable to infect coffee leaves with basidiospores. It is now generally agreed in the literature, that the life cycle is not completed on the coffee tree. It is for this reason that this fungus is assumed to be heteroecious. The plant species which is the alternate host for coffee rust is unknown.

Besides the descriptions of Ward (1882), Thirumalachar & Narasimhan (1947) and Gopalkrishnan (1951), little is known about the development and structure of the rarely occurring teliospores. They have been observed on infected coffee grown in India and Sri Lanka (Nutman & Roberts, 1972), South Africa (Pole Evans, 1909; Gyde, 1932) and Brazil (Sugimori *et al.* 1976).

The discovery of teliospores on locally infected trees prompted a scanning electron microscopy study on their structure, development and germination.

MATERIALS AND METHODS

Infected material. At the end of the winter months, in August, teliospores of *H. vastatrix* were noticed on infected leaves collected from trees of *C. arabica* L. at Ukulinga experimental station near Pietermaritzburg, South Africa.

Induction of basidiospore production. Coffee leaves, containing uredio- and teliosori, were placed on a glass sheet under a bell-jar which had previously been lightly sprayed internally with water. This apparatus was placed overnight in a controlled environment chamber (Conviron®) at 26°C with a 12h photoperiod.

Scanning electron microscopy. Leaf pieces (ca. 5mm x 5mm) were dissected from areas of leaves covered in orange urediosori, among which teliosori had been observed. They were pre-fixed in 3% glutaraldehyde in sodium cacodylate buffer (pH = 6.8 - 7.2), rinsed in buffer, post-fixed in 2% osmium tetroxide and dehydrated in an ethanol series. The specimens were critical point dried using a Hitachi® HCP-2 with carbon dioxide as a transition fluid and mounted on metal stubs. The tissue was gold/palladium-coated in a Polaron® sputter coater. Leaf pieces were viewed with a Hitachi® S-570 operating at either 8 or 10 kV.

Environmental parameters associated with teliosorus induction. The severity of *H. vastatrix* on a few isolated *C. arabica* trees was monitored monthly at Ukulinga during 1989, the year in which the teliospores were noticed. The mean percentage diseased leaf area, used as a rating scale and described by Eskes & Toma-Braghini (1981) (refer to **Table 1**), was calculated from the mean of 20 randomly collected leaves. The total rainfall, mean maximum and minimum temperatures for Ukulinga were obtained from the Agricultural Meteorological Department at Cedara. The relationship between these parameters and percentage diseased area is presented graphically in **Figs. 1** and **2**. The reason for the inclusion of these data is an attempt to link teliosorus induction to environmental parameters.

RESULTS AND DISCUSSION

Teliospores of *H. vastatrix* are described as pedicellate, spherical or napiform and smooth with a terminal papilla (Ward, 1882). Scanning electron microscopy confirmed the morphological details decribed above [**Plate 1** Fig. 1]. Teliospores vary in size from 16-25 μm (length) x 19-22μm (width). Immature teliospores lack

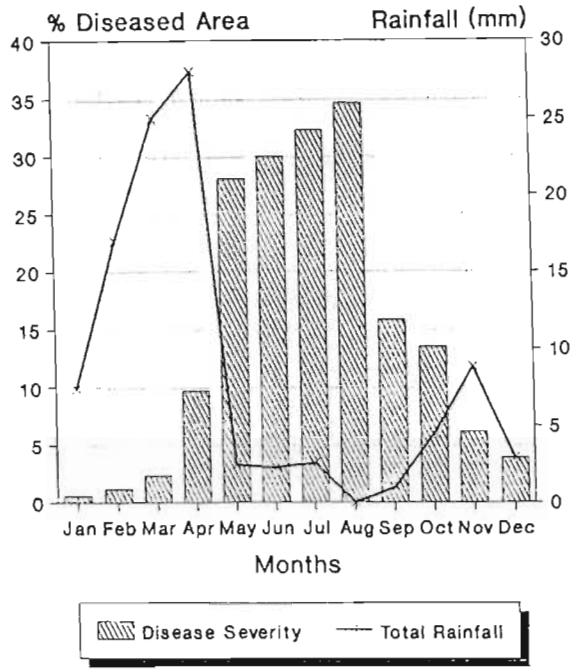


Fig. 1 The relationship between the total rainfall and percentage diseased area on *Coffea arabica* leaves infected with *Hemileia vastatrix* for 1989 at the Ukulinga experimental station, Pietermaritzburg

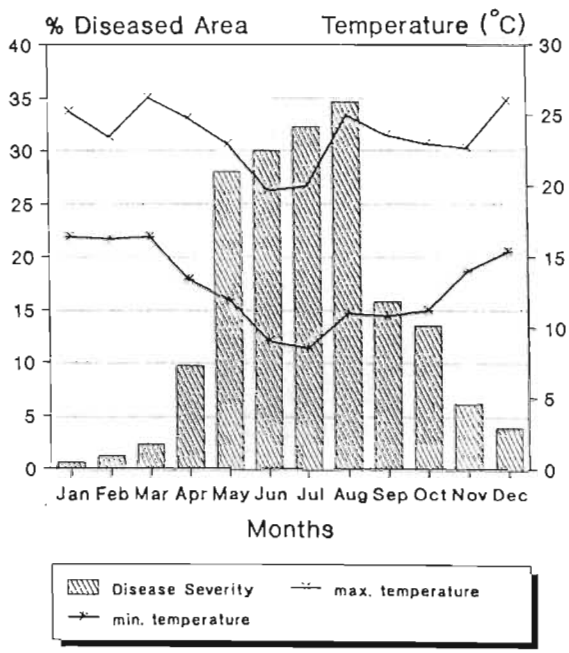


Fig. 2 The relationship between mean minimum and maximum temperatures and percentage of diseased area on *Coffea arabica* leaves infected with *Hemileia vastatrix* for 1989 at the Ukulinga experiment station, Pietermaritzburg

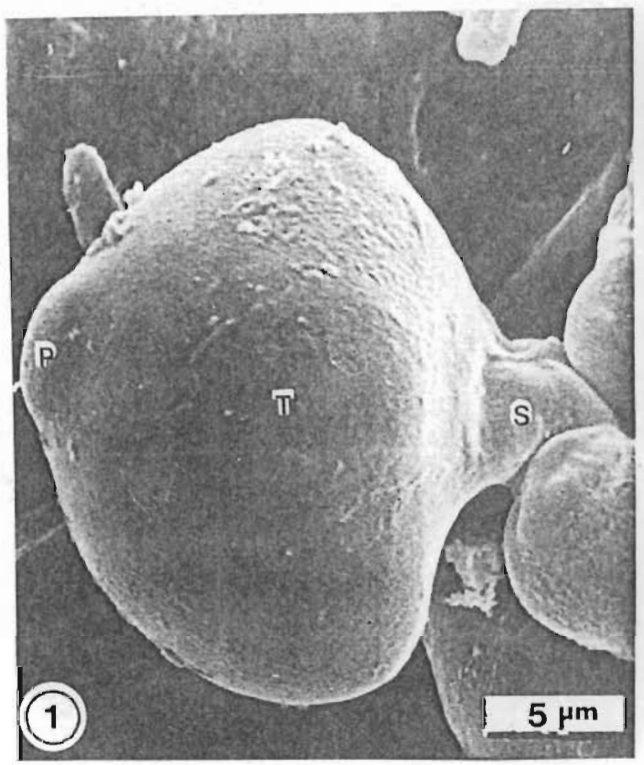
Plate 1

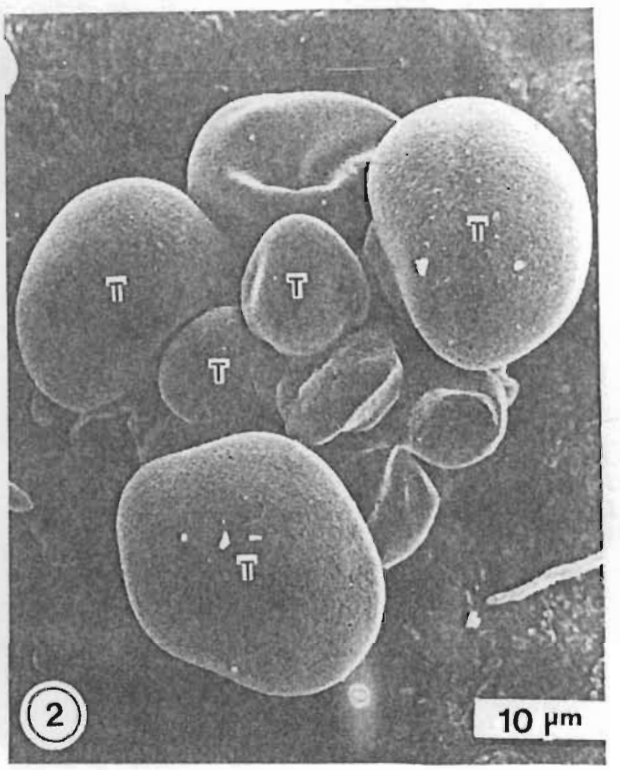
Fig. 1 Napiform teliospore [T] with a terminal papilla [P] borne on a short sporophore [S]

Fig. 2 Immature teliospores [T] in a teliosorus

Fig. 3 Teliosori and a uredio/teliosorus illustrating *in situ* germination of teliospores [T]

Fig. 5 Teliospore germinating *In situ*, illustrating the cylindrical promycelium [arrows]


P
T
S
1
5 µm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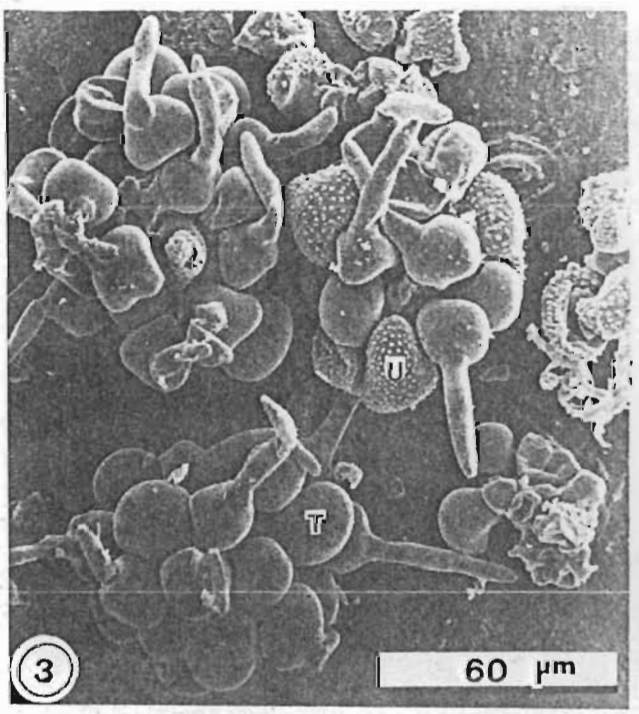
T
T
T
2
10 µm


U
T
3
60 µm

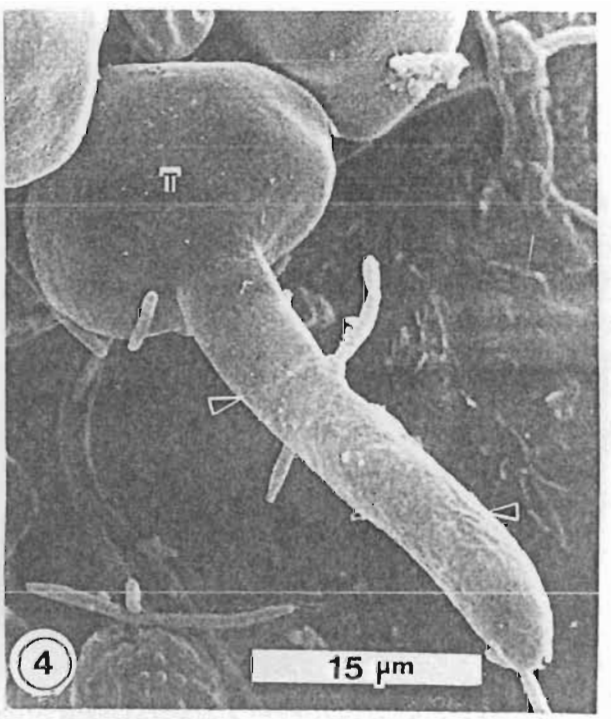

T
4
15 µm

Table 1 Scale developed by Eskes & Toma-Braghini (1981) to calculate the percentage diseased area of *Hemileia vastatrix* on *Coffea arabica* leaves

SCALE VALUE	NUMBER OF LESIONS* PER LEAF	% DISEASED AREA
0	0	0
1	1	0.6
2	2	1.3
3	4	2.5
4	8	5.2
5	15	9.7
6	29	18.5
7	52	33.1
8	84	54.1
9	118	75.6

* In this study "lesion" was understood to mean a colony of urediosori

the terminal papilla [**Plate 1** Fig. 2]. They are borne in clusters on short, ovate teliosporophores (pedicels) which protrude through the stoma in a manner similar to those of urediospores. They are commonly found among the urediospores [**Plate 1** Fig. 3], however, they do occasionally occur in teliosori [**Plate 1** Figs. 2 and 3]. Gopalkrishnan (1951) has also reported that they occur in the urediosorus with, or following, the urediospores, or in separate sori. Although the formation of teliospores could not be investigated, it appears that teliosori and urediosori are derived from the same, or similar, fertile cells in the substomatal chamber. According to a study by Gold & Littlefield (1979) on the *Melampsora lini* (Ehrenb.) Lev./*Linum usitatissimum* L. interaction, few telia developed independently of prior uredial development. This feature would appear to be similar in the *H. vastatrix*/*C. arabica* interaction.

Teliospores germinate *in situ* by producing a promycelium at the apex of the spore [**Plate 1** Figs. 3 and 4]. Ward (1882) reported that the promycelium can also be formed on the hilum side opposite the papilla. This was not observed in the present study. According to Vishveshwara & Nag Raj (1960), and Chinnappa & Sreenivasan (1965), teliospores germinate and produce a cylindrical promycelium from which four spherical basidiospores are formed. The formation of

basidiospores could not be induced in the present study.

Teliospores are reported to be produced on older attached leaves in dry conditions (Chinnappa, 1965; Waller, 1982). The infected specimens examined in this study were from fully expanded leaves, that is, the third-leaf pair and older.

It is evident from **Fig. 1** that disease severity is highest in August, thereafter there is a sharp decrease. From these results it would appear that in the seasonal cycle of *H. vastatrix*, maximum urediospore production culminates in the formation of teliospores. The meteorological data indicated that a dry period of less than 2.5mm rainfall per month for 57 days was recorded prior to the period when the teliospores were first noticed (**Fig. 1**). The average rainfall per month for the 1989 winter months at Ukulinga was 2.1mm (much lower than the recorded 11.3mm for the spring and summer months). Temperature has been implicated in the induction of teliospore formation. Groth & Mogen (1978) reported that teliospore formation of *Uromyces appendiculatus* (Pers.) Unger on *Phaseolus vulgaris* L. can be induced by cold conditions. The mean maximum and minimum temperatures for the winter months at Ukulinga were 21.5°C and 10.6°C, respectively (**Fig. 2**). It would therefore appear that dry, cold conditions are a prerequisite for teliospore production and peak urediosorus generation by *H. vastatrix* at Ukulinga.

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CHAPTER 6

THE EFFECT OF LEAF AGE ON INFECTION OF *COFFEA* SELECTIONS BY *HEMILEIA VASTATRIX*

INTRODUCTION

Plants change in their susceptibility to disease with age. Generally, as they mature there is an increase in resistance (Hyde, 1977) which may be followed by a decrease with further ageing (Parlevliet, 1975). The effect of age on infection does, however, appear to depend on the particular host-pathogen interaction under investigation. For example, Padmanabhan & Ganguly (1954), cited by Eskes & Toma-Braghini (1982) observed that resistance of rice, *Oryza sativa* L., to *Helminthosporium* Link. Fr. decreased with age, but that resistance to *Pyricularia oryzae* Cav. increased with age. Adult plant resistance to cereal rust diseases has been recognized in many host-pathogen combinations and is characterized by an increasing resistance to infection with advancing plant age (Hyde & Elahinia, 1989).

In the case of a perennial host, the age of plant organs may be more important than the age of the whole plant. Sharma *et al.* (1980) studied the *Populus* spp./*Melampsora larici-populina* Kleb. interaction, and found that within a shoot, the latent period and reaction type were related to leaf age: high susceptibility was found to be associated with a short latent period. The developmental stage of *Coffea* berries also appears to influence resistance to *Colletotrichum coffeanum* Noack (Mulinge, 1970; Van der Graaff, 1981): the young green berries being more susceptible than ripe fruit.

There are reports on the influence of leaf age on the resistance of *Coffea* spp. to *Hemileia vastatrix* Berk. & Br. D'Oliveira (1957), cited by Kushalappa & Eskes (1989), mentioned that mature leaves of *C. arabica* L. are more resistant than

succulent young leaves. Rayner (1961), however, found no difference in leaf age effects. More recently, Eskes & Toma-Braghini (1982) reported that the effect of leaf age on resistance varies with particular *Coffea* spp. and *C. arabica* selections. A study on the germination of urediospores on leaves of different ages from the same cultivar revealed that higher germination occurred on young than on intermediate and old leaves (Nutman & Roberts, 1963).

In this chapter the frequency of appressorium formation on the leaf surface, and subsequent sporulation on differently aged leaves of *Coffea arabica* cv. Caturra, and two F6 progenies of a cross between *C. arabica* cv. Caturra and Hybrido de Timor (HDT), were investigated.

MATERIALS AND METHODS

Planting material and conditions. Seeds of *C. arabica* cv. Caturra were obtained from the Tea Research Foundation of Central Africa, Malawi. CENICAFÉ (Centro Nacional de Investigaciones de Café), Colombia, supplied the lines NP-547 and NC-169, the F6 progenies of a cross between *C. arabica* cv. Caturra and HDT. NP-547 gives a resistant, and NC-169 a susceptible, response when inoculated with *H. vastatrix* (G.A. Alvarado, CENICAFE, pers. comm.). HDT is a population derived from a single tree, probably a spontaneous hybrid between *C. arabica* and *C. canephora* L., discovered around 1927 in East Timor (Gonçalves *et al.*, 1977 cited, by Rodrigues, 1984). The majority of HDT selections are resistant to all known races of *H. vastatrix.*

Following germination, seedlings were grown in a greenhouse at temperatures between 15 and 30°C in pre-composted pine bark medium supplemented monthly with N:P:K fertilizer and copper chelate.

Leaf disc preparation. The third plagiotropic branch, from the top of two-year-old

trees of the above lines, was selected. A single branch of each selection was used for a single replication. The experiment was replicated three times. Leaves from each node were collected and their position on the branch noted. Seven leaf pairs were sampled, from the immature shiny first leaf pair to the dark green seventh pair. The method described by Eskes (1982) was used for leaf disc preparation. Leaves were gently rinsed under a stream of tap water in order to remove soil debris. Leaf discs, 2cm in diameter, were punched out using a stainless-steel cork borer. The midvein and leaf margins were avoided. Four leaf discs per each pair of the same age were used in a replication. Discs were placed, adaxial surface down, on foam rubber saturated with tap water in glass trays (29 x 24 x 2.5cm) and inoculated as described below.

Leaf disc inoculation. Urediospores of *H. vastatrix* were freshly collected from infected trees. They were lightly dusted onto the abaxial surface of leaf discs with a camel-hair brush (No. 1). The glass trays with the inoculated discs were initially placed in a dew chamber at 20°C for 24 h in darkness and later transferred to a constant environment chamber (Conviron ®) at 26°C with a 12 h photoperiod.

Fluorescence microscopy. Discs were examined 24 hours-post-inoculation (hpi). The fluorochrome staining procedure described by Tiburzy *et al.* (1983) was used. The leaf samples were examined using a Zeiss research microscope fitted with epifluorescence equipment (light source HBO 50; red suppression filter BG 38; exciter filter BP 390-440; chromatic beam splitter FT 460; barrier filter LP 475). Colour photographs were taken using Ektachrome 160 Professional 35 mm film. Three stages of development of the fungus on the leaf surface were recorded and classified, namely: [1] percentage of germinated urediospores that did not form appressoria; (2) percentage of germinated urediospores that formed appressoria over stomata; and [3] percentage of germinated urediospores that formed appressoria not over stomata. The entire leaf disc surface was scanned and infection structures, excluding those issuing from large clumps of urediospores, counted. Percentage germination could not be determined as many ungerminated urediospores are washed off the leaf

pieces during preparation. The mean percentage of the total counts recorded, from each replicate, was calculated for all three stages of development of the fungus. Differences between means were tested for significance using the ANOVA statistical test.

Latent period, reaction type and urediosorus density. The latent period, the time period from the beginning of incubation to the time at which 50 per cent of the first urediosori appeared, was determined. This involved daily inspections of leaf discs with a hand lens until the urediosori had been produced. A urediosorus was regarded as a clump of spores emerging from one stoma. The disease assessment scale (refer to **Chapter 1 Table 3**) described by Eskes & Toma-Braghini (1981) was used to determine the reaction type. Urediosorus density was calculated with the aid of a Zeiss dissecting microscope. The number of uredosori, in a 1 x 1 mm area of a slit made in cardboard and randomly placed on the infected leaf disc, were counted (n = 10). These latter two parameters were determined for each of the three replications of the experiment eight weeks after inoculation. The value presented is the mean of nine readings in the case of latent period and reaction type, and 90 in the case of urediosorus density. It must be noted that during the eight week period the leaves have aged. Thus leaf pair 1, will at the time of assessing reaction type, latent period and uredosorus density, have aged and probably should more correctly be referred to as Leaf pair 2. This will however depend on the time of the year in which the experiment was conducted. New leaf formation occurs in September under South African conditions. This factor was not taken into consideration when assessing the results.

Ranking. The results obtained for reaction type, urediosorus density and percentage appressoria over stomata were ranked from lowest to highest value. Latent period, percentages of germinated urediospores that did not form appressoria and, those that formed appressoria not over stomata were ranked from highest to lowest value. In this case, a long latent period, high percentage of germinated urediospores that did not form appressoria and a high percentage aborted appressoria were regarded as

indicators of resistance. Thus, a parameter with a low score would indicate resistance and one with a high score, susceptibility.

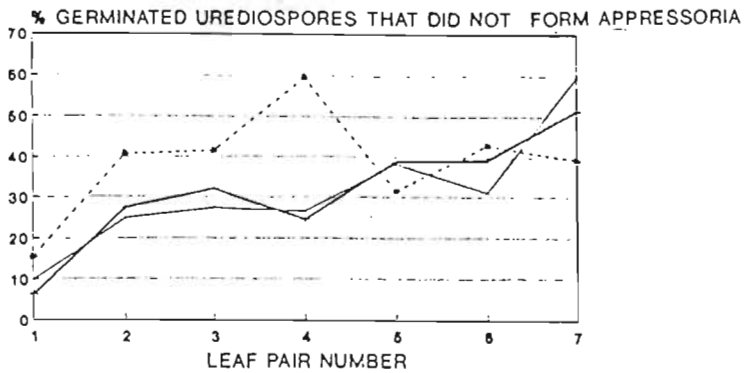
RESULTS

The age of a leaf at the time of inoculation did affect both infection structure development and sporulation on the leaf surface significantly (**Appendix 3** Table A and B).

Fluorescence microscopy. Refer to **Tables 1** and **2**, and **Figs. 1** and **2**. On the leaf surfaces of Caturra and NC-169 there is a decrease in the number of appressoria formed with an increase in leaf age. On the resistant host, NP-547, significantly fewer appressoria are formed on the fourth leaf pair. Appressoria are formed significantly more readily over stomata of leaf pairs two, three and four of the susceptible selections. On the youngest leaf pair a significant high percentage of aborted appressoria was noted compared to percentages obtained for older leaf pairs.

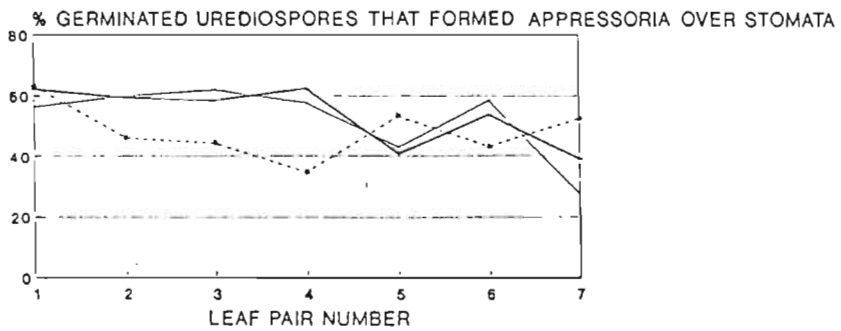
Reaction type, latent period and urediosorus density. The reaction type, in the case of susceptible lines, cv. Caturra and NC-169, was significantly higher in younger than older leaves. The reverse was true of the resistant line, NP-547. The latent period was significantly shorter in the case of NC-169 than on Caturra. In both cases, the length of the latent period increased with increasing leaf age. The urediosori per mm^2 decreased with increasing leaf age on these susceptible selections.

Ranking. Refer to **Table 3**. In general, younger leaves of the susceptible selections, particularly the fully expanded second and third leaf pairs of Caturra, are more susceptible than the older leaf-pairs. This pattern, clearly indicating an effect of leaf age on the infection parameters considered for NP-547, was not shown.



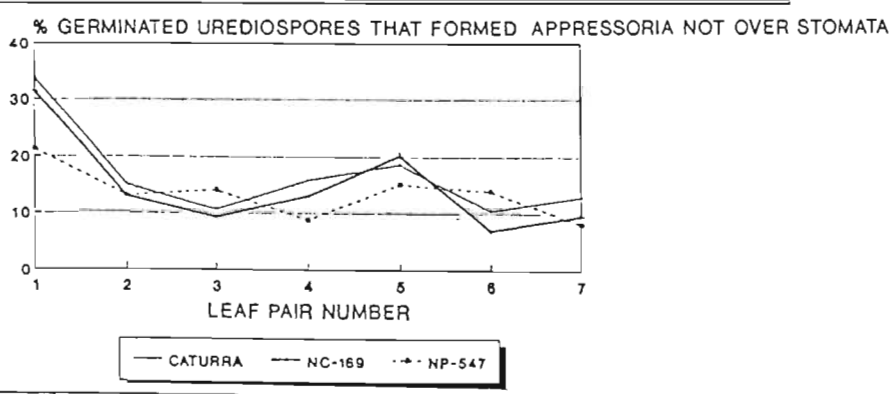
A

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	1.12	3.66	0.31	5.37	3.40	1.67	2.84
NC-169	3.42	2.21	0.52	3.39	6.98	0.85	3.44
NP-547	2.28	1.34	1.17	3.27	3.89	0.69	2.36



B

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	3.78	4.34	0.79	5.46	5.47	3.40	4.03
NC-169	7.35	2.29	1.10	5.76	7.91	1.38	5.69
NP-547	4.77	3.13	1.65	4.52	4.00	1.19	3.33

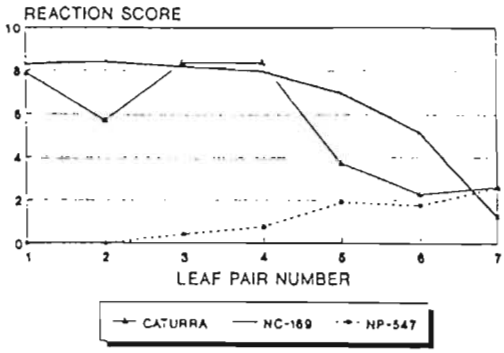


C

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	1.27	0.13	1.15	0.93	2.64	1.50	0.97
NC-169	3.63	0.65	0.64	1.80	4.98	1.00	1.63
NP-547	2.02	2.97	0.82	2.17	2.94	1.90	1.51

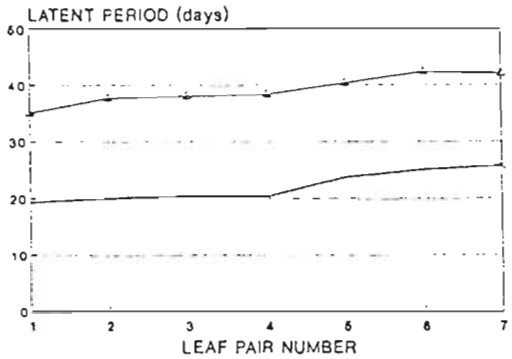
A-C Standard error of the mean presented in table form. Error bars overlapped when drawn on the graph.

Fig. 1 The effect of leaf age on the developmental stages of *H. vastatrix* on the leaf disc surface of *C. arabica* cv. Caturra, and NC-169 and NP-547



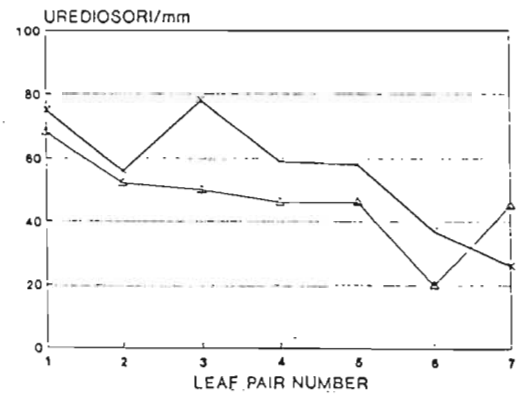
A

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	0.59	0.62	0.60	0.31	0.27	0.27	0.59
NC-169	0.15	0.13	0.39	0.18	0	0.09	1.23
NP-547	0	0	0.22	0.43	0.73	0.55	0.29



B

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	1.00	0.33	0	0.33	0.88	0.33	0
NC-169	0.33	0	0.33	0.88	0.67	0	0.33



C

HOST	LEAF PAIR 1	LEAF PAIR 2	LEAF PAIR 3	LEAF PAIR 4	LEAF PAIR 5	LEAF PAIR 6	LEAF PAIR 7
Caturra	2.65	0.79	2.21	3.12	3.43	2.18	4.31
NC-169	2.42	2.37	1.53	1.43	4.17	2.47	2.84

A-C Standard error of the mean presented in table form. Error bars overlapped when drawn on the graph.

Fig. 2 The effect of leaf age on the reaction type, latent period and urediosorus concentration on the leaf disc surface of *C. arabica* cv. Caturra and NC-169 and NP-547, following inoculation with *H. vastatrix*

Table 1 Percentages of recorded pre-infection stages of *H. vastatrix* on the leaf surfaces of *C. arabica* cv. Caturra, Colombia NC-169 and NP-547

CATEGORY	LEAF PAIR	CATURRA *	NC-169 *	NP-547 *
% germinated urediospores that did not form appressoria	1	9.85 a ** A	6.25 a A	15.53 a B
	2	24.87 b A	27.39 b A	40.71 b B
	3	27.37 b A	32.01 bc B	41.44 b C
	4	26.52 b A	24.49 b A	59.55 c B
	5	38.28 c A	38.82 c A	31.52 b A
	6	31.16 b A	39.29 c B	42.89 b C
	7	59.62 d A	51.43 cd AB	39.43 b C
% germinated urediospores that formed appressoria over stomata	1	56.36 a A	62.25 a A	63.25 a A
	2	59.97 a A	59.49 a A	46.05 b B
	3	61.98 a A	58.63 a A	44.42 b B
	4	57.58 a A	62.43 a A	31.64 c B
	5	43.10 b A	40.98 b A	53.25 ab A
	6	58.33 a A	53.66 ab AB	43.17 b B
	7	27.34 c A	38.93 b AB	52.41 b B
% germinated urediospores that formed appressoria not over stomata	1	33.79 a A	31.50 a A	21.21 a B
	2	15.15 bc A	13.12 ab A	13.24 bc A
	3	10.65 d A	9.28 c A	14.14 bc B
	4	15.90 bc A	13.08 ab AB	8.81 bc B
	5	18.62 c A	20.20 b A	15.23 ab A
	6	10.50 d AB	7.04 c A	13.94 bc B
	7	13.04 b d A	9.64 c AB	8.16 c B

* Values calculated from the mean percentage obtained on three replicates (refer to **Appendix 3**)

** Lower- and upper-case letters indicate significant differences vertically within the category and horizontally between the categories, respectively, according to the LSD 0.05 values

DISCUSSION

The age of leaves at the time of inoculation with *H. vastatrix* influences the susceptibility of *C. arabica* cv. Caturra, NC-169 and NP-547. Susceptibility was associated with a high recorded reaction type score, that is, according to **Chapter 1 Table 3** a seven, eight or nine, shorter latent period and a high number of urediosori/mm^2 (**Table 2**). Thus, the susceptible selections generally showed that,

Table 2 Macroscopic infection parameters on the leaf surface of *C. arabica* cv. Caturra, NC-169 and NP-547 inoculated with *Hemileia vastatrix*

CATEGORY	LEAF PAIR	CATURRA *	NC-169 *	NP-547 *
Reaction type	1	7.87 a A	8.30 a A	0 a C
	2	5.73 b A	8.43 a B	0 a C
	3	8.40 a A	8.23 a A	0.42 a B
	4	8.40 a A	8.00 a A	0.75 ab B
	5	3.73 c A	7.00 a B	1.89 bc A
	6	2.27 c A	5.17 b B	1.78 bc A
	7	2.60 c A	1.23 c A	2.57 c A
Latent period (days)	1	35.00 a A	19.33 a B	
	2	37.67 b A	20.00 a B	
	3	38.00 b A	20.33 a B	
	4	38.33 b A	20.33 a B	
	5	40.33 c A	23.67 b B	
	6	42.33 d A	25.00 bc B	
	7	42.00 d A	25.67 c B	
Uredosori/ 1 x 1 mm	1	68 a A	75 b A	
	2	52 b A	56 a B	
	3	50 b A	78 b B	
	4	46 b A	59 a B	
	5	46 b A	58 a B	
	6	20 c A	37 c A	
	7	45 b A	26 c B	

* Lower- and upper-case letters indicate significant differences vertically and horizontally, respectively, according to the LSD 0.05 values

with increasing leaf age, resistance increased, that is, younger leaves were more susceptible. This observation is confirmed when all six parameters are taken into consideration (**Table 3**). This is in agreement with Waller's (1982) observation that *Coffea* leaves are at their most susceptible when just fully expanded, that is, leaf pairs two and three. In the resistant selection, NP-547, an effect of leaf age on pre-infection parameters was not clearly shown. However, the results from assessing reaction type indicated that resistance is fully expressed in younger leaves and this resistance decreases with increasing leaf age.

Table 3 Ranking of leaf pairs of three *Coffea* selections, Caturra, NC-169 and NP-547, on six infection characters *

SELECTION	LEAF PAIR	CATEGORY						TOTAL	RANK
		1	2	3	4	5	6		
CATURRA	1	5	7	7	7	3	1	30	5
	2	4	6	6	6	6	4	32	6
	3	6	5	5	4	7	6	33	7
	4	6	4	4	5	4	3	26	4
	5	3	3	3	2	2	2	15	2
	6	1	1	1	3	5	7	18	3
	7	2	2	2	1	1	5	13	1
NC-169	1	6	7	6	7	6	1	33	7
	2	7	6	3	5	5	3	29	4
	3	5	4	7	4	4	6	30	5
	4	4	4	5	6	7	4	30	5
	5	3	3	3	3	2	2	16	2
	6	2	2	2	2	3	7	18	3
	7	1	1	1	1	1	5	10	1
NP-547	1	1	-	-	7	7	1	16	5
	2	1	-	-	4	4	5	14	4
	3	3	-	-	3	3	3	12	1
	4	4	-	-	1	1	6	12	1
	5	6	-	-	6	6	2	20	6
	6	5	-	-	2	2	4	13	3
	7	7	-	-	5	5	7	24	7

* Characters were ranked from lowest to highest value in the case of reaction type, urediosorus concentration and appressoria over stomata, and from highest to lowest value in the case of latent period, appressoria that did not form and aborted appressoria

1: reaction type
2: latent period
3: urediosori/mm^2
4: % germinated urediospores that did not form appressoria
5: % germinated urediospores that formed appressoria over stomata
6: % germinated urediospores that formed appressoria not over stomata

In other host-pathogen interactions, other leaf age effects have been noted. According to Cole (1966), leaves of *Nicotiana tabacum* L. do not become infected with *E. cichoracearum* DC. until fully expanded, and the reason for this may be related to

the host cell requirements for nutrients: when the tissue is expanding the pathogen will probably be less able to compete with the host for essential growth substances at this time. Jones & Hayes (1971), investigating the *Avena sativa* L./*Erysiphe graminis* DC. f.sp. *avenae* interaction, found that each leaf exhibits a maximum level of resistance when just fully expanded, and thereafter susceptibility increases. An increase in latent periods at adult plant stages was recorded in the *T. aestivum* L./*Puccinia striiformis* interaction (Hyde & Elahinia, 1989). Pretorius *et al.* (1988) reported that, in the *P. recondita* f.sp. *tritici*/*T. aestivum* interaction, the latent period increased as the adult-plant-resistant cultivar matured. Little effect on latent period was noticed in the susceptible cultivar. In the *Allium porrum* L./*Puccinia allii* interaction, pustule density, pustule length and colony length decreased with increasing leaf age, and there was a tendency for latent period to increase with increasing plant age (Jennings *et al.*, 1990).

According to Eskes & Toma-Braghini (1982), highly susceptible and highly resistant *Coffea* cultivars do not show much variation in resistance to *H. vastatrix* between leaves of different ages. Cultivars Caturra and NC-169 would be regarded as highly susceptible according to the assessment scale developed by Eskes & Toma-Braghini (1981) [**Chapter 1 Table 3**], but findings in the present study indicate that significant differences do exist. Eskes & Toma-Braghini (1982) found that cultivars with complete resistance, as is the case with NP-547, showed symptoms on young leaves (flecks and tumefactions), while adult leaves were symptomless. A fleck is regarded as a "chlorotic spot" while tumefactions are swollen leaf tissue cells visible as dark green spots at the infection site or as dark green circles around chlorotic areas (Rijo, 1972). In the present study, younger leaves were found to be immune while older leaves showed symptoms, that is, flecks and tumefactions, but no sporulation. It thus appears that the effect of leaf age differs between *Coffea* selections. Different rust races or isolates of the same race can have an effect on leaves of different ages (Eskes & Toma-Braghini, 1982).

Although the percentage germination was not determined in the present study, there

are reports which indicate that *H. vastatrix* urediospore germination is higher on young than on intermediate and old coffee leaves (Nutman & Roberts, 1963; Bock, 1962). Russell (1976), investigating urediospore germination of *P. striiformis* West. on adult *T. aestivum* L. leaves with durable resistance, showed that these leaves supported a lower germination percentage than younger leaves. It is possible that the presence of toxic materials from the resident microflora on the leaf surface of older leaves, or the parasitic members of such microflora, prevents urediospore germination. According to Rodrigues *et al.* (1975), resistance of several *Coffea* genotypes was associated with the accumulation of antifungal compounds which diffuse into the infection droplets from host tissue. They found that urediospore germination was reduced and germ tube extension suppressed. Experiments by Martins *et al.* (1986) showed that a non-pathogenic fungus on *Coffea* tissue induced host metabolites that had antifungal activity and reduced germination of *H. vastatrix* urediospores. They noted that this diffusate was more readily produced on older than younger leaves, and observed that germination was higher on younger leaves.

In the present study, higher percentages of aborted appressoria on younger leaves may be due to the presence of immature stomata. Coutinho (unpublished), using scanning electron microscopy, noted that stomata of the first and second leaf pairs were not well developed. Thus, on these leaf pairs it is possible that the germ tube tip fails to recognize the stoma. On older leaf pairs some other factor must operate preventing the recognition of a stoma by a germ tube and leading to the presence of aborted appressoria. According to Dickinson (1949), the formation of appressoria appears to involve responses of the germ tube to the topography of the leaf surface. This has since been confirmed by Dickinson (1970) and Wynn (1976). The germ tubes of *H. vastatrix* appear to lack directional growth, and the contact of the germ tube tip with the stoma appears to be accidental (**Chapter 2**). Thus appressoria may readily abort due to the failure of a germ tube to locate a stoma. Infection structures are known to abort even on the most susceptible cultivar (Lennox & Rijkenberg,

1989). Therefore some mechanism or mechanisms leading to the abortion of appressoria may operate at, or shortly after, germination and may become more effective with increasing leaf age. Such mechanisms may be associated with maturity-induced differences in the cell wall or cuticle.

Eskes & Toma-Braghini (1982) found, when assessing resistance of leaves of different ages, the results differed with the type of inoculation method used. When dry urediospores were applied (as opposed to a spore suspension) the youngest leaves had the highest urediosorus density which declined rapidly with increasing leaf age. Urediospore concentration may also have an effect. Aggregation of too many urediospores of *H. vastatrix* in one area may result in autoinhibition (Musumeci *et al.*, 1973,cited by Kushalappa, 1989). However, low concentrations of urediospores of *H. vastatrix* (10 to 20 spores per droplet) induced less infection that higher concentrations (150 spores per droplet) (Nutman & Roberts, 1963). A spore concentration of 15 to 20 spores/cm^2 of leaf tissue is considered optimum (Bock, 1962). According to Aldwinckle (1975), the number of spores of *Gymnosporangium juniperi-virginianae* Schw. on *Malus sylvestris* Mill., was positively correlated with inoculum concentration, but the lesion size remained constant at different concentrations. Thus, these factors should be taken into consideration when assessing the results obtained in the present investigation.

It must be noted that leaf pair seven was the oldest on the branch of a seedling. In the case of mature trees in the field, ten to fifteen leaf pairs can be counted on a single branch. It would be interesting to compare the effect of age on leaves removed from mature trees under field conditions and, also, from other cultivars or *Coffea* spp.

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CHAPTER 7

CHEMICAL CONTROL OF THE COFFEE RUST PATHOGEN, *HEMILEIA VASTATRIX*

INTRODUCTION

Hemileia vastatrix Berk. & Br., the causal organism of leaf rust on *Coffea* spp., was once such a menace that coffee production in certain regions of the world was abandoned. The advent of chemical control in the 1930s (Mayne, 1971), heralded a new era, and in some regions, for example, in southern Africa, coffee was re-established. Numerous fungicide trials have been carried out, usually by the individual industries of coffee-growing countries (Brodrick, 1971; Javed, 1981; 1982). Today, the application of chemicals is the most widely used method of combating *H. vastatrix*. A wide range of fungicides is commercially available to the grower, and success in preventing and eradicating the disease has been achieved. This success does, however, depend on technical input which in developing countries is often not available (Waller, 1982). Advances in the development of *Coffea arabica* L. selections resistant to rust are being made and the exclusive reliance on chemicals will probably decrease.

The importance of the epidemiology of *H. vastatrix* in developing spray schedules was realized only in the late 1950s (Bock, 1962b). Before chemicals can be applied, an understanding of the relationship between the fungus and the host's seasonal physiological behaviour is essential. The development of coffee rust in the field has been studied and its seasonal periodicity has been reported in India (Mayne, 1930; 1932), Kenya (Bock, 1962a; Rayner, 1962) and in Brazil (Kushalappa & Chaves, 1980). Kushalappa and co-workers, over the past decade, have presented comprehensive models on the development of coffee rust in time and space (Kushalappa, 1981; Kushalappa, *et al.* 1983; 1984; 1986).

Numerous factors influence plant disease epidemics. Meteorological factors such

as rainfall and temperature play major roles in the build-up of *H. vastatrix* (Becker-Raterink, 1984). Other parameters, including, residual inoculum, density of foliage and crop yield are also very important to disease development (Waller, 1982).

In this component of the study, a range of fungicides, mostly systemic, was tested on a rust-infected plantation. A rating scale was developed and used to assess disease incidence. Various epidemiological factors influencing rust development were also examined not only at the fungicide site, but also in all coffee-growing regions of southern Africa.

MATERIALS AND METHODS

Fungicide trial

Site. A plantation of *Coffea arabica* L. on the grounds of the Oakford Priory, near Verulam, Natal, was selected for this experiment. The cultivar and age of the trees were unknown. However, according to the Farm Manager, Mr. Fick (pers. comm.), they were planted in the 1960s. Trees were grown in east/west planted rows on a north-facing slope. The rows displayed a so-called "hedge-row" effect. A medium to high incidence of rust was recorded on the approximately 3m tall trees at the onset of the spraying programme in November 1989. The plantation had, in previous years, been poorly managed. Prior to the last rating (beginning of May 1990), the trees were topped.

Experimental design. Four single-tree plots were randomly selected for each fungicide and each experimental tree was surrounded by four unsprayed trees. The first spray was applied at the beginning of November 1989, and spraying continued for six months.

Fungicides. Selected fungicides (**Table 1**) obtained from various chemical companies were applied at four-weekly intervals. A CO_2 knapsack sprayer (nozzle

D3, 45 core) with a lance extension, recommended by Mr. S. Terry, BAYER (Pty) Ltd., Pietermaritzburg, was used. The spray was applied to all sides of the tree with an up/down motion. Unfortunately, due to the considerable height of the trees it is questionable whether much fungicide reached the top of the trees. The granular fungicide was applied by scattering the chemical around the base of the tree under the canopy.

Table 1 The range of fungicides, their active ingredients and rate of application, selected to control *H. vastatrix* in the field

FUNGICIDE	RATE OF APPLICATION	ACTIVE INGREDIENT	SUPPLIER
Anvil	160ml/ha	hexaconazole	ICI
BAS 480 00F	40 g/100l	triazole	BASF
Bayfidan 250ec	50ml/100l	triadimenol	BAYER
Bayfidan ec + 1% EN600	50ml/100l	triadimenol + armoblen 600	BAYER AKZO
Bayfidan gr	38 g/tree	triadimenol	BAYER
FBC 25/1	250 g/ha	unknown*	FBC
FBC 25/2	500 g/ha	unknown*	FBC
Kocide	200 g/100l	cuprous hydroxide	PLAASKEM
Punch C	50ml/100l	carbendazim/ flusilazol	AGRICURA
Systhane	50ml/100l	myclobutanil	ROHM & HAAS

* Experimental compound

Assessment. Leaf samples were collected at four-weekly intervals from each tree before the next spray was applied. In the case of Replicate 1, a total of 60 leaves were collected from the top, middle and bottom on the north, east, south and west sides of each tree. Twenty leaves, were randomly collected, from all sides and levels, from each tree sprayed in Replicate 2, 3 and 4.

Rating scale. On the basis of disease severity, representative infected leaf samples were selected and were drawn to scale. The regions representing diseased areas as well as control drawings of 0% (no disease) and 100% (diseased) were assessed with a Micro Measurements System 3 Image Analyser ®,

and the percentage diseased area determined (**Table 2**). This system is based on a video camera which counts the pixel numbers of dark (that is, diseased) areas in the field of view, and can therefore measure the total area of all darkened regions on a diagram placed under the camera. A visual rating scale was devised on this basis (**Fig. 1**). Each leaf collected from the field was rated with the aid of this scale, and the results recorded. At the conclusion of the experiment, the mean percentage diseased area was determined for each treatment and statistically analysed using the ANOVA test.

Table 2 Rating scale and percentage diseased area, corresponding to diagrams in **Fig. 1**, used to score coffee leaves infected with *H. vastatrix*

RATING SCORE	PERCENTAGE DISEASED AREA
0	0
1	2.5
3	5.5
5	11.8
7	22.2
9	34.9+*

* Percentage diseased area greater than 34.9 was given a rating score of 9

Evaluation of epidemiological factors influencing rust development in the field. Rainfall, temperature and relative humidity records, for the Verulam district, were obtained from the Weather Bureau in Pretoria. After six fungicide applications (at the end of May 1990) the overall appearance of the trees in the entire test area was evaluated, including treated trees. This was achieved by dividing the area into a grid and evaluating every fifth tree down a row on every alternate row. The overall appearance of the tree and branch die-back were visually assessed using a 0 to 10 scale (0: healthy; 10: 75% of the leaves per tree infected), while overbearing was rated as either present or absent. Overbearing was associated with leaf chlorosis, and a lack of current leaf formation. Rust incidence was rated on a simple visual scale of 0 - no rust, 1 - less than 25% of the

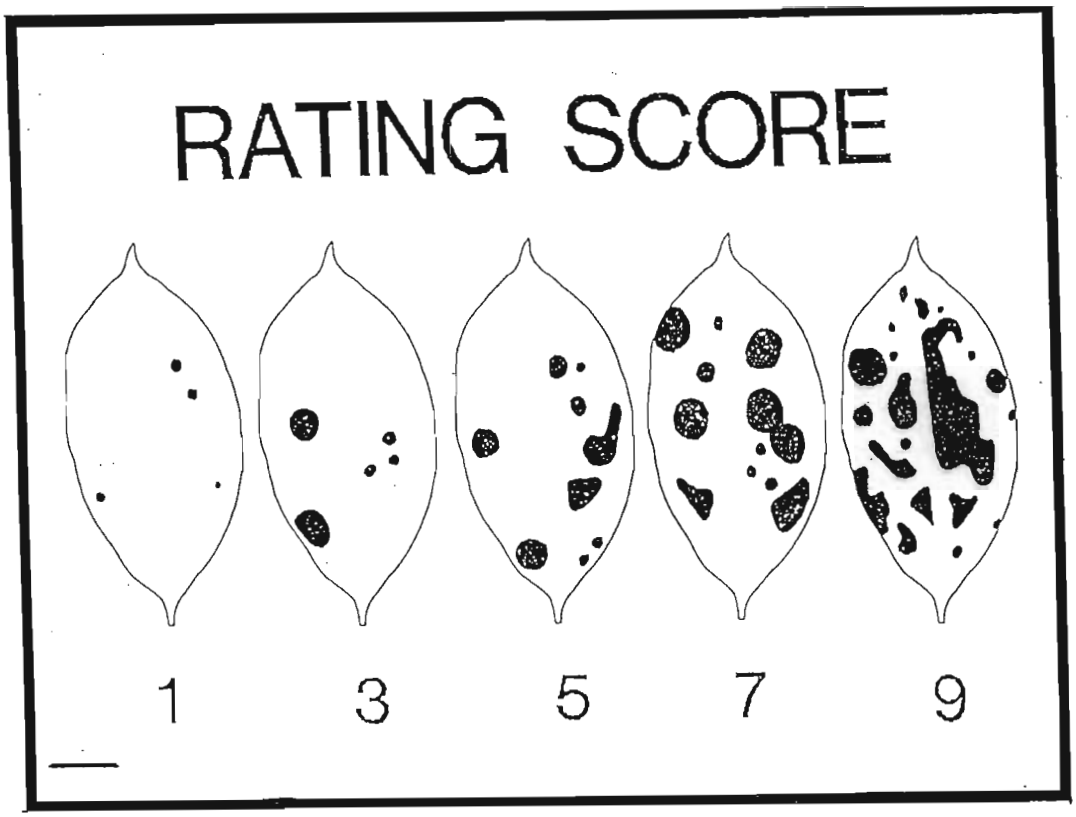


Fig. 1 Visual rating key for estimating disease severity on *Coffea* leaves naturally infected with *H. vastatrix* (bar = 2cm)

leaves on the tree infected, 2 - less than 50% infected, and 3 - 50% and more leaves infected. The experimental site and examples of the parameters measured are presented in Plate 1.

Border rows. The influence of the bordering trees within the hedge-row, that is, down a row, on disease levels of the fungicide-treated trees was evaluated at the conclusion of the experiment. This was achieved by evaluating the disease levels of all trees in the hedge-row, border and treated trees, using the 0 - 3 scale described above.

Latent period. Rayner (1961a) derived a regression equation for the situation in Kenya which related mean daily maximum temperature (x_1) and mean daily minimum temperature (x_2) to the mean latent period (that is, the time to 50% sporulation) as follows:-

$LP = 90.61 - 0.408\ (x_1) - 0.44\ (x_2)$ [temperature in °F].....eq. 1

This basic formula was reassessed in Brazil where certain differences in the numerical values were found to pertain (Moraes *et al.* 1976, cited by Kushalappa


2


4

& Eskes, 1989):-

$LP = 103.01 - 0.98 (x_1) - 2.1 (x_2)$ [temperature in °C].....eq. 2

The difference between these equations is due to other factors that are known to affect the latent period, for example, host physiology. Both equations were used to obtain an indication of the duration of the latent period in the fungicide plot for the months during which the trial was conducted.

Climatic factors influencing rust development in southern Africa. The average rainfall and temperatures recorded for the districts in southern Africa where coffee is grown, were acquired from the Weather Bureau, Pretoria. The data obtained were for the years 1920 to 1989. In an attempt to determine the effect of climatic factors on rust development in southern Africa, a questionnaire was sent to all the farm managers/owners of coffee farms administered by the Coffee Association of South Africa. The month/s they recorded with the highest incidence of rust on their estate was/were noted. For comparative purposes, the relationship between the month/s with the highest rust incidence and the meteorological data for those areas, was analysed. The latent periods, as described above, were also calculated for such estates.

RESULTS

Fungicide trial

Fungicide treatments. The overall treatment effects, the means over each of seven applications, of the fungicides are presented in **Table 3**. **Appendix 4.1** displays the average percentage diseased area for individual replicates for each month over the 24-week period. The systemic fungicides, Punch C, Bas 480 00F and Bayfidan + EN600, proved to be the most effective. Bayfidan ec, Bayfidan granules, Anvil, FBC 25/1 and Systhane were less efficient, while the contact fungicides FBC 25/2 and Kocide were the least effective. All fungicides tested were able to decrease the percentage diseased area. Statistical analysis ($P < 0.05$) of differences in percentage diseased area over the 24 weeks indicated the

time at which the fungicide became effective (see **Appendix 4.2**).

Table 3 Overall treatment effects after seven applications of selected fungicides used to control *H. vastatrix*

FUNGICIDE	% DISEASED AREA
Punch C	0.93 a*
BAS 480 00F	1.02 a
Bayfidan ec + EN600	1.19 a
Bayfidan ec	1.64 ab
Bayfidan granules	1.74 ab
Anvil	1.74 ab
FBC 25/1	1.80 abc
Systhane	2.39 abc
FBC 25/2	2.40 bc
Kocide	2.61 c
Control	5.42 d

* Treatment values with letters in common do not differ significantly at P = 0.05

The effectiveness of each fungicide over the experimental period was compared to the control (**Fig. 2** [**A** to **J**]). In the latter case, the percentage diseased area was relatively low at the start of the experiment and reached a maximum at 12 weeks. The disease level of the control decreased at 16 and 20 weeks and then once again increased. The time period from the onset of the experiment until a fungicide became effective, differed for each. Bayfidan ec together with the penetrant, EN600 [**Fig. 2A**], and the granular formulation of this fungicide [**Fig. 2B**] were significantly effective after one application. BAS 480 00F [**Fig. 2C**], Bayfidan ec [**Fig. 2D**] and Anvil [**Fig. 2E**] only became effective after three applications, while Systhane [**F**] gave significant control after four applications. The percentage diseased area of the Punch C [**Fig. 2G**] treated trees remained at low levels throughout the trial. FBC 25/1 [**Fig. 2H**] and the contact fungicides, FBC 25/2 [**Fig. 2I**] and Kocide [**Fig. 2J**], closely followed the disease pattern of the control but, reduced the percentage diseased area. An analysis of differences in diseased area between plots revealed, in many cases, that the tree in one plot was not equally affected when compared with a tree of the same treatment in another plot. This was especially evident for the trees treated with Bayfidan ec, FBC 25/2, Punch C and Systhane. In the case of the latter two fungicides, the

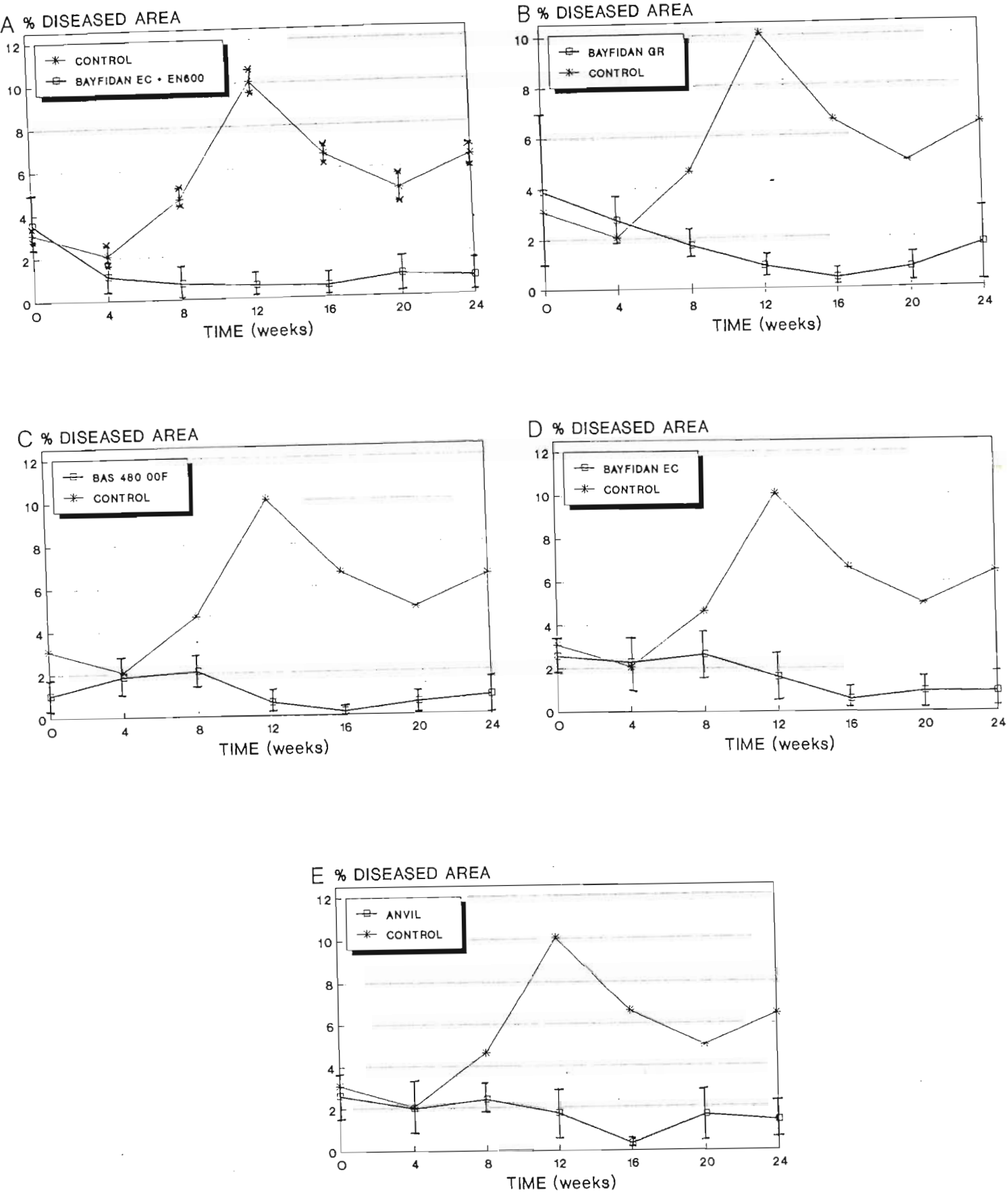
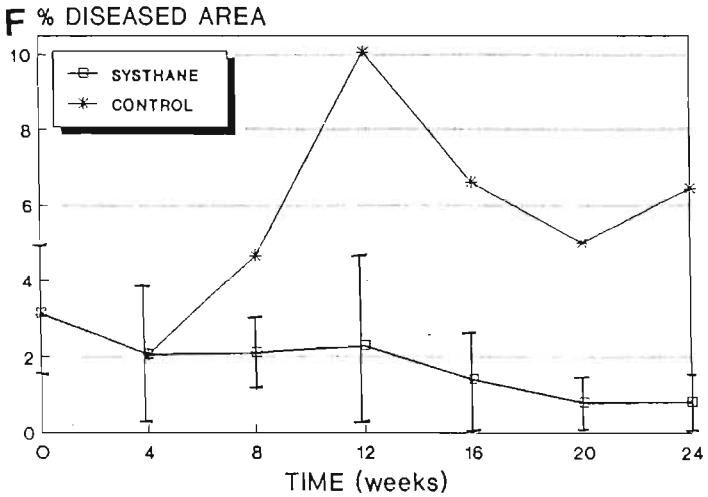
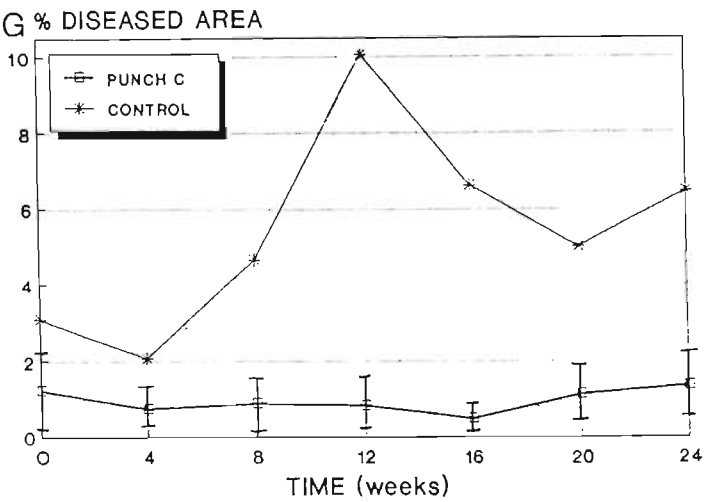
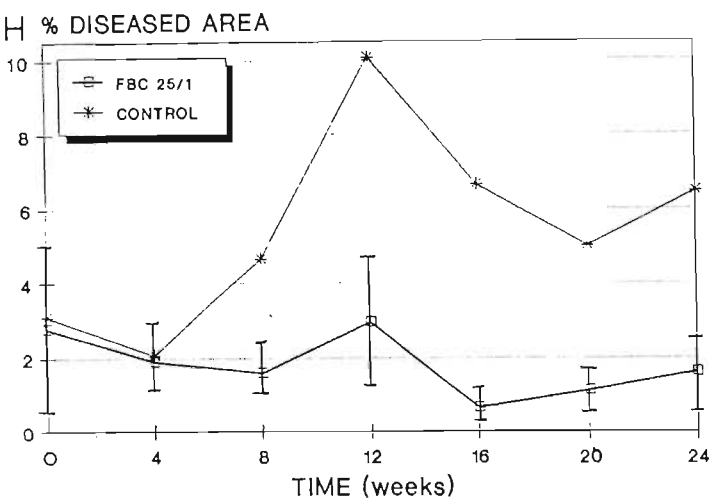
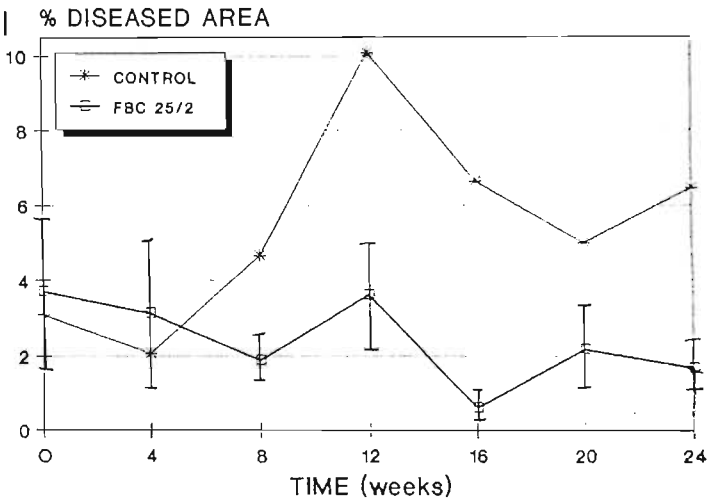


Fig. 2 The percentage diseased area of *Coffea* leaves, naturally infected with *H. vastatrix*, following the application of fungicides at four-weekly intervals for 24 weeks









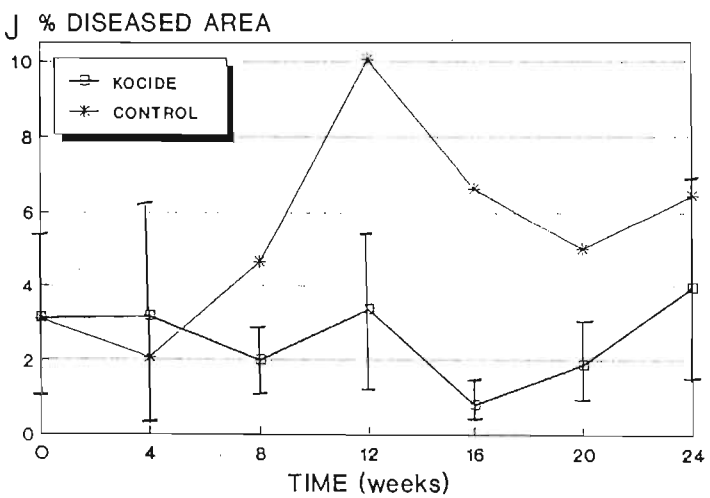


Fig. 2 Continued

FBC 25/2, Punch C and Systhane. In the case of the latter two fungicides, the percentage diseased area in one replication of each treatment was almost zero and the trees appeared very healthy and under no stress.

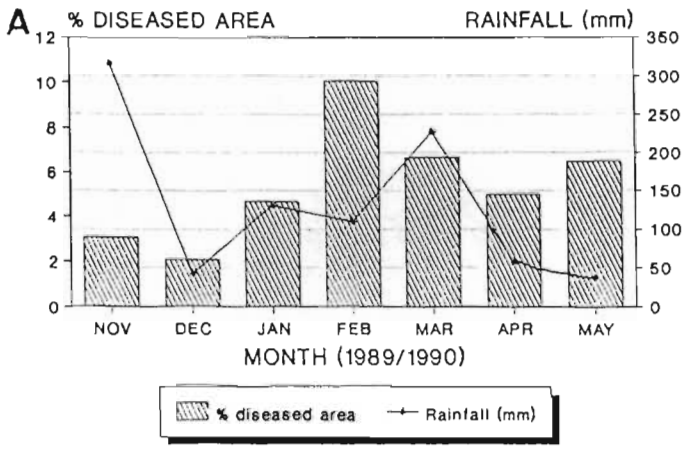
The percentage diseased area, over all treatments, was significantly less at the top of the tree than at either the middle or bottom (**Table 4**). No significant differences ($P < 0.05$) in diseased area were recorded between leaves rated from different sides of the trees (**Table 4**).

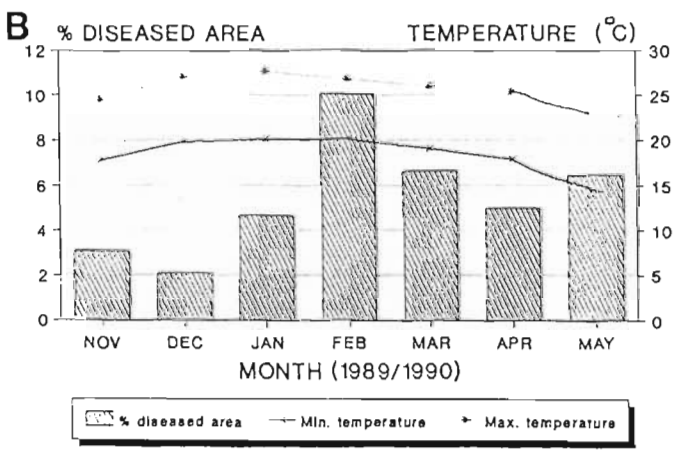
Table 4 Mean percentage diseased area, over all treatments, of leaves rated from different positions of selected trees

POSITION/DIRECTION	% DISEASED AREA	STANDARD ERROR
Top	0.93 a*	0.11
Middle	1.68 b	0.23
Bottom	1.63 b	0.23
North	1.16 a	0.17
East	1.28 a	0.17
South	1.34 a	0.21
West	1.27 a	0.17

* Treatment values with letters in common do not differ significantly at P = 0.05

Evaluation of epidemiological factors influencing rust development in the field. The weather parameters and the percentage diseased area recorded for untreated trees are presented graphically in **Fig. 3** [**A** to **C**]. In order to assess the influence of these parameters, the latent periods for the months during which the fungicide trial was conducted, were calculated on the basis of formulae determined by Rayner (1961a) and Moraes *et al.*, 1976, cited by Kushalappa & Eskes, (1989) (**Table 5**). Although the latent periods cannot be accurately computed, due to the possible influences of other epidemiological factors unique to the situation in southern Africa, they do give an indication of the relative duration





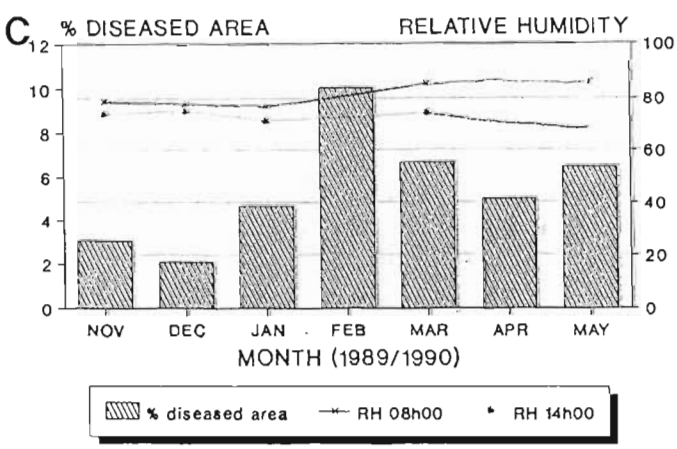


Fig. 3 The percentage diseased area of *Coffea* leaves naturally infected with *Hemileia vastatrix*, and weather parameters

of this period. The relatively high rainfall **Fig. 3 [A]** for November may account for the increase in diseased area in January. Similarly, the high rainfall in March may account for the increase in diseased area in May. The lower minimum temperature **Fig. 3 [B]** in November is optimal for infection structure formation on the leaf surface (De Jong *et al.*, 1987). Relative humidity at 14h00 **Fig. 3 [C]** appeared to have no effect on the course of the epidemic.

Table 5 Latent periods calculated for the seven months of the fungicide trial (from November 1989 to April 1990) using the equations determined by Rayner (1961b) [Eq. 1] and Moraes *et al.* (1976) cited by Kushalappa & Eskes (1989) [Eq. 2]

MONTH	LATENT PERIOD (days)	
	EQUATION 1	EQUATION 2
November	42	31
December	35	28
January	34	28
February	35	28
March	37	29
April	40	31

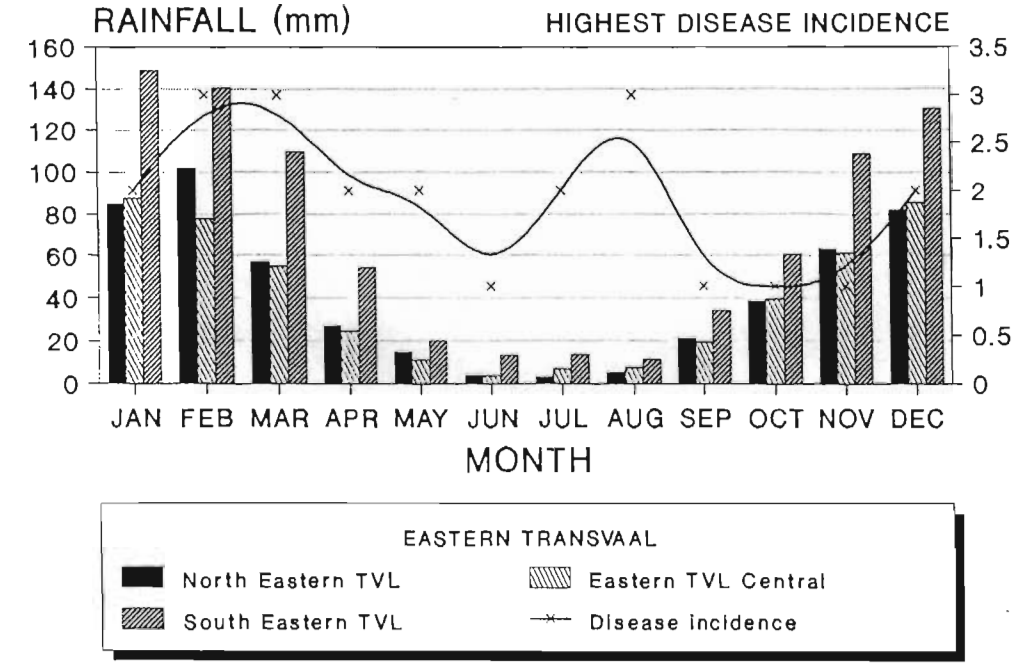
The results obtained for the overall appearance of the trees in the test area are presented in **Appendix 4.3**. The linear correlation coefficient between overall appearance and rust incidence was -0.58. The other two field characteristics recorded (die-back and overbearing) were also not highly correlated with rust incidence (**Table 6**). The overall appearance of the trees was highly negatively correlated with die-back and overbearing. Although the die-back recorded did not correlate with the present rust incidence, it is possibly as a result of a severe rust outbreak the previous year. This would not explain the the lack of correlation between rust incidence and overbearing. In the case of coffee, a high yielding year follows one or two years of low yield (Kushalappa, 1989a). Thus a tree overbearing this year indicates that during the previous year the yield was low. No satisfactory explanation could be found for the lack of correlation between overbearing and low rust incidence.

Table 6 The linear correlation coefficients (r) calculated for the various factors influencing the ratings of individual trees within the plot

INFLUENCING FACTORS		r
Rust incidence	Overbearing	0.53
Rust incidence	Die-back	0.61
Die-back	Overbearing	0.61
Die-back	overall appearance	-0.83
Overbearing	overall appearance	-0.59

The influence of the neighbouring trees within the hedge-row on disease levels was evaluated for each replication (refer to **Appendix 4.4**). A linear correlation coefficient of 0.37 was obtained for the incidence of rust on untreated neighbouring, and treated trees within the hedge-row. It is evident from these results that replicates differed greatly in the above described factors.

Climatic factors influencing rust development in southern Africa. The relationship between the weather parameters and the months with the highest incidence of rust, as reported by farm managers/growers is presented graphically in **Figs. 4, 5 and 6**. The average rainfall patterns and minimum and maximum temperatures, in Natal and the Eastern Transvaal over 12 months, were calculated from the supplied weather data. Eastern Transvaal and Natal have one rainy season per year with a short dry period over the winter months. The information supplied by the farmers revealed that there are two peaks in the annual rust cycle in both regions. The latent periods, as described previously, were calculated for the different regions (**Table 7**). The latent periods during the cooler months of the year are much longer ($\pm$ six weeks) than when the temperature is higher ($\pm$ three weeks).



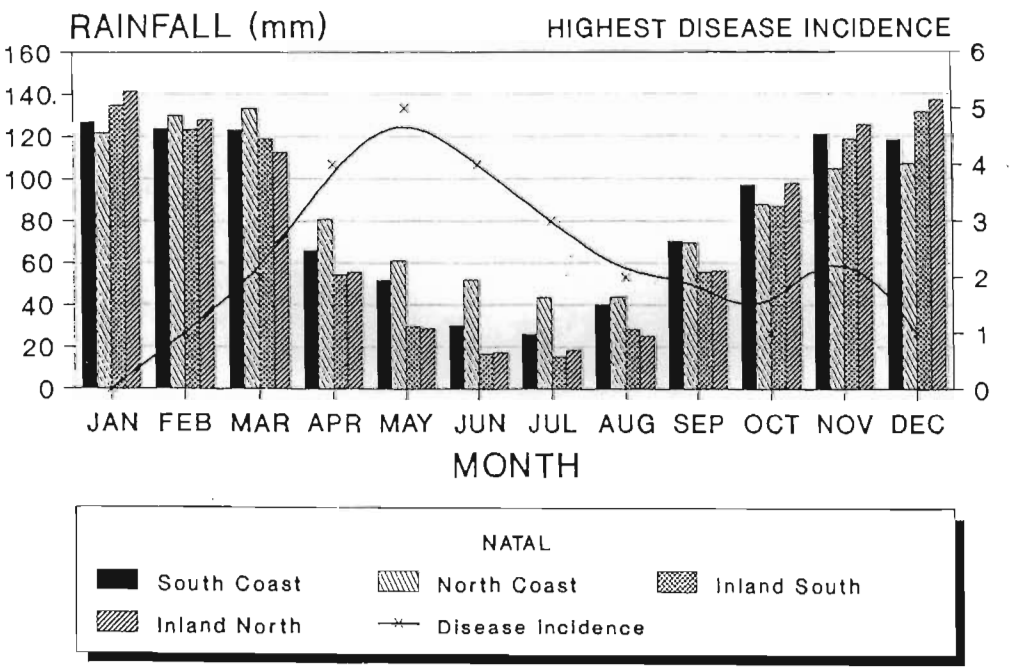


Fig. 4 Relationship between average rainfall, recorded between 1921 and 1989 in the Eastern Transvaal and Natal, and disease incidence as recorded by farm managers

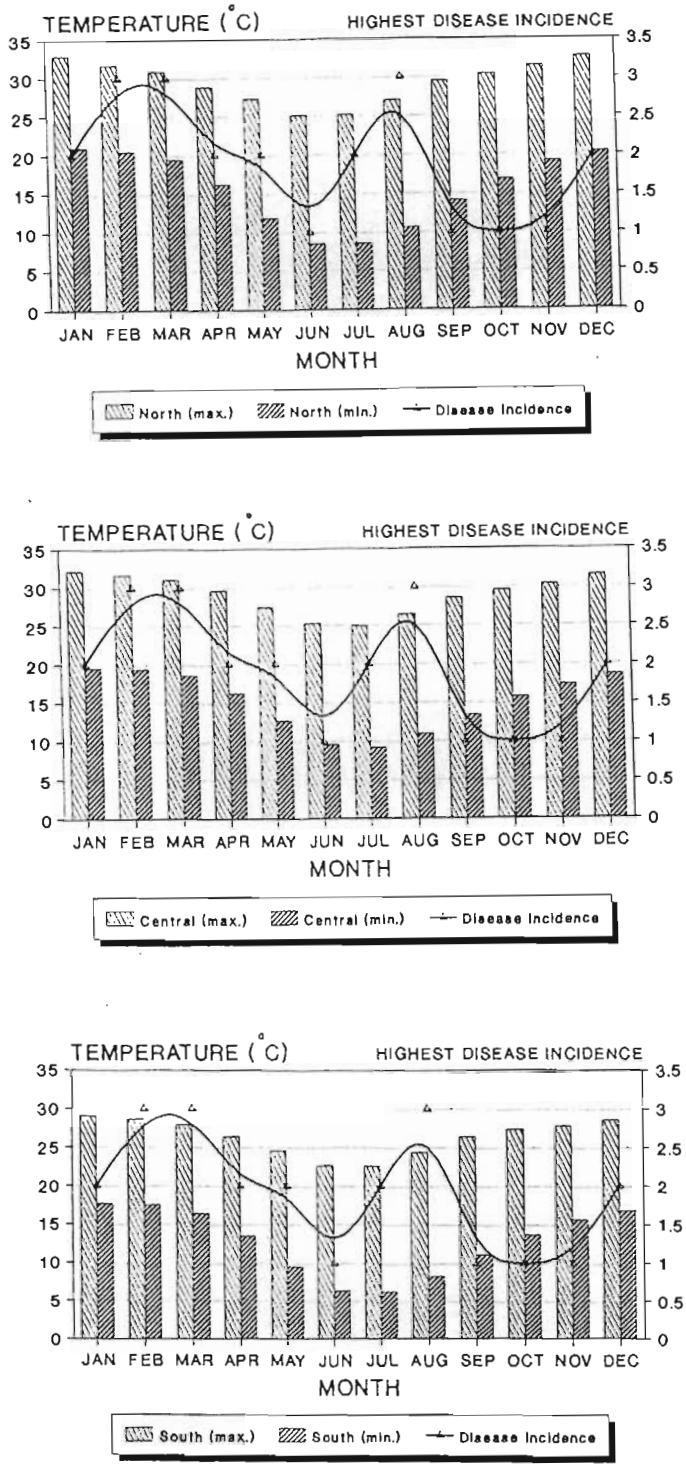
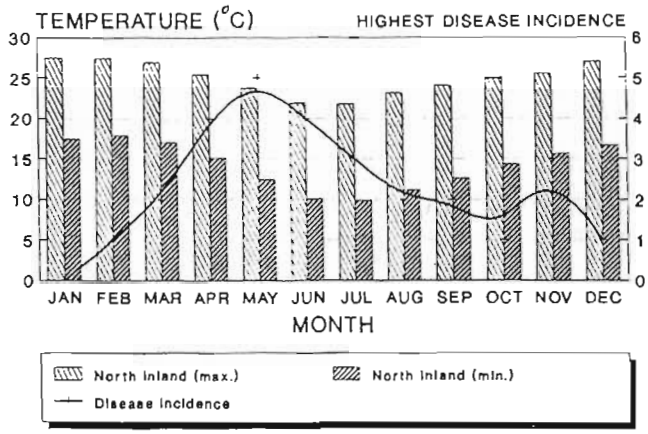
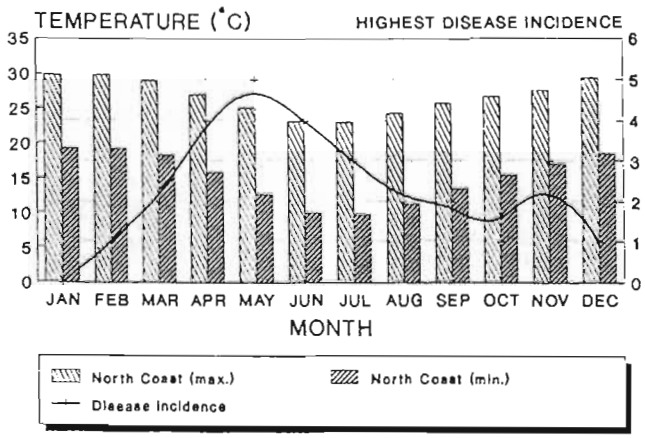
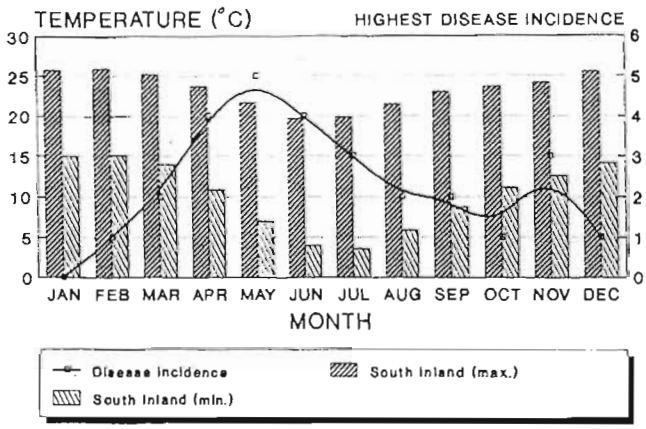


Fig. 5 Relationship between minimum and maximum temperature, recorded between 1921 and 1989 in the Eastern Transvaal, and disease incidence as recorded by farm managers







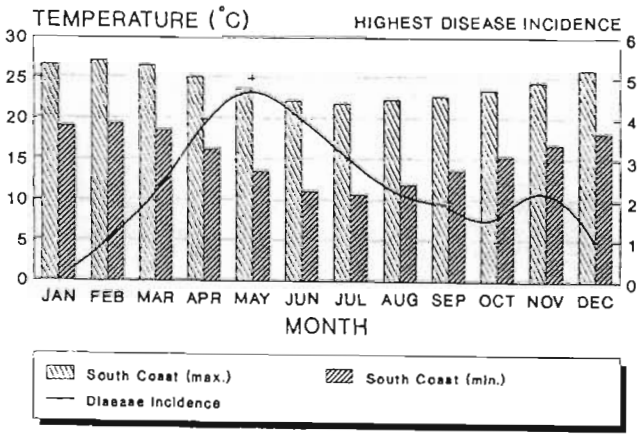


Fig. 6 Relationship between minimum and maximum temperature, recorded between 1921 and 1989 in Natal, and disease incidence as recorded by farm managers

Table 7 Latent periods calculated for 12 months in the Eastern Transvaal and Natal using equations 1 and 2

LOCATION		LATENT PERIOD (days)											
		JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
EASTERN TRANSVAAL													
North	eq.1*	23	24	35	30	34	38	38	35	31	28	25	24
	eq.2**	27	29	32	41	51	61	60	55	45	38	32	29
Central	eq.1	30	31	34	40	50	58	59	54	47	41	37	33
	eq.2	24	25	26	29	33	37	38	35	32	29	27	26
South	eq.1	37	38	41	49	59	68	68	62	54	46	43	40
	eq.2	28	28	30	33	38	42	42	39	35	33	31	29
NATAL													
North-inland	eq.1	39	39	41	46	53	60	61	57	53	49	45	42
	eq.2	29	29	30	33	36	39	40	38	36	34	32	31
North Coast	eq.1	37	36	38	44	51	58	59	56	52	48	44	39
	eq.2	29	28	29	32	35	38	39	38	36	34	32	30
South-inland	eq.1	46	46	49	57	67	75	76	70	62	56	53	48
	eq.2	33	33	34	37	42	46	46	43	40	37	36	34
South Coast	eq.1	33	33	36	43	51	59	60	55	49	44	40	35
	eq.2	26	26	28	31	35	39	39	37	34	32	30	27

* determined using formula described by Rayner (1961b)

** determined using formula described by Moraes *et al.* (1976) cited by Kushalappa & Eskes (1989)

DISCUSSION

Fungicides are initially evaluated using laboratory and greenhouse testing, and based on their success under these conditions the most promising fungicides are evaluated in the field. Some of the fungicides chosen (**Table 1**), had already proved successful commercially in other coffee-growing countries (Okioga, 1978; Javed, 1984). The inclusion of these fungicides, in the present study, was based on the knowledge that the performance of a fungicide can vary under different conditions. Factors such as disease severity and time of fungicide application are known to affect their efficacy (Kushalappa, 1989b). The site chosen for a trial and the time of the first application is therefore of critical importance. To avoid having to inoculate the field, the site chosen in the prevent investigation recorded a high

disease incidence at the onset of the programme. A spray programme against coffee rust, under southern African conditions, is recommended to begin in November and continue for six months (farm managers, pers. comm.).

Although the main objective of disease control is to reduce yield loss in coffee, the evaluation of rust incidence is complicated because of annual variations in berry yield. It is for this reason that only the disease parameter is employed in evaluating fungicide efficiency (Kushalappa, 1989b). Various parameters have been used to quantify disease. According to Kushalappa (1989a), the intensity of coffee rust can be quantified as incidence, proportion of diseased leaves, or severity, the number of "lesions" (affected areas or colonies) per leaf or leaf area infected. In field situations, these parameters have been used successfully (Vine *et al.*, 1973; Eskes & Toma-Braghini, 1981). Relating disease area diagrams to disease progress, as described by Muthappa (1974) and Kushalappa & Chaves (1980), is particularly useful and more relevant than estimating the proportion of diseased leaves or counting the number of "lesions" per leaf. In the present investigation, estimating disease severity by counting the number of "lesions" proved futile as rust colonies often coalesce.

To establish the amount of disease before and following the application of a fungicide, the first step is a random sampling of leaves. A rust epidemic builds up from the lower region of a tree (Rayner, 1961b; Eskes & Toma-Braghini, 1981) and, generally, the disease intensity is low in the top third of a tree as a result of direct sun radiation which decreases the amount of free water available for urediospore germination (Kushalappa, 1989a). Results obtained in the present study (**Table 4**) indicated that the percentage diseased area was significantly higher at the bottom and middle of the tree than on top. In Brazil, the south-eastern side of a coffee tree normally shows a higher degree of attack than the north-western side (Eskes & Toma-Braghini, 1981). At the fungicide site studied in this report, there appeared to be no difference in percentage diseased area between leaves rated from different sides of the same tree (**Table 4**). This was surprising as one would expect the leaves of trees within the hedge-row to have a higher percentage diseased area than the leaves from trees between rows, as disease

severity is positively correlated with host density. According to Becker & Kranz (1977) and Bock (1962a), the lower the host density between trees the slower the rate of disease development.

In the present investigation, the severity of coffee rust at the onset of the spray programme was exceptionally high. Under these conditions, a protectant fungicide, such as Kocide, would be expected to be less successful initially than the curative, systemic fungicides (**Table 1**). The former fungicide would, however, protect the tree from further infection. Generally, systemic fungicides used against coffee rust are more efficient than protective ones (Kushalappa, 1989b). Bayleton and Bayfidan, have been commercially applied to coffee with much success (Javed, 1984; Dr. M. Deall, pers. comm.) and gave good results in the present study. Other systemics, such as Punch C, the experimental product, BAS 450 00F, and Anvil, did show promise as potential control agents. Although, many systemic fungicides performed well under field conditions, both in the present study and those by other researchers (Paul & Patel, 1982; Javed, 1982), the major drawback is their high cost and induction of severe defoliation (Wybou & Stripecke, 1982; Kushalappa, 1989b). In the present study, the amount of defoliation induced by the application of systemic fungicides was not estimated.

One would expect the use of protectant fungicides, such as copper-containing fungicides, to be of limited commercial value since the advent of systemic fungicides. However, according to Waller (1982) and Kushalappa (1989b), systemic fungicides, in general, have shown less consistent and efficient control in the field than copper fungicides; the latter apparently owe their good performance to their greater persistence in the field. Under field conditions in Brazil, copper fungicides have presented the best results in rust control when compared to other fungicides (Muthappa *et al.*, 1989). Besides being highly effective and economical, these fungicides correct copper deficiency, increasing leaf retention and berry yield (Matiello, 1985, cited by Muthappa *et al.*, 1989). Efficacy does, however, depend on the amount of metallic copper, and on the placement of the fungicide at the site of infection, that is, the abaxial surface of the leaf. However, systemic fungicides do have the following advantages over

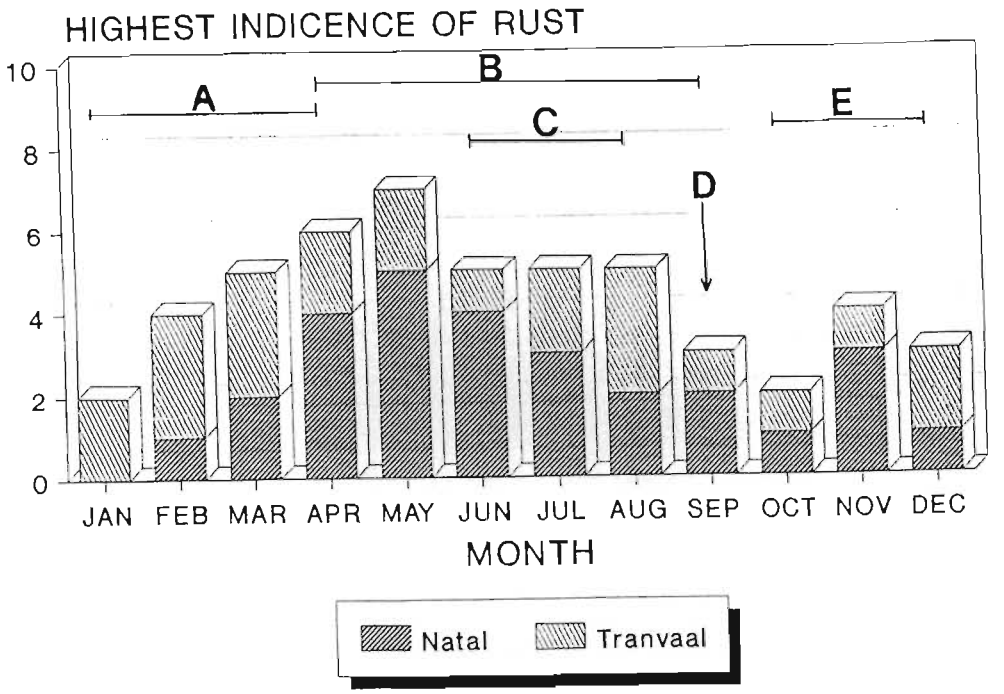
protectant fungicides: quick reduction of infection level, abortion of urediosori, elimination of latent infections, and reduction of the inoculum (Muthappa *et al.*, 1989).

The fungicide trial was not repeated in time due to the high travelling expense involved. In the event of the field trial described in the present study being repeated it is advisable that the possible defoliation induced by systemic fungicides be taken into consideration. The method of disease evaluation used in the present study, that is, determining percentage diseased area on individual leaves, did not compare well with the evaluation of the appearance of the whole tree (**Fig. 6**). It is therefore advised that single leaf as well as whole tree evaluation be carried out during the fungicide application period, and that experiments be conducted for several years to assess the effect of overbearing in subsequent years.

For effective control of coffee rust, a knowledge of climatic conditions is essential. Among physical environmental factors, rain plays the most important role in the rate of rust development (Bock, 1962a; Kushalappa *et al.*, 1983). It provides not only moisture for germination, but also aids in dispersal of urediospores. Consequently, fungicide applications are recommended during the rainy season. Also, the duration of leaf surface wetness influences the amount of penetration and disease severity (Kushalappa, 1989a). According to Guzman & Gomez (1987), five millimetres of rainfall causes canopy saturation and water begins to reach the abaxial surfaces of coffee leaves by upward splashing. In southern Africa, leaf rust incidence lags behind the rainfall pattern (**Fig. 7**) and the latent period would account for the delay in the appearance of disease symptoms. In the East Rift Valley districts of Kenya, two peaks of rust progress were found (Bock, 1962a) and they correspond to the two rainy seasons. The disease progress of coffee rust in southern Africa also follows a bimodal pattern (**Fig. 7**). However, Natal and the Eastern Transvaal Africa have only one rainfall season. Thus factors other than rainfall are likely to be involved in the disease progress. The relationship between disease incidence and dew or the duration, per night and per season, of dew during the year for these regions is not known. It probably does play an essential role in coffee leaf rust epidemics.

The seasonal and daily rhythms, and fluctuations of temperature, influence the rate of disease development (Kushalappa, 1989a). The temperature during the period when free water is present on the leaf is a major factor determining urediospore germination (Kushalappa *et al.*, 1983) and influences appressorium formation (De Jong *et al.*, 1987). Temperature also influences the latent period; the shorter the latent period the greater the number of generations per affected area, and thus the faster the rate of disease development. According to De Jong *et al.* (1987), germination and appressorium formation by *H. vastatrix* occurs at temperatures between 16 and 28°C. The peaks in disease incidence in May/June in Natal and July/August in the Eastern Transvaal, are positively correlated with the optimal temperatures for spore germination in the month prior to these peaks. Similarly, the first peak in disease incidence in the Eastern Transvaal during February/March may be due to optimal temperatures in November/December. This corresponds to the length of the latent period during the hot summer months. However, seasonal fluctuations in temperature may either favour or inhibit infection. At extreme temperatures (< 10°C and > 35°C), "lesion" enlargement is limited (Ribeiro *et al.*, 1978, cited by Kushalappa & Eskes, 1989). According to Becker-Raterink (1984), high temperatures in Brazil lead to the abortion of rust sporulation and a sort of heat-induced dormant period. When the temperatures decrease again, the colonies start sporulating. Similarly, Muthappa (1980) has observed that infections may remain dormant for several months during a dry period, and only actively sporulate at the start of the rainy season. According to the data presented in the present study, the high temperatures experienced in the Eastern Transvaal during the summer months may delay the rust epidemic until the cooler autumn temperatures (**Fig. 8**).

It has long been realised that the physiological condition of the host may exacerbate rust intensity (McDonald, 1930, cited by Kushalappa & Eskes, 1989). This can result in a die-back of bearing branches and feeder roots if trees are carrying a heavy crop, or if defoliation is severe (Waller, 1982). The relationship between months with the highest recorded rust incidence, as determined by the farm managers, and the physiological condition of the host is presented graphically in **Fig. 10**. The increase in disease incidence in the Transvaal and Natal at the



A = maximum berry development and leaf formation
B = harvesting
C = extensive leaf defoliation
D = flowering and new leaf formation
E = leaf formation

Fig. 10 Relationship between host's physiology and incidence of *H. vastatrix* on farms/estates in southern Africa

beginning of the year (January until April) is at a period when berry development and ripening is occurring. High disease levels will place stress on the tree as less photosyntate is available for berry fill. The trees, within the site chosen in the present study, did show overbearing (**Appendix 4.3**). The second increase in September to November is during the time of flowering and the initiation of leaf formation. Both factors require an enormous input from the coffee tree. This physiological stress influences the leaves resistance mechanisms. These factors, together with the climatic stress due to the less than optimum growing conditions for coffee in southern Africa (Joubert, 1985), may account for the observed disease pattern. The increase in leaf abscission, naturally and due to rust infections, has a great effect on yield. Leaf abscission, as a result of rust, can occur even when only one rust "pustule" (affected area) is present on the leaf (Nutman & Roberts, 1970). The effects of the disease on the physiological condition of the host are cumulative and the amount of disease in any year has an effect on the

crop produced the following year.

Once factors influencing coffee rust are known, a predictive system could be employed. Kushalappa *et al.* (1983; 1984; 1986) developed such a system and it has been used effectively to reduce the onset of epidemics and the peak of disease incidence. Although the present study provides a greater understanding of factors influencing coffee rust development under South African conditions, further investigations into the epidemiology of coffee rust in southern Africa may, in the future, permit such a predictive system to operate successfully.

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Table A Mean values and total urediospore counts of the developmental stages of *Hemileia vastatrix* on the leaf surface of susceptible and resistant leaf discs determined using fluorescence microscopy

A. Coffea arabica cv. Caturra

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	18.84	16.41	12.85	16.03
% germinated urediospores that formed appressoria over stomata	69.57	75.38	75.32	73.42
% germinated urediospores that did not form appressoria over stomata	11.59	8.21	11.83	10.54

B. NC-169**

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	33.33	31.42	31.52	32.09
% germinated urediospores that formed appressoria over stomata	56.67	58.74	60.47	58.63
% germinated urediospores that did not form appressoria over stomata	10.00	9.84	8.01	9.28

C. NP-547**

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	44.07	39.64	46.22	43.31
% germinated urediospores that formed appressoria over stomata	41.95	47.56	42.16	43.89
% germinated urediospores that did not form appressoria over stomata	13.98	12.80	11.62	12.80

* Mean percentage of three leaf discs

** F6 Progeny of the cross between *Coffea arabica* cv. Caturra and HDT

Table B Mean values and total urediospore counts of the developmental stages of *Hemileia vastatrix* on the leaf surface of susceptible and resistant leaf pieces cut from attached leaves determined using fluorescence microscopy

A. Coffea arabica cv. Caturra

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	13.70	8.30		11.00
% germinated urediospores that formed appressoria over stomata	82.40	78.20		80.30
% germinated urediospores that did not form appressoria over stomata	3.90	13.50		8.70

B. NC-169**

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	12.10	15.02		13.56
% germinated urediospores that formed appressoria over stomata	71.10	73.30		72.20
% germinated urediospores that did not form appressoria over stomata	16.80	11.68		14.20

C. NP-547**

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*			MEAN
	1	2	3	
% germinated urediospores that did not form appressoria	43.00	34.40		38.70
% germinated urediospores that formed appressoria over stomata	44.80	54.70		49.75
% germinated urediospores that did not form appressoria over stomata	12.20	10.90		11.55

* Mean percentage of three leaf discs

** F6 Progeny of the cross between *Coffea arabica* cv. Caturra and HDT

Table C Mean values for the developmental stages of *Hemileia vastatrix* on the leaf surface of a host and non-host leaf discs determined using fluorescence microscopy

A. Coffea arabica cv. Caturra

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*				MEAN
	1	2	3	4	
% germinated urediospores that did not form appressoria	18.84	16.41	12.85	16.14	16.06
% germinated urediospores that formed appressoria over stomata	69.57	75.38	75.32	73.35	73.41
% germinated urediospores that did not form appressoria over stomata	11.59	8.21	11.83	10.51	10.54

B. Camellia sinensis

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*				MEAN
	1	2	3	4	
% germinated urediospores that did not form appressoria	5.93	15.15	9.69	11.30	10.52
% germinated urediospores that formed appressoria over stomata	45.23	27.27	39.21	37.85	37.39
% germinated urediospores that did not form appressoria over stomata	48.84	57.58	51.10	50.85	52.09

C. Phaseolus vulgaris

DEVELOPMENTAL STAGE ON LEAF SURFACE	REPLICATE*				MEAN
	1	2	3	4	
% germinated urediospores that did not form appressoria	30.90	26.76	21.68	33.14	28.12
% germinated urediospores that formed appressoria over stomata	25.53	30.09	31.65	27.78	28.76
% germinated urediospores that did not form appressoria over stomata	43.57	43.15	46.67	39.08	43.12

* Mean percentage of three leaf discs

Mean values for the developmental stages of *Hemileia vastatrix* on the leaf surface of *Coffea arabica* cv. Caturra discs following treatments with the fungicide Bayfidan®

DEVELOPMENTAL STATE ON LEAF SURFACE	TREATMENT	REPLICATE*			MEAN
		1	2	3	
% germinated uredio-spores that did not form appressoria	A	0	8.70	10.92	6.54
	CONTROL	16.67	18.03	12.97	24.23
	B	27.78	15.56	22.59	21.98
	CONTROL	21.25	25.48	15.74	20.82
	C	8.76	9.17	1.24	20.22
	CONTROL	20.89	11.50	19.37	17.25
	D	10.00	16.37	12.16	12.84
	CONTROL	19.21	18.34	19.45	19.00
% germinated uredio-spores that formed appressoria over stomata	A	88.33	85.74	80.63	84.90
	CONTROL	76.19	72.73	78.40	75.77
	B	66.67	79.17	74.12	73.32
	CONTROL	72.50	71.29	76.67	73.49
	C	74.78	79.75	85.31	79.95
	CONTROL	72.08	78.18	68.10	72.79
	D	76.67	77.37	81.58	78.54
	CONTROL	71.49	69.66	72.86	71.34
% germinated uredio-spores that did not form appressoria over stomata	A	11.67	5.56	8.45	8.56
	CONTROL	7.14	9.24	8.63	8.35
	B	5.55	5.27	3.29	4.70
	CONTROL	6.25	3.23	7.59	5.69
	C	16.46	11.58	13.45	13.83
	CONTROL	7.03	10.32	12.53	9.96
	D	13.33	6.26	6.26	8.62
	CONTROL	9.30	12.00	7.69	9.66

* mean percentage on three leaf discs

A = Inoculated 28 days after second Bayfidan treatment
B = Inoculated 7 days after third Bayfidan treatment
C = Inoculated 14 days after third Bayfidan treatment
D = Inoculated 28 days after third Bayfidan treatment

TABLE A

Mean percentages, on nine leaf discs, of the developmental stages of *Hemileia vastatrix* on the leaf surface of the F6 progeny of a cross between *Coffea arabica* cv. Caturra and Hybrido de Timor (Colombia). Values were determined on leaf discs using fluorescence microscopy.

DEVELOPMENTAL STAGE ON LEAF SURFACE	CULTIVAR/ LINE	REPLICATE			MEAN
		1	2	3	
LEAF 1 (youngest leaf)					
% germinated uredio-spores that did not form appressoria	Caturra	7.76	9.31	12.47	9.85
	NC-169	14.96	0.68	3.10	6.25
	NP-547	11.92	13.70	20.98	15.53
% germinated uredio-spores that formed appressoria over stomata	Caturra	57.24	55.56	56.28	56.36
	NC-169	49.61	75.08	62.07	62.25
	NP-547	68.87	67.12	53.77	63.25
% germinated uredio-spores that formed appressoria not over stomata	Caturra	35.00	35.13	31.25	33.79
	NC-169	35.43	24.24	34.83	31.50
	NP-547	19.21	19.18	25.25	21.21
LEAF 2					
% germinated uredio-spores that did not form appressoria	Caturra	33.34	21.55	19.73	24.87
	NC-169	23.96	26.07	32.13	27.39
	NP-547	41.79	37.47	42.86	40.71
% germinated uredio-spores that formed appressoria over stomata	Caturra	51.38	63.16	65.38	59.97
	NC-169	61.72	61.83	54.92	59.49
	NP-547	39.80	49.34	49.02	46.05
% germinated uredio-spores that formed appressoria not over stomata	Caturra	15.28	15.29	14.89	15.15
	NC-169	14.32	12.10	12.95	13.12
	NP-547	18.41	13.19	8.12	13.24
LEAF 3					
% germinated uredio-spores that did not form appressoria	Caturra	26.60	27.63	27.88	27.37
	NC-169	33.33	31.42	31.52	32.09
	NP-547	44.07	39.64	40.62	41.44
% germinated uredio-spores that formed appressoria over stomata	Caturra	60.47	63.16	62.32	61.98
	NC-169	56.67	58.74	60.47	58.63
	NP-547	41.95	47.56	43.75	44.42
% germinated uredio-spores that formed appressoria not over stomata	Caturra	12.93	9.21	9.80	10.65
	NC-169	10.00	9.84	8.01	9.28
	NP-547	13.98	12.80	15.63	14.14

TABLE A CONTINUED

DEVELOPMENTAL STAGE ON LEAF SURFACE	CULTIVAR/ LINE	REPLICATE			MEAN
		1	2	3	
LEAF 4					
% germinated uredio-spores that did not form appressoria	Caturra	14.32	31.16	34.09	26.52
	NC-169	20.00	32.41	21.07	24.49
	NP-547	60.29	65.89	52.47	59.55
% germinated uredio-spores that formed appressoria over stomata	Caturra	68.18	54.55	50.00	57.58
	NC-169	69.25	50.97	67.06	62.43
	NP-547	26.57	27.70	40.66	31.64
% germinated uredio-spores that formed appressoria not over stomata	Caturra	17.50	14.29	15.91	15.90
	NC-169	10.75	16.62	11.87	13.08
	NP-547	13.14	6.41	6.87	8.81
LEAF 5					
% germinated uredio-spores that did not form appressoria	Caturra	44.13	31.15	39.56	38.28
	NC-169	50.24	23.14	43.08	38.82
	NP-547	32.17	23.72	38.68	31.52
% germinated uredio-spores that formed appressoria over stomata	Caturra	32.47	50.67	46.15	43.10
	NC-169	25.36	50.93	46.64	40.98
	NP-547	58.04	56.41	45.30	53.25
% germinated uredio-spores that formed appressoria not over stomata	Caturra	23.40	18.18	14.29	18.62
	NC-169	24.40	25.93	10.28	20.20
	NP-547	9.79	19.87	16.02	15.23
LEAF 6					
% germinated uredio-spores that did not form appressoria	Caturra	27.31	33.33	32.85	31.16
	NC-169	38.69	40.99	38.20	39.29
	NP-547	42.53	44.31	41.84	42.89
% germinated uredio-spores that formed appressoria over stomata	Caturra	65.00	53.85	56.15	58.33
	NC-169	56.20	51.44	53.35	53.66
	NP-547	41.76	45.54	42.20	43.17
% germinated uredio-spores that formed appressoria not over stomata	Caturra	7.69	12.82	11.00	10.50
	NC-169	5.11	7.57	8.45	7.04
	NP-547	15.71	10.15	15.96	13.94
LEAF 7 (oldest leaf)					
% germinated uredio-spores that did not form appressoria	Caturra	57.93	65.94	54.98	59.62
	NC-169	44.65	58.73	50.91	51.43
	NP-547	35.75	37.54	45.00	39.43
% germinated uredio-spores that formed appressoria over stomata	Caturra	28.12	20.00	33.91	27.34
	NC-169	48.21	28.57	40.00	38.93
	NP-547	54.00	57.23	46.00	52.41
% germinated uredio-spores that formed appressoria not over stomata	Caturra	13.95	14.06	11.11	13.04
	NC-169	7.14	12.70	9.09	9.64
	NP-547	10.25	5.23	9.00	8.16

TABLE B

Replicate and mean values on 27 leaf discs of the reaction type, latent period and calculated uredosori concentration on leaf discs of *Coffea* spp. infected with *Hemileia vastatrix*

A. *Coffea arabica* cv. Caturra

LEAF PAIR	CATEGORY	REPLICATE 1			MEAN
		1	2	3	
1	Reaction type*	9.0	7.0	7.6	7.87
	Latent period**	34	37	34	35.00
	Uredosori/mm²	62	73	70	68
2	Reaction type	4.5	6.5	6.2	5.73
	Latent period	37	38	38	37.70
	Uredosori/mm²	51	53	50	52
3	Reaction type	9.0	9.0	7.2	8.40
	Latent period	38	38	38	38.00
	Uredosori/mm²	45	51	53	50
4	Reaction type	9.0	8.0	8.2	8.40
	Latent period	38	39	38	38.30
	Uredosori/mm²	48	39	51	46
5	Reaction type	4.0	4.0	3.2	3.73
	Latent period	40	39	42	40.30
	Uredosori/mm²	51	37	48	46
6	Reaction type	2.0	2.8	2.0	2.27
	Latent period	42	42	43	42.30
	Uredosori/mm²	17	19	25	20
7	Reaction type	3.5	1.5	2.8	2.60
	Latent period	42	42	42	42.00
	Uredosori/mm²	54	37	43	45

1 Nine leaf discs were used per replicate
* Reaction type determined using the scale described in Appendix A
** in days

TABLE B CONTINUED

B. NC-169 (F6 progeny of the cross between *C. arabica* cv. Caturra and HDT)

LEAF PAIR	CATEGORY	REPLICATE			MEAN
		1	2	3	
1	Reaction type*	8.5	8.0	8.4	8.30
	Latent period**	19	19	20	19.30
	Uredosori/mm²	76	80	70	75
2	Reaction type	8.3	8.3	8.7	8.40
	Latent period	20	20	20	20.00
	Uredosori/mm²	51	58	60	56
3	Reaction type	9.0	7.7	8.0	8.20
	Latent period	20	20	21	20.30
	Uredosori/mm²	76	82	78	78
4	Reaction type	8.3	7.7	7.9	8.00
	Latent period	20	19	22	20.30
	Uredosori/mm²	57	58	62	59
5	Reaction type	7.0	7.0	7.0	7.00
	Latent period	23	25	23	23.70
	Uredosori/mm²	55	67	51	58
6	Reaction type	5.0	5.3	5.2	5.20
	Latent period	25	25	25	25.00
	Uredosori/mm²	31	39	40	37
7	Reaction type	1.0	1.0	1.7	1.23
	Latent period	26	26	25	25.70
	Uredosori/mm²	19	27	30	26

C. NP-547 (F6 progeny of the cross between *C. arabica* cv. Caturra and HDT

LEAF PAIR	CATEGORY	REPLICATE			MEAN
		1	2	3	
1	Reaction type	0	0	0	0
2	Reaction type	0	0	0	0
3	Reaction type	0.75	0	0.50	0.42
4	Reaction type	1.50	0	0.75	0.75
5	Reaction type	3.33	1.33	1.00	1.89
6	Reaction type	2.33	2.33	0.67	1.78
7	Reaction type	3.00	2.67	2.00	2.56

APPENDIX 4.1

Mean percentage of diseased areas of naturally infected leaves with *Hemileia vastatrix* following the monthly application of selected fungicides over a 24-week period

FUNGICIDE	REPLICATION	WEEKS AFTER FUNGICIDE APPLICATION						
		0	4	8	12	16	20	24
ANVIL	1	3.78	1.06	1.70	2.07	0.32	1.22	0.63
	2	2.57	2.26	2.66	2.44	0.14	1.03	1.88
	3	2.81	3.82	2.48	2.32	0.33	1.06	1.40
	4	1.28	0.85	2.84	0.20	0.54	3.42	1.67
BAS 480 00F	1	0.45	0.86	1.76	0.37	0.12	1.08	0.05
	2	0.94	2.44	1.41	0.31	0.14	0.05	0.41
	3	2.04	1.85	2.75	0.60	0.26	0.54	1.14
	4	0.67	2.27	2.48	1.12	0.05	0.59	1.76
BAYFIDAN ec	1	1.83	2.31	0.67	0.39	0.09	0.14	0.11
	2	2.73	3.29	3.62	2.15	0.41	0.77	0.45
	3	2.39	2.67	3.09	2.66	1.10	1.59	1.31
	4	3.31	0.83	3.22	1.13	0.63	1.15	1.74
BAYFIDAN granules	1	2.80	2.61	2.06	0.41	0.18	0.17	0.17
	2	5.75	4.63	2.17	1.10	0.49	1.57	2.70
	3	7.04	2.37	1.33	0.66	0.55	0.36	0.90
	4	0.00	1.39	1.34	1.41	0.40	1.06	3.11
BAYFIDAN + EN600	1	3.03	2.44	1.31	1.09	0.29	0.23	0.46
	2	4.55	1.09	0.03	0.32	1.17	1.83	2.23
	3	5.04	0.08	0.08	0.23	0.17	0.11	0.00
	4	1.57	0.81	1.50	0.75	0.47	1.88	0.66

FUNGICIDE	REPLICATION	WEEKS AFTER FUNGICIDE APPLICATION						
		0	4	8	12	16	20	24
FBC 25/1	1	2.48	0.93	1.55	2.09	0.89	1.76	1.15
	2	2.08	1.55	1.62	4.05	0.28	0.80	1.68
	3	6.38	2.59	0.89	1.06	0.59	0.50	0.77
	4	0.12	2.58	2.37	4.57	0.81	1.40	2.87
FBC 25/2	1	1.80	1.80	2.01	1.58	0.64	0.66	2.26
	2	6.81	2.35	2.19	4.46	0.45	2.46	0.73
	3	1.71	1.79	1.39	3.64	0.60	1.40	2.12
	4	4.49	6.55	1.95	4.81	0.72	4.16	1.58
KOCIDE	1	5.59	1.98	3.01	1.23	0.26	0.41	0.42
	2	3.76	3.38	2.22	5.44	0.66	2.32	5.36
	3	2.57	7.14	1.49	5.44	0.66	1.94	3.26
	4	0.55	0.17	1.32	1.35	1.60	2.83	6.72
PUNCH C	1	0.32	0.25	0.30	0.21	0.10	0.06	0.12
	2	0.65	0.65	0.16	0.93	0.28	1.99	2.10
	3	3.02	1.14	0.62	1.50	0.12	1.74	0.94
	4	0.83	0.91	2.42	0.67	1.42	0.67	2.20
SYSTHANE	1	5.36	1.52	2.83	1.26	0.30	0.40	0.32
	2	0.17	0.05	0.05	0.00	0.00	0.00	0.00
	3	2.35	2.30	3.23	1.24	0.59	0.51	0.75
	4	4.65	4.41	2.38	6.65	4.70	2.21	2.18
CONTROL	1	2.86	1.67	2.58	11.70	7.62	4.66	4.83
	2	2.92	2.21	6.26	12.06	8.00	2.62	5.20
	3	3.54	2.63	5.80	7.84	4.78	4.11	7.79
	4	3.06	1.77	3.98	8.62	6.14	8.62	8.02

APPENDIX 4.2

Statistical differences ($P < 0.05$) in percentage diseased area in time and with replication following the monthly application of selected fungicides over a 24-week period

FUNGICIDE	TIME (weeks)	% DISEASED AREA	REPLICATE	% DISEASED AREA
ANVIL	0	2.61 a	1	1.54 a
	4	2.00 a	2	1.85 a
	8	2.42 a	3	2.03 a
	12	1.76 a	4	1.54 a
	16	0.33 b		
	20	1.68 ab		
	24	1.40 ab		
BAS 480 00F	0	1.03 bc	1	0.67 a
	4	1.86 ab	2	0.81 a
	8	2.10 a	3	1.31 a
	12	0.60 cd	4	1.28 a
	16	0.14 cd		
	20	0.57 cd		
	24	0.84 cd		
BAYFIDAN ec	0	2.57 a	1	0.79 a
	4	2.28 a	2	1.92 ab
	8	2.65 a	3	2.12 b
	12	1.58 ab	4	1.72 ab
	16	0.56 b		
	20	0.91 b		
	24	0.90 b		
BAYFIDAN ec + EN600	0	3.55 a	1	1.26 a
	4	1.11 b	2	1.60 a
	8	0.73 b	3	0.82 a
	12	0.60 b	4	1.09 a
	16	0.53 b		
	20	1.01 b		
	24	0.84 b		
BAYFIDAN granules	0	3.90 a	1	1.20 a
	4	2.75 ab	2	2.63 a
	8	1.73 bc	3	1.89 a
	12	0.90 bc	4	1.24 a
	16	0.41 c		
	20	0.79 bc		
	24	1.72 bc		

FUNGICIDE	TIME (weeks)	% DISEASED AREA	REPLICATE	% DISEASED AREA
FBC 25/1	0	2.77 a	1	1.55 a
	4	1.91 ab	2	1.72 a
	8	1.61 ab	3	1.83 a
	12	2.94 a	4	2.10 a
	16	0.64 b		
	20	1.12 ab		
	24	1.62 ab		
FBC 25/2	0	3.70 a	1	1.54 a
	4	3.12 a	2	3.47 b
	8	1.89 ab	3	1.81 ab
	12	3.62 a	4	2.78 ab
	16	0.60 b		
	20	2.17 ab		
	24	1.67 ab		
KOCIDE	0	3.12 ab	1	1.84 a
	4	3.17 ab	2	3.31 a
	8	2.01 ab	3	3.21 a
	12	3.37 ab	4	2.08 a
	16	0.80 b		
	20	1.88 ab		
	24	3.94 a		
PUNCH C	0	1.21 a	1	0.19 a
	4	0.74 a	2	0.97 ab
	8	0.88 a	3	1.30 b
	12	0.83 a	4	1.30 b
	16	0.48 a		
	20	1.12 a		
	24	1.34 a		
SYSTHANE	0	3.13 a	1	1.71 b
	4	2.07 a	2	0.04 a
	8	2.12 a	3	1.57 b
	12	2.29 a	4	3.88 c
	16	1.40 a		
	20	0.78 a		
	24	0.81 a		
CONTROL	0	3.10 ab	1	5.13 a
	4	2.07 a	2	5.61 a
	8	4.66 bc	3	5.21 a
	12	10.10 d	4	5.74 a
	16	6.64 c		
	20	5.00 bc		
	24	6.46 c		

Scores on selected trees rated on a five-tree grid scale at the site of the fungicide trial 24-weeks after the first treatment. Overall appearance, die-back, overbearing and rust severity were rated

ROW	LINE	OVERALL	OVERBEARING	DIE-BACK	RUST
1	15	8	yes	3	1
1	20	5	yes	1	2
1	25	8	no	1	1
1	30	7	yes	5	2
1	35	4	yes	6	2
1	40	6	yes	3	3
3	5	2	yes	7	1
3	5	3	yes	7	1
3	10	3	yes	8	3
3	15	9	yes	1	0
3	20	4	yes	8	2
3	25	10	yes	0	0
3	30	4	yes	6	1
3	35	1	yes	10	2
3	40	10	no	0	0
5	1	5	yes	5	3
5	5	3	yes	8	2
5	5	5	no	4	0
5	10	5	no	7	1
5	15	3	no	7	1
5	20	1	no	9	3
5	25	1	no	8	3
5	30	9	yes	0	1
5	35	10	yes	0	0
5	40	7	no	5	1
7	1	6	yes	5	2
7	5	5	yes	4	2
7	5	10	no	0	0
7	10	5	yes	7	1
7	15	3	yes	9	2
7	20	2	yes	8	3
7	25	5	yes	6	3
7	30	10	no	0	0
7	35	6	yes	5	1
7	40	10	no	0	1
9	1	8	no	2	1
9	5	3	no	6	1
9	5	10	no	0	0
9	10	3	yes	8	3
9	15	5	no	1	1
9	20	7	no	3	1
9	25	1	yes	0	1
9	30	3	yes	8	2
9	35	5	yes	3	1
9	40	7	yes	5	3
11	20	1	yes	9	1
11	25	5	yes	5	3
11	30	8	no	1	2
11	35	10	no	0	0
11	40	10	no	0	0

Disease severity on a 0 to 3 scale (0 = no infection, 3 = severe infection) on fungicide-treated and border (neighbouring) trees within the four replicates used for the fungicide trial

FUNGICIDE	REPLICATE 1	
	A*	B**
Bayfidan ec	1	1
Bayfican gr	1	0
Systhane	1.5	1.5
Kocide	2	1
Anvil	2	1
FBC 25/2	2	3
FBC 25/1	2	0
Punch C	1	2
BAS 480 00F	1	2
Bayfidan ec + 1% EN 600	0	3
Control	3	3

FUNGICIDE	REPLICATE 2	
	A*	B**
Bayfidan ec	1	2
Kocide	3	3
Bayfidan ec + 1% EN 600	1	2
Bayfidan gr	3	3
FBC 25/2	1	2
BAS 480 00F	2	3
Systhane	0	2
Anvil	2	3
FBC 25/1	3	3
Punch C	3	3
Control	3	3

FUNGICIDE	REPLICATE 3	
	A*	B**
Bayfidan gr	2	2
FBC 25/2	3	2
Systhane	2	3
Bayfidan ec	2	3
Bayfidan ec + 1% EN 600	3	2
Punch C	2	2
Kocide	3	3
Anvil	3	3
FBC 25/1	2	3
BAS 480 00F	2	2
Control	3	3

FUNGICIDE	REPLICATE 4	
	A*	B**
Punch C	2	3
FBC 25/2	2	2
Anvil	2	3
Kocide	3	2
Bayfidan gr	3	3
Bayfidan ec + 1% EN 600	3	3
Systhane	3	3
FBC 25/1	3	3
Bayfidan ec	3	3
BAS 480 00F	2	2
Control	3	3

* Fungicide treated trees

** Neighbouring, untreated tree

Note that the design of the experiment was such that the sequence was as follows: untreated, treated, untreated, etc. down a row