STUDIES ON GREASY SPOT OF CITRUS IN NEW SOUTH WALES

by

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SUMMARY

Greasy spot of citrus in N.S.W. has been noted for many years, although a sudden increase in disease incidence occurred during 1972. In the light of the losses experienced in Florida due to the disease, an investigation in New South Wales of the incidence, distribution, symptomatology, association of fungi and determination of the pathogen was initiated.

Field surveys and examination of herbarium material revealed that greasy spot was found in the inland districts of New South Wales. The variable distribution of the disease, both between and within districts, was related to orchard management, and in particular to the nature of the spray programme.

Symptom development on naturally infected leaves of various ages was defined. Although such careful descriptions have not been published in the literature, it appeared that symptoms of greasy spot in New South Wales contrast in some respects to the disease in Florida and Japan. Histological examination of naturally infected leaves revealed that the raised nature of lesions was due to cell enlargement in the spongy mesophyll. Observations in the field indicated that all commercial varieties of citrus were susceptible, although sweet orange varieties (*Citrus sinensis* Osbeck.) and grapefruit (*Citrus paradisi* Macf.) were more severely affected.

Fungi isolated from lesions included *Colletotrichum gloeosporioides* (Penz.) Sacc., *Septoria citri* Pass. and a previously undescribed *Mycosphaerella* sp. A relationship between fungi isolated and time of sampling, method of isolation and citrus variety was demonstrated. Examination of leaf litter collected under greasy spot infected trees

revealed the *Mycosphaerella* sp. to be the most abundant ascomycete. This fungus was therefore described, and the seasonal changes in it's perithecial maturity established. Peaks of ascospore inoculum would be expected in autumn and spring, which contrasts to the epidemiology of *Mycosphaerella citri* Whiteside in Florida.

The processes of infection and the subsequent pathogenicity of the three fungi were studied. The various infection processes were described and compared with other published descriptions. Symptom production on seedlings of *Citrus sinensis* cv. Ruby Blood and subsequent re-isolation of the fungi demonstrated that *Mycosphaerella* sp. and *Septoria citri* are the pathogens of greasy spot in N.S.W.

As a result of this study, the distribution, host range, symptomatology and fungal pathogenicity of greasy spot in New South Wales have been determined. Preliminary control measures have also been issued, subject to confirmation by spray trials.

PREFACE

The author wishes to acknowledge, with thanks, Professor B. J. Deverall, Department of Plant Pathology and Agricultural Entomology, University of Sydney, for advice during the planning and execution of experiments, and for editorial assistance with the manuscript preparation; Mr. J. Walker and Mrs. P. Barkley, Biology Branch, N.S.W. Department of Agriculture, for advice and encouragement during the investigation; Dr. P. T. W. Wong, Agricultural Research Centre, Tamworth, for editorial assistance, and Mrs. K. Jamieson for preparation of the typescript.

The work presented in this thesis is original and my own except where specifically noted in the text.

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CHAPTER 1
GENERAL INTRODUCTION

The citrus industry in New South Wales (N.S.W.) contributes a considerable proportion to the State's primary production. On a national basis, citrus fruit yielded in excess of 400,000 tonnes in 1977-78, of which 22,000 tonnes were exported fresh to overseas markets earning $6.5 million (Anon. 1978). The industry is therefore an important income earner locally and in export trade.

In order to survive, the citrus industry has needed to overcome many economic and physical threats. For example, a series of extremely wet seasons during the early fifties resulted in the rapid decline of citrus producing areas in N.S.W. The problem was identified as root rot due to the soil borne fungus, *Phytopthora citropthora* (Smith and Smith) Leon. Research showed that the use of *Poncirus trifoliata* as a rootstock afforded complete resistance to the fungus. This result provided the basis for the subsequent consolidation and expansion of the industry throughout the state.

Today, a number of diseases are again posing threats to the livelihood of citrus growers. Greasy spot is one such contemporary disease. Greasy spot has been recognised as a leaf disease of minor significance in N.S.W. since 1946 (Anon. 1946). In 1974, there was a considerable upsurge in disease occurrence in N.S.W. which was closely linked to increased defoliation. The disease was most apparent on grapefruit and some mandarin varieties. The increased disease occurrence coincided with higher than average spring rainfall and prolonged periods of high humidity. As a result of this sudden upsurge in greasy spot, an investigation of its nature and cause was initiated.

(i) Distribution and importance of greasy spot

Greasy spot on citrus has been reported in the literature by workers from various locations. In general, these reports were not authenticated with written and graphic descriptions or with deposited herbarium material. Therefore, such literature reports do not constitute an accurate record of disease occurrence (Walker, 1975). However, a compilation of literature reports of citrus greasy spot has not been previously published and therefore, Table 1 is presented as an indication of the reported world distribution of greasy spot.

Table 1 indicates that greasy spot occurs mainly in humid tropical locations in Asia, the Caribbean and the Americas. However, the disease has been recorded in less humid and cooler areas e.g. central provinces of India.

In Florida, greasy spot has had an important impact on the citrus industry. The disease was recognised in 1896, although changing management practices in the late 1940's and early 1950's resulted in severe economic losses due to greasy spot infection (Fisher, 1961). The principal effect on trees was severe defoliation and consequent yield reduction (Pratt, 1958). Reports also indicated that greasy spot caused a decline in fruit quality by reducing total juice solids (McCoy *et al.*, 1976). Estimates of economic loss due to infection have been in excess of $600 per acre (Lipp, 1974).

Despite the wide distribution and economic impact of greasy spot, comparatively few investigations have been concerned with defining disease symptoms and host range, the determination of its cause and the biology of the disease in the field.

TABLE 1. LITERATURE REPORTS OF GREASY SPOT ON CITRUS

Continental Region	Country	Nature of Report	Reference
Asia	Japan	A disease requiring a regular spray programme	Tanaka, 1968
	China	Caused considerable damage in southern areas	Lin, 1947
	India	First report in the central provinces	Galloway, 1935
	Philippine Islands	Reported as a disease of minor importance	Lee, 1922
Australasia	Australia	A disease in the inland areas of N.S.W.	Wellings & Walker 1976
	Wallis Island	Considered to be a very common disease	Dadant, 1952
Central America	U.S.A.	A disease of increasing importance	Fisher, 1957
	Bermuda Islands	First report of the disease on citrus	Ogilvie, 1926
The Caribbean	Trinidad	A common disease requiring control	Stell, 1937; Knott, 1967
	Porto Rico	Considered an important fungal disease	Cooke, 1924-25
	Venezuala	Reported as a prevalent disease	Knorr & Malaguti, 1964
	Surinam	Considered the most important citrus disease in the country	Childs, 1966
The Mediterranean	Morocco	A common but unimportant disease	Childs & Carpenter, 1960

(ii) Symptomatology of the disease

Although considerable research has been undertaken in Florida, there has been no attempt in recent literature to describe accurately symptoms in the field. However, earlier reports and extension articles indicate that in Florida, greasy spot symptoms were first noticeable as a yellow-green spot which became orange-yellow (Pratt, 1958). These spots became raised and changed colour from dark brown to black, suggestive of a drop of grease (Pratt, 1958). Symptoms were more frequent on leaves of the preceding year's growth and varied from small dots to lesions over 2 mm in diameter (Thompson, 1948). Larger blotches, consisting of coalesced lesions over 2 cm in diameter, occurred under conditions of severe infection (Thompson, 1948). Symptoms appeared on both sides of the leaf, although they were more prominent on the lower surface. Affected leaves often appeared yellow on the upper surface, particularly when infection was heavy (Pratt, 1958; Fisher, 1961). Lesions developing into depressed, necrotic areas have been observed (Fisher, 1961).

Similarly, greasy spot as reported in Japan consisted of dark brown-black, oily pustules surrounded by a translucent halo (Tanaka, 1968). These oily pustules were found mainly on the lower leaf surface and appeared to develop into small brown spots with necrotic centres (Tanaka, 1968). This latter symptom was more apparent towards the end of the season, although various intermediate stages were observed (Tanaka and Yamada, 1952).

It appears, therefore, that symptoms of greasy spot have been similarly described in the United States and Japan, while descriptions from other parts of the world are generally lacking. However, symptoms in the United States and Japan have not been studied on leaves of

different ages. Similarly, microscopic examination of field material has also been neglected in all published reports.

(iii) Aetiology of greasy spot

In Florida, few substantial investigations were carried out prior to 1940. However, most authors agreed that greasy spot was a physiological disorder (Fisher, 1961). Thompson (1948), as a result of use of insecticidal sprays and observations of symptom distribution in orchards, concluded that rust mites (*Phyllocoptruta deivora* (Ashm)) were the cause of greasy spot.

It was not until 1952 that Japanese workers first implicated a fungus as the cause of the disease (Tanaka and Yamada, 1952). A number of fungi were associated with diseased leaves although *Cercospora* and *Phyllostica* spp. were consistently isolated. The *Cercospora* sp. was also observed fruiting in the necrotic brown spots, producing conidia and conidiophores from a sub-epidermal stroma. This stroma later developed into an ascocarp of a *Mycosphaerella* sp. (Tanaka and Yamada, 1952). Pathogenicity tests, using *Cercospora* isolated from fresh lesions and also from cultures derived from *Mycosphaerella* ascospores, proved successful. A new species, *Mycosphaerella horii* (Hara) with a *Cercospora* sp. imperfect stage, was thus described as the casual agent of greasy spot in Japan (Tanaka and Yamada, 1952).

In Florida, Fisher (1957) presented a preliminary report which established the pathogen of greasy spot to be the *Cercospora* stage of a species of *Mycosphaerella*, thus confirming the Japanese reports. In a later paper, Fisher (1961) reported that a fungus was isolated from mesophyll tissue affected by the disease. The fungus was also observed sporulating in lesions which had become depressed and necrotic.

Inoculation with conidia suspended in a sugar solution gave rise to symptoms within 3 weeks on grapefruit seedlings. Re-isolations from lesions on inoculated leaves consistently yielded the original fungus. However, the grapefruit seedlings, which had been raised in growth chambers, did not show the characteristic raised lesions. A suggested explanation is the apparent need for light in symptom development in greasy spot (Calpouzos, 1966; Thompson, 1955). Despite the use of sugar solutions in pathogenicity tests, the results appear conclusive. A new species *Cercospora citri-grisea* (Fisher) was thus described as the pathogen (Fisher, 1961). However, Fisher did not make any mention of the role of the *Mycosphaerella* perfect stage which, in her earlier report, was stated to be the pathogen.

It was not until 1970 that further work by Whiteside continued the elucidation of greasy spot in Florida. He cultured a fungus from ejected ascospores, from surface hyphae on fresh leaves and from greasy spot lesions using Fisher's method (Whiteside, 1970). Macerated mycelia of the fungus were inoculated onto container grown seedlings which were humidified for 3-6 days and then kept in the glasshouse. Symptoms developed slowly, requiring 6-8 weeks on rough lemon seedlings, 2-4 months on sweet orange and up to 6 months on grapefruit. Symptoms produced on the seedlings were considered to be very similar to those appearing on diseased tissue in the field. Although Whiteside failed to re-isolate the fungus from inoculated leaves, the evidence suggests that his fungus was the pathogen.

Whiteside (1970) considered his fungus to be closely related to the cause of greasy spot in Japan (Yamada, 1956). On the advice of F. C. Deighton of the C.M.I., he considered the fungus to be more

closely related to the genus *Stenella* than to the genus *Cercospora* (Whiteside, 1970). However Whiteside did not recognise that Fisher's earlier descriptions of *Cercospora citri-grisea* fitted the same fungus that he was dealing with. Further taxonomic work has shown that the imperfect stage of the greasy spot pathogen in Florida may be designated as *Stenella citri-grisea* (J. Walker, *pers.comm.*).

Whiteside (1970) showed that the perfect stage of the greasy spot fungus was a species of *Mycosphaerella*. He considered the Florida specimens different from the Japanese descriptions of *Mycosphaerella horii* because of the absence of oil droplets in the Florida ascospores. However measurements of ascospores and asci are within the same range in both descriptions and it appears that the asexual stages are also very similar. In addition, Whiteside could not locate authentic type material of *Mycosphaerella horii* with which to compare his isolate of *Mycosphaerella* from Florida (Whiteside, *pers.comm.*). Although *Mycosphaerella citri* (Whiteside) was described as the pathogen in Florida (Whiteside, 1972*a*), further taxonomic work is needed in order to clarify the relationship between the pathogens isolated in Florida and Japan.

Recent work by Van Brussell (1975) has again raised the possible involvement of rust mites in greasy spot aetiology. On the basis of field observations, he postulated that the fungus and the insect had a close relationship in the disease complex. It was suggested that mites feeding on surface cells release cellular fluids and cause mechanical damage which aid fungal penetration. Mites, their cast skins and excrements were also considered to provide a nutrient base which aids fungal survival and penetration. However, experimental evidence for the relationship between the fungus and the insect is

lacking and therefore the hypothesis must be regarded as inconclusive.

(iv) Biology of the greasy spot fungus

There have been comparatively few thorough investigations of the biology and field behaviour of the greasy spot fungus. The majority of the work has been done by Whiteside studying greasy spot in Florida.

The epidemiology of the disease was studied using a number of methods. Symptom development was noted on tagged growth flushes during the spring and summer period. Container grown seedlings exposed in the orchard during this period and then allowed to incubate in the greenhouse developed symptoms (Whiteside, 1970). Examinations of leaf litter showed a marked increase in perithecial maturity during the spring-summer period, followed by a rapid decline in autumn as leaf substrate became exhausted. Results of spore trap data also indicated that air-borne ascospore populations peaked during spring and summer (Whiteside, 1970). It was noted that conidia of *Stenella* sp. were infrequently observed in the spore trap and it was concluded that they assume negligible importance in disease spread (Whiteside, 1970). On the basis of these results, it is clear that infection takes place during the spring-summer period in Florida citrus orchards (Whiteside, 1977). In Japan, symptoms did not develop from mid to late summer inoculations (Tanaka and Yamada, 1952). This may be due to the dry conditions during this period and explains why a single copper spray in spring makes disease control simple and effective (Tanaka, 1968). These results suggest that the greasy spot pathogens in Japan and Florida have a similar behaviour in the field.

Ascospores are liberated following periods of wetting by rain, irrigation or heavy dew (Whiteside, 1970). In addition, Whiteside

(1977) reported that ascospores are ejected high enough above the leaf litter to allow them to be carried by air currents into the tree canopy. This is a common phenomon in infection by Ascomycetes (Ingold, 1965). Ascospores germinated as relative humidity approached 100% and more rapidly as temperatures approached 30°C (Whiteside, 1974). These conditions favouring ascospore germination are commonly experienced during spring and summer in Florida.

The processes of infection were studied on leaves of *Citrus sinensis* (L.) Osbeck. cv. 'Pineapple' inoculated with hyphal fragments (Whiteside, 1972*a*). Ascospores were not used. Following inoculation, hyphal growth was rapid and within 48 hours stomatal penetration was beginning to occur. This resulted in the development of a single or multi-cellular appressorium-like structure in the outer stomatal chamber. A thin hypha then grew from the appressorium and a sub-stomatal vesicle was formed. Two to three weeks later, hyphae began to develop from the vesicle and to grow intercellularly, resulting in considerable hyphal development in the spongy mesophyll after 8 weeks. After 12 weeks, spongy mesophyll cells adjacent to hyphae became hypertrophic and tissue began to collapse and become necrotic (Whiteside, 1972*a*). An incubation period totalling 16 weeks was therefore necessary before symptoms occurred on inoculated seedlings. Less detailed inoculation experiments in Japan also demonstrated that the fungus ramified over the lower leaf surface and penetrated through stomata (Yamada, 1956).

(v) Conclusions

It appears that greasy spot in Japan and Florida are similar in terms of symptomatology and the described pathogens. With this background, an investigation of the occurrence, symptomatology and the association

of fungi with citrus greasy spot in N.S.W. was undertaken, in an attempt to establish the pathogen and ultimately to bring the disease under control.

CHAPTER 2

MATERIALS AND METHODS

1. DISTRIBUTION OF GREASY SPOT

(i) Collection of field material

Fresh leaf material was collected from orchards located in the inland citrus growing districts of N.S.W. Leaves were carefully picked to ensure minimal disturbance of the phylloplane. Collections were stored in plastic bags and kept between 5 and 10^{o}C while in transit to the laboratory. Leaves were examined and processed within a maximum of 5 days of collection. A minimum of 25 leaves were collected from 10 infected trees selected at random throughout each orchard. At each collection site, observations of soil type, irrigation method and general management programme were noted.

In addition to collections made at these field sites, samples were received from the 'Valencia' orange orchard at the Horticultural Research Station, Dareton, N.S.W., (Lower Murray District) throughout the season.

2. SYMPTOMATOLOGY OF THE DISEASE

Each field sample was examined visually in the laboratory and detailed descriptions and measurements of symptoms were made. Leaves were then placed under a dissecting microscope (Nikon Stereoscopic Microscope SMZ-2) and examined at ten times magnification using incident and transmitted light. Detailed notes of characteristics of lesions were made.

3. HISTOLOGY OF DISEASED TISSUE

(i) Stained whole mounts

Pieces of tissue up to 5 mm square were cut from leaves showing symptoms. These pieces were then stained in boiling alcoholic lactophenol

trypan blue for 10 minutes, as described by Jones and Deverall (1977). However, clearing using chloral hydrate was found to be unnecessary. Stained sections were then mounted in 50% aqueous glycerine and the coverslip sealed using Gurr's 'Glyceel'. The slides were examined under a compound microscope (Olympus EHT). Observations of characteristics of lesions and associated phylloplane micro-organisms were noted.

(ii) Transverse sections

Leaves showing symptoms were cut into pieces approximately 5 mm square. These pieces were transferred to glass vials containing formalin-acetic-alcohol fixative (FAA) which was prepared according to Anon. (1968). Fixation of tissue was achieved rapidly by placing the vials in a vacuum chamber for thirty minutes. Tissue pieces were then stored in capped vials, containing FAA, at room temperature until further processing was required.

When sectioning was required, the following procedure was used:

1) tissue pieces washed in running tap water for 4 hours.
2) pieces then allowed to soak in distilled water at $0^{o}C$ for 4 hours.
3) tissue transferred to a 10% aqueous solution of dimethyl sulphoxide (DMSO) and allowed to soak at $0^{o}C$ for 16 hours, or overnight.
4) pieces were then embedded in agar (20g/litre water) containing 2% DMSO. This was achieved by melting a small portion of the agar and then carefully transferring and mounting the tissue in the molten liquid.

The method was a modification of that used by O'Brien *et al.* (1976). The DMSO is an anti-freezing agent which was used to reduce shattering of tissue due to ice crystal formation.

Agar blocks, containing embedded tissue, were transferred to the cooling stage of the freezing microtome (Reichart model fitted with a Mectron 'Frigistor' electronic cooling stage). The block was trimmed and 5-10 micron sections were cut. The sections were transferred from the blade to distilled water in a watch glass. Using a fine pointed needle, individual sections were placed on a glass slide previously coated with egg albumen and flooded with distilled water. When all sections were located, the slide was transferred to a slide dryer to allow the water to evaporate and to ensure that the sections firmly adhered to the slide.

Sections were then stained using Heidenhain's iron haematoxylin (Humason, 1972) according to the following procedure:

1) slides placed in a mordant of 4% aqueous iron alum (30 minutes).
2) followed by a wash in running tap water (5 minutes).
3) sections then stained in haematoxylin (30 minutes).
4) followed by a wash in running tap water (5 minutes).
5) destaining, using 2% aqueous iron alum, was followed under the dissecting microscope until good differentiation was achieved (2-5 minutes).
6) slides were then dipped in distilled water containing 2 drops of ammonia to neutralise the sections.
7) this was followed by a final wash in running tap water (15-30 minutes).
8) slides were then dehydrated through an alcohol series to xylol and then mounted in Canada Balsam.

Sections were examined under the compound microscope and observations of cell structure, lesion formation and fungal hyphae were noted.

4. ISOLATION OF FUNGI FROM DISEASED LEAVES

(i) Isolations from fresh leaves

Two methods were used:

a) Leaf piece technique. This is a modification of the method used by Fisher (1961). The procedure is as follows:

1) both leaf surfaces were swabbed with 95% alcohol and allowed to dry.
2) using a sterile scalpel, a longitudinal cut was made through the leaf tissue, thus exposing a transverse section of the lesion.
3) under a dissecting microscope, mesophyll cells from in and around the lesion were removed with the point of a sterile scalpel blade. These cells were then directly transferred to a sterile petri dish containing potato dextrose agar (PDA).
4) the plates were allowed to incubate for 7-10 days on the laboratory bench (23-25^{o}C).

b) Surface sterilisation of whole lesions, according to the following method:

1) pieces of tissue, 5 mm square, were cut from leaves and placed in a solution of 1:1,000 mercuric chloride in 10% aqueous alcohol. The pieces were shaken in this solution for 60-90 seconds.
2) tissue pieces were then washed in 3 changes of sterile distilled water.
3) a sterile scalpel was used to cut longitudinally through the lesion and the pieces were then transferred to P.D.A.

4) the plates were allowed to incubate as above.

Using both techniques described above, 40 isolations were made from each sample.

(ii) Isolations from leaf litter associated with greasy spot

Samples were collected from the sites previously described and from the 'Valencia' orange orchard at the Horticultural Research Station, Dareton. Where greasy spot lesions were present on fresh leaves, leaf litter from under affected branches was collected. Where no greasy spot was found, the collection was made at random. In both cases, composite samples, from at least 10 trees per orchard were placed in open plastic bags and kept at room temperature until examined. Samples were stored in the laboratory for up to 6 weeks without deterioration of the fungal flora.

Examination of the decaying leaves revealed a *Mycosphaerella* sp. to be the most abundant ascomycete. The following method was devised to determine the maturity and viability of the perithecia and ascospores:

1) pieces of tissue approximately 5 mm square and containing groups of characteristic perithecia were cut from the decaying leaves.
2) the leaf pieces were floated on sterile distilled water for 60 seconds and adhered to the lids of petri dishes containing tap water agar.
3) the plates were allowed to incubate at room temperature overnight and the agar surface was examined for the presence of germinating ascospores.

Fifty leaf pieces, each containing a group of perithecia, were taken from each sample to estimate the percentage of viable *Mycosphaerella* sp. perithecia.

5. PATHOGENICITY OF FUNGI ASSOCIATED WITH GREASY SPOT

The main fungi found consistently associated with greasy spot in the field were tested for pathogenicity. The infection processes of these fungi on citrus leaves were also observed.

(i) Inoculum preparation

Isolates of *Colletotrichum gloeosporioides* (Penz.) Sacc. (DAR 29822, DAR 29823, DAR 29824), obtained from leaves bearing greasy spot symptoms, were maintained on water agar containing sterile carnation leaf pieces. Cultures were regenerated using germinated single conidia. After incubating cultures for 10 days on the laboratory bench at approximately 25°C, conidia were suspended in 50 ml sterile distilled water and centrifuged at 3,000 r.p.m. for 10 minutes. Forty ml of supernatant were carefully decanted and the conidia resuspended in 40 ml of fresh sterile distilled water. Conidia were washed 3 times by this procedure to ensure that there was no nutrient carryover in the conidial inoculum. The final suspension was adjusted to a concentration of 2×10^7 spores per ml using a haemocytometer.

Cultures of *Septoria citri* Pass. (DAR 29826(b), DAR 30594, DAR 30723, DAR 31981), isolated from greasy spot lesions, were maintained by transferring single germinated conidia to potato dextrose agar containing 0.1% vegemite yeast extract (PVDA). After incubating cultures for 10-15 days on the laboratory bench at approximately 25°C, conidial suspensions were prepared as described above and adjusted to a concentration of 2×10^7 spores per ml.

Isolates of *Mycosphaerella* sp. (DAR 30870, DAR 30875, DAR 30877, DAR 30878 and unaccessioned isolate M96) were grown from single germinated ascospores obtained from perithecia on decaying leaves. An additional culture (isolate M8), obtained from greasy spot lesions, was also used in the study. After 10-15 days growth on PVDA, mycelium was aspetically removed

from the surface of the culture and suspended in 10 ml sterile distilled water. The suspension was then macerated in an "Omni-Mix" blender for 10 seconds.

(ii) Inoculation of excised leaves

Initial studies of spore germination were carried out on excised unexpanded leaves of *Citrus sinensis* Osbeck cv. Ruby Blood. Leaves were placed in sterile petri dishes containing sterile moist filter paper.

Conidial suspensions of *Colletotrichum gloeosporioides* and *Septoria citri*, prepared as described above, were sprayed onto the leaves leaving a uniform distribution of fine droplets on the surface. Leaf pieces containing ascocarps of *Mycosphaerella* sp. were wetted, as described previously, and adhered to the lid of a petri dish. Ascospores were thus ejected onto the leaf surface which had been previously wetted with a fine mist of sterile distilled water. The inoculated leaves were then allowed to incubate in the petri dishes on the laboratory bench (23-25^{o}C).

(iii) Inoculation of seedlings and trees

Container grown seedlings of *Citrus sinensis* cv. Ruby Blood, and container grown budded trees of *C.sinensis* cv. Lanes Late Navel and *C.paradisi* Macf. cv. Marsh were used in inoculation experiments. Branches were pruned to allow young growth to develop. When these leaves were approximately 6 weeks old, inoculum was applied using a compressed gas spray applicator to the lower leaf surface. Each seedling or tree branch received a uniform application of 50 ml of inoculum. In each series of experiments, sterile water was sprayed on leaves to serve as a control treatment.

Following inoculation, plants were kept at 100% relative humidity for 10 days at 25°C. This was achieved using an industrial humidifier (Defensor 2002) to supply a constant fine mist to plants in an enclosed, clear-plastic chamber. After 10 days, the plants were then subjected to morning and evening periods of mist for 4 hours duration. This continued for a further 14 days after which plants were returned to the glasshouse. Ten weeks after inoculation, a portion of the seedlings and trees were planted in nursery rows in the field during autumn.

(iv) Sampling techniques

At regular intervals after each inoculation, samples were taken to monitor the processes of infection. Leaf pieces, 5 mm square, were stained in alcoholic lactophenol-trypan blue and mounted in 50% aqueous glycerine, as described previously. In addition, transverse sections were cut freehand with a sharp razor blade and mounted in 0.1% acid fucshin in lactophenol. This latter technique provided a more rapid method for immediate determination of fungal penetration.

6. DOCUMENTATION

Specimens collected in the field were pressed, details noted and accessioned in the N.S.W. Department of Agriculture Biology Branch Herbarium (Herb. DAR). Symptom descriptions and slides were filed with the specimens from which they were made. Accession numbers quoted in the Appendix will allow future workers to trace the specimens, the descriptions and the slides on which results are based.

CHAPTER 3

DISTRIBUTION OF GREASY SPOT IN N.S.W.

A number of field collecting trips to the inland citrus growing districts of N.S.W. were made during 1977. Fresh leaf material was collected during autumn-early winter and again in spring. Specimens collected in previous seasons, and accessioned in Herb. DAR, were closely examined to determine the wider distribution of greasy spot in N.S.W.

1. OCCURRENCE WITHIN N.S.W.

Field collections and specimens accessioned in Herb. DAR were examined in order to determine the distribution of greasy spot in N.S.W. Results are presented for each of the major citrus producing districts.

(i) Coastal areas

A number of herbarium specimens which were labelled 'greasy spot' had been collected from orchards in this district. Symptoms ranged from superficial speckling to large, black tarry spots. It was apparent that gross visual symptoms had been used for the diagnosis. However, all specimens, which were examined microscopically, failed to show characteristic features, such as stomatal necrosis and chlorotic haloes. In addition, fungi were rarely isolated by the original collectors of the specimens.

(ii) Central west

The principal citrus growing district is centred around Narromine. Herbarium specimens collected during October 1976 revealed the presence of greasy spot in close association with Septoria fruit spot. All specimens revealed typical symptoms of greasy spot, as described later

in this chapter, on a range of citrus varieties including orange, grapefruit and lemon. However, the Herbarium notes revealed that no fungi were isolated from lesions on leaves.

(iii) Murrumbidgee Irrigation Area (M.I.A.)

Citrus production in this area is centred around Griffith, Leeton and Narrandera. Field collections and observations in these areas showed greasy spot to be scattered, both between and within orchards. Symptoms were found on trees growing on a range of soil types, from Cudgel sands to the heavier loam soil of Stanbridge, and under differing management practices. Typical symptoms were found on all specimens, and a characteristic range of fungi were isolated as described later in this chapter.

(iv) Mid-Murray irrigation districts

These districts consist of scattered groups of orchards located along the Murray River. From field collections, greasy spot was again noted to be variable in distribution both between and within orchards. However, typical symptoms were found on the whole range of commercial varieties and a characteristic range of fungi were isolated.

(v) Lower-Murray irrigation districts

Commercial citrus production in this area is found in irrigation districts located along the lower reaches of the Murray River in south-west N.S.W. Some districts are located further north along the Darling River. Specimens collected from a range of citrus varieties in these districts showed characteristic symptoms and yielded a similar range of fungi. Infection, in terms of disease incidence, leaf drop and tree debilitation, appeared to be most severe in these districts.

The distribution of greasy spot within N.S.W. is illustrated in Figure 1, and has been based on authenticated records of disease occurrence.

2. DISTRIBUTION WITHIN DISTRICTS

In an attempt to understand the variable distribution of the disease throughout the inland growing districts, field site characteristics were observed and are presented in Table 2.

Results suggest that management is the main factor determining disease distribution within the M.I.A. and the mid-Murray locations. In the M.I.A., furrow irrigation, lack of fungicide spray programme and bare soil between trees were associated with increased disease incidence. In the mid-Murray, orchard neglect and consequent lack of tree vigour were associated with increased severity. The orchards sampled at Barham, in the mid-Murray, showed little to no disease incidence due to good orchard management including routine autumn copper sprays.

However, greasy spot in the lower-Murray locations was generally abundant. In these districts, disease was more prevalent in situations of low fungicide usage, although symptoms were quite prevalent even in orchards with high management.

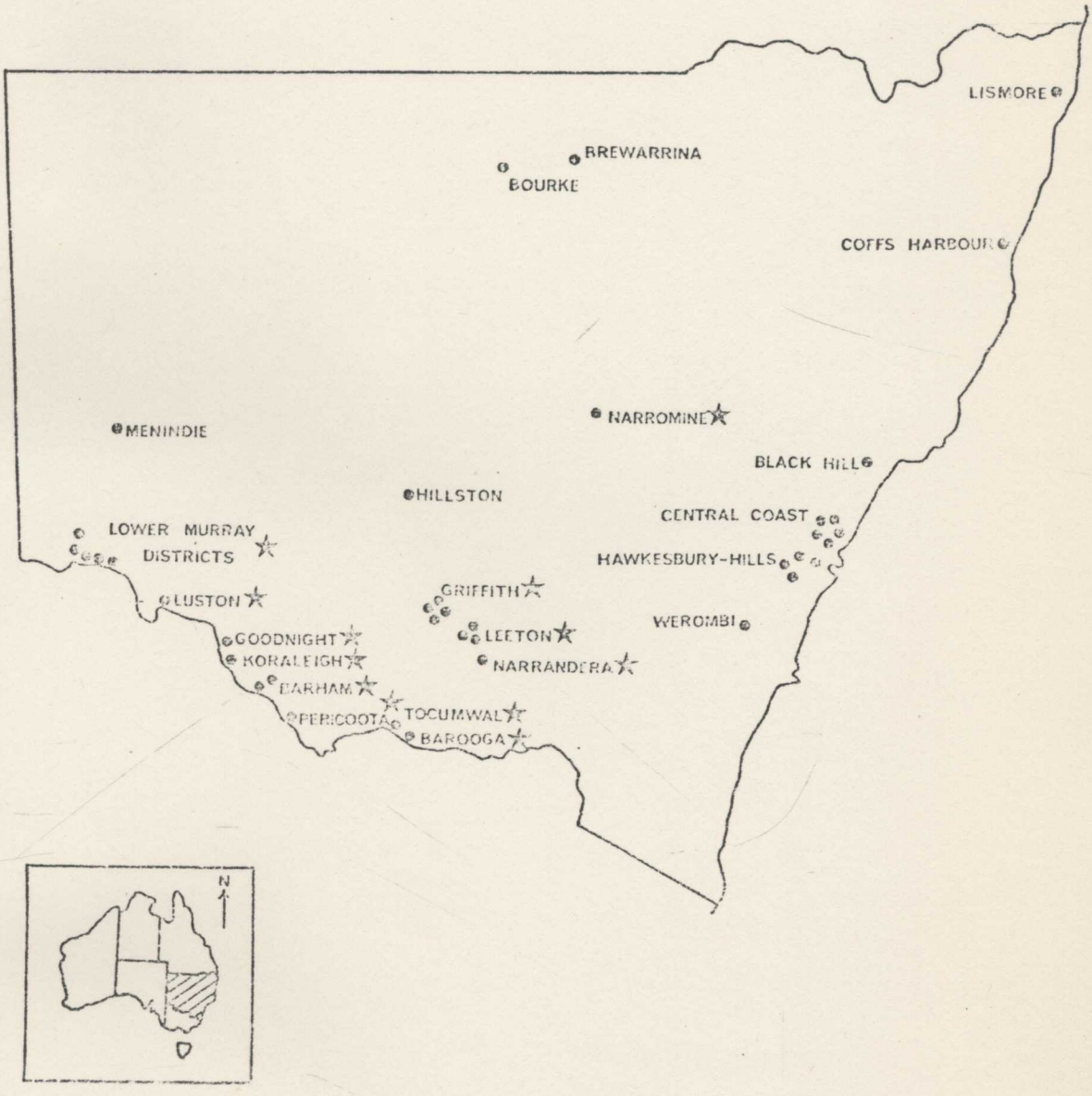


FIGURE 1. Occurrence of greasy spot in the citrus producing districts of New South Wales. The distribution is based on specimens lodged in Herb. DAR.

★ Indicates districts recorded with greasy spot.

TABLE 2. Results of observations made at field collection sites.

District	Orchard	Soil Type	Irrigation Method	Disease* Incidence	Management
M.I.A.	Mr. P.Clancy, Cudgell	Sandy Loam	Overhead	++	i) Vegetative cover between rows. ii) Little under tree cultivation resulting in accumulated leaf litter. iii) Copper oxychloride sprayed in autumn for Septoria control.
M.I.A.	Mr.V.Protheroe, Wamoon	Sandy Loam	Furrow	+++	i) Blanket herbicide applied leaving bare soil between rows. ii) Accumulated leaf litter. iii) No fungicides applied.
M.I.A.	Mr.J.Naylor, Stanbridge	Loam	Overhead	++	i) Vegetative cover between rows. ii) Considerable snail activity resulting in minimal leaf litter. iii) No fungicides applied.
Mid-Murray	Mr. Bailey, Barooga	Loam	Overhead	++++	i) Vegetative cover between rows. ii) Accumulated leaf litter. iii) No fungicides applied.
Mid-Murray	Mr. Backhaus, Barooga	Sandy Loam	Overhead	++++	i) Vegetative cover between rows. ii) Accumulated leaf litter. iii) No fungicides applied.
Mid-Murray	Mr. L. Keamy, Barooga	Sany Loam	Overhead	++	i) Vegetative cover between rows. ii) Leaf litter accumulation variable, depending on snail activity. iii) Bordeaux mixture applied in autumn.

TABLE 2. (continued)

District	Orchard	Soil Type	Irrigation Method	Disease* Incidence	Management
Mid-Murray	Mr. F. Durrand	Sandy Loam	Overhead	-	i) Inter-row cultivation leaving bare soil between rows. ii) Minimal leaf litter under trees. iii) Copper oxychloride applied in autumn for Septoria control.
Mid-Murray	Mr. N. Eagle, Barham	Sandy Loam	Overhead	-	i) Inter-row cultivation leaving little vegetative cover between rows. ii) Leaf litter accumulation variable, although generally abundant. iii) Copper oxychloride applied in autumn.
Mid-Murray	Mr. B. O'Farrel, Barham	Sandy Loam	Overhead	-	i) Inter-row cultivation. ii) Leaf litter generally abundant. iii) Copper oxychloride applied in autumn.
Lower-Murray	Mr. N. Kirwin, Morquong	Sandy Loam	Overhead	++++	i) Inter-row cultivation. ii) Abundant leaf litter. iii) Copper oxychloride applied during autumn and spring.
Lower-Murray	Mr. J. Mills, Buronga	Sandy Loam	Overhead	++++	i) Herbicides applied between rows leaving bare soil. ii) Abundant leaf litter. iii) No fungicides applied.
Lower-Murray	Mr. J. Whyte	Clay Loam	Furrow	+++	i) Inter-row cultivation. ii) Accumulated leaf litter. iii) Copper sulphate applied in autumn for snail control.

*Disease incidence :
- No symptoms could be found.
+ Diseased leaves occasionally detected.
++ Disease confined to leaves around the base of the tree.
++++ Diseased leaves observed throughout the three canopy.

CHAPTER 4

SYMPTOMATOLOGY AND HISTOLOGY

1. SYMPTOMATOLOGY OF THE DISEASE

Notes and observations for each specimen examined have been filed in Herb. DAR and are listed in the Appendix. As a result of these 11 detailed studies the pattern of symptom development in the field became apparent.

Initial spots became noticeable to the unaided eye on the underside of leaves as small, black specks up to 0.5 mm in diameter. At this stage, chlorotic spots associated with the specks occasionally appeared scattered on the upper surface of the leaf (Plate 1a). This was particularly noticeable in the field on grapefruit. When viewed in transmitted light under the dissecting microscope, bright chlorotic haloes were found consistently around smaller black specks on the lower leaf surface (Plate 1b).

Lesions appeared to develop into more extensive necrosis, with individual spots measuring 1-2 mm in diameter. The spots became distinctly raised, giving the appearance of oil drops (Plate 1c). Chlorosis in surrounding tissue remained evident.

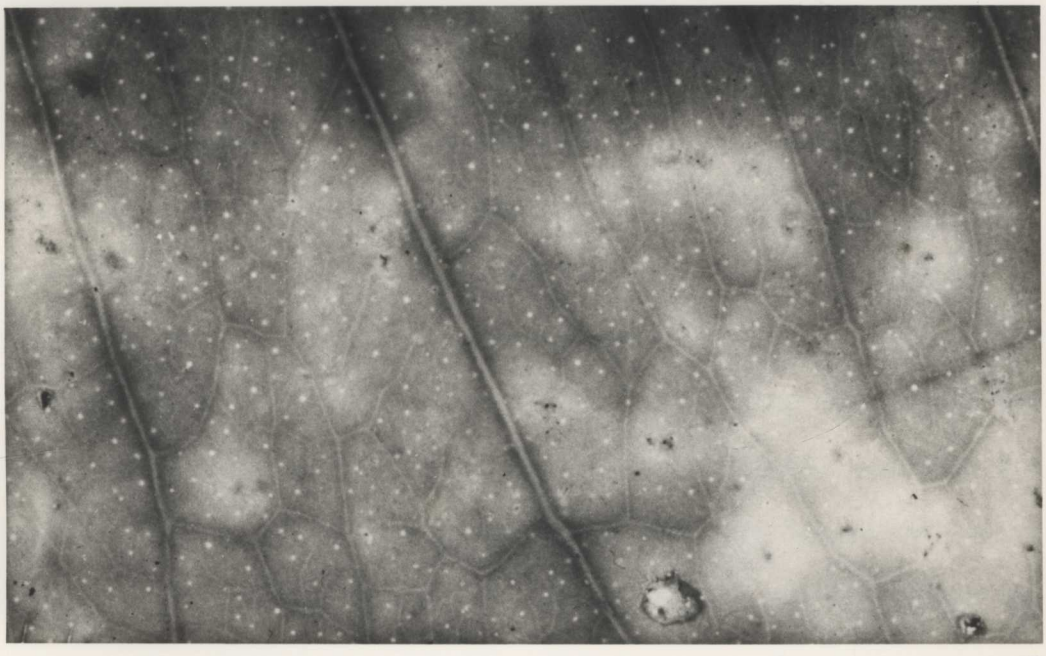
The final stage occurred as extensive, blister-like lesions up to 3 mm in diameter (Plate 1c). Individual lesions were less raised than the intermediate stages described above and the chlorotic haloes were less distinct. In many cases, necrosis extended through the entire leaf tissue resulting in black spots on the upper and lower leaves.

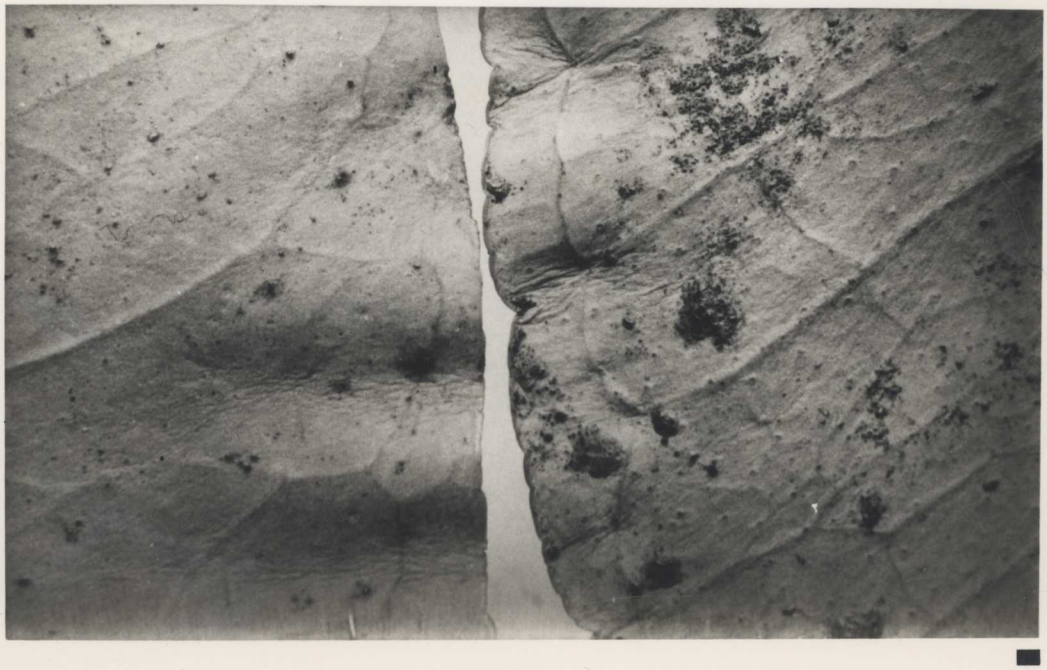
The three stages of symptom development were often found together on leaves which were greater than 8 months of age. Symptoms were not found on immature growth flushes. Leaves of 4 to 8 months showed

PLATE 1a Small black specks on the undersurface of a grapefruit leaf. Chlorotic spots associated with these specks occasionally appear on the upper surface of grapefruit leaves.
(Bar represents 1 cm).

PLATE 1b Chlorotic haloes surrounding small black specks when viewed in transmitted light.
(Bar represents 1 mm).

PLATE 1c Raised spots on the left develop into more extensive, blister-like lesions on the right.
(Bar represents 1 mm).





predominantly young and intermediate stages, while those greater than 12 months tended to have a predominance of the final stages of symptom expression.

Lesion numbers on leaves were variable, ranging from masses of spots to a few lesions. The spots were either uniformly distributed over the leaf surface or confined to one side of the mid-rib. Occasionally, lesions occurred in small discrete areas scattered over the leaf surface, suggesting a prior distribution of water droplets containing infective propagules.

Symptoms were observed on all commercial varieties of citrus. Table 3 shows the incidence of greasy spot in Lower Murray orchards during surveys from 1975 to 1977. In this area, where disease occurrence is very high, all the common commercial varieties of citrus show a high incidence of infection, generally ranging from 70 to 90%. Observations made in the arboretum at the Horticultural Research Station, Dareton, showed that some citrus types are probably immune to infection, despite being surrounded by diseased trees. These varieties, which were *Microcitrus* sp., *Citrus macrophylla*, *Citremon* sp., *Poncirus trifoliata* selection and *Erymocitrus glauca*, are not used in commercial fruit production.

2. HISTOLOGY OF DISEASED TISSUE

(i) Stained whole mounts

Small specks, which were invisible to the naked eye but were observed under the dissecting microscope, proved to be necrotic stomatal guard cells. Fungal activity associated with these necrotic guard cells was variable, although mycelium was in general noted to be present (Plate 2a). Hyphae were noted to penetrate guard cells and sometimes

TABLE 3. Incidence of greasy spot in Lower-Murray orchards during 1975, 1976 and 1977 surveys.

Variety	Orchards surveyed showing greasy spot	Total number of orchards surveyed	% Orchards showing greasy spot
'Valencia' orange	16	22	72.7
'Navel' orange	9	10	90
Grapefruit	13	19	68.4
Mandarin	12	15	80
Lemon	6	7	85.7
Tangelo	5	6	83.3

to produce swollen structures in the outer stomatal chamber. Mesophyll tissue immediately below these necrotic guard cells occasionally retained the trypan blue stain.

When symptoms first became visible to the naked eye, necrosis was observed in epidermal and mesophyll cells adjacent to necrotic stomata. Mycelium was observed on the surface, and swollen hyphal structures were apparent in stomata. Retention of stain in cells surrounding the necrotic area rarely occurred. No hyphae were observed penetrating beyond the necrotic stomates.

As lesions reached the intermediate stage of symptom expression, individual necrotic areas comprised a number of necrotic guard cells and intervening epidermis and mesophyll. Mycelium on the surface was rare and no hyphae were observed in mesophyll tissue.

Large blister-like spots on older leaves consisted of extensive necrotic epidermis and mesophyll cells, with surrounding individual necrotic stomata. Mycelium was rare to absent on the leaf surface, and was not observed in the mesophyll. Remnants of hyphal structures were occasionally seen in stomatal chambers.

Mycelium observed on the leaf surface could not be identified, although dark appresoria characteristic of *Colletotrichum gloeosporioides* were noted around lesions and also on symptomless tissue (Plate 2a).

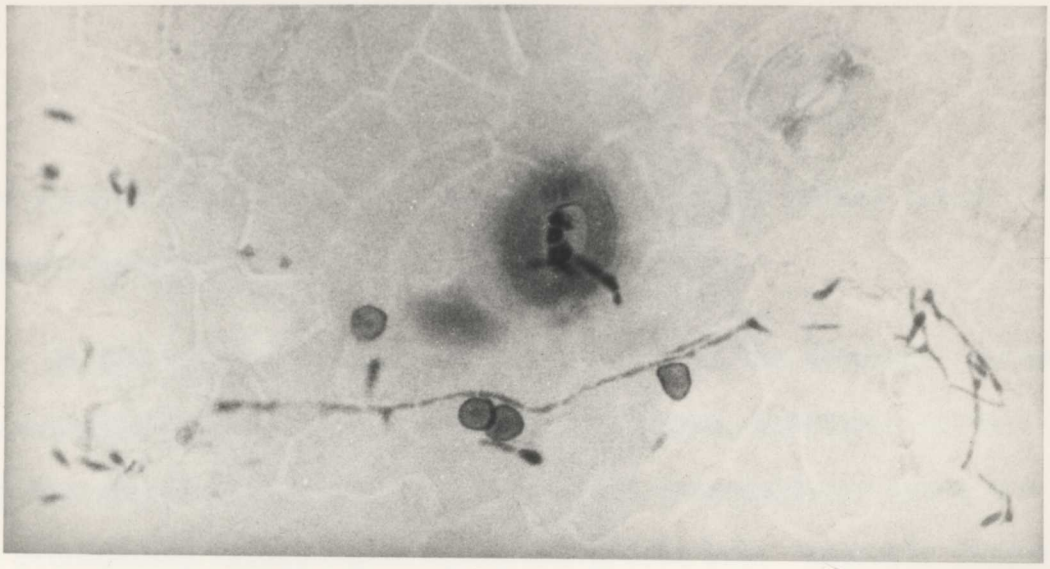
(ii) Transverse sections

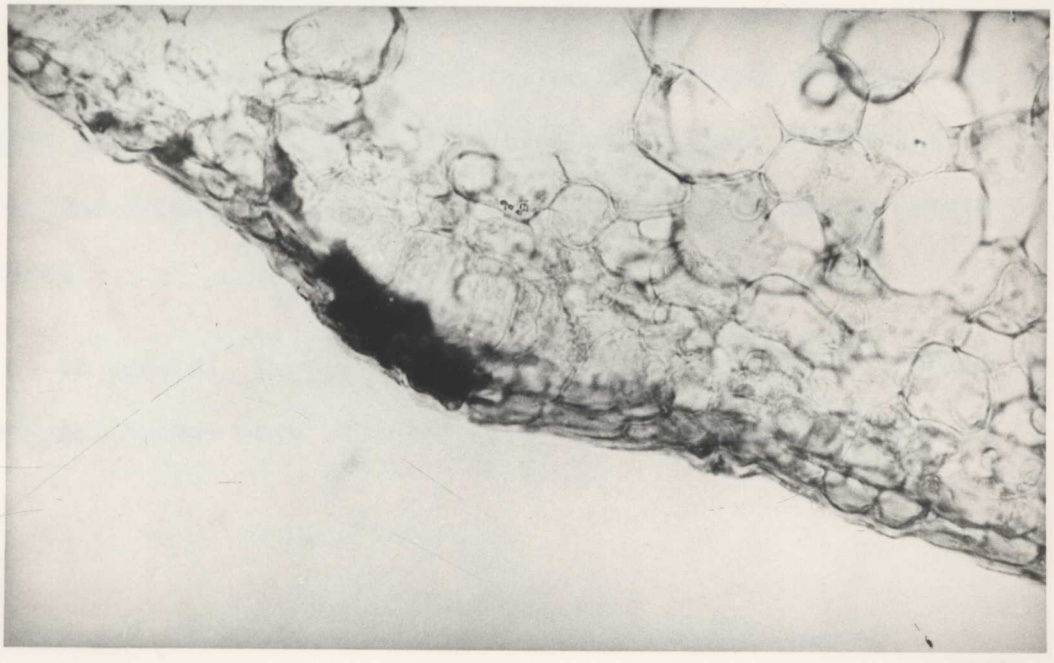
Sections through small, early stage leaf spots revealed necrosis beginning in epidermal and adjacent mesophyll cells. Occasionally, symptoms of cellular disorganisation and the beginning of cell enlargement were noted in tissue surrounding the necrotic epidermis (Plate 2b). This

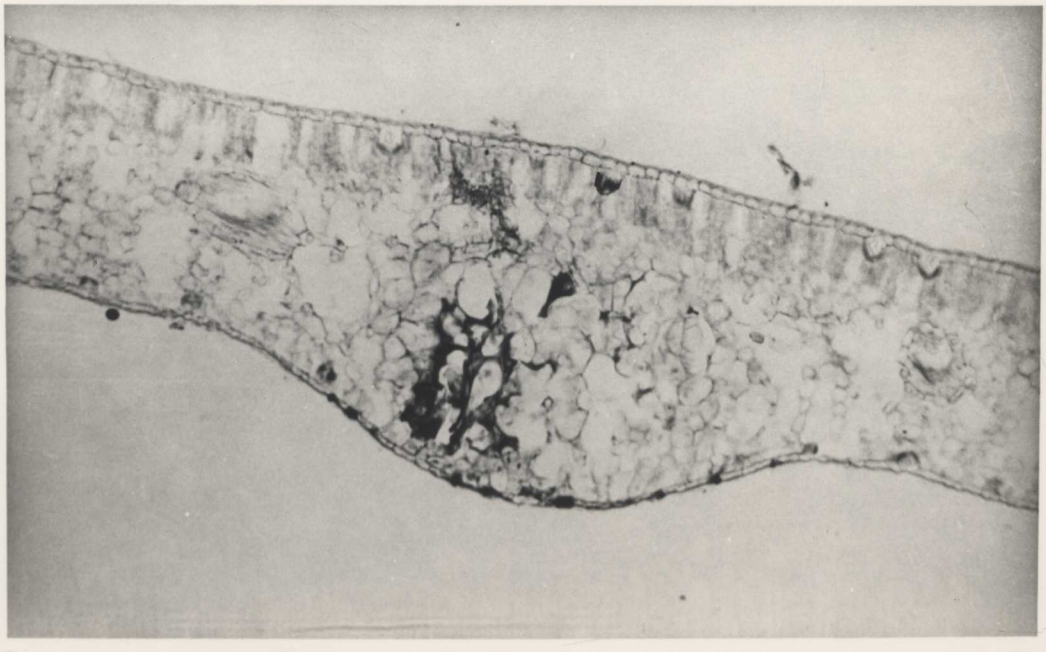
PLATE 2a Stained whole mount of small black specks showing necrotic stomatal guard cells and fungal infection structures. *Colletotrichum gloeosporioides* appressoria can be seen on the healthy tissue. (Bar represents 10μ).

PLATE 2b Transverse section through an early stage spot showing some cellular disorganisation in advance of necrosis. Cell enlargement is also beginning in the immediate mesophyll tissue. (Bar represents 10μ).

PLATE 2c Transverse section through a raised black spot showing cell enlargement and extensive necrosis in affected mesophyll tissue. (Bar represents 10μ).







could explain the chlorotic haloes and the slightly raised nature of the early symptoms.

As lesions became more advanced, cells in the mesophyll became more extensively affected. These cells enlarged, sometimes up to 3-4 times the normal size, resulting in reduced intercellular space and a more pronouncely raised lower epidermis (Plate 2c). Necrosis became more extensive with cell death.

Finally, necrosis reached the palisade layer and upper epidermis At this stage, the extent of elevation of the spot diminished, possibly due to the breakdown of cell walls and consequent loss of turgor pressure.

In general, fungal hyphae were rarely observed in stained sections. Occasional hyphae were observed slightly in advance of necrosis.

CHAPTER 5
ISOLATION OF FUNGI FROM DISEASED LEAVES

1. ISOLATIONS FROM FRESH LEAVES

Results of isolations from fresh field material collected from the previously described field sites and using two methods of surface sterilisation are presented in Table 4 and Table 5. These results are illustrated graphically in Figure 2 and Figure 3. Fungi isolated were generally restricted to 3 species, plus a collection of miscellaneous fungi which were regarded as saprophytic.

Colletotrichum gloeosporioides was commonly isolated in spring and autumn sampling using mercuric chloride surface sterilisation. However, the isolation frequency appeared to be less in the lower-Murray districts using this technique. The use of the leaf-piece procedure noticeably reduced the isolation frequency of *C.gloeosporioides* from all districts at both times of sampling.

Septoria citri was isolated less frequently. However, results in Chapter 3 indicated that greasy spot was less prevalent in orchards where Septoria fruit spot was under control with fungicides. In addition, the latent infection of tissue by *S.citri* may result in a lower isolation frequency. Therefore, this fungus was still considered to be closely associated with the disease. Results indicate that mercuric chloride surface sterilisation produced a slightly greater isolation frequency, compared to the leaf-piece technique. The fungus was more commonly isolated from the lower-Murray district and, with one exception, was more prevalent in the spring sampling.

Mycosphaerella sp. isolates were identified on cultural morphology

TABLE 4. Isolation frequency of fungi from greasy spot lesions using mercuric chloride surface sterilisation.

District	Site	Variety	Date	% Fungi Isolated* C	S	M	Others
M.I.A.	1 Naylor	'Valencia' orange	30.3.77	50	-	-	20
			3.10.77	65	10	-	-
	2 Clancy	'Valencia' orange	30.3.77	65	-	-	-
			3.10.77	90	-	-	-
	3 Protheroe	Grapefruit	30.3.77	70	-	-	15
			3.10.77	100	-	-	-
Mid-Murray	4 Keamy	Grapefruit	31.3.77	100	-	-	-
			4.10.77	45	-	10	-
	5 Bailey	'Navel' orange	31.3.77	80	-	-	-
			4.10.77	55	-	-	-
	6 Backhaus	'Valencia' orange	31.3.77	100	-	-	-
			4.10.77	70	-	-	-
Lower-Murray	7 Kirwin	Grapefruit	24.5.77	30	-	30	5
			6.10.77	-	5	15	15
	8 Mills	Grapefruit	24.5.77	30	10	20	10
			6.10.77	-	5	10	-
	9 Whyte	Grapefruit	24.5.77	80	-	5	-
			6.10.77	16	-	-	-

*Number of colonies developing from 20 lesions plated on PDA expressed as a percentage.

C = *Colletotrichum gloeosporioides*

S = *Septoria citri*

M = *Mycosphaerella* sp.

TABLE 5. Isolation frequency of fungi from greasy spot lesions using the leaf-piece technique

District	Site	Variety	Date	% Fungi Isolated* C	S	M	Others
M.I.A.	*1* Naylor	'Valencia' orange	30.3.77	-	-	-	-
			3.10.77	-	5	-	-
	2 Clancy	'Valencia' orange	30.3.77	-	-	-	-
			3.10.77	-	-	-	5
	3 Protheroe	Grapefruit	30.3.77	-	-	-	-
			3.10.77	10	-	-	-
Mid-Murray	*4* Keamy	Grapefruit	31.3.77	-	-	-	5
			4.10.77	-	-	-	-
	5 Bailey	'Navel' orange	31.3.77	-	-	-	-
			4.10.77	15	-	-	-
	6 Backhaus	'Valencia' orange	31.3.77	-	-	-	-
			4.10.77	5	-	-	-
Lower-Murray	*7* Kirwin	Grapefruit	24.5.77	-	-	10	-
			6.10.77	-	-	-	-
	8 Mills	Grapefruit	24.5.77	-	-	-	-
			6.10.77	-	5	20	-
	9 Whyte	Grapefruit	24.5.77	-	-	-	-
			6.10.77	-	-	-	-

*Number of colonies developing from 20 lesions plated on PDA expressed as a percentage.

C = *Colletotrichum gloeosporioides*
S = *Septoria citri*
M = *Mycosphaerella* sp.

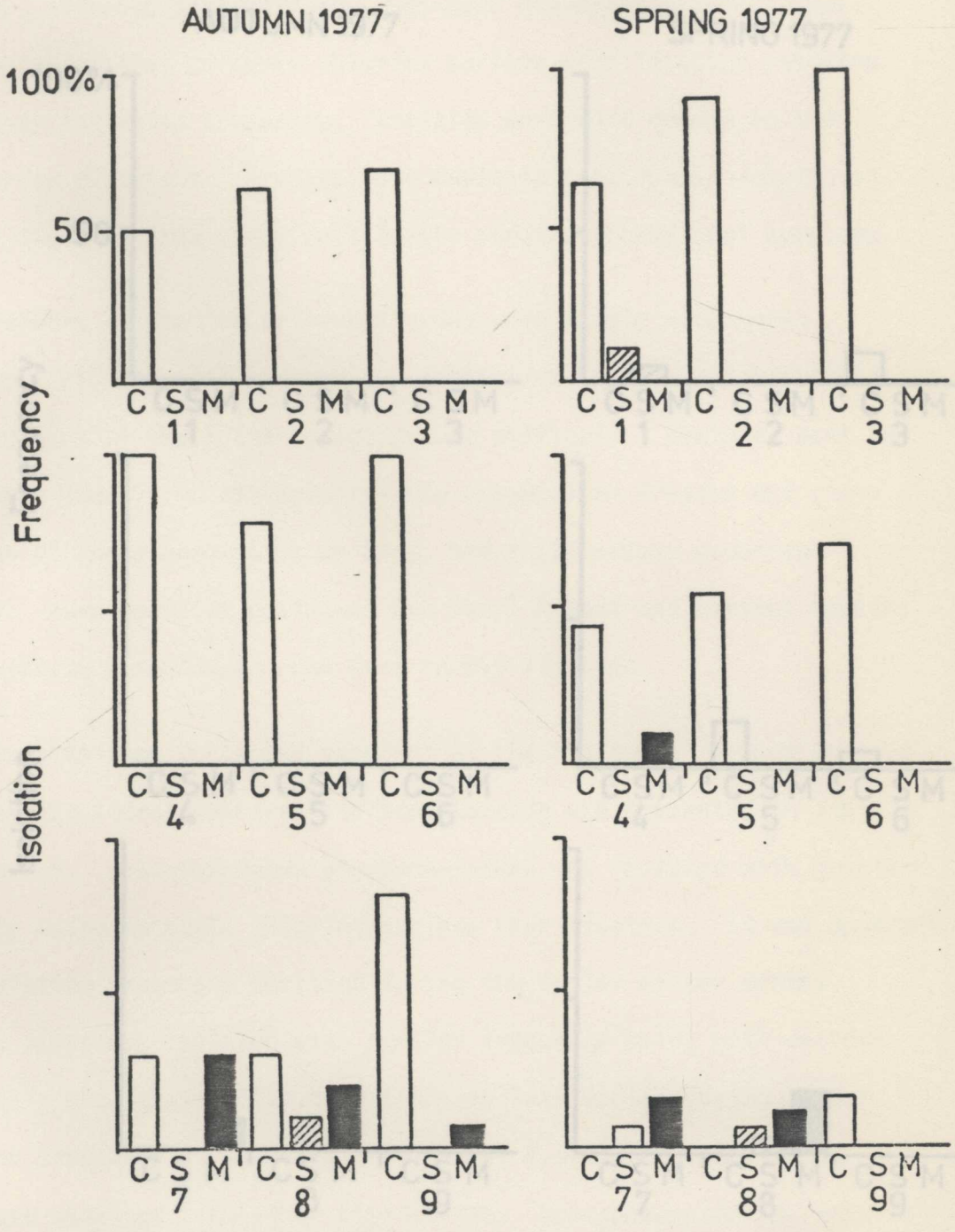


FIGURE 2. Fungi isolated from greasy spot affected leaves at 2 times of the year using mercuric chloride surface sterilisation.

Isolation frequency represents the number of colonies growing from lesions expressed as a percentage of 20 lesions plated on PDA. The horizontal axis indicates the 3 main genera of fungi isolated from various hosts at different locations, according to the accompanying legend.

C = *Colletotrichum gloeosporioides*

S = *Septoria citri*

M = *Mycosphaerella* sp.

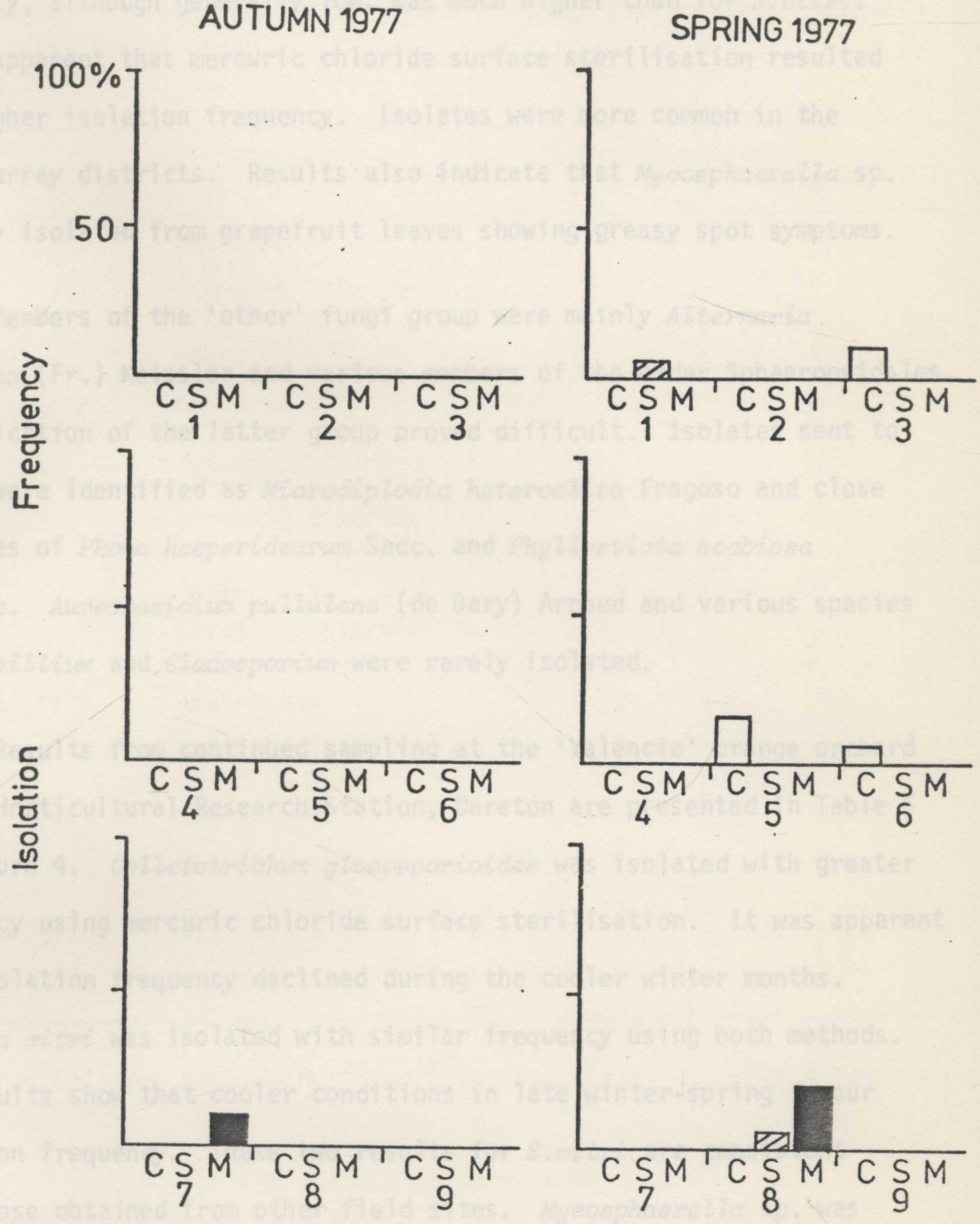


FIGURE 3. Fungi isolated from greasy spot affected leaves at 2 times of the year using the mesophyll cell culture technique.

Isolation frequency represents the number of colonies growing from lesions expressed as a percentage of 20 lesions plated on PDA. The horizontal axis indicates the 3 main genera of fungi isolated from various hosts at different locations, according to the accompanying legend.

C = *Colletotrichum gloeosporioides*

S = *Septoria citri*

M = *Mycosphaerella* sp.

in comparison with those obtained from ascospore cultures. Isolation frequency, although generally low, was much higher than for *S.citri*. It was apparent that mercuric chloride surface sterilisation resulted in a higher isolation frequency. Isolates were more common in the lower-Murray districts. Results also indicate that *Mycosphaerella* sp. was only isolated from grapefruit leaves showing greasy spot symptoms.

Members of the 'other' fungi group were mainly *Alternaria alternata* (Fr.) Keissler and various members of the order Sphaeropsidales. Identification of the latter group proved difficult. Isolates sent to C.M.I. were identified as *Microdiplodia heteroclita* Fragoso and close relatives of *Phoma hesperidearum* Sacc. and *Phyllosticta scabiosa* McAlpine. *Aureobasidium pullulans* (de Bary) Arnaud and various species of *Penicillium* and *Cladosporium* were rarely isolated.

Results from continued sampling at the 'Valencia' orange orchard at the Horticultural Research Station, Dareton are presented in Table 6 and Figure 4. *Colletotrichum gloeosporioides* was isolated with greater frequency using mercuric chloride surface sterilisation. It was apparent that isolation frequency declined during the cooler winter months. *Septoria citri* was isolated with similar frequency using both methods. The results show that cooler conditions in late winter-spring favour isolation frequency. These two results for *S.citri* are consistent with those obtained from other field sites. *Mycosphaerella* sp. was not isolated from 'Valencia' orange leaves which is also consistent with the previous results.

2. ISOLATIONS FROM LEAF LITTER ASSOCIATED WITH GREASY SPOT

Examinations of leaf litter samples collected from under trees infected with greasy spot, revealed a number of fungi including

TABLE 6. Isolation frequency of fungi from greasy spot lesions sampled in the 'Valencia' orange orchard at the Horticultural Research Station, Dareton.

Date	Method of Isolation	% Fungi Isolated* C	S	M	Others
January 1977	leaf-piece	5	-	-	-
	mercuric chloride	35	-	-	-
March 1977	leaf-piece	-	-	-	-
	mercuric chloride	80	-	-	-
May 1977	leaf-piece	-	-	-	-
	mercuric chloride	65	-	-	-
July 1977	leaf-piece	-	5	-	10
	mercuric chloride	30	20	-	-
September 1977	leaf-piece	-	10	-	5
	mercuric chloride	10	10	-	-
December 1977	leaf-piece	-	-	-	-
	mercuric chloride	100	-	-	-
February 1978	leaf-piece	5	-	-	-
	mercuric chloride	75	-	-	-

*Number of colonies developing from 20 lesions plated on PDA expressed as a percentage.

C = *Colletotrichum gloeosporioides*

S = *Septoria citri*

M = *Mycosphaerella* sp.

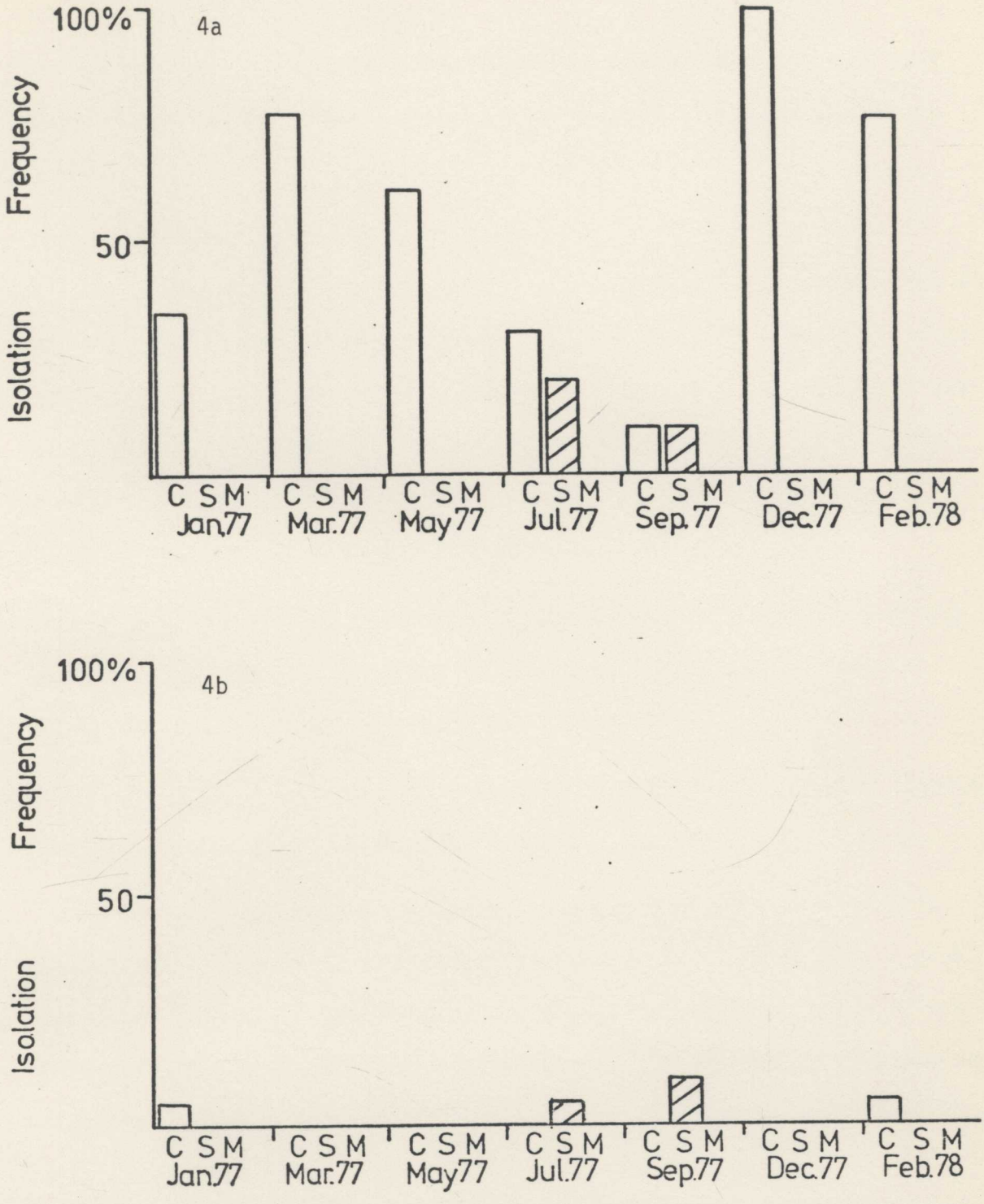


FIGURE 4. Fungi isolated from Valencia orange leaves affected with greasy spot. The leaves were collected from the Horticultural Research Station, Dareton (Lower-Murray district). Figure 4a shows results using mercuric chloride surface sterilisation. Figure 4b shows results using the mesophyll cell culture technique.

Isolation frequency represents the number of colonies growing from lesions expressed as a percentage of 20 lesions plated on P.D.A. The horizontal axis indicates the 3 main genera of fungi isolated at diffetent times of the year, according to the accompanying legend.

C = *Colletotrichum gloeosporioides*

S = *Septoria citri*

M = *Mycosphaerella* sp.

Colletotrichum gloeosporioides and *Septoria citri*. However, the most abundant ascomycete was a *Mycosphaerella* sp. previously unrecorded in N.S.W.

(i) Description of *Mycosphaerella* sp.

Ascocarps found on decomposing citrus leaves, occurring mainly on the undersurfaces of leaves in dense groups 1-10 mm in diameter; often but not always, associated with greasy spot lesions. The ascocarps are subepidermal, erumpent and consist of an ascostroma with 1-3 separate pseudothecia, broadly ellipsoidal in shape, 65-110 (mostly 80-85) µ in diameter, each with a papillate ostiole; paraphyses absent.

Asci bitunicate, grouped in fascicles within the pseudothecium; generally fusiform to obclavate in shape, 31.5-40.5 x 5-7.5 (mostly 35-40 x 5-6.5) µ, and containing 6-8 ascospores.

Ascospores hyaline with a single non-constricting central septum; fusiform to obclavate in shape 8-13 x 2.5-3 (mostly 10-11 x 2.5) µ.

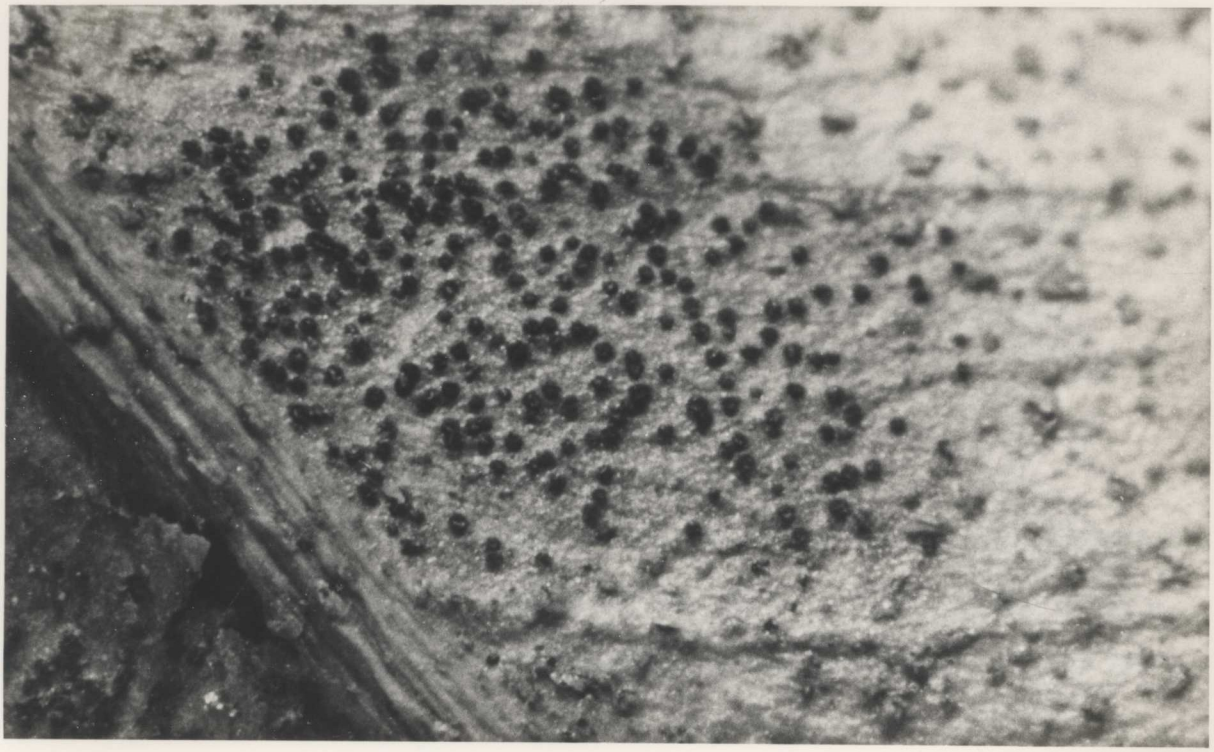
In culture, single germinated ascospores produce dark grey to greenish-black colonies with occasional white aerial mycelium; a pink diffusing pigment is sometimes produced particularly when cultures are incubated in the light; growth rate is slow, 17-28 (mostly 22-26)mm in diameter at 25°C after 14 days on ¼ P.D.A.; no fruiting structures developed in culture.

Specimens examined in Herb.DAR: On *Citrus paradisi*, Buronga N.S.W., v 1977, C. Wellings (30870)*; on *Citrus sinensis*, Morquong N.S.W., v 1977, C. Wellings (30874); on *Citrus paradisi*, Buronga N.S.W., v 1977, C. Wellings (30875)*; on *Citrus sinensis*, Barooga N.S.W., iv 1977, C. Wellings (30877)*; on *Citrus sinensis*, Barooga N.S.W., iv 1977, C. Wellings (30878)*.

*indicates cultures examined.

PLATE 3a Appearance of *Mycosphaerella* sp. ascocarps on decaying citrus leaf litter.
(Bar represents 1 mm).

PLATE 3b Transverse section through *Mycosphaerella* pseudothecia showing typical Loculoascomycete characters, and papillate ostiole.
(Bar represents 10µ).



Macro and micro characters of the *Mycosphaerella* sp. are illustrated in Plate 3.

(ii) Epidemiology of *Mycosphaerella* sp.

Perithecia of the *Mycosphaerella* sp. were assessed for maturity according to the described method during 1977. Table 7 shows results for assessments of perithecial maturity at the described field sites during autumn and spring. It appears that there is little relation between time of sampling and perithecial maturity for either 'Valencia' orange or grapefruit. In general, ascospores were found to be viable during both autumn and spring. By cross reference to Table 2 it can be concluded that *Mycosphaerella* sp. was consistently found fruiting on leaf litter under trees infected with greasy spot. This was particularly noticeable in mid-Murray locations where litter examined from greasy spot-free orchards were noted to have no perithecia of *Mycosphaerella* sp.

The results of continual assessment of perithecial maturity at one location are presented in Table 8 and Figure 5. A chi-square test on this data was significant at $P > 0.001$. This indicates that the peaks of perithecial maturity during autumn and spring represent real trends.

TABLE 7. Assessment of perithecial maturity on leaf litter collected in N.S.W. orchards during 1977.

District	Site	Variety	% Viable Perithecia* Autumn 1977	Spring 1977
M.I.A.	Naylor	'Valencia' orange	4	2
	Clancy	'Valencia' orange	2	2
	Protheroe	Grapefruit	48	36
Mid-Murray	Bailey	'Valencia' orange	74	14
	Backhaus	'Valencia' orange	12	30
	Keamy	Grapefruit	42	76
	Keamy	Lemon	4	4
	Durrand	'Valencia' orange	No perithecia	
	Eagle	'Valencia' orange	"	
	O'Farrel	'Navel' orange	"	
Lower-Murray	Kirwin	'Valencia' orange	54	24
	Kirwin	Grapefruit	4	60
	Mills	Grapefruit	38	20
	Whyte	Grapefruit	30	48
	Whyte	'Valencia' orange	0	42

*The number of leaf litter segments each containing characteristic groups of *Mycosphaerella* sp. perithecia, which ejected viable ascospores under the described conditions are expressed as a percentage of the total of 50 segments tested.

TABLE 8. Assessment of perithecial maturity on leaf litter collected from the 'Valencia' orange orchard at the Horticultural Research Station, Dareton.

Date	% Viable Perithecia*
November 1976	12
December 1976	12
January 1977	0
March 1977	28
May 1977	12
July 1977	10
September 1977	46
December 1977	10

χ^2 87.4 with 7 degrees of freedom is significant at $P > 0.001$.

*The number of leaf litter segments, each containing characteristic groups of *Mycosphaerella* sp. perithecia, which ejected viable ascospores under the described conditions are expressed as a percentage to the total of 50 segments tested.

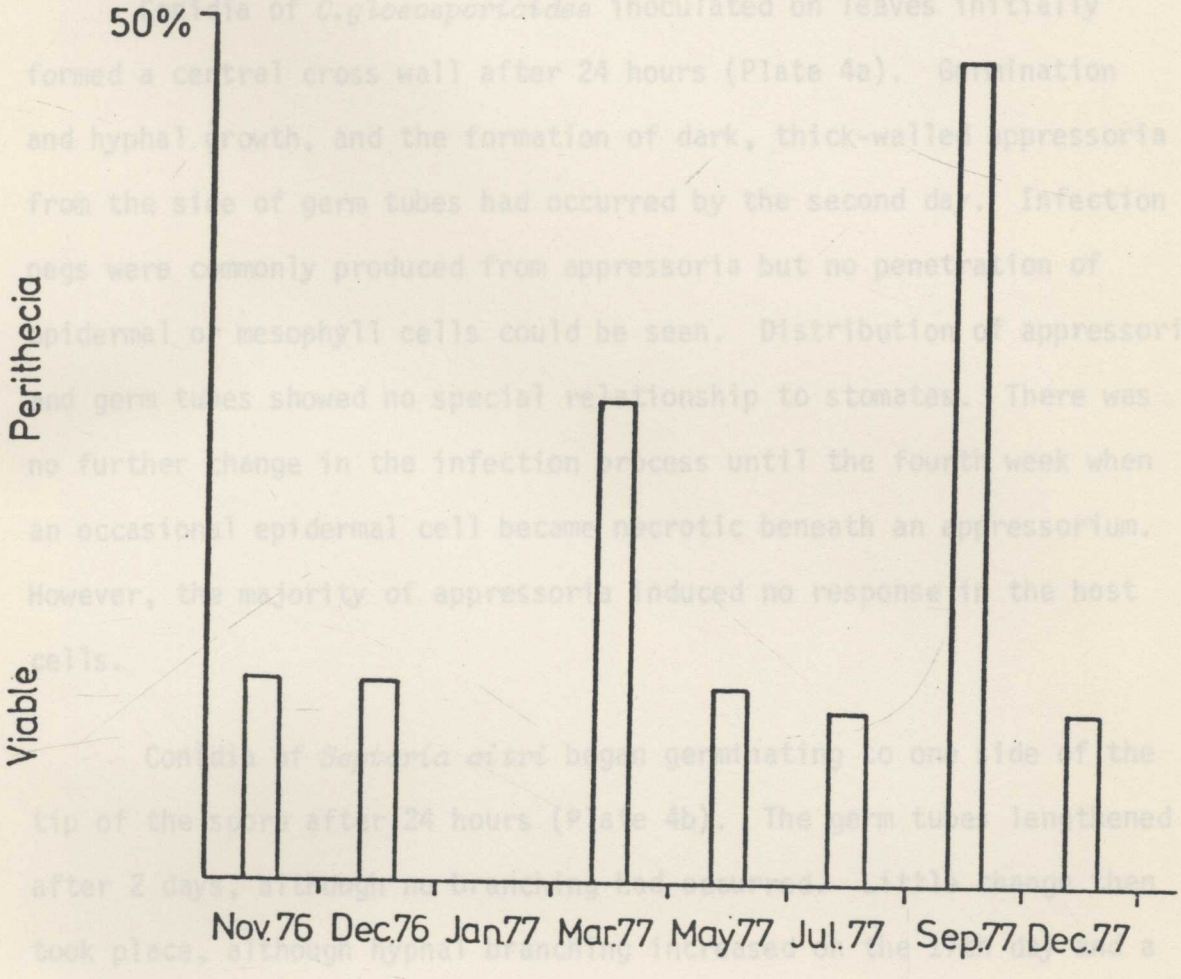


FIGURE 5. Percentage of viable perithecia sampled from Valencia orange leaf litter. The leaves were collected from the Horticultural Research Station, Dareton (Lower-Murray district).

Viable perithecia indicates the number of leaf litter segments each containing characteristic groups of *Mycosphaerella* sp. perithecia, which ejected viable ascospores under test conditions. The number is expressed as a percentage of the total of 50 segments tested.

CHAPTER 6

PATHOGENICITY OF FUNGI ASSOCIATED WITH GREASY SPOT

1. INOCULATION OF EXCISED LEAVES

Conidia of *C.gloeosporioides* inoculated on leaves initially formed a central cross wall after 24 hours (Plate 4a). Germination and hyphal growth, and the formation of dark, thick-walled appressoria from the side of germ tubes had occurred by the second day. Infection pegs were commonly produced from appressoria but no penetration of epidermal or mesophyll cells could be seen. Distribution of appressoria and germ tubes showed no special relationship to stomates. There was no further change in the infection process until the fourth week when an occasional epidermal cell became necrotic beneath an appressorium. However, the majority of appressoria induced no response in the host cells.

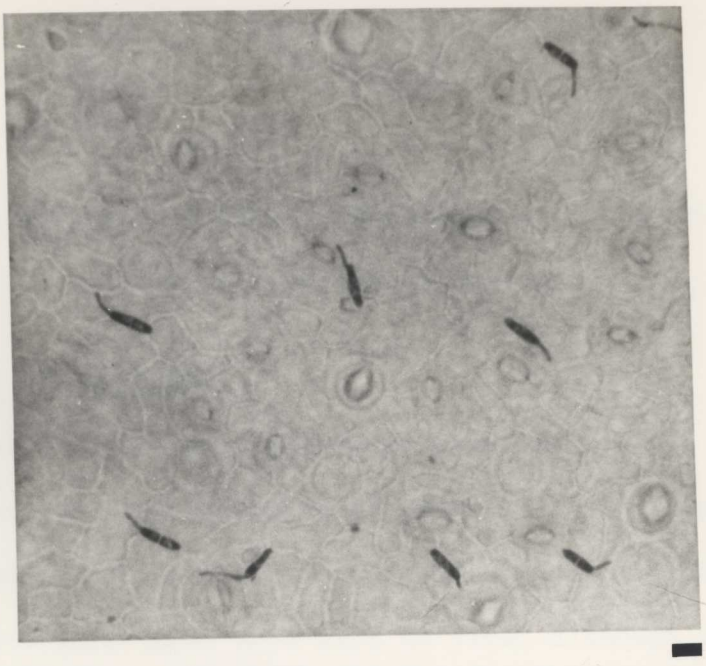
Conidia of *Septoria citri* began germinating to one side of the tip of the spore after 24 hours (Plate 4b). The germ tubes lengthened after 2 days, although no branching had occurred. Little change then took place, although hyphal branching increased on the 17th day and a small number of hyphae began to directly penetrate stomata (Plate 4c). However, after 5 weeks no further developments occurred and no response to the limited stomatal penetration was observed.

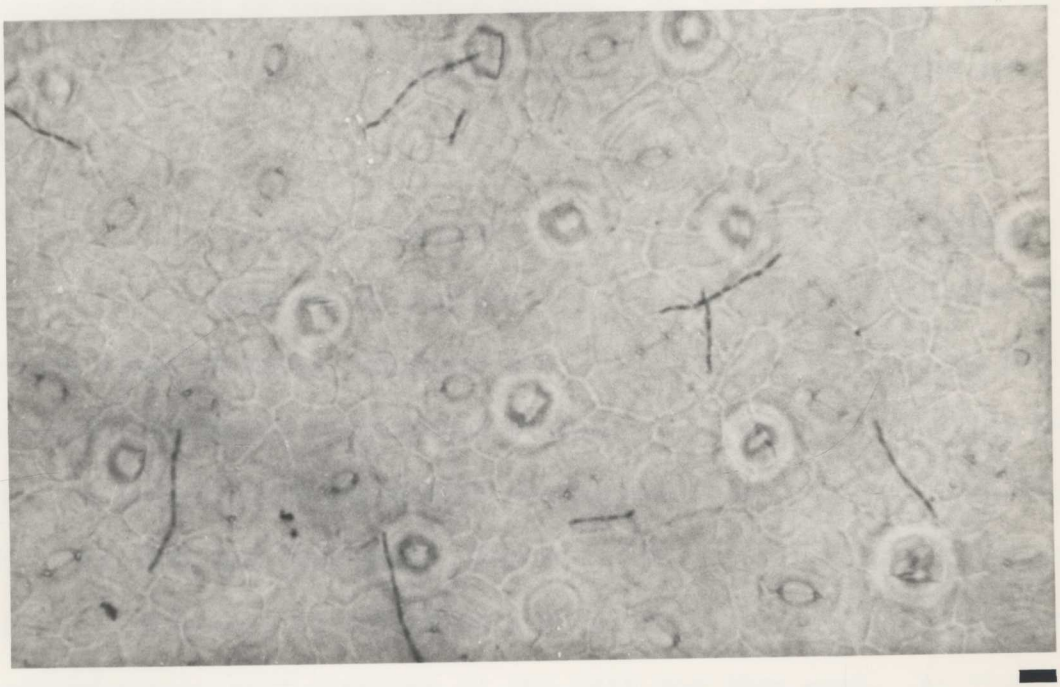
Mycosphaerella ascospores ejected onto the leaf surface showed bi-polar germination after 3 days. Germ tubes grew randomly over the surface, showing no directional growth towards stomata. After 1 week, considerable hyphal branching had occurred with some hyphae developing swollen tips while others appeared to directly penetrate stomata. Little change occurred after 3 weeks, with no cellular reaction to

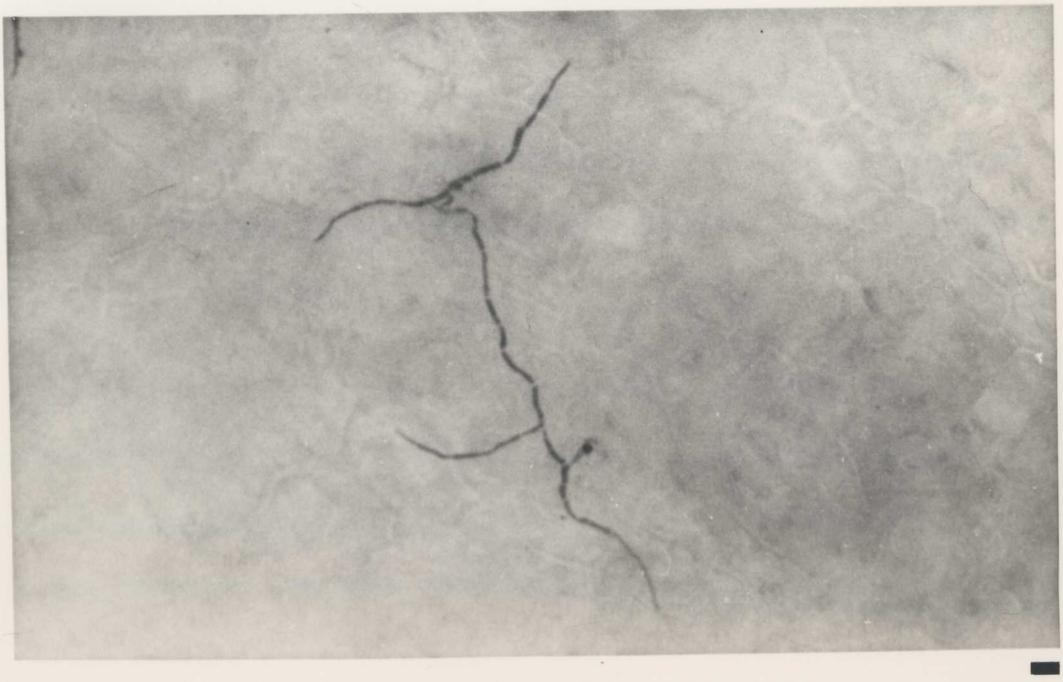
PLATE 4a Germination of *Colletotrichum gloeosporioides* on leaves after 24 hours. Conidia form cross walls before the germ tube emerges.
(Bar represents 10μ).

PLATE 4b Germination of *Septoria citri* on leaves after 24 hours.
(Bar represents 10μ).

PLATE 4c *Septoria citri* on inoculated leaves occasionally show a direct penetration of stomatal cavities.
(Bar represents 10μ).







occasional stomatal penetration, no formation of distinctive infection structures and no sporulation from the surface hyphae.

2. INOCULATION OF SEEDLINGS

The three isolates of *C.gloeosporioides* applied to sweet orange seedlings showed the same processes of germination and appressorium formation as was observed on excised leaves. However, after the 6th week, conidia and germ tubes were hard to find, although appressoria remained evident on leaves for 4 months. At the end of this period, no cellular reaction and therefore no observable symptoms had been produced.

All the isolates of *Septoria citri* showed a similar patten of germination as observed on excised leaves. Hyphae ramified over the leaf surface after 15 days. Isolates DAR 31981 and DAR 30723 began to form infection structures in stomates at week 6 and week 12. Isolate DAR 30594 produced no infection structures and hyphae became difficult to find after the 12th and 18th week. However, the former two isolates showed a greater frequency of infection structures at week 18 and both had produced greasy spot symptoms at the 24th and 30th week. At these stages, necrosis of guard cells and some adjacent epidermis and mesophyll had occurred in response to infection, although hyphae could not be seen penetrating beyond stomata. With these two isolates, only one of three inoculated seedlings showed symptoms and in both cases *Septoria citri* could not be re-isolated.

Inoculum of *Mycosphaerella* sp. used in seedling inoculations consisted of mycelial fragments in contrast to ascospores used on excised leaves. However, after the 2nd week hyphae had developed from the fragments and were ramifying over the leaf surface, although

no conidia were produced. A limited number of unicellular infection structures were observed in stomatal cavities with all isolates. However, at this stage there was no cellular response to the infection. The number of infection sites had increased by the 4th week (Plate 5a), with the structures becoming multicellular and stomatal guard cells showing some necrosis at the 10th week (Plate 5b). By week 18, necrosis of invaded stomata was very common and greasy spot symptoms were formed by 4 of the 6 isolates. The fifth isolate caused symptoms at the 24th week, but isolate DAR 30877 failed to show any infection structures and yielded no symptoms after the 30th week. *Mycosphaerella* sp. was re-isolated in 3 cases from 5 where leaf tissue was showing symptoms. Transverse sections of leaves showing symptoms at the 30th week revealed that the raised lesions contained cells with increased size. Hyphae were observed in transverse sections in stomatal cavities and occasionally in mesophyll cells. Plate 5c illustrates symptoms produced on sweet orange seedlings 30 weeks after *Mycosphaerella* sp. inoculation.

Table 9 summarises pathogenicity data for all fungi tested. *Mycosphaerella* sp. can be regarded as strongly pathogenic, while *Septoria citri* was less so. As a result of these tests *Colletotrichum gloeosporioides* appears to have no role as a pathogen in greasy spot of citrus.

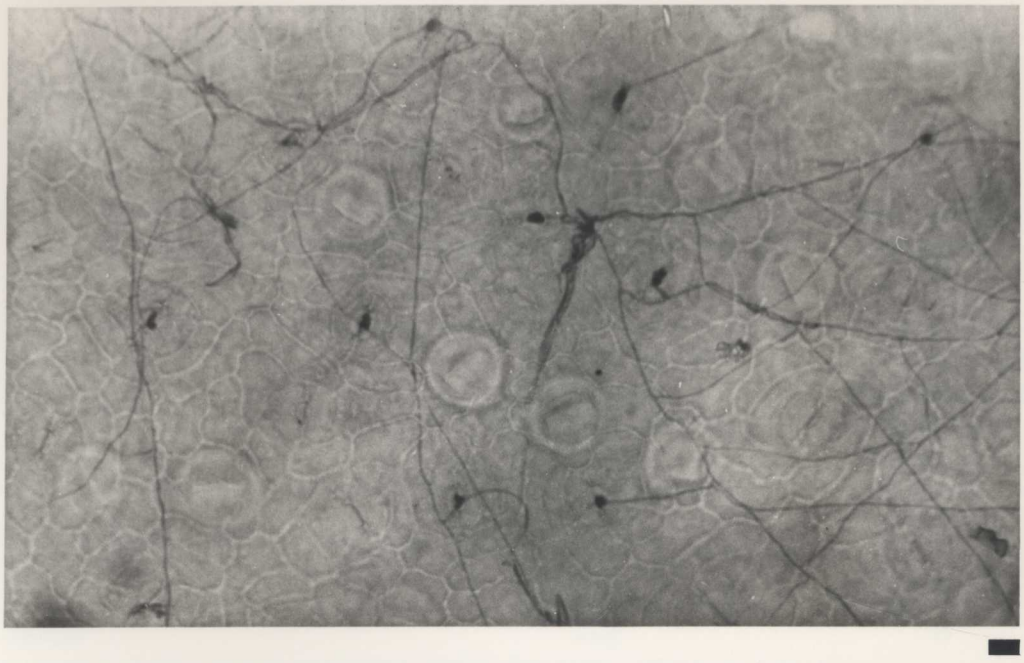
3. INOCULATION OF SEEDLINGS AND TREES IN THE FIELD

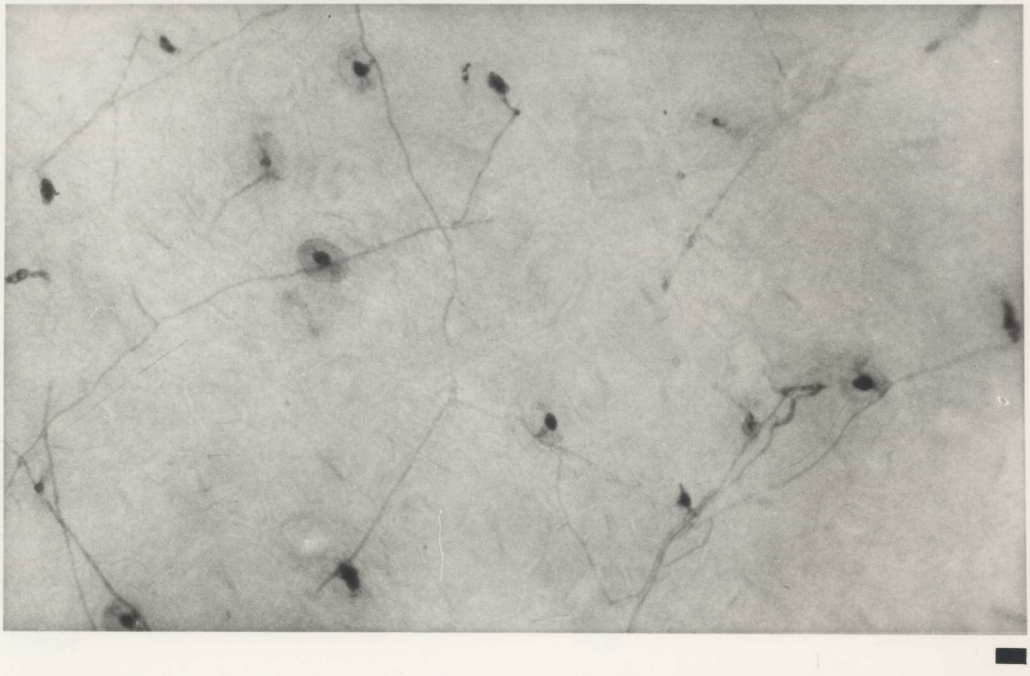
Container grown seedlings and trees were inoculated in the glasshouse with isolates of *C.gloeosporioides*, *S.citri* and *Mycosphaerella* sp. and then planted in the field. However, in all cases, conidia and hyphae could not be found on the leaf surface and no symptoms developed after the 30th week.

PLATE 5a Infection structures of *Mycosphaerella* sp. observed in stomatal cavities 4 weeks after inoculation.
(Bar represents 10µ).

PLATE 5b Invaded stomatal guard cells showing necrosis 10 weeks after inoculation with *Mycosphaerella* sp.
(Bar represents 10µ).

PLATE 5c Symptoms produced on sweet orange leaves (cv. Ruby Blood) following inoculation with *Mycosphaerella* sp.
(Bar represents 1 cm).





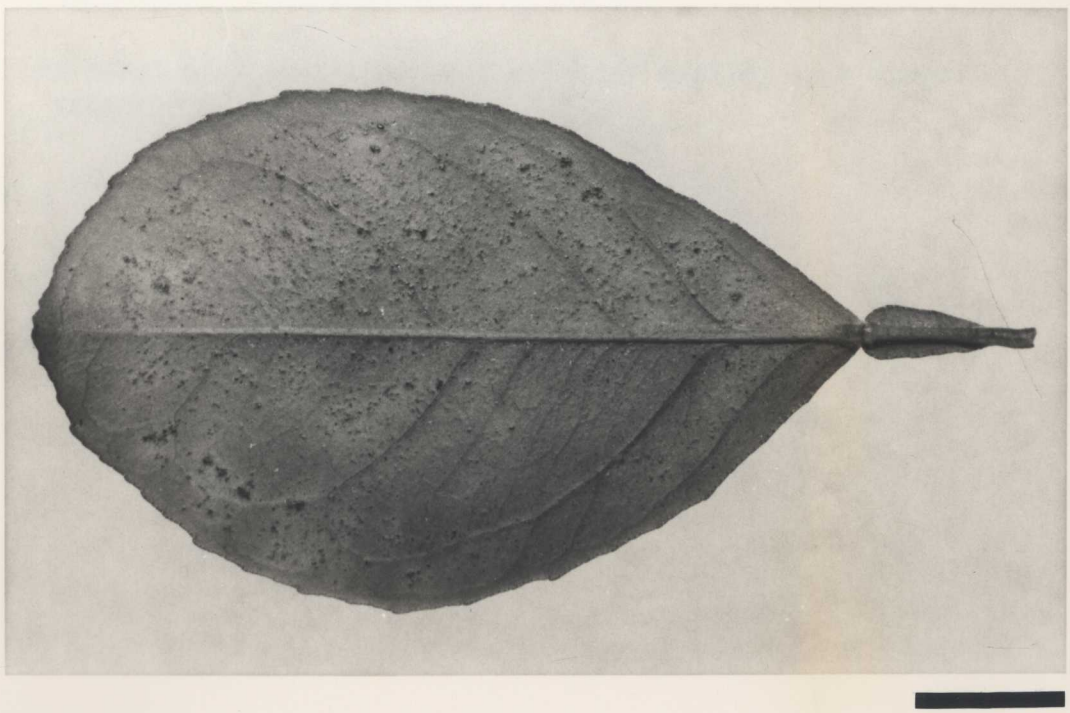


TABLE 9. Re-isolation of fungi and symptom induction after incubation of inoculated sweet orange seedlings in the glasshouse.

Isolate	Re-isolation from leaf tissue after 30 weeks	Number of seedlings* exhibiting greasy spot symptoms after 30 weeks
Colletotrichum gloeosporioides		
DAR 29822	-ve	0
DAR 29823	-ve	0
DAR 29824	-ve	0
Septoria citri		
DAR 30594	-ve	0
DAR 29826 (b)	-ve	0
DAR 30723	-ve	1
DAR 31981	-ve	1
Mycosphaerella sp.		
DAR 30870	+ve	1
DAR 30875	+ve	3
Isolate M96	+ve	2
Isolate M8	-ve	2
DAR 30878	-ve	2
DAR 30877	-ve	0

*A total of 3 seedlings were used for testing each fungal isolate.

CHAPTER 7

DISCUSSION

1. DISTRIBUTION OF GREASY SPOT

Greasy spot, on a world basis, has been reported from humid, tropical climates and also cooler, temperate areas. In these investigations, the disease was found to occur in the temperate citrus growing districts of N.S.W. In addition, the disease defined in this study was found to be present only in the western irrigation districts of the state. The warm and generally dry conditions in these districts contrast to the extremely humid and hot growing areas in Florida (Table 10).

The variable distribution of greasy spot between and within the inland districts in N.S.W. was attributed to orchard management and in particular to the nature of the spray programme. The survey of individual orchards indicated that autumn copper sprays, including Bordeaux mixture or copper oxychloride, would be useful in controlling leaf infection.

2. SYMPTOMATOLOGY OF THE DISEASE

Symptoms of greasy spot in N.S.W. have been described here in some detail. Overseas descriptions have been generally brief and ill-defined. However, greasy spot in N.S.W. appears to contrast in symptom expression to greasy spot described in Florida.

In N.S.W., the initial signs of infection appear as small black specks with chlorotic haloes. In Florida, lesions are first noticeable as yellow green spots which become orange yellow and subsequently develop into black raised spots (Pratt, 1958; Whiteside *pers.comm.*). As these lesions enlarge and coalesce under severe infection, blisters over 2 cm in diameter occur in Florida (Thompson, 1968). Lesions of

TABLE 10. Climate statistics for Mildura (Lower Murray) and Apalachicola (Florida).

Month	Mean Daily Temperature (°C)		Mean Precipitation (mm)	
	Lower Murray	Florida	Lower Murray	Florida
January	24.1	12.8	19	80
February	24.4	13.8	23	99
March	21.6	16.1	18	115
April	16.9	19.7	14	109
May	13.4	23.8	26	73
June	10.4	26.8	27	135
July	10.1	27.5	23	201
August	11.8	27.5	26	197
September	14.4	26.1	24	217
October	17.5	21.8	25	62
November	20.7	16.3	21	66
December	23.3	13.2	18	75
Annual	17.4	20.4	264	1,429

these dimensions are extremely rare in N.S.W. Symptoms occur almost exclusively on the lower leaf surface in N.S.W., whereas both upper and lower surfaces may show necrosis in Florida (Pratt, 1958; Fisher, 1961). In the later stages of symptom expression in Florida, lesions become necrotic and the pathogen sporulates in the centres (Fisher, 1961). In N.S.W., lesions rarely become necrotic and no potential pathogens have been observed on the lesions.

Disease symptoms in Japan were concluded to be similar to those described in Florida. The development of brown necrotic centres in advanced lesions (Tanaka, 1968) again contrasts to the symptoms in N.S.W. Therefore, on the basis of symptom descriptions, greasy spot in N.S.W. appears distinct from greasy spot in Florida and Japan.

Varietal susceptibility is generally similar between locations. In Florida, all commercial varieties of citrus were susceptible (Whiteside, 1972*a*), with lemons and tangeloes noted to be more severely infected than grapefruit and oranges (Whiteside, 1977). In N.S.W., all commercial varieties showed a high incidence of infection when exposed to high levels of inoculum in the field. However, some selections of *Poncirus trifoliata* (L.) Raf. were noted to be free of the disease in N.S.W., while Florida reports indicate this variety to be susceptible (Whiteside, 1972*a*). Fisher (1969) found that different root stocks were responsible for varying symptom expression in Florida. This phenomenon has not been observed in N.S.W. orchards. However, these field observations from N.S.W. and overseas may vary according to seasonal conditions, thus making strict comparisons difficult.

3. HISTOLOGY OF DISEASED TISSUE

Histological examination of field material has not been reported for greasy spot overseas. Results in N.S.W. have shown that fungal structures were associated with initial symptoms of stomatal necrosis. As lesions became more advanced, mycelium was less apparent on the lower leaf surface. These observations agree with the study of Whiteside (1972*a*) on artificially inoculated leaves. Transverse sections of naturally infected leaves in N.S.W. showed that hyphae very rarely penetrated beyond the stomatal cavity. However, artificial inoculations by Whiteside (1972*a*) demonstrated considerable fungal activity in the mesophyll tissue.

The process of lesion development began with invasion of stomata and subsequent necrosis of guard cells. Chlorotic haloes surrounding the initial spots which were observed with the naked eye, may have been due to the cellular disorganisation which occurred slightly in advance of necrosis. The disruption of cells may also explain the noticeable retention of stain in cells surrounding the necrotic stomata. Characteristic raised, black lesions finally develop due to cell enlargement and subsequent necrosis in the mesophyll, which agrees with the inoculation experiments of Whiteside (1972*a*).

The increase in cell size in the absence of fungal hyphae in the affected tissue appears to present a problem in interpreting how the change in growth occurred. There has obviously been a change in the hormonal balance of tissues, resulting in a stimulus for cells to enlarge. Auxins are a group of growth promoting compounds and indole acetic acid (IAA) has long been known to promote cell enlargement.

Many fungi have the ability to synthesise IAA from tryptophane *in vitro* and "it is likely that this auxin is often produced in diseased tissues" (Wood, 1967). However, Pegg (1976) questions this circumstantial evidence for the synthesis of IAA *in vivo*, stating that more critical studies of the function of host and fungus are needed in hyperauxiny syndromes. It is well known, however, that hypertrophy found in many diseases can be produced when IAA is applied to healthy tissue. Thus it is conceivable that changed balances in IAA, whether resulting from fungal synthesis or the interaction of fungus and host, may occur when hyphae enter stomata. This would then cause localised effects on cell growth at a small distance from the site of invasion.

4. ISOLATION OF FUNGI FROM DISEASED LEAVES

Isolations from fresh greasy spot lesions collected in the field and examination of leaf litter associated with greasy spot infections revealed a range of fungi to be potential pathogens.

The most frequent fungus isolated from fresh diseased leaves was *Colletotrichum gloeosporioides*. Although this species was originally described from citrus, its taxonomic status remains somewhat confused. *C.gloeosporioides*, sensu von Arx (1957), is a variable species encompassing over 600 described species which were reduced to synonymy. However, further work has shown some distinct groups within this previously widely defined species (J. Dingley per J. Walker, *pers.comm.*).

This fungus has been attributed as the cause of a number of diseases, including leaf spots, fruit rots and anthracnose of leaves and stems, on a wide host range, including citrus (Mordue, 1971). In the U.S.A., *C.gloeosporioides* is primarily a fruit pathogen of citrus

causing tear stain and fruit spot, although it has been implicated as a secondary invader of damaged tissue (Klotz, 1973). In addition, Fisher (1961) regarded the fungus as a contaminant in greasy spot studies. In Japan (Yamada *et al.*, 1965), *C.gloeosporioides* is also considered to be primarily a fruit infecting pathogen causing anthracnose tear stain. In Australia, citrus diseases associated with *C.gloeosporioides* have received little attention. Adam *et al.* (1949) noted that the fungus remained in a latent phase in citrus fruit. He also described spots which were associated with Septoria spot lesions.

This study has shown that *C.gloeosporioides* is closely associated with greasy spot lesions on citrus leaves in N.S.W. It was shown that the frequency of isolation increased during the warmer period of the year. This observation is consistent with *in vitro* studies which showed that the optimum temperature for growth was 29^{o}C and that disease incidence increases with higher temperature and moist conditions (Mordue, 1971).

Examination of under-tree leaf litter showed that the fungus was an initial coloniser of decaying citrus leaves in N.S.W., indicating its prevalence as a regular inhabitant of the phyllosphere. This observation, plus the common association of the fungus with a number of citrus disorders (Klotz, 1973) casts doubts on the significance of *C.gloeosporioides* as a primary pathogen.

Septoria citri was also isolated from greasy spot lesions. A number of species of *Septoria* have been described on citrus in Australia (McAlpine, 1899). In N.S.W., Noble (1932) was the first to observe Septoria damage on fruit and foliage, and considered the pathogen to

be *Septoria depressa* McAlp. However, a study of available specimens of *Septoria* spp. on citrus throughout the world concluded that all species should be reduced to synonomy under *Septoria citri* (Laundon, 1973).

On a world basis, *S.citri* causes a well known fruit disease called Septoria spot. The fruit symptoms have been well documented in Australia (Anon. 1976) and overseas (Klotz, 1973). Leaf symptoms in Australia have been described as "brown, slightly raised typically round spots" (Adam, 1930). The spot is predominantly on the under surface of the leaf, is generally of buff colour, and may be surrounded by a halo of light green tissue (Adam, 1930). The fruiting bodies are occasionally seen in the necrotic centres of larger leaf lesions. Previous records in N.S.W. Department of Agriculture, Biology Branch Files (No. 64, 1932-) indicate the association of "black, tarry lesions" on the undersurface of leaves infected with *S.citri*. On the basis of symptom expression and isolations from diseased tissue, it appears that greasy spot and Septoria spot could be closely related.

In addition, Septoria spot is found only in inland citrus producing districts in N.S.W. (Anon. 1976). This trend also occurs in overseas growing districts where the disease is found to be more prevalent in inland districts and less important on the coast (Klotz, 1973; Fawcett and Klotz, 1940). The distribution of *Septoria citri* on citrus in N.S.W. is identical to greasy spot distribution.

Results of isolation from fresh tissue showing symptoms indicates that *S.citri* is more active in winter-spring than at other periods during the season. This could be explained in two ways. Following infection during late summer-autumn (Fawcett and Klotz, 1940; Woglum

and Lewis, 1941), the fungus remains latent in fruit tissue until symptoms appear the following late winter-spring. The factor which induces symptom expression is thought to be temperature and in particular damage to tissue resulting from frosting (Anon. 1949). Therefore, the increased isolation frequency during late winter-early spring is probably due to renewed mycelial activity in the leaf tissue following the emergence from the latent state. Alternatively, the cooler temperatures may have reduced the growth of *C.gloeosporioides*, thus allowing *S.citri* a greater opportunity to develop from lesions.

The study of fungi developing on leaves showing greasy spot symptoms revealed the presence of *Septoria citri*. The fungus may be expected to develop, irrespective of any relationship to greasy spot, due to its characteristic latent infection behaviour, although this phenomenon has not been demonstrated in leaves.

The third fungus associated with this disease is a *Mycosphaerella* sp., which was previously unrecorded on citrus in Australia. Various species of *Mycosphaerella* have been described as pathogens of citrus greasy spot overseas. Table 11 gives the range in dimensions of morphological features of these *Mycosphaerella* spp. All dimensions presented fall within a similar range, although lack of statistical detail makes conclusions difficult. The description of the Florida species (Whiteside, 1972*a*) did not mention the presence of an ascostroma, bitunicate asci and absence of paraphyses. However, these characters are necessary requirements for classification in the Loculoascomycete class (Talbot, 1971), and therefore may be assumed to have been present. Japanese drawings (Yamada, 1956) indicate that ascospores are constricted at the septum and show the presence of oil droplets. This is in contrast

TABLE 11. Dimensions of *Mycosphaerella* spp. described from citrus.

Source	Ascocarp diameter (μ)	Ascus length (μ)	Ascus width (μ)	Ascospore length (μ)	Ascospore width (μ)
M.horii, Japan (Yamada, 1956)	-	48.3 (29.3-58.5)*	5.8 (2.7-10.6)	12.0 (7.9-15.9)	2.9 (2.3-3.7)
M.citri, Florida (Whiteside, 1972*a*)	58-90	25-35	5.0-5.5	8.5 (6.2-11.2)	2.5 (2.2-2.8)
Mycosphaerella sp. N.S.W.	80-85 (65-110)	35-40	5.0-6.5	10.5 (8.0-12.5)	2.5 (2.5-3.0)

*Bracketed figures indicate the upper and lower limits of the dimensions.

to Florida and N.S.W. descriptions. In cultural characters, N.S.W. and Florida isolates are similar as both form dark-coloured and slow growing colonies. However, the lack of conidiation in N.S.W. cultures contrasts to the *Stenella* conidia readily produced by Florida cultures (Whiteside, 1972*a*).

Further taxonomic work is needed to determine the relationship between *Mycosphaerella horii* Hara, *Mycosphaerella citri* Whiteside and the undetermined *Mycosphaerella* sp. from N.S.W. The examination of authenticated collections and type material may well show that these fungi are very similar.

Epidemiological data of *Mycosphaerella* sp. in N.S.W. contrasts somewhat to overseas results. Whiteside (1970) demonstrated a rapid rise of perithecial maturity and ascospore populations during late spring to mid summer, followed by a sudden drop by the end of summer. On this basis, he concluded that the peak infection period in Florida orchards occurred during spring-mid summer. Results obtained in N.S.W. showed that inoculum in lower Murray orchards rose during autumn and spring. However, the different patterns of spore release most probably reflect adaptation to different climatic regimes, rather than intrinsic differences in behaviour of the *Mycosphaerella* spp. Thus, ascospores are released during hot humid conditions favouring infection in Florida. Similarly, ascospore release during autumn and spring in N.S.W. coincides with moist conditions and leaf growth flushes which combine to favour leaf infection.

The isolation frequency of *Septoria citri* and *Mycosphaerella* sp. was low, in the order of 5 to 20%. However, this is consistent with the histological examination of field material which showed hyphae to be rare beyond the stomatal cavity of affected tissue.

Other fungi associated with greasy spot were isolated infrequently and were generally regarded as saprophytes. For example, Japanese studies indicate that various species of *Phoma* saprophytically colonise dead twigs in citrus trees (Yamada *et al.* 1965). It is interesting to note, however, that *Aureobasidium pullulans* may be involved in "greasy spot-like" disease in Japan, although this has not been confirmed (M. Koizumi, per P. Barkley, *pers.comm.*).

5. PATHOGENICITY OF FUNGI ASSOCIATED WITH GREASY SPOT

(i) Processes of infection

Observations of the germination and formation of appressoria and infection pegs by *C.gloeosporioides* generally agrees with *in vitro* studies (Richardson and Thorn, 1962). Studies involving inoculation of orange leaves with *C.gloeosporioides* (Vares Megino, 1975) also agree with the results presented here, although no mention was made of the formation of a cross wall in the conidium prior to germination. The noted persistence of appressoria and the absence of conidia and germ tubes after 4 weeks confirms earlier studies which also showed that *Bacillus* sp. on citrus leaves lyse conidia and germ tubes of *C.gloeosporioides* and result in stimulated appressorium formation (Lenne and Parberry, 1976). This contrasts with work on *Colletotrichum acutatum* Simmonds where phylloplane bacteria stimulated appressorium formation by competing for available nutrients (Blakeman and Parberry, 1977).

Results of spore germination and infection by *Septoria citri* indicated that penetration and infection occurs through stomata, although further growth through mesophyll tissue was not observed. There have been no other reports of histological studies on leaves or fruit. However, the only report of pathogenicity testing of *S.citri* on fruit, indicated that predisposing damage by cold injury or non-fungicidal sprays was

necessary to establish infection (Klotz and De Wolfe, 1969). Such conditions were not necessary in these experiments. It was apparent, however that symptom production depended on the successful colonization and establishment of infection structures in stomata by the 6th and 12th weeks.

The processes of germination and infection by the N.S.W. *Mycosphaerella* sp. agree closely with the results of *M.citri* in Florida (Whiteside, 1972*a*). In both cases, the technique of using macerated cultures was the same. The incubation period under glasshouse inoculation conditions also agrees with Florida reports (Whiteside, 1972*a*). Transverse sections of lesions on inoculated leaves showed occasional penetration of hyphae into the mesophyll which agrees with Whiteside (1972*a*).

However the germination of ascospores on excised leaves did not result in any development of the infection structures which were observed following inoculation with macerated cultures. The pattern of bi-polar germination of ascospores and the formation of swollen hyphal tips agrees with *in vitro* studies in Florida (Whiteside, 1974).

The decline of surface hyphal activity of *S.citri* and *Mycosphaerella* sp. with time, and the persistence of infection structures in necrotic stomates agrees closely with observations on naturally infected leaves. However, the occasional observation of hyphal activity in the mesophyll of artificially infected leaves is in contrast to observations on transverse sections of naturally infected leaves.

(ii) Pathogenicity of the fungi

As a result of symptom development on artificially inoculated

leaves and subsequent re-isolation of the fungus, the *Mycosphaerella* sp. is concluded to be a pathogen causing greasy spot of citrus in N.S.W. Although *Septoria citri* could not be re-isolated, the production of symptoms on test seedlings also implicates this fungus as a pathogen. Early reports in N.S.W. (Fraser, 1957) indicate that *Septoria citri* could have a *Mycosphaerella* sp. sexual stage, which would thus explain the ability of both fungi to cause the disease. However, this relationship has not been finally demonstrated.

The failure of symptoms to develop on seedlings and trees transplanted into field plots at Rydalmere could be explained by the adverse climate, since greasy spot has not been recorded from coastal areas. The close proximity of these plots to car and industrial pollution may also have been responsible for the absence of infection.

6. GENERAL CONCLUSIONS

Greasy spot on citrus in N.S.W. has been found to be caused by a previously undescribed *Mycosphaerella* sp. and *Septoria citri*. Overseas reports (Tanaka and Yamada, 1952; Whiteside, 1970) also revealed *Mycosphaerella* spp. to be the pathogen. However, the asexual stage of the *Mycosphaerella* species are different. In Japan and Florida, a dematiaceous hyphomycete in the genus *Stenella* forms the asexual state (Yamada, 1956; Whiteside, 1972*a*). In N.S.W., the pycinidial fungus *Septoria citri* has been found to be pathogenic and could well be the asexual stage. Therefore the relationship of sexual and asexual stages of the pathogens in N.S.W., Florida and Japan requires further taxonomic study.

The disease was defined in the field and found to be confined to the inland irrigation districts. Septoria spot of citrus fruit has the

same distribution as greasy spot. In addition, fungicidal control of Septoria spot in orchards was associated with reduced greasy spot incidence. These results suggest that greasy spot may be an alternative leaf symptom of Septoria spot.

Various fruit blemishes associated with greasy spot in the field were considered to be ill-defined and associated with a multiplicity of causes. Therefore, this study concentrated on the leaf disease, although greasy spot rind blotch has been characterised in Florida (Whiteside, 1972*b*).

There is some evidence which suggests that another greasy spot may occur in coastal N.S.W. One specimen in the Biology Branch Herbarium (DAR 33641) collected from the sub-tropical north coast region in 1973, showed sparse sporulation of a *Stenella*-like fungus. However, this has not been subsequently recorded and all examined specimens did not agree with symptoms defined from inland orchards. Further work in coastal orchards may reveal a low incidence of greasy spot-like disease which could show similarities with overseas descriptions.

Field surveys on disease occurrence and intensity revealed a number of methods of minimising infection. The contemporary practice of blanket herbicide application between rows is associated with leaf litter accummulation. Under tree cultivation reduces leaf litter and consequent carryover of inoculum. Routine use of copper sprays in autumn for Septoria spot control was also associated with reduced greasy spot incidence. This is consistent with epidemiological data which showed a peak of ascospore maturity during the autumn period. These control recommendations have been used by extension officers and are awaiting confirmation from spray trials being conducted at Mildura and Dareton.

These studies of greasy spot in N.S.W. have resulted in the definition of symptoms and host range, the determination of disease distribution and the establishment and behaviour of the pathogen. Comparison with overseas descriptions leads to the conclusion that greasy spot in inland N.S.W. shows different symptomatology, while exhibiting some similarities in the behaviour of the causal fungi.

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APPENDIX Accessions of Specimens Examined and Deposited in Herb.DAR.

Specimens examined in Herb.DAR are listed with the host species, location and date of collection and accession number.

1. DISTRIBUTION OF GREASY SPOT

The following specimens were examined to determine the distribution of greasy spot in N.S.W.

(i) Coastal districts

C.reticulata	Somersby	15.9.1976	(28119)
C.reticulata	Maroota	8.1974	(24846)
C.aurantiifolia	Mullumbimby	11.10.1973	(24071)
C.aurantiifolia	Broken Head	8.9.1976	(27988)
C.aurantiifolia	Wollongbar	2.7.1976	(27922)
C.sinensis	Pitt Town	13.3.1978	(30593)
C.aurantiifolia	Broken Head	6.1973	(33641)*

**Stenella* sp. associated with leaf spots

(ii) Central West

C.sinensis	Narromine	18.10.1976	(18017)
C.paradisi	Narromine	18.10.1976	(28018)
C.limon	Narromine	18.10.1976	(28020)
C.sinensis	Narromine	18.10.1976	(28021)
C.paradisi	Narromine	18.10.1976	(30570)

(iii) M.I.A.

C.sinensis	Cudgell	28.1.1975	(27611)
C.paradisi	Yanco	22.1.1975	(26515)
C.sinensis	Leeton	22.1.1975	(25426)
C.sinensis	Yanco	22.1.1975	(25425)
C.sinensis	Yanco	22.1.1975	(27612)
C.grandis	Yanco	20.11.1973	(24070)
C.sinensis	Beelbangura	5/57	(5114)
C.sinensis	Stanbridge	31.3.1977	(30708)
C.paradisi	Wamoon	31.3.1977	(30711)
C.sinensis	Cudgell	31.3.1977	(30712)
C.sinensis	Cudgell	2.10.1977	(29822)
C.sinensis	Stanbridge	2.10.1977	(29827)
C.paradisi	Wamoon	2.10.1977	(29828)

(iv) Mid-Murray Irrigation Districts

C.paradisi	Peericoota	5.3.1975	(25403)
C.limon	Barham	6.3.1975	(25405)
C.sinensis	Goodnight	7.3.1975	(25404)
C.sinensis	Barooga	4.3.1975	(25406)
C.sinensis	Barooga	1.4.1977	(30705)
C.sinensis	Barham	1.4.1977	(30706)

C.sinensis	Barooga	1.4.1977	(30707)
C.paradisi	Barooga	1.4.1977	(30709)
C.sinensis	Barooga	1.4.1977	(30710)
C.sinensis	Barham	4.10.1977	(29826)
C.sinensis	Barham	2.10.1977	(30720)
C.sinensis	Barooga	2.10.1977	(30724)

(v) Lower-Murray Irrigation Districts

C.sinensis	Balranyld	27.2.1970	(19901)
C.sinensis	Dareton	5/75	(25017)
C.sinensis	Ellerslie	9.3.1975	(25407)
C.paradisi	Pomona	11/74	(25049)
C.grandis	Buronga	12/72	(24069)
C.paradisi	Buronga	25.5.1977	(30713)
C.sinensis	Morquong	25.5.1977	(30714)
C.paradisi	Pomona	25.5.1977	(30715)
C.sinensis	Morquong	5.10.1977	(29821)
C.paradisi	Pomona	5.10.1977	(29824)
C.paradisi	Buronga	5.10.1977	(30722)
C.paradisi	Morquong	5.10.1977	(30723)

2. SYMPTOMATOLOGY OF THE DISEASE

Detailed observations and notes of fresh specimens were made and have been filed with the following dried specimens.

C.sinensis	Stanbridge	2.10.1977	(29827)
C.paradisi	Buronga	5.10.1977	(30722)
C.sinensis	Pomona	5.10.1977	(29824)
C.paradisi	Wamoon	2.10.1977	(29828)
C.paradisi	Morquong	5.10.1977	(30723)
C.paradisi	Barooga	2.10.1977	(29823)
C.sinensis	Buronga	25.5.1977	(30713)
C.paradisi	Pomona	25.5.1977	(30715)
C.sinensis	Morquong	25.5.1977	(30714)
C.sinensis	Dareton	14.12.1977	(30883)
C.sinensis	Dareton	7.2.1978	(30879)

3. HISTOLOGY OF THE DISEASE

Permanent slides were made of stained lesions and transverse sections of lesions. The slides plus detailed notes were filed in Herb. DAR with the specimen from which they were made.

C.paradisi	Barooga	2.10.1977	(29823)
C.sinensis	Dareton	14.12.1977	(30883)
C.sinensis	Dareton	7.2.1978	(30879)
C.sinensis	Pomona	5.10.1977	(29824)
C.sinensis	Stanbridge	2.10.1977	(29827)
C.paradisi	Morquong	5.10.1977	(30723)
C.paradisi	Buronga	5.10.1977	(30722)
C.sinensis	Cudgell	31.3.1977	(30712)*
C.paradisi	Dareton	28.4.1977	(30719)*

C.paradisi	Barooga	1.4.1977	(30709)*
C.paradisi	Pomona	25.5.1977	(30715)*
C.paradisi	Wamoon	2.10.1977	(29828)**

*Transverse sections only.

**Stained whole mounts only.