

## Etiology and Epidemiology of Citrus Greasy Spot

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### ABSTRACT

A *Mycosphaerella* fungus found producing perithecia in abundance on decomposing fallen citrus leaves in all Florida citrus groves examined was confirmed as the cause of citrus greasy spot. This fungus also produced rough-walled *Cercospora*-like conidia on unbranched conidiophores developing singly from hyphae growing extramatrically on living citrus leaves. In a spore trap operated continuously from February to November 1969, ascospores were trapped after rain and on nights with heavy dews. Ascospore numbers were relatively small during the winter, increased through the spring, and reached a peak in June. In blocks of grapefruit, orange, and Milam lemon, a subsequent

decline in ascospore numbers was associated with rapid decomposition of leaf litter during July and August and the absence of any substantial leaf drop after early spring to replenish the perithecial substrate. In a grove of true lemons, however, ascospore numbers were still high in September due to continued leaf drop. Conidia were trapped in relatively small numbers at all locations, and represented only a minor source of inoculum. The amount of disease that developed on container-grown rough lemon trap plants placed outdoors for 2-week periods from April to November correlated closely with the number of ascospores discharged over each exposure period. *Phytopathology* 60:1409-1414.

Greasy spot is an important disease of citrus leaves in Florida, often causing severe defoliation. For many years, the true nature of greasy spot remained controversial, the disease having been variously attributed to mite injury, physiogenic factors, and unidentified pathogens. The earlier literature on this subject has been reviewed by Fisher (3). The first convincing evidence that greasy spot is caused by a fungus was obtained by Tanaka & Yamada (5) in Japan, where a *Cercospora*-type fungus with *Mycosphaerella horii* Hara as the perfect stage was described as the causal agent. In 1957, Fisher (2) reported that greasy spot in Florida is caused by a *Cercospora*, and later proposed the name *C. citri-grisea* (3).

In addition to the more characteristic greasy lesion, another symptom, described as a "small round brown spot", was reported in Japan (5, 6) also to be caused by the greasy spot fungus. Similar small spots have also been reported in Florida on certain types of citrus (3). In these spots, Yamada (6) found ascocarps of a fungus described as *Mycosphaerella horii* Hara and concluded that these represented the perfect stage of the greasy spot fungus. Yamada also described an external growth of the fungus on the leaf surface, with conidiophores branching out singly from the hyphae and producing *Cercospora*-type conidia at the tips. He found this sporulating mycelial growth on leaves that were visibly infected and on leaves with no evidence of greasy spot.

Although little information had previously been obtained in Florida on sources of inoculum, indirect evidence about infection periods had been provided by the results of fungicide spraying experiments. Fisher (3) reported satisfactory control of greasy spot with oil, copper, or zineb applied between mid-June and early August. Cohen (1) found that the disease could be controlled by copper fungicides only when applied after the completion of leaf expansion in the flush to be protected.

During an examination of decomposing fallen citrus leaves at Lake Alfred, Florida, in June 1968, and later in every grove examined, the author found numerous perithecia of a *Mycosphaerella* fungus. Evidence confirming the identification of these perithecia as the perfect stage of the greasy spot fungus is presented in this paper. Also reported are studies on the etiology and epidemiology of the disease, with particular reference to the seasonal availability of spore inoculum.

**MATERIALS AND METHODS.**—Isolation of the fungus from fallen leaves was readily achieved by attaching a leaf, after wetting, to the top of a petri dish and allowing the ascospores to be ejected from perithecia onto an agar surface. Cultures were obtained from greasy spot lesions by removing small portions of mesophyll tissue with the point of a scalpel blade in a manner similar to that described by Fisher (3). To confirm the identity of the mycelial growth on the leaf surface, portions of hyphae were picked up under a stereoscopic microscope with a fine needle and transferred to the agar medium. All cultures were grown and maintained in the dark on Difco cornmeal agar (CMA). Production of the *Cercospora*-like conidia, essential for identification purposes, was generally good on CMA; but some variations in sporulating ability occurred among isolates. All new isolations produced conidia, but sporulation decreased on older colonies and often ceased after repeated transfer and subculturing.

All pathogenicity tests were undertaken in the greenhouse. Inoculum was prepared by fragmenting 7- to 14-day-old agar colonies in a Waring Blendor for 10 sec, using about 1 cm<sup>2</sup> of colony to 20 ml water. Larger fragments were then removed by filtering through cheesecloth. In preliminary tests, symptoms often failed to appear on leaves that were too old at the time of inoculation. Consequently, only potted plants carrying recent flushes of growth were used for pathogenicity tests. The inoculum was applied to the lower leaf surface with an atomizer, and plants were then covered

individually with clear polyethylene bags. Moist conditions were maintained by atomizing plants periodically with water, and the bags were removed 3-6 days later. Thereafter, the foliage was kept dry constantly because occasional natural infection had been observed in the greenhouse where leaves had been wetted during watering.

The availability of ascospore inoculum was assessed at different sites by using a spore trap and by direct examination of leaf samples from the grove floor.

A spore trap, basically similar to that designed by Schenk (4), was operated continuously from 20 February to 10 November 1969, with the air inflow through an orifice 1 m above ground level maintained at about 10.5 liters/min. The trap was situated, except for three separate 1-week periods, under the canopy of four closely planted Milam lemon trees chosen because of their proximity to headquarters. The trap was moved temporarily to a Valencia orange grove for 1 week in July and 1 week in late August, and to a young lemon grove for 1 week in September.

Spore trap slides were coated with silicone stopcock grease, except on those days when it was desired to check the identity of trapped ascospores and *Cercospora*-type conidia. For the latter purpose, the slides were coated with glycerine so that adhering spores could be removed by washing with a small jet of sterile water for single spore isolation and culture.

Spore counts were made at  $\times 1,000$  magnification over three traverses, each 180  $\mu$  wide, after mounting the slides with lactophenol-cotton blue. The efficiency of the trap as defined by the proportion of spores retained on the coated slide was unknown, and volumetric estimates of spore load could not be calculated. Daily spore counts over a 540- $\mu$  width of deposit were, therefore, used in compiling the results, thereby providing relative data on changes in air-borne inoculum concn.

For assessing seasonal changes in inoculum availability on the fallen leaves, samples of leaf litter were collected at ca. monthly intervals under selected trees in three citrus groves at the Citrus Experiment Station, Lake Alfred, which included the blocks of Milam lemon and Valencia orange where the spore trap was located and a more distant grapefruit grove where no spore trapping was attempted. No quantitative assessments were made on leaf litter in the true lemon grove, where the spore trap was operated for 1 week in September. The leaf litter was collected and composited from 10 randomly chosen quadrates, each measuring 1 ft<sup>2</sup>, from within the confines of the noncultivated area under the tree canopy.

Because perithecia developed only after leaves reached a sufficiently advanced stage of decomposition,

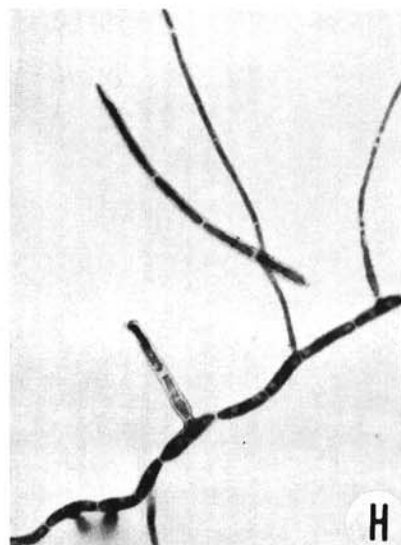
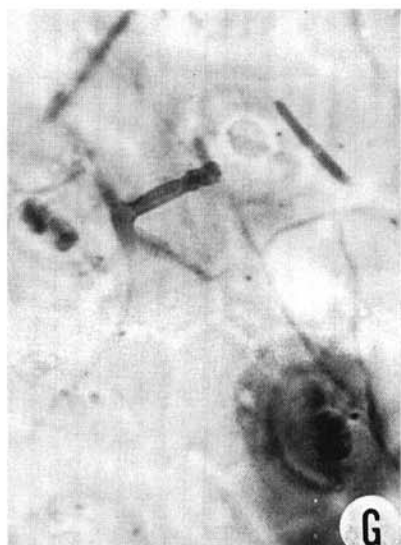
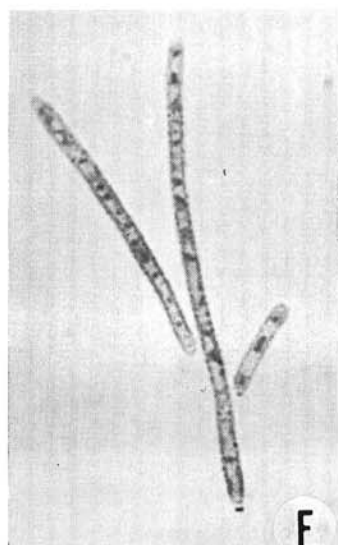
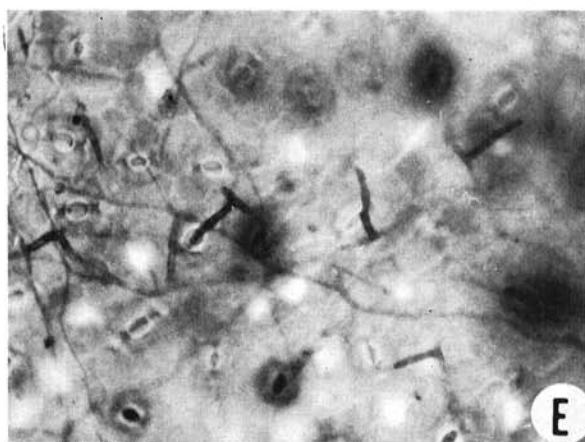
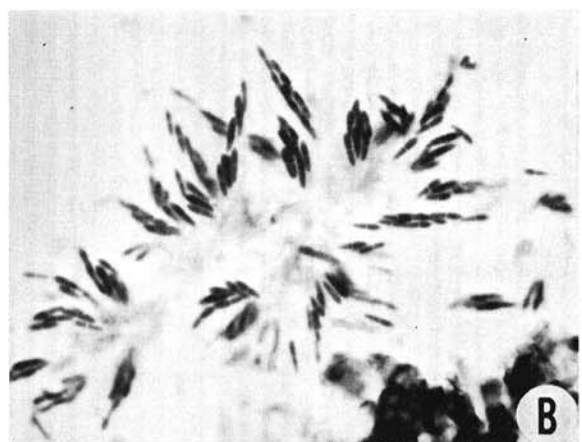
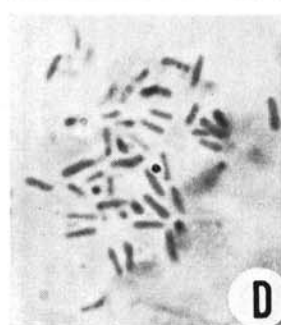
it was possible without constant microscopic examination to be reasonably accurate in determining perithecial maturity merely by observing the degree of leaf decomposition. After preliminary sorting on this basis, suspect leaves were then examined more closely for perithecia with  $\times 10$  magnification. The samples were graded into four categories: (i) recently fallen leaves with little or no decay and no perithecia evident; (ii) leaves in early stages of decomposition with perithecia mostly immature; (iii) leaves in a more advanced stage of decomposition with well-defined mature perithecia; and (iv) decomposing leaves apparently free from perithecia. After further decomposition leading to leaf fragmentation and veinal skeletonization, ascospore discharge generally ceased and such leaves were, therefore, excluded from the counts. No attempt was made to assess the number of perithecia on each leaf.

In 1968, information on time of infection was obtained by measuring the amount of disease on tagged flushes of rough lemon and Duncan grapefruit that developed at different times during the season. In 1969, a more accurate measure of the seasonal infection pattern was obtained by exposing outdoors four container-grown rough lemon plants, 25 feet from the nearest citrus tree. After 14 days, the plants were returned to the greenhouse for development of greasy spot symptoms. To insure a high level of uniformity, only plants with long and continuously growing shoots were selected as traps, and young growth above the uppermost fully expanded leaf was cut back at the time of exposure.

**RESULTS.—Fungus morphology.**—On decomposing citrus leaves, *Mycosphaerella* perithecia developed in abundance in characteristic densely packed groups (Fig. 1-A). These perithecia were produced mostly on the underside of the leaf, measured 58-90  $\mu$  in diam, remained covered by the epidermis, and contained paraphyses and numerous asci with biserially arranged hyaline ascospores, 7.2-10.5  $\times$  2.3-2.8  $\mu$  (Fig. 1-B, C). Spermogonia containing rod-shaped spermatia, mostly 3.5  $\times$  0.9  $\mu$ , were often observed prior to perithecial development (Fig. 1-D).

On living citrus leaves, an external mycelial growth bearing conidiophores with conidia similar to those described by Yamada (6) was observed both outdoors and after inoculating plants in the greenhouse. These conidiophores were produced singly from the surface mycelium and were darker brown than the hyphae (Fig. 1-E, G) except at the tip, which was paler in color and bore scars indicating the points of conidial attachment. Conidia varied considerably in size, 6-50  $\times$  2.0-3.5  $\mu$ , contained up to nine indistinct septa (sometimes none), and were pale yellowish-brown in color. A feature which proved very important for iden-

**Fig. 1.** Morphology of the citrus greasy spot fungus. **A)** Decomposing fallen grapefruit leaf after clearing with 10% NaOH solution, showing perithecia of *Mycosphaerella* arranged in densely packed groups. The larger, more widely spaced bodies present on the clearer tissue are perithecia of *Guignardia* sp. **B)** Asci and ascospore contents expressed after exerting pressure on a perithecium. **C)** Appearance of 2-celled ascospores after staining lightly with cotton blue, showing faint vacuolate appearance of cell contents. **D)** Spermatia extruded from perithecial initials. **E)** Growth of mycelium with conidiophores on undersurface of rough lemon leaf. **F)** Conidia from CMA culture after staining lightly with cotton blue. **G)** Conidiophore and detached conidium on undersurface of rough lemon leaf. **H)** Conidiophore and detached conidium from CMA culture after staining deeply with cotton blue.



tification purposes was the roughened conidial surface (Fig. 1-F).

On CMA, colonies were slow-growing and brownish-green in color. The hyphae were thin and showed very rough walls, particularly when young. Conidia were present on unbranched conidiophores (Fig. 1-H), but no perithecia were formed on this medium. Cultures arising from isolations made from external hyphae on the leaf surface were identical to those derived from ascospores and from mycelium in greasy spot lesions.

*Pathogenicity tests.*—All inoculations carried out with fresh isolates were successful, but virulence was sometimes reduced or even lost in cultures maintained for long periods. The incubation period in the greenhouse varied considerably on different types of citrus: usually 6-8 weeks on rough lemon, 2-4 months on sweet orange, and up to 6 months on grapefruit leaves. Apart from the fact that the spots produced in the greenhouse tended to remain yellow or orange for a longer period before darkening, the appearance of diseased tissue was similar to that observed outdoors.

*Ascospore discharge from fallen leaves.*—Large numbers of leaves fell from the Milam lemon, Marsh grapefruit, and Valencia orange trees during winter and early spring, reaching a peak in March-April, as represented by the counts in Fig. 2 for the recently fallen leaves. Perithecia were present on decomposed leaves throughout the period of examination, but relatively few were mature during the winter and early spring. Perithecial numbers increased through April, May, and June and declined rapidly in July. The decline was associated with rapid decomposition of the fallen leaves and the absence of any substantial leaf drop during the summer to replenish the perithecial substrate.

A more accurate appraisal of inoculum availability is revealed by the spore trap data shown in Fig. 3 together with weekly rainfall totals. Most of the ascospore release occurred immediately after rain. However, some discharge was recorded during nights with heavy dews, and this explains the numbers trapped during weeks when no rain was recorded. The seasonal pattern of ascospore availability corresponds closely with that predicted from the fallen leaf estimates. The smaller number of ascospores trapped in the orange grove as compared with that in the Milam lemon grove is related to differences in the total number of fallen leaves at the two sites. The situation in the true lemon grove was clearly very different from that at the other two spore trap locations and in the grapefruit grove. In this lemon grove, the extremely high spore count as late as mid-September (Fig. 3) was attributed to continued leaf drop through the spring and summer, and to the high level of greasy spot infection in this type of citrus.

*Availability of conidial inoculum.*—Weekly totals of trapped conidia (Fig. 3) were always very low and insufficient to establish the existence of a definite release pattern, except that conidia were detected only from June through September. Periodic microscopic examinations through the summer revealed extramatricular growth of the greasy spot fungus on most

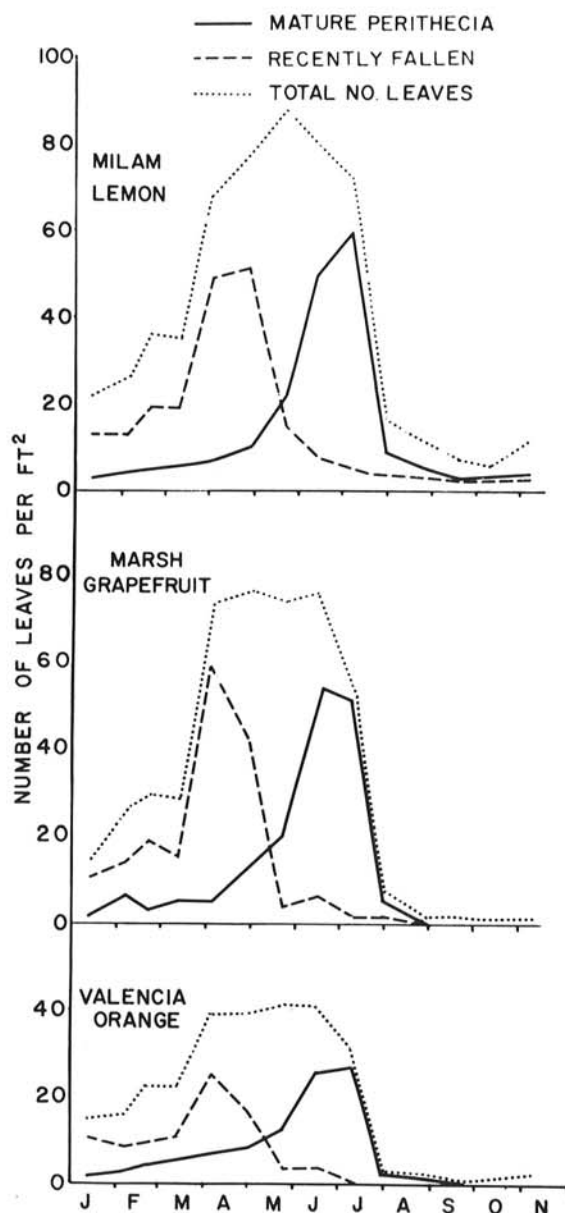
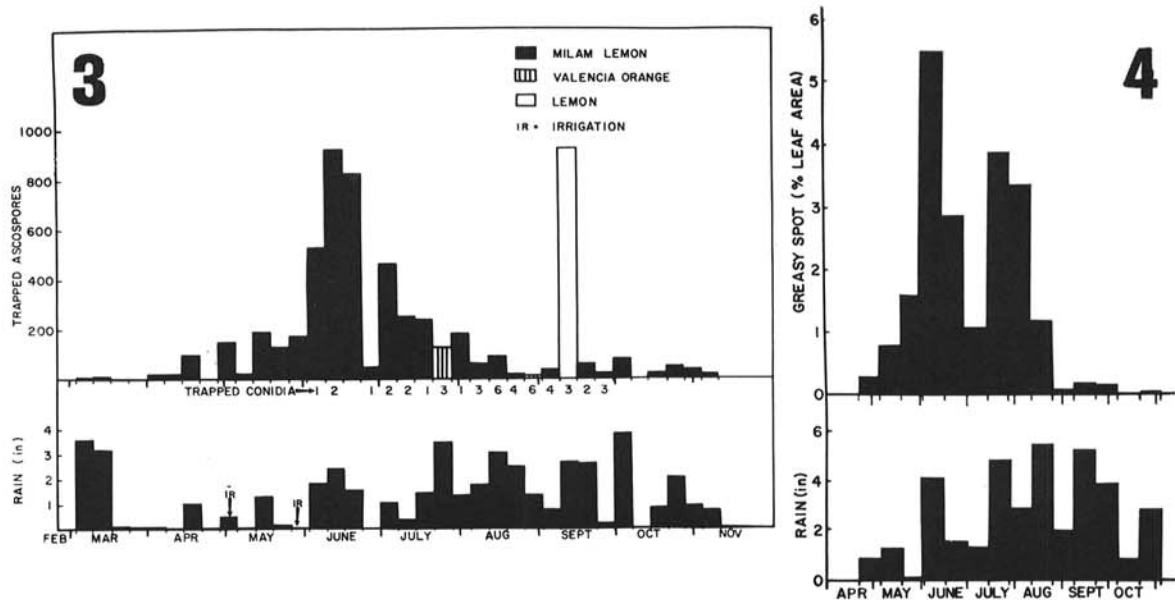


Fig. 2. The seasonal relationship between leaf fall, leaf decomposition, and perithecial development on the leaf litter under trees of three types of citrus. Leaves in the 'recently fallen' category included those showing little or no decomposition and with no visible signs of perithecial initiation.

leaf samples, but this was less extensive on orange and grapefruit leaves than on true lemon and rough lemon leaves. Conidiophores were more numerous on rough lemon than on the other types of citrus.

*Time of infection.*—In the 1968 experiment (Table 1), similar amounts of disease appeared on flushes that developed during March through May; but lesser amounts appeared on later flushes, with no symptoms at all appearing on the August and September rough lemon flushes or on the September grapefruit flush.



**Fig. 3-4.** 3) Rainfall amounts and numbers of ascospores and conidia trapped over each weekly period from 24 February to 10 November 1969. The spore trap was located under an isolated group of Milam lemon trees except for the periods 21-28 July and 25 August-1 September when it was operated in a nearby Valencia orange grove, and 8-15 September in a lemon grove. The Milam trees were irrigated by overhead sprinklers on 1 May and 31 May. 4) The incidence of greasy spot on rough lemon trap plants, 3 months after returning to the greenhouse, following a 14-day exposure outdoors during the period 21 April to 3 November 1969, with rainfall totals for the corresponding periods. The infection percentages were based on the extent of both necrotic and chlorotic tissue in relation to the total leaf area on the uppermost 15 leaves of each of four plants exposed.

In the 1969 experiment involving the use of container-grown trap plants, the data presented in Fig. 4 indicate the combined effects of air-borne inoculum potential and the suitability of the climatic conditions for infection over each exposure period. The highest disease rating on these trap plants occurred during the period of peak ascospore discharge.

**DISCUSSION.**—Although citrus greasy spot in Florida is now known to be caused by a *Mycosphaerella*, it is impossible without further taxonomic study to identify the species involved. In Japan, the causal fungus has been designated *Mycosphaerella horii* Hara; but the fungus found in Florida differs from this species in several respects. The four clearly defined large oil globules in the ascospores described by Yamada (6)

were not seen in Florida material, and differences have also been noted in ascospore shape and size. Yamada's identification in Japan was based on *Mycosphaerella* perithecia seen occasionally in "small round brown spots", a symptom that the author does not consider to be caused by the greasy spot fungus in Florida.

But from Yamada's description of the *Cercospora*-type spores it seems probable that the fungus causing greasy spot in Florida is identical to that causing the disease in Japan. The imperfect state of the fungus as described in both Japan and Florida is certainly rather distinctive in that the rough-walled, *Cercospora*-like spores are produced on conidiophores produced singly from hyphae growing over the surface of the leaf and not from conidiophores derived from a stroma em-

**TABLE 1.** Incidence of greasy spot on different leaf flushes developing during 1968 on rough lemon and Duncan grapefruit trees

Month flush originated	Rough lemon			Grapefruit		
	No. flushes examined <sup>a</sup>	Avg. no. leaves/flush	% Leaf area diseased	No. flushes examined <sup>a</sup>	Avg. no. leaves/flush	Avg. no. spots/leaf <sup>b</sup>
March	8	3.6	74	13	6.8	3.3
April	10	4.2	73			
May	11	5.6	62	12	8.7	4.3
June	16	5.4	34	10	11.2	2.5
July	10	7.4	38	12	9.0	0.5
August	17	7.2	0			
September	13	6.9	0	9	9.7	0

<sup>a</sup> Data obtained from tagged flushes on a single tree.

<sup>b</sup> Only a small proportion of leaf area diseased and relatively few spots/leaf compared with rough lemon.

bedded within the leaf in a manner generally characteristic of the *Cercospora* genus. In fact, according to F. C. Deighton, Commonwealth Mycological Institute, England (*personal communication*), the imperfect state of the greasy spot fungus in Florida might be classified not as a *Cercospora* but as a member of the currently little-known *Stenella* genus.

Evidence presented in this paper suggests that conidia play only a minor role in the dispersal of the greasy spot fungus in view of the small numbers deposited in the spore trap and the association of peak infection with maximum ascospore discharge. If conidia represent an important source of inoculum in creating a secondary infection cycle, a persistent or even increased level of infection would have been expected throughout the wet summer period.

The time of maximum ascospore discharge is likely to vary from year to year, and even from one locality to another, according to variations in rainfall amount and distribution. Dry weather not only prevents substantial ascospore release but, if prolonged, also results in delayed perithecial development. Periods of wet weather, on the other hand, tend to hasten perithecial development, increase the amount of ascospore discharge, and cause a more rapid leaf decay, thereby causing earlier perithecial exhaustion. Below-normal rainfall in spring and early summer could delay peak ascospore discharge until July or even later, whereas an earlier peak discharge would be anticipated following above-average spring rainfall. Furthermore, prolonged wet spells in June or July might shorten the period of abundant ascospore discharge due to rapid decay of the fallen leaf substrate. If, however, the inoculum supply is replenished by further leaf drop, as happened in the lemon grove, ascospore discharge can be maintained at a high level for a longer period.

The earliness of leaf drop probably has little influence on perithecial maturation because decomposition of fallen leaves, and consequently the perithecial development, is relatively slow during the winter. The time of peak ascospore discharge is therefore more likely to be influenced by seasonal variations in spring and early summer rainfall rather than by the amount and distribution of rainfall in the earlier part of the year.

The knowledge that ascospores are present in abundance on dead fallen leaves and represent a major source of inoculum suggests certain improvements in the control of greasy spot by providing a basis for more timely fungicide application. It also indicates a need for burying leaf litter on the grove floor by cultivation, or for eradicating this source of inoculum by other possible methods.

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