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STUDIES OF A STRAIN OF ALTERNARIA CITRI PIERCE, THE CAUSAL ORGANISM OF BROWN SPOT OF EMPEROR MANDARIN

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SUMMARY

Brown spot of the Emperor of Canton mandarin in Queensland was found to be caused by a distinct strain of *Alternaria citri* Pierce. In morphological and cultural characters the fungus is identical with *A. citri*, but differs in pathogenicity and the production of toxic filtrates when cultured on liquid medium.

No evidence of cuticular or stomatal penetration by the germinating spores was found, and hyphae were not found in young lesions. When the fungus was cultured on Czapek's sucrose nitrate solution a toxic filtrate was produced which induced the same disease symptoms and showed the same host specificity as the pathogen itself. Non-pathogenic isolates of *A. citri* did not produce this host-specific toxic filtrate.

Chloroform and Celite treatments increased the susceptibility of mature fruit to damage, presumably by removing a barrier of waxes which was impermeable to the toxin.

The host range in Queensland was found to include Emperor of Canton mandarin, Sovereign mandarin and Calamondin.

I. INTRODUCTION

Brown spot of *Citrus reticulata* Blanco cv. Emperor of Canton (commonly called Emperor mandarin) is confined to Queensland and New South Wales. In Queensland the disease is restricted to the Howard-Burrum, Palmwoods and Elimbah districts. It was first recorded in Queensland at Howard in 1928 (Mandelson and Blackford 1938).

The disease was first described by Cobb (1903), who found *Alternaria* sp. and *Phoma* sp. to be associated with diseased fruit tissue. In 1917 Darnell-Smith claimed that *Colletotrichum gloeosporioides* Penz. was the causal organism. Noble *et al.* (1937) suspected that a bacterial pathogen might be involved. Mandelson and Blackford (1938) considered the disease to be caused by a species of *Gloeosporium*. Later, Kiely (1964) demonstrated that a strain of *Alternaria citri* Pierce was responsible. Investigations reported here support this conclusion.

The studies were concerned also with the mode of action of the pathogen.

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II. ISOLATION AND PATHOGENICITY TESTS

In the autumn of 1962 a species of *Alternaria* was found constantly associated with brown spot lesions on Emperor mandarin foliage at Palmwoods. Isolations of the fungus from unsterilized surface tissues were readily made onto potato dextrose agar, and pathogenicity was determined by inoculating rapidly expanding leaf tissue and young fruit of the Emperor mandarin. An aqueous spore suspension was sprayed onto Emperor seedlings under conditions of saturated humidity. Pathogenic isolates induced typical leaf spots within 16 hr following inoculation. The association was confirmed by reisolating the *Alternaria* from these lesions.

Young fruit were also inoculated both in the laboratory and in the field. Discrete lesions typical of the disease were again visible within 16 hr. In the field many of the young fruit dropped within 3 days of inoculation.

The pathogen was cultured on potato dextrose agar, corn meal agar and water agar. Small squares were cut from the edge of advancing colonies and placed on young leaves of Emperor mandarin seedlings growing under saturated humidity. Discrete lesions became visible with all three media within 24 hr following inoculation with mycelium (Figure 1). This contrasts with the experience of Kiely (1964), who was unable to secure infection of young leaves with actively growing mycelial tips on agar blocks under conditions of saturated humidity.

The mature leaves were resistant to the pathogen.

As fruit developed there was a decrease in susceptibility, particularly after the stage where the oil glands became prominent.

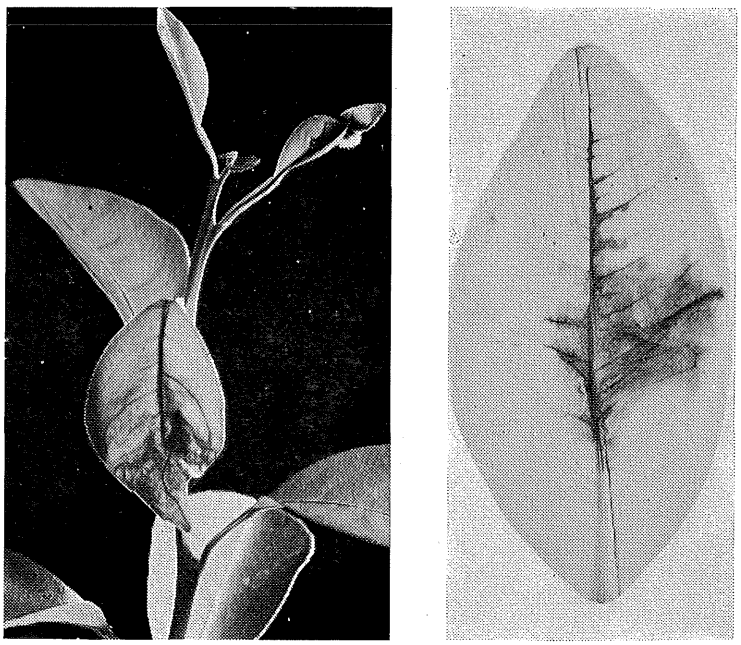


Fig. 1.—Left, infection of young Emperor mandarin leaf produced by actively growing mycelial tips on agar block; note vein necrosis. Right, natural infection.

III. HOST RANGE

In Queensland, brown spot lesions have been observed in the field on Emperor mandarin, Calamondin (*Citrus mitis* Blanco) and *C. reticulata* cv. Sovereign. Green-house tests with the pathogenic *Alternaria* sp. confirmed the susceptibility of these three cultivars.

In addition, pathogenicity tests have revealed the susceptibility of *C. reticulata* cv. Dancy; Wheeny grapefruit (*Citrus paradisi* Macf. hybrid); Sampson, San Jacinto, Thornton, Seminole and Orlando tangelos (*C. paradisi* x *C. reticulata* hybrids); and Wekiwa tangelo (*C. paradisi* x Sampson tangelo). Brown spot symptoms on these varieties have been reported from New South Wales (Anon. 1953, 1959, 1960).

The following citrus varieties available for testing in Queensland have proved to be resistant: Villa Franca lemon (*Citrus limon* (L.) Burm.); Meyer lemon (*C. limon* hybrid); Imperial, Kara, King of Siam and Beauty of Glen Retreat mandarins (*C. reticulata* cultivars); Valencia Late and Joppa oranges (*Citrus sinensis* (L.) Osbeck); Marsh grapefruit (*C. paradisi*), and oval kumquat (*Fortunella margarita* (Lour.) Swing.).

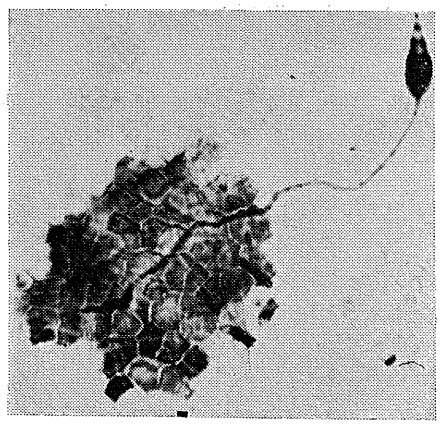
Although the Emperor brown spot pathogen was isolated on one occasion from leaf spots on leaves of rough lemon (*C. limon* hybrid) growing adjacent to a block of Emperor mandarins, repeated inoculations with the Emperor mandarin pathogen failed to initiate infection on the leaves of this variety. Leaf spots have been observed on the leaves of rough lemon trees growing at Gayndah and Byfield, districts where brown spot of the Emperor mandarin has not been recorded. Similar leaf spots have been found in Queensland on Rangpur, Kusaie and Jatti Khatti limes. An *Alternaria* which was morphologically and culturally identical with the Emperor brown spot pathogen was consistently isolated from these leaf spots. These isolates proved to be pathogenic to rough lemon but not to Emperor mandarin.

IV. HISTOLOGICAL STUDIES OF LEAF SPOTS

Following inoculation, the pathogen was rarely reisolated from 24-hr-old leaf spots when the leaf tissue was first surface-sterilized with 0.1% mercuric chloride for 45 sec or with 0.35% sodium hypochlorite. When lesions were not surface-sterilized the pathogen was readily reisolated. Studies were initiated to determine the mode of penetration of the pathogen.

Using a technique described by Bock (1964), a thin film of nail varnish suitably thinned with acetone was brushed lightly on a leaf surface which had been inoculated with an aqueous spore suspension. After drying, the thin membrane was peeled off to display a perfect cast of the epidermis with the oil glands and stomatal openings. The germinated *Alternaria* spores were also lifted off with the membrane and stained with lactophenol containing 1% cotton blue. This technique failed to reveal any penetration by the fungus either directly through the cuticle or *via* the stomatal openings, even though necrotic areas on the leaf were clearly visible. Appressoria were not formed.

Inoculated leaf tissue was also placed in equal parts of glacial acetic acid and absolute ethyl alcohol until the chlorophyll was removed. The material was then washed in water, transferred to 10% "Teepol" solution, and boiled for 3 min. After cooling, the leaf tissue was transferred to lactophenol containing 1% cotton blue, and simmered for 2 min. The material was then cleared in a saturated aqueous solution of chloral hydrate and mounted in lactophenol containing 1% cotton blue. Microscopic examination of the leaf surface again failed to reveal any penetration by the germ-tubes through either cuticle or stomatal openings. The germ-tubes did not grow in any particular direction on the leaf surface, and in many cases they were seen to have grown over stomatal openings without penetrating them (Figures 2 and 3).



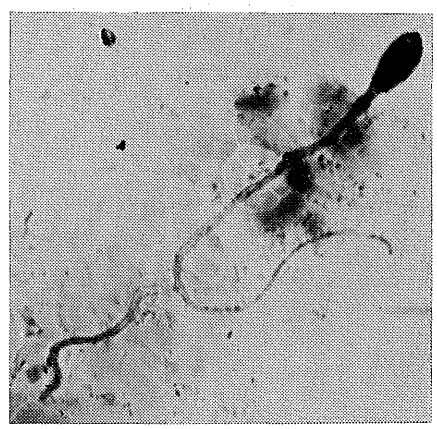


Fig. 2.—Germinating spore of *A. citri* on surface of Emperor mandarin leaf, 30 hr after inoculation. Note cell necrosis without host penetration. (x 450.)

Fig. 3.—Germinating spore of *A. citri* on surface of Emperor mandarin leaf, showing germ-tube growing over stomatal opening without penetrating it. (x 550.)

Numerous sections prepared with a freezing microtome failed to reveal any mycelium within the host tissues of 24-hr-old leaf and fruit lesions.

V. TOXIN PRODUCTION BY THE BROWN SPOT PATHOGEN

As direct penetration by the brown spot pathogen through the cuticle and stomata of leaves and fruit did not occur, and since discrete lesions were visible within 16 hr of inoculation, the possibility of a phytotoxin being involved in disease development was considered.

Production of a toxin-like substance was investigated by growing the fungus on Czapek's sucrose nitrate solution (Riker and Riker 1936). Agar blocks with mycelium of the pathogen were aseptically floated on 25-ml aliquots of Czapek's solution in 100-ml Erlenmeyer flasks. The cultures were grown at room temperature for 12-18 days. The culture solution was then decanted off and

filtered through a Buchner funnel, using Whatman No. 42 filter papers. To ensure that the filtrate did not contain spores or mycelial fragments, a small portion was plated onto potato dextrose agar. These plates when incubated remained sterile. This method was adopted as a standard procedure for filtrate production.

To determine the toxic activity of the culture filtrate, the cut ends of young terminal shoots of citrus varieties were immersed in the filtrate. The filtrate was also sprayed onto young foliage and immature fruit with a nebulizer.

When terminal twigs of the brown spot susceptible citrus varieties, including Emperor and Sovereign mandarins, Calamondin, Dancy tangerine and Sampson tangelo, were immersed in the filtrate, vein necrosis of leaves resulted within 30 hr (Figure 4). Vein necrosis was eventually followed by collapse of intercostal tissue. Sterile Czapek's solution was used as a control. All shoots immersed in it remained quite healthy. Shoots from brown spot resistant citrus varieties, including rough lemon, did not develop vein necrosis when immersed in the filtrate, and remained unaffected.

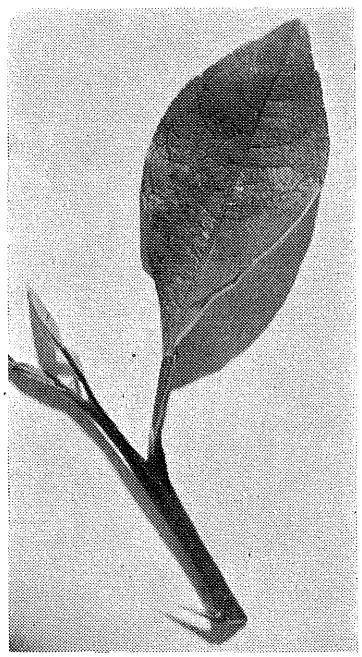


Fig. 4.—Vein necrosis produced by inserting cut end of young Emperor mandarin twig in culture filtrate.

When the filtrate was sprayed onto young terminal shoots and young fruit of Emperor mandarin and Calamondin, typical brown spot symptoms appeared within 24 hr (Figures 5 and 6). Isolations from these spots failed to reveal a pathogenic organism. There was no reaction when the toxic filtrate was sprayed onto citrus varieties shown to be resistant to brown spot by spore inoculation. Emperor mandarin fruit and Calamondin fruit were not affected by spraying with sterile Czapek's solution.

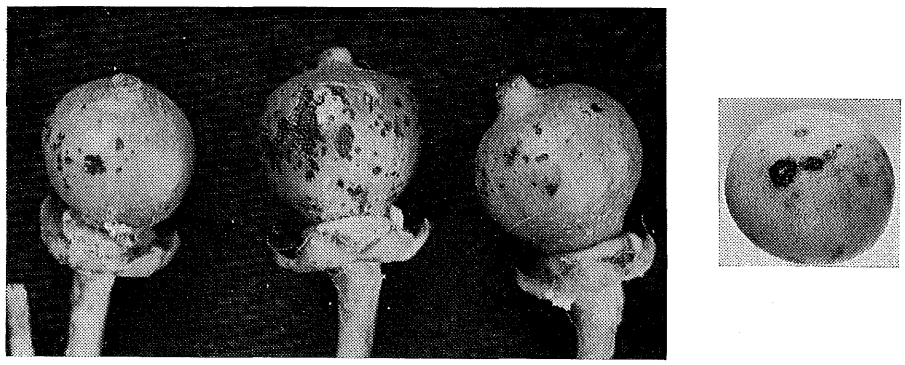


Fig. 5.—Left (three fruit), lesions produced by spraying young Emperor mandarin fruit with culture filtrate. Right, natural infection.

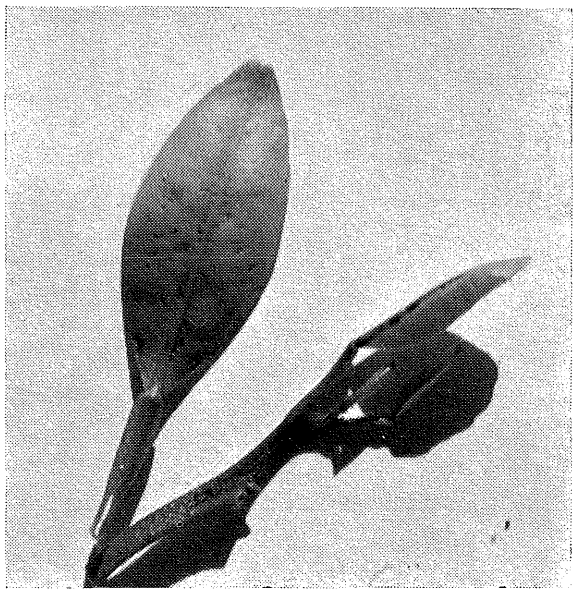


Fig. 6.—Lesions produced by spraying young Emperor mandarin twig with culture filtrate.

A toxic filtrate was also produced when the pathogen was cultured on a modified Czapek's solution in which the sucrose was replaced by glucose.

Non-pathogenic isolates of *Alternaria* from citrus did not produce a toxic filtrate when cultured on Czapek's medium. Also, filtrates produced by *A. solani* Sorauer, *A. passiflorae* Simmonds, *A. dauci* (Kühn) Groves and Skolko and *A. longipes* (Ell. and Ev.) Mason did not contain the toxin-like substance.

The toxic filtrates were heat-labile, being destroyed by autoclaving and by boiling for 1 min. To test whether this loss of toxic activity was caused by the high pH value of the filtrate (pH approximately 8·0), the reaction was adjusted with $\frac{N}{10}$ HCl solution to a range of pH values prior to autoclaving. Toxin instability was not affected.

The dilution end point for toxic activity by the crude culture filtrates was determined. Cultures were grown as previously described at room temperature for 15 days and filtered through a Buchner funnel. Serial dilutions were made and the activity of the filtrate tested by the usual methods. Sterile Czapek's solution was again used as a control (Table 1).

TABLE 1

DILUTION END POINT FOR TOXIC ACTIVITY

Filtrate Dilution	Vein Necrosis of Leaves when Twigs Immersed in Filtrate			Lesions Produced when Filtrate Sprayed on Young Growth		
	Replicate			Replicate		
	A	B	C	A	B	C
Nil	+	+	+	+	+	+
1/10	+	+	+	+	+	+
1/100	−	−	−	+	+	+
1/1000	−	−	−	−	−	−

A dilution of 1/100 produced spotting when sprayed on young leaves, but did not produce vein necrosis when soft terminal shoots were immersed in the filtrate.

VI. MATURE HOST RESISTANCE

Spore inoculation tests revealed that mature leaves were resistant to the brown spot pathogen and that fruit became less susceptible as they approached maturity. Only very small scattered lesions developed on laboratory-inoculated fruit which had passed the stage of development where the oil glands became prominent.

Evidence suggests that the symptoms associated with the disease are the result of penetration of the tissue by a toxic substance produced by the germinating *Alternaria* spores and that resistance of mature tissue to penetration may be due to the presence of a mechanical barrier impermeable to the toxin. As Fernandes, Baker, and Martin (1964), working with apples, had removed cuticular waxes by washing leaves and fruits with chloroform at room temperature, and Bock (1964) had reduced the resistance offered by a thick cuticle to penetration of onions by *Alternaria porri* (Ell.) Cif. by rubbing carborundum powder over the leaf surface, similar measures were tested with mandarin fruit.

Six-month-old Emperor and Beauty of Glen Retreat mandarin fruit were removed to the laboratory and surface-sterilized with 0·35% sodium hypochlorite. Some fruit were then rubbed in a marked zone with a chloroform-soaked pad for approximately 3 sec and the chloroform was allowed to evaporate. Other fruit were lightly rubbed in a marked zone with "Celite" powder, care being taken not to rupture the oil glands. The remainder of the fruit were untreated. All

fruit were then inoculated by spraying with an aqueous spore suspension, toxic filtrate and sterile Czapek's solution (Table 2 and Figure 7). All treatments on Beauty of Glen Retreat fruit, a brown spot resistant variety, failed to produce a reaction.

TABLE 2

EFFECT OF PRETREATMENT OF EMPEROR FRUIT SURFACE WITH CHLOROFORM OR CELITE PRIOR TO INOCULATION WITH SPORES AND FILTRATE

Treatment	Inoculation Method	Reaction
1 (*a*) Untreated	Sterile Czapek's solution ..	No reaction
(*b*) Untreated	Aqueous spore suspension	Scattered pin-point lesions produced
(*c*) Untreated	Culture filtrate	Scattered pin-point lesions produced
2 (*a*) Celite abrasion	Nil	No reaction
(*b*) Celite abrasion	Sterile Czapek's solution ..	No reaction
(*c*) Celite abrasion	Aqueous spore suspension	Complete necrosis of treated area—fruit turned yellow within 5 days
(*d*) Celite abrasion	Culture filtrate	Complete necrosis of treated area—fruit turned yellow within 5 days
3 (*a*) Rubbed with chloroform	Nil	No reaction—except for slight bronzing in 1 out of 12 fruit
(*b*) Rubbed with chloroform	Sterile Czapek's solution ..	No reaction
(*c*) Rubbed with chloroform	Aqueous spore suspension	Complete necrosis of treated area—fruit turned yellow within 5 days
(*d*) Rubbed with chloroform	Culture filtrate	Complete necrosis of treated area—fruit turned yellow within 5 days

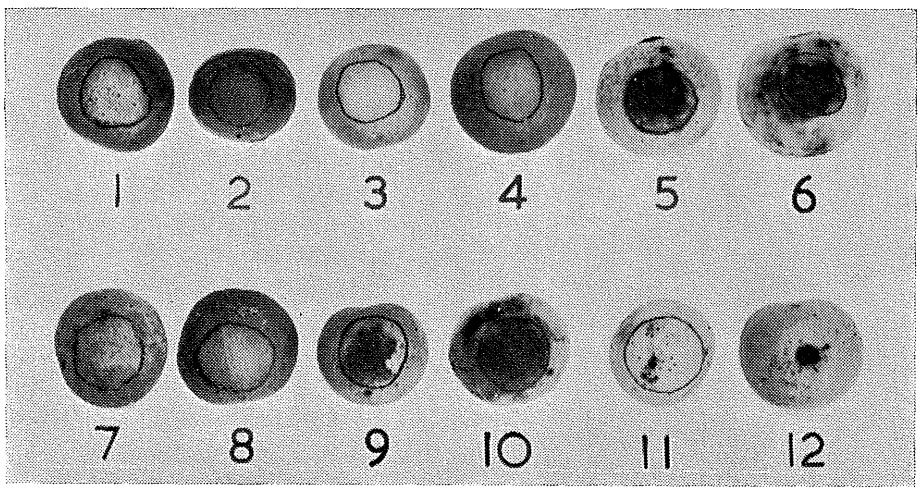


Fig. 7.—Lesions produced on 6-month-old Emperor mandarin fruit by the following treatments: 1, spore inoculation; 2, sterile culture solution; 3, Celite abrasion; 4, Celite abrasion + sterile culture solution; 5, Celite abrasion + spore inoculation; 6, Celite abrasion + culture filtrate; 7, chloroform treatment; 8, chloroform treatment + sterile culture filtrate; 9, chloroform treatment + spore inoculation; 10, chloroform treatment + culture filtrate; 11, culture filtrate; 12, 6-month-old fruit which had been inoculated 52 days previously in the field with an aqueous spore suspension.

On fruit of Emperor mandarin, application of aqueous spore suspension or culture filtrate following treatment with either Celite powder or chloroform resulted in complete necrosis of treated areas. This suggests that increasing resistance with age is associated with thickening of the cuticle.

VII. IDENTITY OF THE PATHOGEN

Methods and materials.—Isolates of *Alternaria* from various citrus varieties were divided into two groups on the basis of pathogenicity tests and the production of a toxic filtrate when cultured on Czapek's sucrose nitrate solution. Only the pathogenic isolates were capable of producing the host-specific toxic filtrate.

Morphological and cultural characters were studied on artificial culture medium. As the Queensland isolates had been shown not to sporulate well on Czapek's agar, the medium favoured by Bliss and Fawcett (1944) as the standard laboratory medium for the identification of *Alternaria citri* Pierce, namely corn meal agar, which favours abundant sporulation with little aerial mycelium, was used, and cultures were grown on this agar at 26°C for 15-20 days.

To facilitate the measurement of consecutive spores in the spore chain, adhesive cellulose tape was lightly pressed onto the cultures and the resultant adhering spores examined under the microscope. Three measurements were made of each spore, namely the length (including the apical beak, if present), the width, and the length of the apical beak. The number of transverse septations was also noted.

Many isolates did not sporulate on corn meal agar and were subsequently discarded. Saltations occurred frequently in culture, the new modifications generally appearing as sectors with sterile mycelium.

Identity with A. citri.—Mason (1928) divided the *Alternaria* species into two groups based on the length of the apical beak and the length of spore chains in culture. In the *Alternaria tenuis* group he included species with short beaks seldom more than half the length of the spore and which in culture formed conidia in long chains. The majority of spores placed in this group had 3–5 transverse septa, and in culture fell within the limits 20–50 x 10–14μ. Long-beaked species were placed in the *Alternaria solani* group.

Bliss and Fawcett (1944) described *A. citri* as being similar to the modern conception of *A. tenuis* Nees, but not to the original illustration of Nees. They considered the name *A. tenuis* invalid and suggested that the short-spored, short-beaked species be tentatively grouped about some other species such as *A. citri.*

McAlpine (1899) described a *Macrosporium citri* on leaves of lemon. The description given is:—septa 0–6, beak 15μ, length up to 60μ, breadth 15–20·05μ. Mason (1928) regarded McAlpine's fungus as an *Alternaria.*

In the original description of *A. citri* published by Pierce in 1902 (Bliss and Fawcett 1944), an adult spore measurement of 25–40 x 15–25μ with 4–6 transverse septa is given.

Rudolph (1917) described *Alternaria citri* var. *Cerasi* as being practically indistinguishable from *A. citri* and spore measurements are given as 15·3–57·8 x 6·8–15·3μ. Rudolph also described a short, stout, hyaline-subhyaline isthmus which is usually 3·4 x 3·4μ.

Doidge (1929) described *A. citri* on rough lemon leaves in South Africa. When cultured on prune agar, a spore measurement 15–60 x 10–20μ was obtained.

Bliss and Fawcett (1944) mentioned the great polymorphism within *A. citri*. They took into account the effect of different substrates on the development of the fungus. When cultured on Czapek's agar, spores measured 8·1–81·0μ x 6·3–24·3μ. Beaks were up to 54·0μ in length (90·7% up to 7·6μ). Half of the spores had three transverse septa, the number of such septa ranging from 0 to 11 and being commonly 1–4. When cultured on corn meal agar, a spore measurement of 7·6–49·4 x 7–13μ was obtained. Apical beaks between 5 and 7μ long were present on 90% of the spores.

The conidial measurements obtained in the present study are summarized in Table 3. The apical beaks were short, hyaline, bluntly pointed structures at the narrow distal ends of the spores (Figure 8). There were no significant differences in spore measurements between the isolates from the pathogenic and the non-pathogenic groups. All isolates fell within the range described by Bliss and Fawcett (1944) and are identical with *Alternaria citri* Pierce.

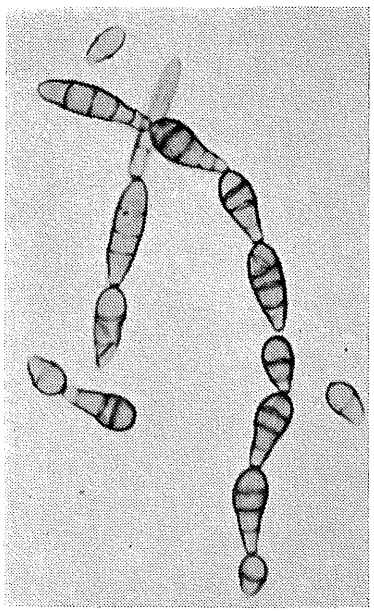


Fig. 8.—Conidia produced by pathogenic strain of *A. citri* on C.M.A. Note the short, hyaline, apical beaks. (x 500.)

TABLE 3

SPORE DIMENSIONS OF VARIOUS ISOLATES OF *Alternaria citri*

Isolate Designation	Source of Isolate	No. of Spores Measured	Spore Length (μ)		Spore Width (μ)		No. of Transverse Septa	Length of Apical Beak (μ)	
			Range	Mean	Range	Mean		Range	Mean
Pathogenic									
A	Emperor mandarin—Woombye. From leaf spot	213	8·1–43·2	20·06	5·4–10·8	6·75	1–5	1·4–10·8	4·26
B	Emperor mandarin—Woombye. From fruit lesion ..	178	8·1–35·1	20·65	5·4–13·5	8·50	1–5	1·4– 8·1	3·20
C	Emperor mandarin—Palmwoods. From leaf spot	148	8·0–42·0	22·30	5·0–12·0	7·70	1–6	2·0–11·0	3·20
D	Emperor mandarin—Palmwoods. From leaf spot	105	10·0–40·0	24·30	5·0–11·0	7·40	1–5	2·0–10·0	3·90
Non-pathogenic									
A	Calamondin—Palmwoods. From rotting fruit	175	8·1–45·9	21·46	5·4–13·5	9·30	1–6	1·4– 8·1	3·10
B	Culture of *A. citri* from University of California	198	5·4–35·1	14·70	5·4–12·15	8·10	1–4	0·6– 5·4	1·80
C	Emperor mandarin—Palmwoods. Fruit lesion	133	10·0–52·0	22·00	6·0–12·0	8·60	1–7	2·0–12·0	4·10
D	Navel orange—Gayndah. From navel end of fruit ..	83	14·0–40·0	28·80	6·0–16·0	9·80	1–4	2·0–10·0	4·10

Isolates were forwarded to the Commonwealth Mycological Institute, where they were examined by Dr. M. B. Ellis. All isolates were grown on plates of malt agar and potato dextrose agar under standard conditions and in slide cultures on malt agar. Dr. Ellis considered that there were no cultural or morphological differences which warranted their separation into more than one species but agreed that the pathogenic group should be kept apart as belonging to a distinct strain. This is in accordance with the conclusion reached by Kiely (1964).

VIII. DISCUSSION

The brown spot disease of Emperor mandarin was found to be caused by a distinct strain of *Alternaria citri* Pierce, thus supporting the findings of Kiely (1964).

The germinating conidia induced cell necrosis without penetrating the host tissue through cuticle or stomata. This behaviour is similar to that shown by *Botrytis* sp. on onion leaves (Segall and Newhall 1960).

The fungus was shown to produce a host-specific toxic filtrate, and it is suggested that a phytotoxin is involved in the etiology of the disease.

Toxin production by *Alternaria* sp. has been discussed by Ludwig (1960). Pringle and Scheffer (1964) observed that there are only three examples where a phytotoxin produced *in vitro* displays the same host specificity as the pathogen itself. These are the phytotoxins produced by *Helminthosporium victoriae* Meehan and Murphy, *Periconia circinata* (Mangin) Sacc., and the Japanese pear pathogen *Alternaria kikuchiana* Tanoka. In each case the phytotoxin induced symptoms identical with those produced by the pathogen. This, as well as host specificity, was observed for the toxic filtrates produced by the brown spot pathogen, *Alternaria citri* Pierce f. sp.

Hiroe and Aoe (1954) found that non-pathogenic isolates of *A. kikuchiana* did not produce toxic culture filtrates, and that there was a correlation between the relative virulence of different pathogenic isolates and the toxicity of their respective culture filtrates. Luke and Wheeler (1955) also found that, among pathogenic isolates of *H. victoriae,* differences in pathogenicity correlated with differences in toxin production, and demonstrated a lack of toxin production by non-pathogenic isolates. It has been shown in the present studies that non-pathogenic isolates of *A. citri* did not produce a toxic filtrate. However, as a simple quantitative bioassay was not available, no attempt was made to correlate toxin production with the relative virulence of various pathogenic isolates.

Dimond and Waggoner (1953) contended that phytotoxic culture filtrates should not be ascribed a role in the etiology of a disease unless the phytotoxin has been also isolated from naturally infected host tissues.

Pringle and Scheffer (1964) indicated that there is a greater likelihood of establishing a phytotoxin produced *in vitro* as being important in disease development if it displays the same host specificity as the pathogen itself.

No attempt has been made in the present studies to isolate the toxic metabolite from infected host tissue. However, the specificity of the toxic filtrates, the toxin production by pathogenic isolates only, and the necrosis of host tissue without penetration by the germinating conidia, all indicate that the toxin is the primary disease incitant.

The brown spot pathogen is probably, like other strains of *A. citri,* a very weak parasite, and would be unable to infect healthy plant tissue were it not for the toxic secretion which kills the host cells in advance of the fungus.

IX. ACKNOWLEDGEMENTS

The advice of Mr. B. L. Oxenham and Mr. J. H. Simmonds is gratefully acknowledged. Mr. William Manley (Illustrator of the Department of Primary Industries) prepared the photomicrographs. Dr. M. B. Ellis, of the Commonwealth Mycological Institute, examined isolates of *Alternaria citri.*

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