Histopathology of Citrus Greasy Spot and Identification of the Causal Fungus

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ABSTRACT

The fungus causing greasy spot of citrus in Florida is designated as *Mycosphaerella citri* sp. nov., and the imperfect state, which produces long cylindrical conidia on simple conidiophores arising from extramatricular hyphae, is classified as a *Stenella*.

Hyphae penetrate citrus leaves and rind through stomata, and commonly cause death of guard cells. Fungal growth within the host is intercellular. In orange rind, the hyphae reach only a few cells beneath the substomatal chamber, and therefore cause little necrosis.

Additional key words: ontogeny of conidiophores.

After penetrating stomata on leaves, the hyphae grow very slowly through the adjacent compacted layer of mesophyll, but grow more rapidly through the large air spaces of the spongy mesophyll. Cells of the spongy mesophyll become hypertrophic and eventually necrotic. The extent of injury resulting from each stomatal penetration is very limited, and a concentration of many penetrations is required to produce macroscopic symptoms.

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Greasy spot of citrus in Florida is caused by a *Mycosphaerella* fungus that produces perithecia in abundance on decomposing fallen citrus leaves (3). This fungus also produces asexual spores on conidiophores that arise from extramatricular hyphae growing over living citrus leaves, but these spores are produced sparsely, and are considered to play only a minor role in epidemiology (3). The imperfect state of this fungus is similar, and perhaps identical, to one of several asexual spore forms described in a report on the etiology of greasy spot in Japan (5).

Greasy spot in Florida had previously been attributed to *Cercospora citri-grisea* Fisher, a fungus that produces fasciculate conidiophores arising from a stroma within small brown spots in citrus leaves (1). The description of this fungus does not fit the imperfect state of the *Mycosphaerella* found later and established as the cause of greasy spot in Florida (3). Nor does this *Mycosphaerella* fit the description of *Mycosphaerella horii* Hara, which is stated to be the cause of greasy spot in Japan (5), and which is the only *Mycosphaerella* previously recorded on citrus that has been associated with a greasy spot symptom.

The purpose here was twofold: to obtain (i) a better understanding of the sequence of symptom development to aid disease identification; and (ii) more information on the morphology and ontogeny of the extramatricular and intramatricular fungal growth. This helped to clarify the taxonomic status of the fungus. The further details obtained, along with other data previously published (3, 4), form the basis for considering the greasy spot fungus in Florida as a new species of *Mycosphaerella*.

MATERIALS AND METHODS.—Development of the extramatricular fungal growth, path of host penetration, and production of disease symptoms were studied in the greenhouse on inoculated leaves of container-grown plants of Pineapple sweet orange (*Citrus sinensis* [L.] Osbeck). Inoculum from cornmeal agar cultures was prepared as previously described (3) and sprayed on both leaf surfaces. The plants were covered with transparent polyethylene bags and kept moist by spraying with water once a day. After 7 days, the bags were removed, and thereafter the foliage was kept dry. Inoculated leaves were detached after various periods and fixed in Formalin:acetic acid:alcohol (FAA). To observe the extramatricular growth and host penetration, I stripped portions of epidermis from the leaf surface by cutting with a razor blade and mounted in lactophenol-cotton blue. The path of penetration and other histopathological features were observed in transverse sections of leaves. For this purpose, portions of leaf were fixed in FAA, dehydrated in tertiary butyl alcohol, embedded in paraffin wax, sectioned at 10-μ thickness with a rotary microtome, and stained with Heidenhain's iron hematoxylin and orange G.

After greasy spot symptoms appeared, some of the inoculated leaves were picked, allowed to dry for 3 days, then placed in a cheesecloth bag on the greenhouse bench and sprinkled with water daily to promote perithecial development. Portions of these leaves were removed periodically, fixed in FAA, embedded in paraffin wax, sectioned transversely, and stained with iron hematoxylin. For studies on mature perithecia and ascospores, the material was mounted in lactophenol containing acid fuchsin or cotton blue.

Fruit inoculations were performed as previously described (4). Microtome sections of the rind were stained with iron hematoxylin to determine the extent of hyphal penetration beneath the stomata.

RESULTS AND DISCUSSION.—*Development of extramatricular fungal growth*.—The inoculum applied to the sweet orange leaves consisted mostly of

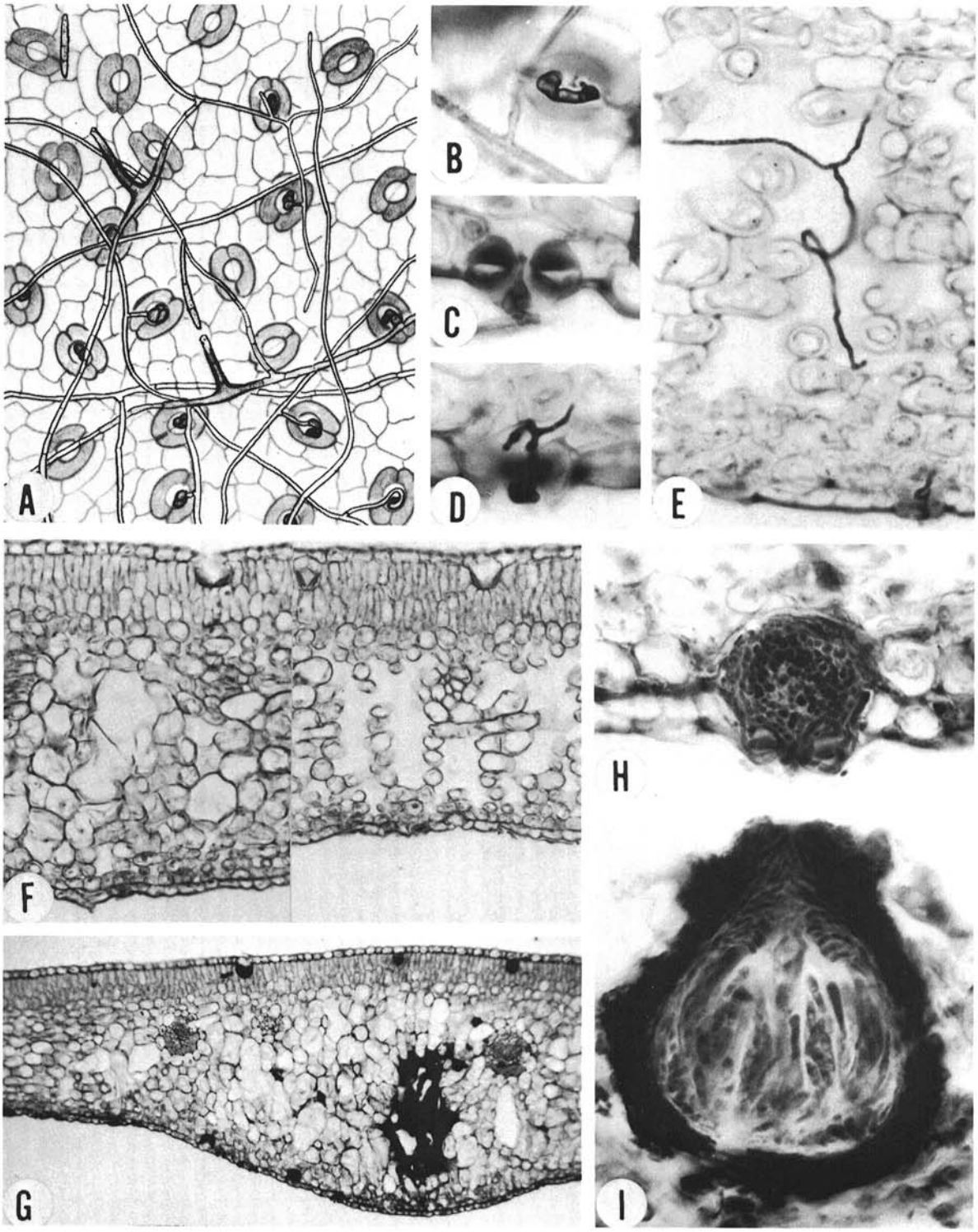

Fig. 1. Morphology of *Mycosphaerella citri* and histopathology of inoculated sweet orange leaves. **A)** Drawing of extramatricular growth showing conidiophores, detached conidia, and hyphal growth into stomata (X 380). **B)** Surface view of multicellular appressoriumlike structure in outer stomatal chamber (X 900). **C)** Transverse section of leaf showing persistent structure in outer stomatal chamber, hypha growing between guard cells and formation of substomatal vesicle (X 900). **D)** Hypha growing between cells beneath substomatal chamber (X 900). **E)** Transverse section of leaf showing hypha growing through stoma, and mycelial growth in spongy mesophyll resulting from this penetration (X 400). **F)** Hypertrophied mesophyll tissue in portion of leaf that was originally the same thickness as that shown in healthy portion on right. Note normal appearance of palisade mesophyll (X 150). **G)** Transverse section through blistered portion of leaf showing hypertrophied spongy mesophyll and early stages of necrosis (X 80). **H)** Perithecial primordia developing beneath stoma in decomposing leaf (X 700). **I)** Mature perithecium (X 700).

hyphal fragments, but also contained a few conidia. Both forms of inoculum produced a ramifying mycelial growth over the leaf surface (Fig. 1-A). When the first examinations were made, 2 days after enclosing the inoculated plants in polyethylene bags, a few conidiophores had already formed, and these were present on the upper as well as the lower leaf surfaces.

Extramatricular mycelial growth of the greasy spot fungus was also found on both surfaces of leaves in citrus groves, particularly during the humid summer period. In the greenhouse, however, atmospheric conditions were generally too dry, even during the summer, to permit development of superficial hyphae. The extramatricular fungal growth virtually ceased on the inoculated sweet orange leaves after the polyethylene covers were removed.

Path of fungal penetration.—Penetration of fruit rind and leaves was observed to occur only through stomata. Because the upper epidermis of citrus leaves is devoid of stomata, penetration is restricted to the lower surface. On the inoculated sweet orange leaves, the hyphae grew over the leaf surface mostly at random, and even passed over some stomata without penetrating. In other cases, hyphal tips that were sufficiently close to stomata grew towards the outer stomatal chamber as though responding to a stimulus. On reaching the outer stomatal chamber, the hyphal tip became swollen and formed an appressoriumlike, thick-walled structure which sometimes consisted of only a single cell, but more often was comprised of two or more cells (Fig. 1-B, C, D). This structure persisted long after the remainder of the extramatricular growth had disintegrated, and was still discernible when greasy spot symptoms appeared.

A thin hypha grew out from the appressoriumlike structure and formed a small vesicle in the substomatal chamber (Fig. 1-C). Some stomatal penetration had already occurred by the time the leaves were first examined 48 hr after inoculation.

Growth of intramatricular hyphae and effects on host tissues.—There was a delay of 2-3 weeks before hyphal growth progressed beyond the formation of the vesicle in the substomatal chamber. The hypha commonly branched one or more times in the substomatal chamber, and then grew very slowly between the cells of the tightly packed, one- to three-celled layer of mesophyll immediately above the stomata (Fig. 1-D). Eight weeks after stomatal penetration, a few hyphae had grown through to the spongy mesophyll. In the large air spaces of this tissue, growth proceeded more rapidly, and some hyphae soon reached the base of the palisade parenchyma which, however, they seldom penetrated. The hyphae branched only occasionally in the mesophyll (Fig. 1-E). The extent of hyphal penetration was very limited, and never exceeded 150 μ in a lateral direction from the actual point of leaf penetration. Large numbers of leaf sections were examined to determine whether any hyphae had grown from the substomatal chamber of the invaded stoma to that of a neighboring stoma. No hyphae were seen to follow such a path, and it therefore seems unlikely that extramatricular growth could originate from hyphae growing within the host.

The first indication of injury to host tissues was observed 8 weeks after inoculation, consisting at this time of dead guard cells around some of the penetrated stomata. Changes in the appearance of internal tissues were not observed until ca. 12 weeks after inoculation. Cells of the spongy mesophyll became hypertrophic, causing a disappearance of the air spaces (Fig. 1-F). The cell enlargement was of such magnitude that the epidermis became elevated, this effect being more pronounced on the lower side of the leaf. This hypertrophy is responsible for the development of the minute blisters that are the first external symptoms of greasy spot disease. After another 4 weeks, groups of dead cells appeared in the hypertrophied mesophyll (Fig. 1-G). Only after extensive necrosis had occurred in the spongy mesophyll did the palisade layer become involved. For this reason, visible necrotic symptoms appear more clearly during early stages of disease development when leaves are viewed from the lower surface.

Because of limited intramatricular fungal growth, and the fact that only cells in close proximity to the hyphae become hypertrophied and later necrotic, the amount of injury caused by a single stomatal penetration is insufficient to produce macroscopic symptoms. For the development of a visible greasy spot symptom, a concentration of many stomatal penetrations is necessary.

In diseased sweet orange rind, hyphal penetration was restricted to a depth of from one to two cells into the flavedo beneath the substomatal chamber, and only a few cells became necrotic. The differences in extent of hyphal penetration and amounts of injury to tissues in infected leaves and rind may be related to the absence of large air spaces in the latter, in contrast to the spongy mesophyll of leaves.

Perithecial development.—Perithecial primordia were first observed in incubated leaves 10 days after leaf detachment, developing at this time mostly from hyphae lying immediately beneath the stomata (Fig. 1-H). In later stages of leaf decomposition, some perithecia also formed beneath epidermal cells away from stomata and beneath the epidermis on the upper leaf surface, but in aggregate, the perithecia were much more numerous on the underside of the leaf. Perithecial distribution over the leaf showed little relationship to the location of visible greasy spot symptoms. The greasy spot fungus was often isolated from tissues underlying the epidermis of leaves and rind that appeared healthy to the naked eye, indicating that the fungal penetration can be more extensive than visual disease symptoms might indicate.

Identification of causal fungus.—According to F. C. Deighton, Commonwealth Mycological Institute, Kew, England (*personal communication*), the imperfect state of the greasy spot fungus resembles more closely the genus *Stenella* than *Cercospora*. The original description of *Stenella* by Sydow (2) was based on a fungus, *S. araguata* Syd. nov. sp., found

infecting leaves of *Pithecolobium lanceolata* Benth. in Venezuela. This fungus was described as producing one- to three-celled, colored, cylindrical conidia on simple, colored, septate conidiophores arising from colored hyphae growing over the surface of the leaf. Sydow described the superficial hyphae as arising from a nearly hyaline intramatricular mycelium, whereas the extramatricular mycelium of the greasy spot fungus has not been observed to originate in this manner. In view of the close similarities in other respects and a possibility that an incorrect interpretation of the origin of extramatricular mycelium may have been made by Sydow, it seems reasonable to regard the imperfect state of the greasy spot *Mycosphaerella* as a *Stenella*.

The fungus previously described as the cause of greasy spot in Florida, *Cercospora citri-grisea* Fisher, was reported to produce conidiophores arising from a stroma in small brown round spots (1). There was no report of inoculations having been made with any cultures derived from this source. These spots, as described by Fisher, are distinct from greasy spot, and I have obtained no evidence that they could represent even an atypical expression of greasy spot disease. A fungus isolated by Fisher from the mesophyll tissue in greasy spot lesions did produce typical greasy spot symptoms in inoculation tests (1). Insufficient information on the morphology of this fungus in artificial cultures was published for identification purposes, but in view of the inoculation results, it seems likely that this was the greasy spot fungus. However, the description of *C. citri-grisea* apparently was not based on this fungus, but on another fungus found sporulating only in small brown round spots. The imperfect state of the greasy spot fungus, here described, differs in conidiophore ontogeny and morphology from the fungus described by Fisher. Because of these discrepancies, *C. citri-grisea* is unacceptable, even as a synonym, for the *Stenella* state of the greasy spot *Mycosphaerella*.

In view of the nature of its imperfect state and the fact that the *Mycosphaerella* in Florida differs greatly from the description of *M. horii* Hara (3), a new name is proposed for the fungus causing greasy spot in Florida, described as follows:

Mycosphaerella citri Whiteside sp. nov.

Perithecia copiosa in foliis dissoluentibus, caespitosa, crebriora in latere infero, subepidermalia, globosa cum ostiolo conico, muro atro, 58-90 μ diametro. Asco crebri, obclavati, 25-35 X 5.0-5.5 μ. Ascosporae hyalinae, aut rectae aut leniter curvatae, naviculares oblonge, non colligatae ad septum, guttulatae inaequaliter, 6.2-11.2 X 2.2-2.8 μ, plerumque 8.5 X 2.5 μ. Status conidicus *Stenella* est, conidiophora proficiscentia ex hyphis extramatricalibus, recta, simplicia, obscuriora colore quam hyphae, cum cellis 2-5, 12-40 X 2.0-3.5μ. Conidia confuse septata, cum septis 0-9, mure asperulo, cylindracea, 6-50 X 2.0-3.5 μ subolivacea-brunnea.

In foliis laesiones subbrunneas-atras, oleosas, elevatas, inaequales, cum marginibus diffusis cinctis limbo galbano producunt. In fructu *Citrus sinensis* (L.) Osbeck maculas minutas atras in flavedo producunt.

Specimen typicum in foliis *Citrus paradisi* Macf. prope "Lake Alfred, Florida 17.7. 1969", a J. O. Whiteside lectus; in Herb. Commonwealth Mycological Institute IMI No. 141543 depositum.

Mycosphaerella citri sp. nov. Perithecia produced in densely packed groups on decomposing leaves, more numerous on underside of leaf, subepidermal, globoid with conical ostiole, 58-90 μ in diam., wall black and containing numerous obclavate asci 25-35 X 5.0-5.5 μ (Fig. 1-I). Ascopores hyaline, straight or slightly curved, oblong-naviculate, not constricted at septum, irregularly guttulate when mounted in lactophenol-cotton blue, 6.2-11.2 X 2.2-2.8 (mostly 8.5 X 2.5) μ.

Conidial state a *Stenella*; conidiophores sparsely produced, simple, erect, arising from extramatricular hyphae, darker in color and less rough-walled than hyphae, 2- to 5-celled, with apical cell paler in color and bearing scars left by detachment of conidia, 12-40 X 2.0-3.5 μ. Conidia indistinctly septate, rough-walled, pale olive-brown, 0-9 septate, cylindrical-acicular, 6-50 X 2.0-3.5 μ.

Extramatricular hyphae branched, rough-walled, olive-brown with mostly indistinct septa, epiphytic or with hyphae penetrating stomata, with persistent appressoriumlike structure in outer stomatal chamber. Intramatricular hyphae occasionally branched, smooth-walled, hyaline, and conspicuous in air spaces of spongy mesophyll.

On cornmeal agar forming slow-growing, brownish-green colonies. New isolates often produce conidiophores and conidia, similar in appearance to those formed on living leaves and fruit, except that in some cultures the conidia are catenulate. No perithecial development was observed on this medium.

Infection of citrus leaves results in the formation of raised, light-brown to black oily lesions with diffuse margins, surrounded by a yellowish-green border of variable width that is more pronounced during the earlier stages of symptom development. Infected fruit develop black specks between the oil glands due to death of guard cells and a few underlying cells. Leaf symptoms have been recorded on all locally grown commercial *Citrus* and *Fortunella* cultivars and on *Aeglopsis chevalieri* Swing. and *Murraya paniculata* (L.) Jack. Fruit symptoms are authenticated to date only on sweet orange.

Type locality, Lake Alfred, Florida. Leaf material and cultures deposited in Herbaria of the Commonwealth Mycological Institute, Kew, England, and the University of Florida.

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