Factors Affecting Postharvest Development of Collectotrichum gloeosporioides in Citrus Fruits

G. Eldon Brown

Plant Pathologist III, Florida Department of Citrus, University of Florida, Institute of Food and Agricultural Sciences, Agricultural Research and Education Center, P.O. Box 1088, Lake Alfred 33850.

Florida Agricultural Experiment Stations Journal Series Paper No. 5480.

Appreciation is extended to L. K. Schmidt for technical assistance, and to T. A. Wheaton for ethylene analyses.

Accepted for publication 18 October 1974.

ABSTRACT

Appressoria of *Colletotrichum gloeosporioides* were often very numerous in localized areas on the surface of citrus fruits. Most appressoria did not produce infection hyphae, and those hyphae that were formed caused only a latent infection before fruit maturity. Such hyphae were thin, thread-like, less than 1.0 μm in diameter, and were observed within or beneath the cuticle, intercellularly in the epidermis, or inter- and intracellularly in the upper two-to-four cell layers of the flavedo. Ethylene-degreening treatment of mature Robinson tangerines stimulated appressoria to form infection hyphae, thereby causing an increased incidence of anthracnose. This effect was greater when an ethylene concentration of 50 μliters/liter of air rather than 5 μliters/liter of air were used. Washing the tangerines before degreening removed many of the appressoria and thereby reduced anthracnose severity. Infections established before harvest frequently did not provide sufficient latent inoculum to induce anthracnose.

Phytopathology 65:404-409

Additional key words: anthracnose, latent infection, ethylene.

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., has been described as a minor decay of oranges, *Citrus sinensis* (L.) Osbeck and grapefruit, *C. paradisi* Macfadyn (5). On some of the mandarin-type fruit, however, anthracnose can become economically important (12). Robinson tangerine, which originated from a cross between Clementine mandarin (*C. reticulata* Blanco) and Orlando tangelo (*C. reticulata* × *C. paradisi*), is particularly susceptible to the disease. On fruit of Robinson tangerine, anthracnose not only occurs following rind injury, but it may also develop from latent infection in intact peel.

Spores of *C. gloeosporioides* commonly germinate on the surface of citrus fruit and form appressoria which in turn produce infection hyphae that remain latent within the upper three cell layers of the flavedo portion of the peel (1). Such infections usually remain latent even after the fruit reaches maturity. In fact, in oranges and grapefruit anthracnose generally only occurs if the peel is injured or the fruit is overly mature.

Two factors greatly affect the incidence of anthracnose on the mandarin-type citrus fruits. Degreening, a method of improving fruit color by removing chlorophyll from the peel with ethylene, can cause an increase in anthracnose (12). In this procedure, fruit are exposed in degreening rooms to ethylene concentrations of 1-5 μliters/liter of air at 28-30 C and 90-96% relative humidity (RH) (16) for a period of 12 hours to 4 days, depending upon the time required to remove the green color from the fruit peel. The other factor is washing and this can cause a substantial decrease in the incidence of anthracnose (7, 13, 14) if the fruit are washed before being degreened. Washing after degreening, which is the current commercial practice, does not reduce anthracnose. Fruit washed before degreening frequently developed less anthracnose than fruit washed after degreening even where a postharvest fungicide was applied (7, 13, 14).

This study was initiated to observe the penetration of *C. gloeosporioides* through noninjured peel of citrus fruit, and to determine why degreening and washing alter the incidence of anthracnose.

MATERIALS AND METHODS.—The process of citrus peel penetration by *C. gloeosporioides* was studied in Robinson and Dancy tangerines, Orlando tangelos, Marsh Seedless grapefruits, and Valencia oranges. Degreening and washing experiments were performed only with Robinson tangerines.

Fruit were degreened in rooms maintained at 28-30 C and 90-96% RH (16) with ethylene concentrations of 0, 5, or 50 μliters/liter of air. Relative humidity was determined with a hygrometer and ethylene concentrations were verified periodically using a gas chromatograph equipped with a flame ionization detector. Fruit were washed on transverse rotary synthetic bristle brushes for approximately 40 seconds with a biodegradable, nonfungicidal soap which was later removed by rinsing with water.

For studies of spore germination, appressorium formation, and infection hypha growth, fruit were held at 26 C and supported on plastic rings in plastic dish pans (30 × 28 × 13 cm). Inoculations were made with a composite suspension of spores derived from seven isolates of *C. gloeosporioides* cultured on autoclaved orange twigs. Drops of spore suspension were deposited on washed and dried fruit at the equator on areas originally observed to be substantially free of appressoria formed by natural infections in the grove. An atmosphere of near 100% RH was obtained by pouring 50 ml of water into the bottom of the dish pan and covering the pan with a 0.2-mm-thick sheet of polyethylene film.

Germination of spores and formation of appressoria were observed by removing these structures from fruit using clear nail polish (17), and staining them with cotton blue-lactophenol. To determine whether infection hyphae had been produced, the fruit surface was first sterilized with 1.0% sodium hypochlorite. Spores, germ tubes, and appressoria present on the rind surface were thus killed before isolating for *C. gloeosporioides*. Isolations were made by taking five pieces of peel, each approximately 1.0 mm^2, from each of 12 fruit in each treatment. Peel of

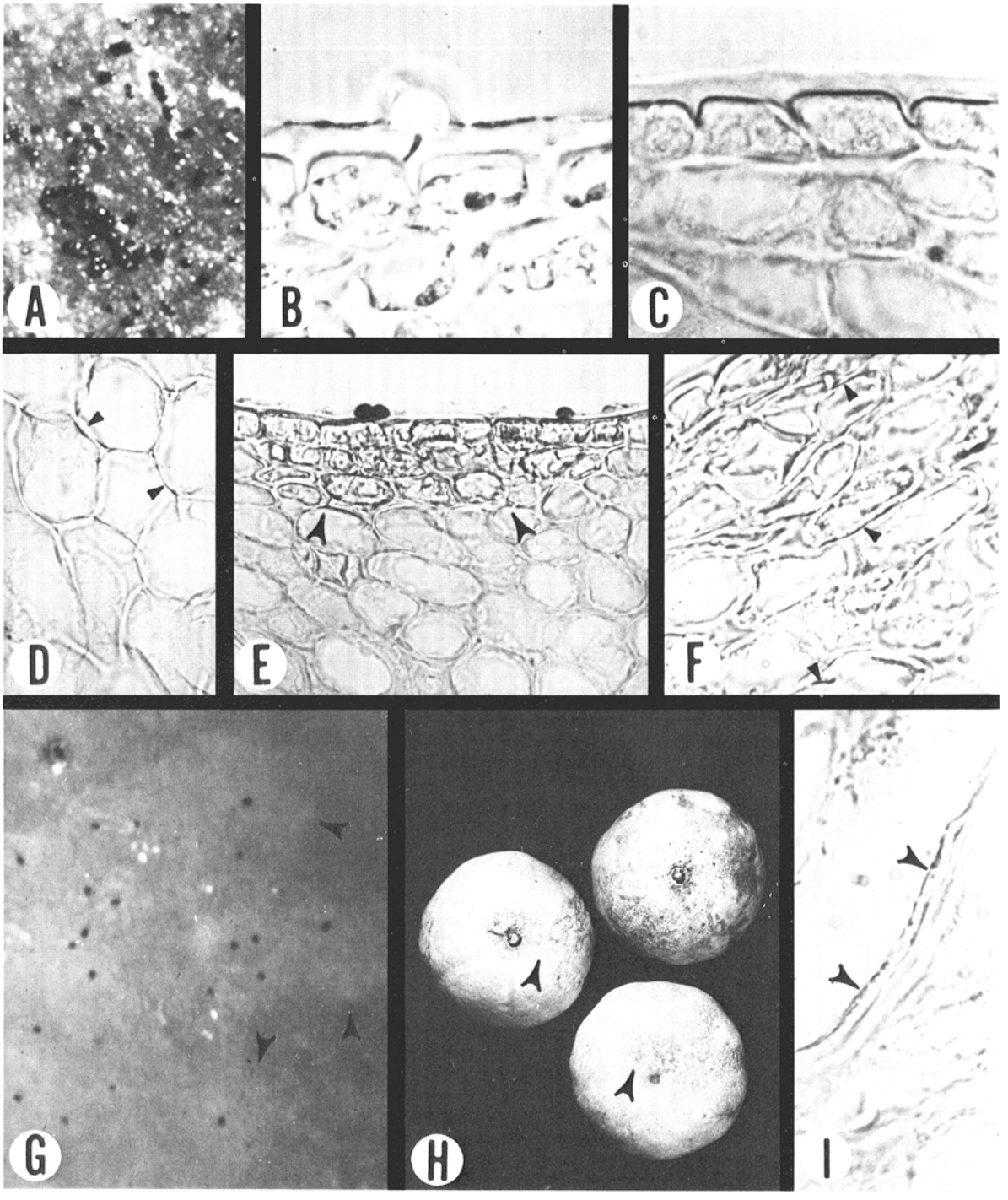

Fig. 1-(A to I). Stages of development of *Colletotrichum gloeosporioides* in citrus fruits. **A)** Appressoria on the surface of an immature Orlando tangelo (× 190). **B)** An appressorium with its infection hypha in the cuticle of a Valencia orange (× 1,250). **C)** Infection hypha growing under the cuticle and between epidermal cells of an Orlando tangelo (× 1,125). **D)** Single infection hypha entwining the walls of cells within the flavedo of an Orlando tangelo (× 500). **E)** Early stage of necrosis caused by infection hyphae of *C. gloeosporioides* in flavedo cells in the peel of a Robinson tangerine (× 320). **F)** Growth of infection hyphae within the peel of a Robinson tangerine (× 1,125). **G)** Halos around individual appressoria caused by removal of the red-orange pigments from the flavedo of a Robinson tangerine during the initial stage of anthracnose (× 125). **H)** Robinson tangerines in advanced stages of anthracnose. **I)** Developing hypha that has originated from a thread-like infection hypha (× 1,125).

TABLE 1. Influence of relative humidity (RH) after inoculation on formation of infection hyphae by appressoria of *Colletotrichum gloeosporioides* on peel of Robinson tangerines

Treatments	Fruit surface preparation	Tissue pieces yielding *C. gloeosporioides*[a] (%)
Check[b]	NSS[d]	27
	SS[e]	17
RH 100% for 1 day, then 50% for 2 days[c]	NSS	95
	SS	28
RH 100% for 3 days[c]	NSS	100
	SS	68
RH 100% for 1 day, 50% for 2 days, then 100% for 1 day[c]	NSS	100
	SS	25

[a]Each value was determined by plating five pieces of tissue/fruit from 12 fruit.
[b]Isolations made immediately from noninoculated fruit.
[c]Inoculated fruit were held under these conditions before isolations were made.
[d]Surface not sterilized before isolations were made.
[e]Surface-sterilized before isolations were made.

TABLE 2. Removal of *Colletotrichum gloeosporioides* from the peel of Robinson tangerines by washing after 0, 1, 2, 3, or 4 days of degreening with different concentrations of ethylene

Days of degreening	Percent removal by washing after degreening at ethylene concentrations of[a] (μliters/liter of air)		
	0	5	50
0	63	...	...
1	50	66	30
2	25	21	0
3	52	37	0
4	64	6	0

[a]Each value was determined by plating 120 pieces of tissue. Sixty pieces were taken from 12 fruit before, and again after, washing.

TABLE 3. Influence of time of washing either before or during degreening on the incidence of anthracnose (caused by *Colletotrichum gloeosporioides*) in Robinson tangerines

Time of washing[b]	Percentage fruit with anthracnose during degreening[a] for	
	3 days	4 days
Before degreening	1	1
After 1 day of degreening	16	27
After 2 days of degreening	31	51
Not washed	29	48

[a]Fifty μliters ethylene per liter of air.
[b]In each treatment 175 fruit were used.

inoculated fruit was taken from the inoculation site (approximately 10 mm in diameter) and that of noninoculated, nonwashed and washed fruit was removed from five equidistant regions at the fruit equator. A medium composed of 2.0% Bacto-agar, 0.5% dextrose, and 100 μg/ml novobiocin was used to isolate *C. gloeosporioides*.

To observe penetration and development of infection hyphae within the fruit peel, infected tissue was embedded in paraffin and sectioned at thicknesses of 6-15 μm with a rotary microtome. Cuticular penetration was best observed by viewing nonstained sections with phase contrast microscopy. Subcuticular growth of the infection hyphae could only be viewed by staining sections with thionin and orange G (10), and observing the hyphae under brightfield illumination.

RESULTS.—*Observations of appressorium formation and development of infection hyphae.*—Appressoria, 5-8 μm in diameter, were frequently observed on the surface of fruit collected from citrus groves. They were encountered on all cultivars examined, and tended to be concentrated in localized areas of the peel (Fig. 1-A). In one instance, 1,200 appressoria were observed on 1.0 mm^2 of the surface of a Robinson tangerine. The appressoria were usually more numerous around the stem-end than at the equator of citrus fruit.

In the inoculation tests, up to 50% of the spores formed germ tubes with appressoria within 3 hours at 26 C and 100% RH. After 22 hours, more than 90% of the spores had germinated and formed appressoria. Within 24 to 48 hours, some of the appressoria had formed infection hyphae, as evidenced by recovery of *C. gloeosporioides* from surface sterilized peel. However, sectioning of inoculated peel showed that relatively few appressoria had produced infection hyphae by this time. This delay in development of infection hyphae was particularly noticeable in sections taken from naturally infected mature fruit. After the infection hyphae had penetrated the cuticle, removal of the appressorium by surface sterilization did not diminish the recovery of the fungus from the peel, indicating that once penetration occurs, the appressorium is not needed for further subsistence.

Formation of infection hyphae by appressoria was affected by RH (Table 1). Surface sterilization consistently reduced the recovery of *C. gloeosporioides* from both naturally infected and inoculated fruit. Recovery from inoculated nonsurface-sterilized peel was essentially complete, whether fruit were held following inoculation for 1 or 3 days at 100% RH. On the other

hand, surface-sterilized tissue from inoculated fruit did not yield much more of the fungus than did the surface-sterilized check, except when the inoculated fruit were held for 3 days at 100% RH (Table 1). Recovery of *C. gloeosporioides* from inoculated peel was not increased by returning the fruit to 100% RH for a day following 2 days at 50% RH (Table 1). Additional studies with naturally infected Orlando tangelos stored 3 days at either 50 or 100% RH showed that no additional latent infections were established during storage other than those which already existed at the time of harvest.

Effect of washing and degreening fruit on the development of infection hyphae and on the incidence of anthracnose.—Washing effectively removed appressoria of *C. gloeosporioides* from the fruit surface. Occasionally, some regions of washed fruit were observed to contain appressoria where the surface was either missed or only brushed lightly. Removal of *C. gloeosporioides* from naturally infected fruit by washing was affected by the degreening period and the concentration of ethylene used while degreening (Table 2). Degreening room conditions of 28-30 C and 90-96% RH without ethylene did not encourage formation of infection hyphae, as removal of *C. gloeosporioides* by washing was just as effective at 4 days as at the beginning of the experiment. When ethylene was added to the atmosphere, however, the formation of infection hyphae increased with time. This effect was greater when fruit were degreened with 50 rather than 5 μliters ethylene/liter of air. After 2 days of degreening with 50 μliters/liter of air, no removal of *C. gloeosporioides* was attained by washing the fruit (Table 2) and by 3 days, several fruit were exhibiting early symptoms of anthracnose.

In a subsequent experiment, Robinson tangerines were degreened for 4 days with either 5 or 50 μliters ethylene/liter of air and the incidence of anthracnose was recorded. The increase in anthracnose by degreening with 50 μliters ethylene/liter of air was quite striking, the incidence of decay being more than double that at 5 μliters/liter (19% vs. 45% after 3 days of degreening and 26% vs. 60% after 4 days of degreening).

Next, an experiment was performed with Robinson tangerines to compare the effectiveness of a pre-degreening wash with later times of washing for anthracnose prevention. In this test, the fruit were exposed to 50 μliters ethylene/liter of air because of the more rapid development of disease at this concentration. Washing before degreening almost completely prevented the occurrence of anthracnose during 4 days of degreening (Table 3). Washing after 1 day of degreening was less effective in this respect, and washing after 2 days did not reduce disease incidence below that in unwashed fruit.

As mentioned previously, the distribution of appressoria over the fruit surfaces tended to be uneven, with only localized areas having a high concentration of appressoria. Furthermore, areas of the peel of Robinson tangerines with more than 200 appressoria/mm^2 were usually the first areas of the peel to develop anthracnose during degreening. To determine the appressorium density needed to induce disease, fruit were inoculated with different concentrations of spores and observed for development of anthracnose during 5 days of degreening (Table 4). Fruit with an average of 283 or more appressoria/mm^2 of fruit surface developed anthracnose within 3 days. Those with an average of 156 appressoria/mm^2 of surface developed anthracnose after 4 days, while fruit with an average of 99 appressoria or less had none after 5 days.

TABLE 4. Effect of inoculum (*Colletotrichum gloeosporioides* spore suspension) concentration on the occurrence of anthracnose during degreening of Robinson tangerines

Appressoria/mm^2 of fruit surface[b]	Percentage fruit with anthracnose during degreening[a] for		
	3 days	4 days	5 days
314	25	58	83
283	8	42	58
156	0	17	42
99	0	0	0
54	0	0	0

[a] 50 μliters ethylene per liter of air.
[b] Average number of appressoria on the surface of 12 inoculated fruit in each treatment.

Histology of latent infection and decay.—Infection hyphae of *C. gloeosporioides* in peel were very thin and thread-like, being less than 1.0 μm in diameter. These latent hyphae persisted within and beneath the cuticle, and in the intercellular spaces of the epidermis (Fig. 1-B, 1-C). Frequently, they had also penetrated beneath the epidermis by the time of fruit maturity, and were latent within three or four cell layers of the flavedo (Fig. 1-D). Degreening with 50 μliters ethylene/liter of air did not stimulate these infection hyphae to cause anthracnose of noninjured, mature Dancy tangerines, Orlando tangelos, Marsh Seedless grapefruits, or Valencia oranges. Even on noninjured, nondegreened, mature Robinson tangerines, the infection hyphae did not normally develop further and cause anthracnose. Growth of the infection hyphae in the peel of degreened Robinson tangerines, however, was quite extensive (Fig. 1-E, 1-F). Hyphae grew inter- and intracellularly, usually along the cell wall. The first visible symptoms occurred after the infection hyphae had colonized the peel. Silver-grey halos, due to the removal of the red-orange pigments from the flavedo, formed around many of the individual appressoria (Fig. 1-G). As the disease progressed (Fig. 1-H), infected tissue contained both the thread-like infection hyphae and the larger, 2-5 μm in diameter, hyphae.

Ontogeny of the infection hypha was difficult to interpret from observations of prepared sections. The thin infection hypha appeared to enlarge gradually into the larger hypha which retained the dark-staining thionin in its cell walls (Fig. 1-I). When mature, these hyphae did not retain thionin in their walls, and thus were not readily detected in stained sections. Infection hyphae developed into the larger hyphae only in degreened Robinson tangerines.

DISCUSSION.—*C. gloeosporioides* persists on the surface of mature citrus fruit mostly as latent appressoria. This form of latency is apparently not unique to citrus fruit. It has also been reported for isolates of this fungus on avocados (2) and papayas (15). However, the infection

process on mango (11) is reported to be different. On this host, the fungus forms an appressorium and immediately produces an infection hypha. This penetrates the cuticle and remains latent as a small knot of mycelium beneath the cuticle or within the epidermal cell wall. Callose and cell proliferation, as observed beneath latent appressoria of *C. gloeosporioides* on papayas (15), were not detected beneath appressoria on the surface of citrus fruit.

Recovery of the fungus subsequent to surface sterilization as proof of latent infection in papayas has been questioned (15) since disinfectants may be ineffective against appressoria formed in outer stomatal chambers. However, with citrus fruit, appressoria were observed only in one instance in stomata of the peel. Also, the histological studies described here showed that infection hyphae had indeed been formed by some of the appressoria. Adam et al. (1) in their studies with Washington Navel oranges in Australia, made no distinction between appressoria with and without infection hyphae. Apparently, they believed that all appressoria on mature fruit had formed such hyphae. Formation of infection hyphae was favored by holding inoculated fruit at 100% RH for 3 days. These results suggest, presuming a similar response with attached immature fruit, that infection hypha formation may be affected by the duration of high humidity or wetness existing on the surface of immature fruit in the grove following spore dissemination, germination, and appressorium formation.

The shape of the infection hyphae produced by appressoria of *C. gloeosporioides* during penetration varies with its particular host. In avocados (2), an infection hypha several μm in diameter is produced during penetration of the cuticle and epidermis. However, during parasitism of immature poplar and lemon leaves (3, 8) and mango fruit (11), the infection hypha is quite narrow and constricted while penetrating the cuticle, but it rapidly enlarges as it grows subcuticularly or into the epidermal cells. The thread-like hypha produced during the infection of citrus fruit is similar to the hypha formed by *Gloeosporium perennans* (4) during penetration of the cuticle and cortical cells of apples. Such extensive development of the thread-like infection hypha following penetration, as observed here during infection of degreened Robinson tangerines, apparently has not been observed with any of the other hosts of *C. gloeosporioides*.

Washing removed many of the appressoria of *C. gloeosporioides* from the surface of citrus fruit. As only a few of the appressoria had formed a true latent infection, this was an effective means of separating the organism from its host. As a result, the remaining inoculum was often insufficient to induce disease even in highly susceptible Robinson tangerines. Exposure of unwashed Robinson tangerines to ethylene, which is required for degreening, caused rapid penetration of the peel by the infection hyphae. This explains why washing before, as opposed to after, degreening reduces anthracnose (7, 13, 14). The manner in which ethylene induces the appressorium to form the infection hypha is yet to be determined. Ethylene may stimulate formation of infection hyphae by exerting a direct effect on the appressoria. Also, it could hasten or alter physiological changes in the peel which allow or induce formation of infection hyphae. Such changes in Robinson tangerines not only allow penetration of the cuticle, but they also allow extensive colonization of the peel and eventual decay of the fruit. A concentration of 50 μliters ethylene/liter of air stimulated more rapid development of anthracnose than did 5 μliters/liter of air. A similar response to ethylene concentration has also been observed with other citrus fruit decay (9).

Washing before, rather than after, degreening has been suggested (7, 13) as a commercial practice to reduce anthracnose in the mandarin-type citrus fruits. At present, commercial packinghouses in Florida are not designed for such a practice. More importantly, results have been obtained, although only with oranges and grapefruit (6), showing that washing retards the removal of chlorophyll during degreening. The additional degreening time required can thus lead to an increase in other postharvest decays and peel disorders. However, more recent work (7) suggests that this problem may not be as serious as originally believed. Probably, as recommended by Smoot and Melvin (12), the most practical approach to keeping anthracnose at a minimum in susceptible cultivars is to harvest the fruit when it has sufficient color break so that no more than 36 hours of degreening are necessary. Also, as shown in this study, ethylene should be held at recommended concentrations of 1 to 5 μliters/liter of air (16) during degreening, so as not to encourage the development of *C. gloeosporioides* or other pathogens (9).

LITERATURE CITED

1. ADAM, D. B., J. MC NEIL, B. M. HANSON-METZ, D. F. MC CARTHY, and J. STOKES. 1949. The estimation of latent infection in oranges. Aust. J. Sci. Res. B2:1-18.
2. BINYAMINI, N., and M. SCHIFFMANN-NADEL. 1972. Latent infection in avocado fruit due to Colletotrichum gloeosporioides. Phytopathology 62:592-594.
3. DEY, P. K. 1933. Studies in the physiology of the appressorium of Colletotrichum gloeosporioides. Ann. Bot. 47:305-312.
4. EDNEY, K. L. 1958. Observations on the infection of Cox's Orange Pippin apples by Gloeosporium perennans Zeller & Childs. Ann. Appl. Biol. 46:622-629.
5. FAWCETT, H. S. 1936. Citrus diseases and their control. McGraw-Hill, New York and London. 656 p.
6. GRIERSON, W., and W. F. NEWHALL. 1960. Degreening of Florida citrus fruits. Fla. Agric. Exp. Stn. Bull. 620. 80 p.
7. JAHN, O. L., R. H. CUBBEDGE, and J. J. SMOOT. 1970. Effects of washing sequence on the degreening response and decay of some citrus fruits. Proc. Fla. State Hortic. Soc. 83:217-221.
8. MARKS, G. C., J. G. BERBEE, and A. J. RIKER. 1965. Direct penetration of leaves of Populus tremuloides by Colletotrichum gloeosporioides. Phytopathology 55:408-412.
9. MC CORNACK, A. A. 1971. Effect of ethylene degreening on decay of Florida citrus fruit. Proc. Fla. State Hortic. Soc. 84:270-272.
10. SADIK, S., and P. A. MINGES. 1964. Thionin for selective staining of necrosis in plants. Proc. Am. Soc. Hortic. Sci. 84:661-664.
11. SIMMONDS, J. H. 1941. Latent infections in tropical fruits discussed in relation to the part played by species of

Gloeosporium and Colletotrichum. Proc. Roy. Soc. Qd. 52:92-120.

12. SMOOT, J. J., and C. F. MELVIN. 1967. Postharvest decay of specialty hybrid citrus fruits in relation to degreening time. Proc. Fla. State Hortic. Soc. 80:246-250.
13. SMOOT, J. J., and C. F. MELVIN. 1972. Decay of degreened citrus fruit as affected by time of washing and TBZ application. Proc. Fla. State Hortic. Soc. 85:235-238.
14. SMOOT, J. J., C. F. MELVIN, and O. L. JAHN. 1971. Decay of degreened oranges and tangerines as affected by time of washing and fungicide application. Plant Dis. Rep. 55:149-152.
15. STANGHELLINI, M. E., and M. ARAGAKI. 1966. Relation of periderm formation and callose deposition to anthracnose resistance in papaya fruit. Phytopathology 56:444-450.
16. WARDOWSKI, W. F., and A. A. MC CORNACK. 1973. Recommendations for degreening Florida fresh citrus fruits. Fla. Coop. Ext. Service Circ. 389, Institute of Food and Agricultural Sciences, University of Florida, Gainesville.
17. WASTIE, R. L. 1972. Secondary leaf fall of Hevea brasiliensis: meteorological and other factors affecting infection by Colletotrichum gloeosporioides. Ann. Appl. Biol. 72:283-293.