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The Use of a Benomyl-Resistant Mutant to Demonstrate Latency of *Colletotrichum gloeosporioides* in Avocado Fruit

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Abstract

A benomyl-resistant mutant of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., generated by irradiating a wild-type isolate of the fungus with ultraviolet light, was used as a marker organism to demonstrate latency under field conditions. This mutant could be easily distinguished from wild-type isolates of *C. gloeosporioides* on the basis of growth rates on benomyl-amended media, and was as virulent in avocado fruit as wild-type isolates. Through the use of this mutant in field inoculations of avocado fruit, it was possible to demonstrate conclusively the existence, for the first time, of latency in the life cycle of *C. gloeosporioides* in this host. It was also shown that the fungus was able to remain latent for periods of at least 6 months.

Keywords: latency, benomyl-resistance, anthracnose, *Colletotrichum gloeosporioides*, avocado, *Persea americana*.

Introduction

Latency is defined as a quiescent or dormant parasitic relationship which, after a time, can change into an active one (Verhoeff 1974). The existence of latent infections of *Colletotrichum* spp. in many subtropical and tropical fruits has long been recognized. Various methods have been used to demonstrate latency of such infections. In early studies, an isolation technique was commonly used to demonstrate latency. In this technique, isolations were made from the surface sterilized peel of apparently disease-free fruit. Wardlaw (1931) isolated *C. musae* from the peel of symptomless banana fruit. Similarly, Baker (1938) isolated *C. gloeosporioides* from the peel of avocado, mango and citrus fruit. At the time, these findings were considered to be good evidence for the existence of latent infection, although Simmonds (1941) pointed out that the technique does not prove the existence of latent infection because it does not show that the fungus resumes parasitic activity when conditions become more favourable.

In recognizing this inadequacy, Simmonds (1941) proceeded to demonstate latency of *C. musae* in banana fruit using an artificial inoculation technique. Banana fruit were inoculated at various stages of development with conidia of *C. musae*. The inoculated areas on each fruit were marked by circles. Fruit were harvested at maturity, ripened and assessed for symptom development within the circled areas. The development of anthracnose lesions within these areas was

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taken as evidence for latent infection. Simmonds (1941) claimed that natural and artificial infections of *C. musae* could be distinguished on the basis of their position and timing of appearance. Using this inoculation technique, Simmonds concluded that infections of *C. musae* could be established shortly after bunch emergence and remain latent for a period of approximately 5 months before developing into typical anthracnose lesions during fruit ripening.

More recently, the inoculation technique was used by Binyamini and Schiffmann-Nadel (1972) to demonstrate latency of *C. gloeosporioides* in avocado fruit. To minimize fruit contact with natural sources of inoculum, inoculated fruit were enclosed in paper bags until they were harvested at maturity.

Dickman and Alvarez (1983) also used an inoculation technique to demonstrate latency of *C. gloeosporioides* in papaw. Entire columns of papaw fruit were spray-inoculated with conidia of *C. gloeosporioides*. Inoculated fruit were harvested when mature, ripened and observed for development of anthracnose symptoms. They found that *C. gloeosporioides* could infect fruit at early stages of maturity and remain latent until fruit reached the climacteric phase.

The main limitation of the inoculation technique is the difficulty in distinguishing between natural and artificial infections. The aim of the work reported in this paper was to develop a new technique for studying latency which would readily distinguish between natural and induced infections using a fungicide-resistant mutant of *C. gloeosporioides*. Benomyl resistance was chosen as a marker for this study because it is relatively easy to obtain, stable, and arises from a single chromosomal gene mutation (Hastie and Georgopoulos 1971). The growth of wild-type strains of *C. gloeosporioides* is strongly inhibited by benomyl (Bollen 1972; Muirhead 1974). Benomyl interferes with fungal mitosis by binding to tubulin, the building block of microtubules (Davidse and de Waard 1984). Resistance to benomyl is apparently conferred by a change in tubulin structure (Davidse and Flach 1978; Sheir-Neiss *et al.* 1978). This paper describes the generation of a benomyl-resistant mutant of *C. gloeosporioides* which could be readily distinguished from wild-type isolates of this fungus and therefore be used subsequently to demonstrate latency in avocado fruit under field conditions.

Materials and Methods

Generation of Benomyl-Resistant Mutants

Conidia of *C. gloeosporioides* (BRIP 19780) were obtained by gently scraping with a wire loop the surface of 7-day-old cultures growing on oatmeal agar (OMA). Suspensions of conidia in sterile distilled water were filtered through muslin to remove most of the mycelial fragments and adjusted to a concentration of $2 \cdot 5 \times 10^5$ conidia/mL using a haemocytometer. Plastic petri dishes each containing 10 mL of the suspension were exposed to ultraviolet (UV) irradiation at 254 nm wavelength (30 W tubes at a distance of 38 cm) for 5 min. This UV irradiation exposure time was chosen on the basis of a preliminary experiment which showed that a 5 min exposure to UV irradiation resulted in a 90% kill of conidia. Drops (0·2 mL) of the treated suspension were spread over potato dextrose agar (PDA) plates containing either 0, 1 or 5 μg/mL benomyl. Preliminary studies had shown that the growth of wild-type isolates of *C. gloeosporioides* was almost completely inhibited by 1 μg/mL benomyl. Plates were incubated at 25°C under 12 h/day irradiation from near UV light (peak at 365 nm, range 310–410 nm) and 12 h/day darkness. Colonies which grew on benomyl-amended media were regarded as benomyl-resistant. Benomyl-resistant colonies of *C. gloeosporioides* were subcultured onto PDA and OMA and held at 25°C under near UV light (12 h/day).

Growth Characteristics of the Benomyl-Resistant Mutants In Vitro

Plugs of mycelium 5 mm in diameter were taken from the margin of 7-day-old cultures of three benomyl-resistant mutants and six wild-type isolates of *C. gloeosporioides* (ex avocado) growing on PDA. Plugs were transferred to PDA plates containing either 0, 1 or 10 μg/mL benomyl. Plates were incubated at 26±0·5°C under near UV light (12 h/day). Colony diameters were measured 7 days after inoculation.

Pathogenicity of the Benomyl-Resistant Mutants in Avocado Fruit

Avocado fruit cv. Reed were washed to remove traces of copper-based fungicide and then wiped with 70% (v/v) ethanol to reduce the epiphytic microflora. Paper discs saturated with a concentrated suspension of benomyl (2 mg/mL) were placed on the freshly cut surface of fruit pedicels to control stem end rot caused by *Dothiorella aromatica*. Conidium suspensions (4×10^5 conidia/mL) were prepared from 7-day-old cultures of three benomyl-resistant mutants of *C. gloeosporioides* and the parental wild-type isolate (BRIP 19780). Fruit were inoculated by placing 25 μL drops of each conidium suspension onto an area of unwounded peel. Inoculated fruit were incubated under high humidity (≥ 95% RH) at 24±1°C. At 8–12 days after inoculation, fruit were removed and lesion diameters were measured. The firmness of fruit was estimated by applying gentle thumb pressure (firmness scale: 1, hard; 2, first detectable softening; 3, between first detectable softening and eating-ripe; 4, eating-ripe; 5, over-ripe). Isolations were made from the advancing margins of lesions onto PDA. Isolates growing on PDA were subcultured onto benomyl-amended PDA. Plates were incubated at 25°C under near UV light (12 h/day) and colony diameters were measured 6 days after inoculation.

Fruit Inoculations in the Field using a Benomyl-Resistant Mutant of C. gloeosporioides

On the basis of *in vitro* growth characteristics, the benomyl-resistant mutant BRIP 19779 was selected for use in field inoculations. Suspensions of conidia (10^6 conidia/mL) were prepared as previously described. Tween-20 wetting agent was added to conidium suspensions at a rate of 0·35 mL/L.

All fruit inoculations were conducted on the cultivar Fuerte on two neighbouring avocado orchards at Maleny (26° 46′S., 152° 51′E.), south-east Queensland. No attempt was made to control diseases on orchard 1, whereas trees on orchard 2 were regularly sprayed with copper oxychloride for anthracnose control.

Fruit were inoculated at monthly intervals, commencing in November when fruit were approximately 1·5–2 cm in length. The final inoculation was performed in April of the following year when fruit were mature. Fruit were inoculated by saturating a 1 or 2 cm^2 (depending on fruit size) piece of sterile blotting paper in the prepared conidium suspension of *C. gloeosporioides*, and placing this onto an area of unwounded peel. The paper squares were attached to the surface of fruit using waterproof tape, and the location of each square was marked on the surface of fruit. An equal number of fruit was inoculated with water containing wetting agent only (controls). Following inoculation, each fruit along with a few leaves on the same shoot was enclosed in a plastic bag containing a small amount of wet cotton wool to help maintain a high humidity around the inoculation site. A white paper bag was then placed on the outside of the plastic bag to protect fruit from high temperatures. Plastic and paper bags were removed after 48 h.

Preharvest Sampling of Fruit

At 24, 48, 72 and 96 h after each inoculation, three inoculated fruit and three control fruit were removed from orchard 1 and taken to the laboratory within 2 h. Slices of peel approximately 3 mm long×3 mm wide×200 μm thick were cut from the inoculated area of each fruit using a razor blade. Four slices of peel were cut from each fruit. Peel slices were surface sterilized in 1% (v/v) sodium hypochlorite for 1 min, rinsed 3 times in sterile distilled water, and dried on sterile blotting paper. Surface sterilized peel slices were transferred to PDA plates which were incubated at 25°C under near UV light (12 h/day) for 4 days. Mycelial plugs 3 mm in diameter were taken from colonies growing from peel slices and transferred to

PDA containing 0, 1 or 10 μg/mL benomyl. Colony diameters were measured 3 and 6 days after inoculation. On the basis of these growth rates, isolates were classified as being either benomyl-resistant or benomyl-sensitive.

Preharvest Symptom Development

At various intervals after inoculation, observations were made on the development of disease symptoms on fruit still attached to the tree.

Postharvest Sampling of Fruit

All inoculated fruit remaining on trees in May were harvested, brought to the laboratory within 2 h and placed in storage at 20°C. Fruit were sampled at various stages during ripening. Fruit firmness was the parameter used to define different stages of ripeness and was measured using an Instron universal testing machine (12 mm diameter cylindrical probe, crosshead speed 20 mm/min, probe penetration 2 mm). In a preliminary experiment, changes in the firmness of Fuerte avocado fruit during ripening at 20°C were measured. From these results (Fig. 1), five firmness categories were devised as follows: (1) $\geq$ 53 newtons (N) (hard fruit); (2) 40–52 N; (3) 27–39 N; (4) 14–26 N; (5) $\leq$ 13 N (soft fruit). First detectable softening, as judged by using gentle thumb pressure, coincided with firmness readings in the range of 15–20 N.

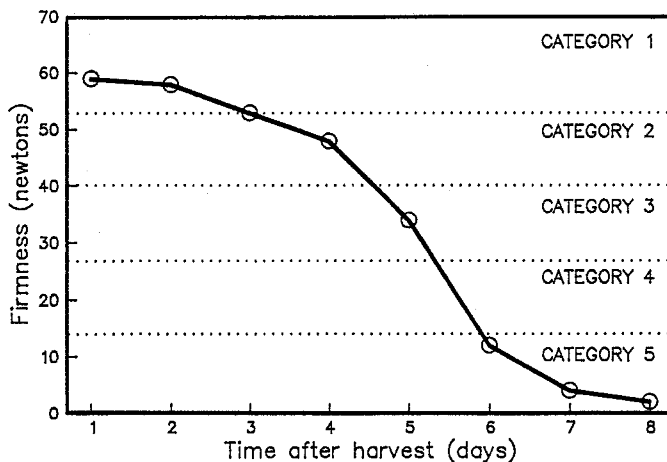

Fig. 1. Postharvest firmness changes in 'Fuerte' avocado fruit ripened at 20°C.

Each day, a subsample of field-inoculated fruit was removed from storage for measurement of firmness. Two firmness readings were taken for each fruit. Fruit were sampled when they attained the desired degree of firmness. For each inoculation time (i.e. November–April) and each inoculum concentration (i.e. 0 or 10^6 conidia/mL), 3–5 fruit were sampled per firmness category, depending on the total number of fruit available. Where fruit were soft and lesions had formed in the inoculated area, isolations were made by plating out small pieces of diseased pulp tissue from lesion margins onto PDA. In all other fruit (unripe fruit and ripe fruit with no lesions), peel slices were taken from the inoculated areas of fruit as previously described and transferred to PDA. Colonies of *C. gloeosporioides* growing from peel or pulp pieces were subcultured onto benomyl-amended media as previously described.

Results

Generation of Benomyl-Resistant Mutants

A total of 10 colonies of *C. gloeosporioides* was found growing on benomyl-amended media after treatment of conidia with UV irradiation for 5 min. Three

of these mutants (BRIP Nos 19777, 19778 and 19779) were chosen for further study on the basis of growth rates on benomyl-amended media and ability to produce abundant conidia.

Growth Characteristics of the Benomyl-Resistant Mutants in vitro

The mycelial growth of three benomyl-resistant mutants and six wild-type isolates of *C. gloeosporioides* on benomyl-amended media is shown in Table 1. On PDA containing no benomyl, the growth rates of mutants BRIP 19777 and 19779 were significantly lower ($P < 0{\cdot}05$) than that of the parental wild-type isolate (BRIP 19780), whereas the growth rate of mutant BRIP 19778 was higher. On PDA containing 1 μg/mL benomyl, the growth rates of all mutants were significantly higher than those of all wild-type isolates of *C. gloeosporioides*. Mutant BRIP 19778 grew the fastest on 1 μg/mL benomyl. On PDA containing 10 μg/mL benomyl, mutants BRIP 19777 and 19779 grew significantly faster than all wild-type isolates of *C. gloeosporioides*, but mutant BRIP 19778 did not.

Table 1. Mycelial growth of benomyl-resistant mutants and wild-type isolates of *C. gloeosporioides* at 26°C on benomyl-amended PDA

Means followed by the same letter within columns do not differ at $P = 0{\cdot}05$ using the l.s.d. test

Isolate	Colony diameter (mm)[A]		
Benomy concentration (μg/mL):	0	1	10
BRIP 19777 (mutant)	65·1c	18·3c	11·5b
BRIP 19778 (mutant)	80·0a	64·3a	0·8d
BRIP 19779 (mutant)	66·3c	46·9b	38·8a
BRIP 19780 (parental wild-type)	73·3b	4·5de	2·0cd
BRIP 19768 (wild-type)	73·3b	2·9de	2·0cd
BRIP 19769 (wild-type)	80·0a	2·9de	2·5cd
BRIP 19770 (wild-type)	71·5b	1·9de	2·5cd
BRIP 19773 (wild-type)	72·3b	5·1d	4·4c
BRIP 19774 (wild-type)	71·5b	1·0e	1·0d

[A] Mean of four replicates at 7 days after inoculation.

Pathogenicity of the Benomyl-Resistant Mutants in Avocado Fruit

There were no significant differences between the mean diameters of lesions in fruit inoculated with any of the mutants or the wild-type isolate. On benomyl-amended media, the growth characteristics of all isolates obtained from lesions were consistent with the growth characteristics of isolates used to inoculate fruit. The pathogenicity of mutants BRIP 19777, 19778 and 19779 in avocado fruit cv. Reed was established in this experiment.

Selection of a Benomyl-Resistant Mutant for Use in Field Inoculations of Fruit

On the basis of growth characteristics on PDA containing 10 μg/mL benomyl, mutant BRIP 19779 was selected for use in field inoculations.

Preharvest Sampling of Field-Inoculated Avocado Fruit

The benomyl-resistant mutant of *C. gloeosporioides* (BRIP 19779) was isolated from the peel of inoculated avocado fruit at each sampling time (24–96 h) after

inoculation (Table 2). Out of a total of 72 fruit sampled from November to April, only three fruit (4·2%) did not yield the mutant. As expected, the mutant was not isolated from the peel of uninoculated control fruit.

Table 2. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of field-inoculated avocado fruit cv. Fuerte sampled between 24 and 96 h after inoculation

Month of inoculation	No. fruit yielding mutant/Total no. fruit sampled[A]							
Time after inoculation (h):	24		48		72		96	
	C[B]	I	C	I	C	I	C	I
November	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
December	0/3	3/3	0/3	3/3	0/3	2/3	0/3	3/3
January	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
February	0/3	3/3	0/3	3/3	0/3	3/3	0/3	3/3
March	0/3	3/3	0/3	2/3	0/3	3/3	0/3	3/3
April	0/3	3/3	0/3	3/3	0/3	3/3	0/3	2/3

[A] Sampled from orchard 1 only.
[B] C, uninoculated control fruit; I, inoculated fruit.

Preharvest Symptom Development in Field-Inoculated Avocado Fruit

Fruit inoculated in November showed no symptoms of disease in the marked area prior to harvest. Some of the fruit inoculated at each of the other times, however, did show symptoms of disease before harvest. These symptoms appeared as small, dark, circular or irregularly shaped lesions on the surface of fruit ranging from 1 to 5 mm in diameter. Unlike typical anthracnose lesions, these lesions were restricted to the peel of fruit and did not extend into the flesh. Because the diameter of these lesions rarely exceeded a few millimetres, they

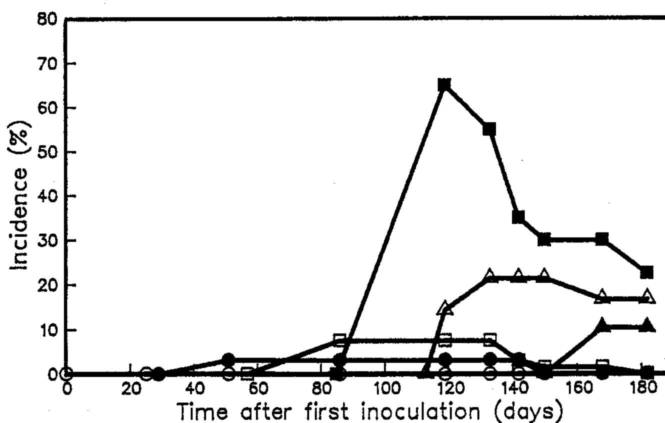

Fig. 2. Incidence of limited anthracnose lesions in avocado fruit cv. Fuerte inoculated with *C. gloeosporioides* at various stages of development (orchard 1 and 2 data combined). ○ November-inoculated fruit; ● December-inoculated fruit; □ January-inoculated fruit; ■ February-inoculated fruit; △ March-inoculated fruit; ▲ April-inoculated fruit.

have been referred to as 'limited lesions'. They were not associated with any macroscopically visible wounds in the fruit peel, although they were often formed around lenticels. In fruit where these limited lesions formed, symptoms usually appeared in the inoculated area within 10 days of inoculation.

The incidence of these limited lesions in inoculated avocado fruit is shown in Fig. 2. Fruit inoculated in February clearly showed the highest incidence of limited lesions. A high incidence of these lesions was also recorded in March-inoculated fruit. In many cases, the incidence of limited lesions decreased during the later part of the season. This is because some of the fruit displaying these symptoms fell from trees at various times. In some cases, it appeared that the limited lesions might be the cause of fruit fall. In other cases, however, the cause of fruit fall was spreading preharvest anthracnose lesions which resulted from natural infections of *C. gloeosporioides* through skin injuries.

Postharvest Sampling of Field-Inoculated Avocado Fruit

Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of fruit sampled at various stages of ripeness is shown in Table 3. The rate of mutant recovery from peel was very high in fruit from each of the inoculation times. Out of a total of 90 inoculated fruit sampled, only 10 fruit (11%) did not yield the mutant from peel.

Table 3. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of field-inoculated avocado fruit cv. Fuerte sampled at various stages of ripeness (orchard 1 and 2 data combined)

Month of inoculation	No. fruit yielding mutant/Total no. fruit sampled									
	FC[A]: 1		2		3		4		5	
	C[B]	I	C	I	C	I	C	I	C	I
November	0/4	1/3	0/4	3/4	0/4	3/3	0/4	2/2	0/3	—[C]
December	0/4	2/4	0/4	4/4	0/4	4/4	0/3	4/4	0/5	2/2
January	0/4	3/4	0/4	4/4	0/4	3/4	0/4	4/5	0/4	—[C]
February	0/4	3/3	0/4	3/3	0/4	2/2	0/5	3/3	0/4	—[C]
March	0/4	4/4	0/4	3/4	0/4	3/3	0/4	2/2	0/4	1/1
April	0/4	4/4	0/4	5/5	0/4	5/5	0/4	0/1	0/5	3/3

[A] FC, firmness category: 1, $\geq$ 53 N (hard); 2, 40–52 N; 3, 27–39 N; 4, 14–26 N; 5, $\leq$ 13 N (soft).
[B] C, uninoculated control fruit; I, inoculated fruit.
[C] Pulp isolations made only (see Table 4).

Table 4. Recovery of the benomyl-resistant mutant of *C. gloeosporioides* from diseased pulp of field-inoculated avocado fruit cv. Fuerte (orchard 1 and 2 data combined[A])

	No. fruit yielding mutant/Total no. fruit sampled												
Month of inoculation:	Nov.		Dec.		Jan.		Feb.		Mar.		Apr.		Total
FC[B]:	4	5	4	5	4	5	4	5	4	5	4	5	
	1/2	2/4	—	3/11	—	13/14	—	6/6	—	9/9	2/2	5/5	41/53

[A] Inoculated fruit only.
[B] FC, firmness category: 4, 14–26 N, 5, $\leq$ 13 N.

Recovery of the benomyl-resistant mutant from the diseased pulp tissue of field-inoculated fruit sampled at firmness categories of 4 and 5 (softening fruit) was also very high (Table 4). Out of a total of 53 inoculated fruit sampled, only 12 fruit (23%) did not yield the mutant from diseased pulp. The lowest rate of mutant recovery from diseased pulp was recorded in December-inoculated fruit.

The benomyl-resistant mutant was not isolated from the peel or pulp of any uninoculated control fruit.

Discussion

The latency of *C. gloeosporioides* in avocado fruit cv. Fuerte was demonstrated clearly in this study. The isolation of the benomyl-resistant mutant of *C. gloeosporioides* from the peel of fruit between 24 and 96 h after inoculation confirmed the presence and the viability of the fungus in or on the peel of fruit. The isolation of the mutant from the peel of field-inoculated fruit after harvest indicated that the fungus was still viable after several months. The isolation of the mutant from the diseased pulp of field-inoculated fruit confirmed that the latent fungus was able to resume growth and cause disease in ripe fruit. It was shown that the fungus could remain latent in or on the peel of avocado fruit for at least 6 months. This is consistent with the findings of Binyamini and Schiffmann-Nadel (1972).

Performance of the benomyl-resistant mutant as a marker organism under field conditions was excellent at all times. Of all the isolations made from the peel or diseased pulp of uninoculated control fruit, no isolate of *C. gloeosporioides* was able to grow on benomyl-amended media to the same extent as the benomyl-resistant mutant. Occasionally, *C. acutatum* was isolated from the peel of uninoculated control fruit, but this too was clearly distinguishable from the mutant on the basis of growth rates and cultural characteristics.

Fruit inoculated in December on both orchards showed a relatively low rate of mutant recovery from diseased pulp. This occurred despite the fact that isolations made from peel after inoculation and after harvest confirmed the existence of the fungus in a viable state in or on the peel of December-inoculated fruit. For some reason, the fungus did not develop further to cause disease lesions. The lesions that were present in the marked area of December-inoculated fruit must have been caused by natural infections of *C. gloeosporioides*. Simmonds (1941) stated that 'areas of artificially inoculated banana peel have yielded isolations of *Gloeosporium musarum* (≡ *C. musae*) after surface sterilization from areas free from any lesion although the time for the normal appearance of such had definitely passed'. Simmonds (1941) could not offer an explanation as to why some latent infections of *C. musae* did not develop further to cause disease lesions.

Although anthracnose is predominantly a postharvest disease of avocado, Fitzell (1987) reported that lesions can develop in immature, unripe fruit still on the tree. Two types of preharvest anthracnose lesions were described by Fitzell (1987). 'Type 1' lesions were described as large (1–4 cm diameter), spreading lesions which were usually associated with some form of peel injury. 'Type 2' lesions, on the other hand, were small (0·1–0·5 cm diameter), limited lesions which were rarely associated with peel injury, but in 69% of cases were associated with fruit lenticels. The small, limited anthracnose lesions which we observed in

field-inoculated avocado fruit prior to harvest closely fitted Fitzell's description of Type 2 lesions. In the studies conducted by Fitzell (1987), however, these Type 2 lesions were never observed in artificially inoculated avocado fruit. Because such lesions were able to be reproduced in the field study reported here without artificial wounding of the fruit peel, infection must have occurred either through areas of undamaged peel or through lenticels.

The timing of appearance of limited lesions in this field study is worthy of comment. Limited lesions occurred in fruit inoculated between December and April, which correlates well with the periods of highest rainfall during the season. The highest incidence of these lesions, however, occurred in fruit inoculated in February. Although the monthly rainfall during February was high (129 mm), there were many other months when rainfall was much greater (e.g. April, 858 mm). Perhaps other factors such as duration of fruit surface wetness, which was not monitored in this field trial, play an important role in the development of limited lesions. Fitzell (1987) suggested that the appearance of Type 2 lesions may be related to a low concentration of antifungal compounds in the peel of fruit, allowing either direct lenticel infections (no latent phase) or normal latent infections to proceed prematurely. There is very little published information available on preharvest changes in the concentration of antifungal compounds in the peel of avocado fruit. It is quite possible that levels of these compounds vary at different stages during fruit development. Such changes could help to explain the seasonal variation in fruit susceptibility to infection.

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