Environmental Factors Affecting Infection of Citrus Leaves by Mycosphaerella citri

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ABSTRACT

Penetration of citrus leaves by *Mycosphaerella citri* sufficient to cause visible symptoms of greasy spot, requires prolonged or frequently repeated periods of near 100% relative humidity (RH), combined with high temp. At RH near the required min of ca. 92%, ascospore germination and germ tube growth was greater on sucrose- or honeydew-coated glass than on clean glass. A preinoculation spray of sucrose greatly increased disease severity because of its nutritional effects in promoting extramatrical hyphal growth and, hence, the number of stomatal penetrations. The observed association of greasy spot with honeydew-excreting pests in a greenhouse, in which the atmosphere was otherwise too dry for infection, was attributed to the hygroscopicity and sucrose content of the honeydew.

Appressoria developed only in the outerstomatal chamber, and were produced in greater numbers when the ventral surface of the inoculated leaves was dried off periodically, than when it was kept continuously wet. In vitro, stomatal guttation fluid sometimes caused hyphal tips to swell as though inducing appressorium formation. If this fluid represents the actual appressorium-inducing agent, then certain environmental conditions might also influence infection indirectly by promoting stomatal guttation.

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Mycosphaerella citri Whiteside, the cause of citrus greasy spot in Florida, penetrates the host tissue only through stomata (8), which on citrus leaves are confined almost entirely to the ventral leaf surface. The invading hyphae do not penetrate very far laterally through the leaf, and the effects on the host tissue remain very localized. A high density of penetrations is therefore required to produce visible disease symptoms. The number of potential penetrations arising from the germination of a single spore can be increased considerably under favorable conditions by the development of a ramifying extramatrical mycelial growth (8). Thus, it is possible for disease symptoms to appear even in citrus groves where the ascospore load was relatively low during the infection period.

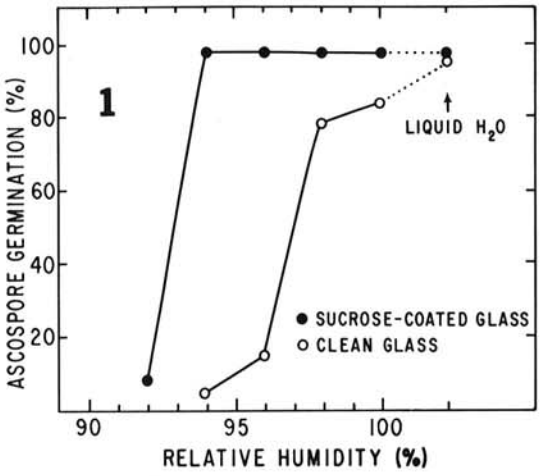
Environmental factors can be expected to influence the over-all amount of infection not only by determining the time and amount of ascospore discharge from the decomposing fallen citrus leaves (7), but also by affecting ascospore germination, extramatrical mycelial growth, formation of appressoria, and perhaps the actual process of stomatal penetration.

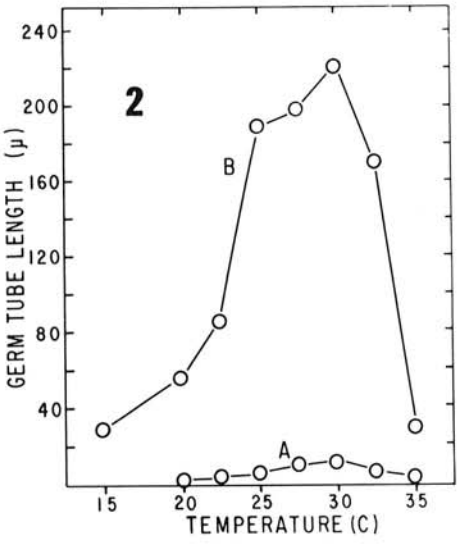
This paper reports on the effects of light, temp, relative humidity (RH), and stomatal guttation fluid on ascospore germination, germ tube growth, and appressorium formation. Also reported are the effects of some factors on the overall amount of leaf penetration as expressed by the eventual severity of the greasy spot symptoms. Because of an observed association of greasy spot in the greenhouse with honeydew-excreting insects, studies were also made to determine the possible action of honeydew on pathogen behavior.

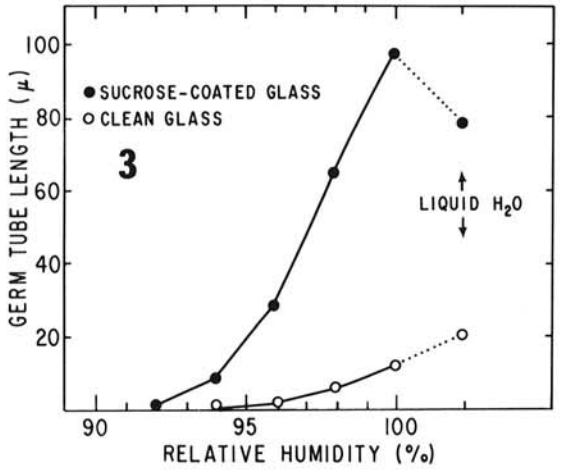
MATERIALS AND METHODS.— Naturally infected leaves were picked from citrus trees, air-dried for 2-3 days and thereafter wetted and dried daily to promote development of perithecia. The leaves were then placed in a wind tunnel spore trap as described by Brook (2) and sprayed with water. The ascospores were impacted onto clean microscope slides or onto slides smeared with 10% sucrose solution or moist honeydew, the latter being obtained from greenhouse-grown rough lemon (*Citrus jambhiri* Lush.) plants that had become infested with white fly (*Dialeurodes* spp.) or mealy bug (*Pseudococcus citri* Risso). Sucrose, which is a major constituent of honeydew (3), was substituted in most tests for honeydew, because the latter contained numerous fungal contaminants. For studies on ascospore germination and germ tube growth on cornmeal agar, pieces of leaf bearing perithecia were wetted and attached to the cover of a petri dish, so that the ascospores were discharged onto the agar surface.

For tests on the effects of RH on ascospore germination and germ tube growth, microscope slides, containing either nongerminated or germinated ascospores, were supported in modified desiccator jars over the appropriate solutions of sulfuric acid (6) or saturated salt solutions (9) in incubators at 25 ± 0.5 C.

Stomatal droplet water was obtained from container-grown rough lemon and sweet orange (*Citrus sinensis* (L.) Osbeck) plants after treating them in the following manner. Water-soluble material was first removed from the leaf surface by inverting the plants and submerging the leaves in deionized water. After the leaves had dried, the plants were covered with polyethylene bags that had been sprayed lightly on the inside with water and placed in a dark incubator at 30 C for 4 h, during which time the RH reached 100%. The temp was then reduced to 20 C over the next 1-2-h period. This caused droplets of water to appear at the stomatal openings. These droplets apparently represented exudations from the mesophyll tissue. After the polyethylene bags were removed, the stomatal droplets disappeared in less than 10 sec at an ambient 30 to 40% RH. Observations made through a stereoscopic microscope revealed that this was due mainly to withdrawal into the leaf and not to evaporation. Stomatal guttation has previously been described for several plants by Bald (1), but this is believed to be the first published report of this phenomenon occurring with citrus leaves. If the plants







remained covered too long, the leaves also became wetted by condensation of dew. Therefore, to avoid dilution of the stomatal guttation fluid by condensation water, it had to be collected as quickly as possible. To collect the guttation fluid, a microscope slide was brushed very lightly against the ventral surface of recently expanded leaves. Droplets that adhered to the slide after it was withdrawn were swept to the corner of the slide with a glass rod. This was repeated on several leaves and the composite drop was then placed in the middle of a deposit of freshly-discharged or germinating ascospores. Before the drop had a chance to evaporate, the slides were placed in a damp chamber to study the effects of the guttation water on spore germination and germ tube growth.

All inoculation studies were made on container-grown rough lemon plants, selected because of their high susceptibility to greasy spot and short incubation for this disease. Plants with single recently expanded shoots were pruned back to the uppermost fully expanded leaf and the data were obtained only from the remaining top 10 leaves. Disease ratings were determined as the percentage leaf area diseased, which included the yellowish green areas around the spots as well as the necrotic tissue.

Inoculum consisted of a suspension of fragmented hyphae obtained from 28-day-old cultures grown in horizontally held 1,000-ml prescription bottles containing 50 ml of modified Fries' liquid medium (5). The mycelium was collected on cheesecloth, washed with deionized water, and fragmented with water in a Waring Blendor. The suspension was poured through cheesecloth to remove excessively large fragments and more water was added to provide a total of 50 ml water to the mycelial contents of each prescription bottle. One-quarter of this suspension was first sprayed lightly onto the ventral leaf surface and allowed to dry. This was repeated three times, until all the inoculum had been used, thereby providing a more uniform deposit than would have been possible with a single spraying. After the leaf surface had dried, the plants were covered with polyethylene bags that had been sprayed very lightly on the inside with water.

The period that the plants were kept covered with the polyethylene bags after spraying with inoculum is hereafter referred to as the inoculation period. It was only during this period that environmental factors were critically controlled. Thereafter, the plants were held in a greenhouse in which there was no humidity control and in which the temperature range was 20 to 33 C.

Relative humidity and temp within the polyethylene bags

Fig. 1-3. Reaction of *Mycosphaerella citri* ascospores and germ tubes therefrom to in vitro environmental factors. **1)** Effect of relative humidity (RH) and liquid water on germination of ascospores deposited on clean and sucrose-coated glass. Germination counts were made after 24 h at 25 C. **2)** Effect of temperature on linear growth of germ tubes that emerged from ascospores deposited on cornmeal agar. (A) Growth after 6 h. (B) Ascospores germinated first for 7 h. Shown here is the net increase in germ tube length over the following 16-h period. **3)** Effect of RH and liquid water on germ tube growth at 25 C. Ascospores were germinated for 6 h at 25 C and then exposed to the different RH levels. The data shown represent the mean net increase in germ tube length over the following 22-h period.

TABLE 1. Survival of ascospores of *Mycosphaerella citri* on clean(C) and sucrose-coated(S) glass when exposed to different relative humidity (RH) regimes immediately after perithecial discharge

Exposure time (h)	Germination[a] %			
	30-90% RH[b]		29% RH[c]	
	C	S	C	S
49	70	94	96	90
102	0	95	89	92
142	0	81	78	95

[a] After spraying the clean slides with 10% sucrose solution and the sucrose-coated slides with water and holding them at 100% RH at 25 C for 24 h.

[b] Placed in the shaded part of a greenhouse with max temp range, 22 to 33 C.

[c] Held over saturated solution of $CaCl_2 \cdot 6H_2O$ at 25 C.

that covered the plants during the inoculation period were monitored periodically by inserting the appropriate sensor element of a Hygrodynamics, Inc. Model 15-3001 Electric Hygrometer-Indicator.

For studies on the effect of light on appressorium formation and leaf penetration, the plants were held in a growth room having a 12-hr photoperiod, during which the temp rose gradually from 15 to 28 C, and a 12-hr dark period over which the temp dropped from 28 to 15 C. Plants were placed in inoculation chambers constructed with either clear or black polyethylene sheeting. Light was supplied by cool-white fluorescent and incandescent lamps which provided an intensity of ca. 7532 lx (700 ft-c) within the clear polyethylene bags. The temp within the clear polyethylene chambers during the light period was 1-2 C higher than within the dark chambers.

For examination of extramatrical hyphal growth and appressoria, small tangential sections, each ca. 5-mm square, were cut from the ventral leaf surface and mounted in lactophenol-cotton blue. To observe hyphal penetration into the substomatal chambers and mesophyll tissue, the tangential sections were stained with Heidenhain's iron hematoxylin.

Data on ascospore germination and germ tube growth were based on a min of 100 counts and replicated at least twice. A min of four plant replicates was used in the inoculation experiments. At least four tangential sections (one/leaf) were cut from each plant for the appressoria counts.

RESULTS.—*Ascospore germination and survival.*—Light was not required for ascospore germination or germ tube growth. All tests were, therefore, made in the dark, thereby ensuring greater control of temp and RH. Germination usually reached 90 to 100% in distilled water, deionized water, 10% sucrose solution, and in dew water collected at 0800 h from the ventral surface of citrus leaves in the field. However, it never exceeded 28%, and was frequently zero, in stomatal guttation fluid.

The time to 50% ascospore germination on cornmeal agar was 17 hr at 15 C, 6 to 7 hr at 20 C, 6 hr at 25 C, and 4 hr at 30 C.

Relative humidity requirements for ascospore germination depended on whether the ascospores were deposited on clean glass or on sucrose- or honeydew-coated glass. Ascospore germination on sucrose-coated slides remained at near 100% at RH down to ca. 94%, but then declined rapidly, reaching zero at 90 to 92% RH (Fig. 1). The results on honeydew-coated slides were almost identical and consequently are not shown on Fig. 1. On clean glass, however, germination started to decrease as soon as the RH dropped below 100% and it reached zero at 92 to 94% RH.

For survival tests (Table 1), ascospores were impacted upon clean or sucrose-coated slides and held either at a constant 29% RH or in a greenhouse in which the maximum diurnal RH ranged 30 to 90% RH and the temp ranged 22 to 33 C. On sucrose-coated slides, most of the ascospores remained viable after 142 h of exposure to either regime. At a constant 29% RH, ascospores survived almost as well on clean glass as on sucrose-coated glass; but in the greenhouse, viability was lost much sooner on clean glass.

Growth and survival of ascospore germ tubes and hyphae.—Some measure of the effect of temperature and RH on germ tube growth is inherent in the germination data. However, in view of the importance of extramatrical hyphal growth in greasy spot epidemiology, the study was

TABLE 2. Survival of germ tubes growing from ascospores of *Mycosphaerella citri* on clean (C) and sucrose-coated (S) glass following exposure to relative humidities (RH) below those required for growth

Initial period of germination (b)	Time exposed to reduced RH (h)	Germ tubes which resumed growth[a] (%)			
		30-90% RH[b]		29% RH[c]	
		C	S	C	S
7	48	0	12	30	68
	96	0	0	16	71
	144	0	0	3	10
24	24	0	80	58	72
	72	0	50	22	67
	120	0	0	10	54

[a] Percentage of ascospores from which the germ tubes continued to grow out (either from the apical, basal, ro both cells) after spraying the clean slides with 10% sucrose and the sucrose-coated slides with water and holding them at 25 C and 100% RH for 48 h.

[b] Placed in shaded part of greenhouse. Indicates maximum diurnal range of RH. Temp range 22 to 33 C.

[c] Held over saturated solution of $CaCl_2 \cdot 6H_2O$ at 25 C.

TABLE 3. Effect of a light-dark regime versus continuous darkness, during the four-day period of inoculation of rough lemon leaves with *Mycosphaerella citri*, on appressorium formation, stomatal penetration, and greasy spot severity

Treatment[a]	Stomata with appressoria (no./mm²)	Appressoria with hyphae growing into mesophyll[b] (%)	Disease severity[c] (%)
Light 12 h, dark 12 h	66	25.3**[e]	44.6**
Continuous dark[d]	55	4.1	16.6

[a] Growth room temperature rose to 28 C by end of photoperiod and dropped to 15 C by end of dark period.
[b] Counts made 20 days after inoculation.
[c] Disease severity based on percentage leaf area showing necrotic and chlorotic symptoms 67 days after the end of the inoculation period.
[d] Plants held in same growth room but covered with black polyethylene.
[e] ** = Significantly different at $P = 0.01$.

taken a step further to determine the effect of certain factors on growth and survival of the germ tubes following the initial germination phase.

In one of the tests, the ascospores were first germinated on cornmeal agar by holding all the plates at 25 C for 7 h, by which time 92% of the ascospores had produced germ tubes in excess of 2 μ, with an average germ tube length of 8 μ. The plates were then held at five different temp from 15 to 35 C for 16 h. During this 16-h period, the net increase in linear growth of germ tubes rose rapidly from 15 to 25 C and reached a peak at ca. 30 C (Fig. 2). The ultimate effect of temp on germ tube growth was greater than that indicated by the measurements made only 6 h after depositing ascospores on the agar surface and exposing them to the different temp levels.

The effect of RH on linear growth of germ tubes is shown in Fig. 3. The ascospores were germinated first at 30 C for 6 h, by which time 81% had germinated, and the mean germ tube length was 2 μ. Germ tube growth was more rapid on sucrose-coated glass than on clean glass. On both surfaces, growth decreased rapidly as the RH dropped below 100% and ceased at ca. 92%.

Germ tubes survived longer when exposed to a constant 29% RH than when subjected to fluctuating humidity and temp in the greenhouse (Table 2). They also survived longer when sucrose was present. Under both environmental conditions, older germ tubes survived longer than younger ones.

Effect of various factors on appressoria formation.— Although light was not required for ascospore germination and hyphal growth, there remained the possibility that appressorium formation might be influenced by this factor. Therefore, studies were made on the effect of alternating 12-h light and 12-h dark periods versus continuous darkness on appressorium formation during the inoculation period. The results (Table 3) showed that appressorium formation occurred just as freely in the dark as in the light.

Hyphae did not appear to grow over the leaf surface in any definite pattern and apparently reached the stomata by chance. However, after reaching the rim of the outer stomatal chamber, they often turned into the chamber as though responding to some localized stimulus. Once within this chamber, the tip of the invading hypha became swollen and formed an appressorium.

The possibility that appressorium formation might be stimulated by stomatal guttation fluid was then examined. Drops of guttation fluid were placed in the middle of a deposit of germinating ascospores on a microscope slide. Within the area contacted by the guttation fluid (or its deposit if the water had evaporated), germ tube growth usually ceased. In a few cases, however, some of the germ tubes lying at or just beyond the edge of the guttation fluid or deposit sometimes developed swollen tips, as shown in Fig. 4. In these tests, no attempt was made to sterilize the guttation fluid and it often teemed with bacteria after 12 to 18 h. Thus, it could not be definitely concluded that the stomatal fluid had in itself caused the germ tube tips to swell. Nevertheless, no such swellings were produced when nonsterile drops of water or 2% sucrose solution were placed in contact with the germ tubes.

The evidence suggested that stomatal guttation fluid in some manner might be involved in stimulating appressorium formation. It was considered that such a stimulus might be disturbed if the ventral surface of the leaf remained wet for long periods. Studies were, therefore, made on the effect of continuous wetting versus intermittent wetting on the numbers of appressoria produced on inoculated leaves. These experiments were carried out in the dark to obtain more critical temp control, and, hence, improved control of RH and dew deposition.

The leaves on 16 plants were sprayed with inoculum and allowed to dry before the plants were covered individually with polyethylene bags. These plants were placed in an incubator at 25 C for 24 h. After this time, both leaf surfaces were wet with condensation water. The covers were then removed from all the plants. On eight plants, the bags were immediately replaced before the leaves had a chance to dry. On the other plants, the leaves were allowed to dry at an ambient RH of 35 to 50% before the covers were replaced. In the wet treatment, RH within the bags returned to 100% in less than 30 min and the leaf surface remained continuously wet. When the leaves

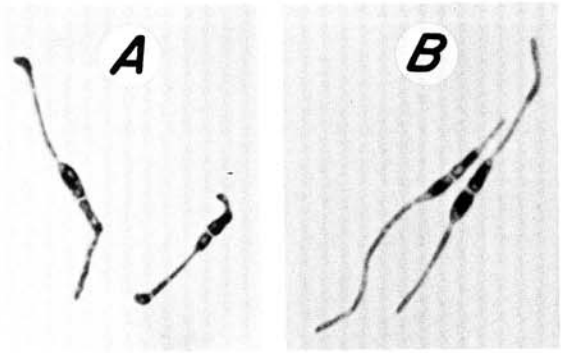


Fig. 4-A, B. A) Swellings on tips of germ tubes formed after placing a drop of stomatal guttation water on germinating ascospores of *Mycosphaerella citri*. **B)** Appearance of germ tubes when ascospores were germinated on a sucrose-coated or clean glass surface (×936).

TABLE 4. Effect of continuous wetting versus daily wetting and drying of the ventral surface of rough lemon leaves, during the 11-day period of inoculation with *Mycosphaerella citri*, on appressorium formation and greasy spot severity

Wetness regime	Stomata[a] traversed by hyphae (no./mm²)	Stomata with appressoria (no./mm²)	Stomata with traversing hyphae that produced appressoria (%)	Disease severity[b] (%)
Continuously wet[c]	214	14	6.5	3.5
Dried daily[d]	249	92**[e]	36.5**	19.7**

[a] Includes only those stomata with hyphae growing over the opening of the outer stomatal chamber.

[b] Disease severity based on percentage leaf area showing necrotic and chlorotic symptoms 82 days after the end of the inoculation period.

[c] Because of droplet formation, the lower leaf surface was not covered with a continuous film of water. Polyethylene was removed only momentarily each day.

[d] Plants uncovered daily and lower leaf surface allowed to dry off completely before replacing cover.

[e] **=Significantly different at $P=0.01$.

were allowed to dry before the covers were replaced, it took ca. 4 h for the RH to return to saturation point and for dew deposition to commence. These uncovering and re-covering procedures were repeated daily on the respective groups of plants for another 9 days. The results (Table 4) showed that even though a similar number of stomata had been traversed by hyphae during the inoculation period under each wetness regime, more appressoria were formed where the leaf surfaces dried periodically.

Effect of various factors on the penetration of stomata by infection pegs.— After an appressorium had formed in the outer stomatal chamber, a very short infection peg grew from it towards the substomatal cavity and formed a small vesicle immediately below the stomatal pore. The appressorium then slowly enlarged, became multicellular, and eventually filled up the outer stomatal chamber. Sometimes the appressoria did not produce infection pegs. In such cases, the appressoria enlarged very little and usually remained unicellular.

Provided that the foliage surface was kept free from infestation by honeydew-excreting insects, the extramatrical fungal growth virtually ceased after the plants were returned to the greenhouse bench, and after the polyethylene covers were removed. Consequently, no further appressoria were formed after this time. However, stomatal penetration by infection pegs formed from existing appressoria sometimes continued after the plants were placed in the greenhouse. Therefore, disease-severity ratings alone only partly reflected the amount of stomatal penetration that occurred under those specific environmental conditions imposed during the inoculation period. Nevertheless, by comparing the number of appressoria produced per unit area with disease severity, it was possible to determine to a limited extent how much of the stomatal penetration actually had occurred during or after the inoculation period.

In the experiment on the effect of leaf wetness on appressorium formation, the disease-severity readings corresponded closely to the number of appressoria (Table 4). There were, however, considerable discrepancies between the disease-severity readings and appressorial counts in the experiment comparing the effect of total darkness versus a daily light-dark regime on infection (Table 3). The results of histological examinations made 20 days after inoculation on tangential sections of leaf, supported the suspicion that more hyphal penetration had occurred on plants exposed to light during the inoculation period than on those held continuously in the dark during this period.

TABLE 5. Effect of inoculum concentration and presence of sucrose on leaf surface, during the 4-day inoculation period, on infection of rough lemon leaves by *Mycosphaerella citri*

Concentration of inoculum	Disease severity 60 days after inoculation (%)	
	Leaves sprayed with sucrose[a]	Leaves not treated
Full strength[b]	38.2w[c]	22.3x
Diluted 1:9	12.7y	1.7z

[a] Leaves were sprayed with 5% sucrose and allowed to dry prior to inoculation on same day.

[b] Fragmented mycelium from each 1000-ml prescription bottle suspended in 50 ml water.

[c] Disease readings followed by different letters differed significantly from each other according to Duncan's multiple range test.

Effect of inoculum concentration and sucrose sprays on disease severity.— A study was made of the overall effect of inoculum concentration and an exogenous supply of sucrose on disease severity. The ventral leaf surface on some plants was sprayed with 5% sucrose and the other plants were left untreated. Later the same day, the plants were sprayed with either a low or high concentration of inoculum. After the 4-day inoculation period, the remaining sucrose on the treated leaves was removed by immersing the foliage in water for 5 min. Disease severity was affected greatly by inoculum concentration, and the presence of sucrose on the leaf surface caused a substantial increase in infection, particularly at the lower inoculum level (Table 5).

DISCUSSION.— Near 100% RH is essential for spore germination and hyphal growth of *M. citri*. Liquid water does not apparently provide any additional advantage to the fungus and it may even reduce infection by interfering with appressorium initiation. Rainfall has a major effect

in determining the time of ascospore discharge, but is probably less important than prolonged high atmospheric humidity in determining the amount of ascospore germination and extramatrical mycelial growth. The results indicated that if ascospores are discharged following a short-lived daytime rainshower, after which the RH immediately drops, the ascospore could remain viable for limited periods until conditions again become favorable for germination. In summer, the ascospores would seldom be exposed to desiccating conditions for very long because leaf conditions favorable for germination occur almost every night. Ascospore and germ tube survival rates are, therefore, likely to be much higher during summer than at other times of year.

Mature perithecia can be found on fallen citrus leaves throughout the year, but relatively few are present during the fall and winter (7). Their numbers increase through the spring and reach a peak in early summer (7), at which time climatic conditions become more favorable both for frequent ascospore discharge and for extramatrical hyphal growth. Nevertheless, it must be recognized that ascosporic inoculum can be released at other times of year and the fate of ascospores that are released at such times also requires consideration. Outside the main summer period, June through September, rainfall in Florida is sporadic and is generally associated with the convergence of warm, moist maritime air with rapidly moving cold fronts from the north. Thus, even though ascospores may have been discharged during the resulting rainfall, the associated drop in temp and RH would soon cause a cessation of any germ tube growth.

Even the surface of apparently clean leaves carries sufficient nutrient for extramatrical growth of *M. citri*. This may be partly due to exudations from the leaves themselves. Nevertheless, an additional supply of nutrient, provided naturally as honeydew, or artificially as sucrose, greatly stimulates growth of extramatrical hyphae.

No precise information is currently available concerning the impact that honeydew-secreting insect infestations might have on greasy spot epidemiology. Certainly, severe greasy spot can occur even when such infestations are at a low level. There are, however, some observational data to support a possible relationship between greasy spot severity and certain insect infestations. Griffiths (4) was apparently the first worker to report that greasy spot symptoms often appear around the remains of insect pests. He suggested that the disease followed insect injury. However, in the light of more recent knowledge concerning greasy spot etiology (8), it seems more likely that the insects act by providing an additional source of nutrients for extramatrical fungal growth, through their excreta and perhaps also through the decomposition of their own bodies. Greasy spot is uncommon in our greenhouses, mainly because the RH is generally too low for ascospore germination and for extramatrical hyphal growth. When the disease does appear, it is found mostly on those leaves that have become infested with white fly or mealy bug, both honeydew-producing pests. The results of the in vitro tests on germ tube growth (Fig. 3) and the leaf inoculation tests (Table 5) showed that a deposit of sucrose, and hence honeydew, probably increases extramatrical fungal growth and leaf penetration partly because of its nutritional value and partly because of its hygroscopic properties.

The results showed that although just as many appressoria developed in continuous darkness as under the daily light-dark regime, the actual penetration of the stomata by the infection pegs derived from appressoria may have been favored by light. This might have been associated with the effects of light in increasing the size of the stomatal aperture. There is, however, another possible explanation: The inability to control temp as accurately within the clear polyethylene chambers as within the dark chambers could have accounted for the differences in numbers of infection pegs produced in the light versus the dark. Even slight temperature fluctuations within the closed atmosphere around the plants greatly affected RH and leaf wetness; these may have been the actual factors that affected infection peg production.

If guttation fluid is the agent that stimulates appressorium formation, then the nighttime regimes of RH and air temperature could have an important influence on greasy spot epidemiology through their effects on stomatal guttation. This effect would be in addition to that imposed more directly through the effects of RH and temp on ascospore germination and extramatricial fungal growth. Feasibly, guttation fluid might have its greatest stimulatory action on appressorium initiation during the earlier part of the night before it becomes diluted with dew.

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