Aberrant Behavior of *Mycosphaerella citri* on Freeze-Killed Citrus Leaf Tissue and Its Taxonomic and Epidemiologic Implications

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ABSTRACT

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Conidia of *Mycosphaerella citri* normally develop only on single conidiophores produced sparsely from extramatrical hyphae on citrus leaves, and ascocarps usually develop only on decomposing fallen leaves. No fruiting structures were observed on or in greasy spot lesions on living leaves. However, when leaves carrying latent infections of *M. citri* were locally injured by freezing, the fungus sometimes produced fasciculate conidiophores and ascocarps on the resulting necrotic spots and patches while the leaves were still alive and attached to the tree. Similar fasciculate conidiophores were described previously for *Cercospora citri-grisea* on necrotic citrus leaf tissue. Therefore, *C. citri-grisea* is a binomial for the anamorph of *M. citri*. The number of airborne conidia released from fasciculate conidiophores on freeze-induced leaf spots was infinitesimal compared with the number of ascospores released from ascocarps on fallen leaves.

Additional key words: Stenella sp.

Greasy spot lesions, caused by *Mycosphaerella citri* Whiteside, consist of hypertrophied spongy mesophyll that becomes gum-impregnated and necrotic (Fig. 1A,B). The lesions are raised, particularly on the underside of the leaves, and remain swollen and firmly attached to adjacent healthy tissue, even when old. No fruiting structures of *M. citri*, either sexual or asexual, have been observed in greasy spot lesions on living leaves, but ascocarps are produced abundantly on decomposing fallen citrus leaves (5,6).

Following a report by Tanaka and Yamada (4) that a *Cercospora*-type fungus was associated with the greasy spot (black melanose) disease in Japan, Fisher sought a *Cercospora* fungus as a possible cause of greasy spot in Florida and in 1961 described a new species, *Cercospora citri-grisea*, which produced fasciculate conidiophores on depressed necrotic leaf spots (1). Fisher considered the necrotic spots to be an advanced form of greasy spot in which the originally swollen tissue had shrunk and become depressed (1).

I was unable to reconcile *C. citri-grisea* as the anamorph of *M. citri* (5–7) for several reasons. First, fruiting structures of *C. citri-grisea* were described as occurring only in depressed necrotic spots (1), but I found no evidence that greasy spot disease could produce such spots (5), either through transformation of existing lesions, as reported by Fisher (1), or as a direct result of infection. Second, the only asexual fruiting structures I observed for *M. citri* were conidiophores that arose from extramatrical hyphae on the phylloplane (5,6), whereas conidiophores of *C. citri-grisea* were described as fasciculate and derived from a stroma in the host tissue (1). Third, conidia of *M. citri* are noticeably verrucose, but no such feature was mentioned in the description of *C. citri-grisea* (1). Finally, the successful pathogenicity tests Fisher reported were not made with conidial isolates from depressed necrotic spots but with isolates obtained from hyphae present in the spongy mesophyll of typical greasy spot lesions (1). Thus, no evidence was presented that the hyphae present in greasy spot lesions and the fasciculate conidiophore fruiting structures belonged to the same fungus.

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In Florida, leaves of most citrus cultivars, including sweet orange and grapefruit, remain remarkably free from the type of depressed necrotic spots with light-colored centers that are typical of lesions induced by *Cercospora* spp. In fact, the first time I observed necrotic spots with conidiophores similar to those described by Fisher was in March 1980, after a severe and unusually late freeze.

This article reports on the identification of the fasciculate conidiophores and conidia present on the freeze-induced spots as *C. citri-grisea*, the confirmation of this fungus as the anamorph of *M. citri*, the occasional presence of fasciculate conidiophores of this fungus on leaf tissue killed by other agents, and the potential importance of conidia from fruiting structures on freeze-induced necrotic tissue to the epidemiology of greasy spot.

MATERIALS AND METHODS

Single-spore isolates were made from conidia produced on fasciculate conidiophores on necrotic leaf spots, from ascospores present in ascocarps in the same spots, and from ascospores released from ascocarps in dead, fallen citrus leaves. All isolates were maintained on cornmeal agar (CMA).

Inoculum for pathogenicity studies was cultured in a modified Fries solution (2) for 14 days without being shaken. The mycelium was collected by straining the medium through cheesecloth and was fragmented in 2% sucrose in distilled water for 5 sec in a Waring Blendor. Larger fragments were removed by straining the resulting suspension through a 500-μm (32-mesh) sieve. Because some isolates of *M. citri* can lose virulence quickly (5), six to 12 single-spore isolates were combined in preparing inoculum.

The inoculum was sprayed onto the lower surface of grapefruit (*Citrus paradisi* Macf.) and rough lemon (*Citrus jambhiri* Lush.) leaves. To promote extramatrical growth and stomatal penetration, the inoculated plants were exposed to near 100% relative humidity in a moist chamber, continuously for the first 3 days and only at night for the next 11 days. Thereafter, the plants were maintained in the greenhouse for symptom development.

A Kramer-Collins 7-day drum spore sampler (G. R. Electric Mfg. Co., Manhattan, KS 66502) was operated continuously from 1 April to 30 September 1980 in a grapefruit grove that showed freeze-induced leaf spots and patches. The spore trap tape was mounted on slides, stained with cotton blue in lactophenol, and viewed at $\times$1,000 magnification for conidia and ascospores of *M. citri*.

RESULTS AND DISCUSSION

In the grapefruit grove at Lake Alfred, FL, where the observations were made, no freeze injury to foliage occurred during the winter of 1979–1980 until the night of 2–3 March, when the temperature reached a minimum of −4.4 C and remained below 0 C for 11 hr. While the air temperature was below 0 C, high winds prevented frost formation. On the following night, the temperature dropped below 0 C only briefly, reaching a minimum of −2.8 C, which caused no further injury. Normally, an advective arctic front-induced freeze of the severity of experienced on 2–3 March would be followed, when the winds calmed, by one or more nights of severe frost, causing rapid necrosis of leaf tissue, particularly in the vicinity of greasy spot lesions, and most injured leaves would drop 1–3 wk later. After the 1980 freeze, relatively few leaves dropped. Most affected leaves showed only localized injury (Fig. 1C), and some necrotic spots were as small as 2 mm in diameter, which is unusual for freeze or frost injury on citrus leaves.

The location or absence of greasy spot symptoms on some of the affected leaves had been recorded previously as part of other studies made in this grove. The freeze-induced spots developed on areas of the leaf that had been healthy, and the appearance of preexisting greasy spots was not altered by this freeze; thus, the necrotic spots did not develop from existing greasy spot lesions.

By the end of March, two fungi with fasciculate conidiophores and conidia resembling those of *Cercospora* spp. were observed on some of the necrotic spots, especially the smaller spots. One of the fungi, which was uncommon, produced smooth-walled, distinctly septate conidia and formed colonies on CMA unlike those of *M. citri*. Isolates of this unidentified fungus were not pathogenic to grapefruit or rough lemon leaves.

The other fungus formed verrucose, indistinctly septate conidia and produced colonies on CMA identical to those of *M. citri*. The stromata from which the fasciculate conidiophores arose on the necrotic leaf tissue (Fig. 1D,E) later formed spermogonia (Fig. 1F) and ascocarps. Most stromata in the necrotic spots did not produce conidiophores but developed directly into spermogonia and ascocarps, as stromata on decomposing fallen leaves do. Stromata were produced on both leaf surfaces; therefore, they were not necessarily associated with stomata, which are lacking in the upper

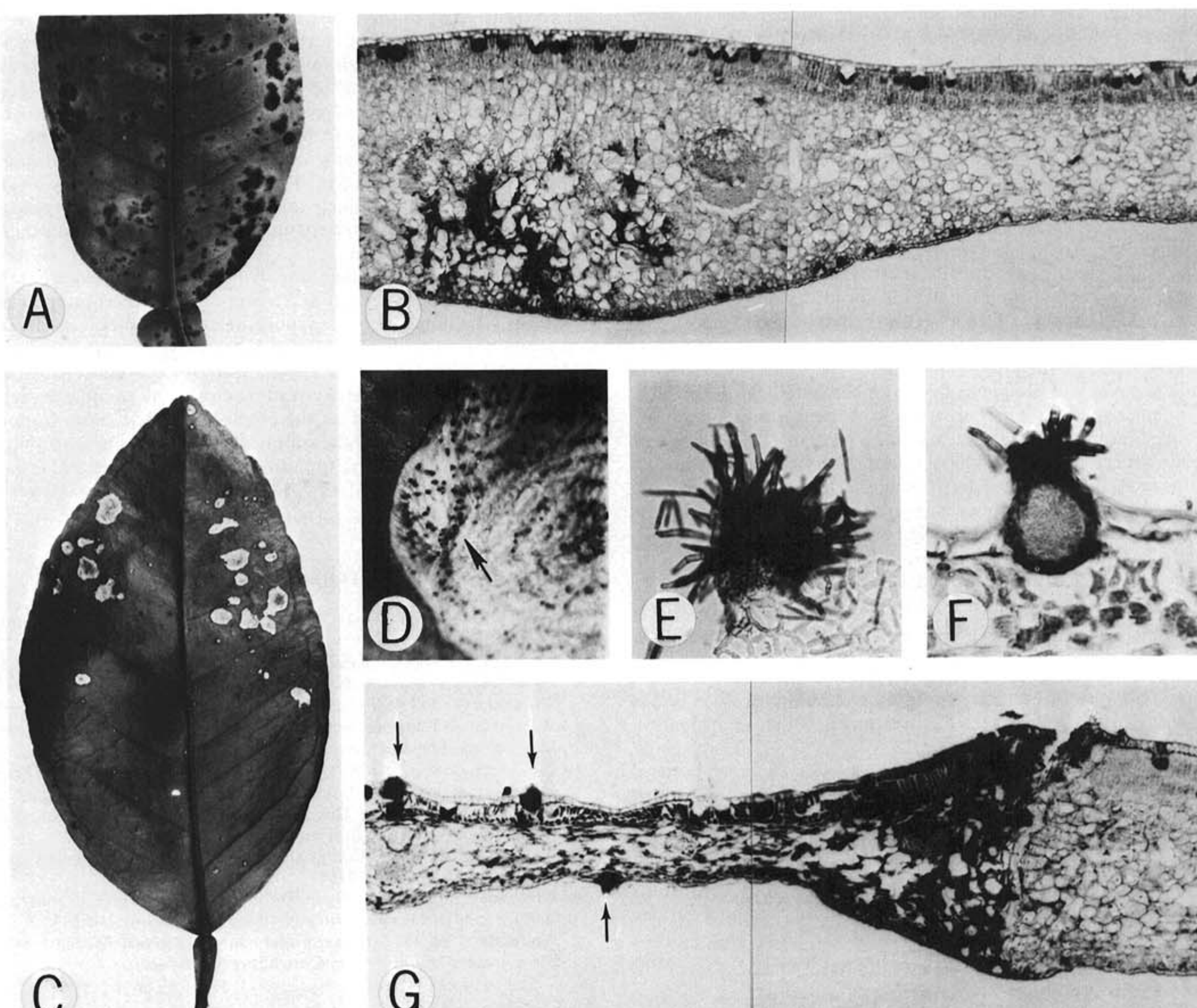

Fig. 1. Infection of grapefruit leaves by *Mycosphaerella citri*. **A,** Typical greasy spot symptoms on the underside of the leaf. **B,** Transverse section through greasy spot lesion showing gumming in the hypertrophied spongy mesophyll; normal leaf tissue at right (dark structures immediately beneath epidermis are crystal idioblasts). **C,** Sunken, light-colored necrotic spots caused by a one-night advective freeze in March 1980. **D,** Enlargement of freeze-induced spot viewed from upper side of leaf, with a group of stromata of *M. citri* (arrow) (the larger, mostly concentrically arranged structures are acervuli of *Colletotrichum gloeosporioides*) (×25). **E,** Fasciculate conidiophores arising from stroma in freeze-killed leaf tissue and detached conidia (×260). **F,** Conidiophore remnants on spermogonium (×410). **G,** Transverse section through dry ice-induced lesion on previously inoculated leaf, showing amphigenous distribution of stromata and spermogonia (arrows) (living tissue on right is separated from freeze-induced necrotic tissue by periderm layers).

epidermis of citrus leaves.

Fungal isolates obtained from verrucose conidia borne on fasciculate conidiophores in freeze-induced leaf spots, from ascospores discharged from ascocarps in the same leaf lesions, and from ascospores discharged from ascocarps in decomposing fallen leaves all produced greasy spot symptoms on inoculated grapefruit and rough lemon leaves. All isolates produced appressoria in the outer stomatal chamber, as is characteristic of *M. citri* (6). Time from inoculation to symptom appearance was 4–6 wk on rough lemon and 8–12 wk on grapefruit. The fungus was reisolated from plants inoculated with each set of isolates.

After determining that *M. citri* could, under certain conditions, produce fasciculate conidiophores, I then considered whether these were the structures that Fisher had described as *C. citri-grisea* (1). The necrotic spots and patches on type material of *C. citri-grisea* on lemon (*Citrus limon* (L.) Burm.) leaves in the University of Florida herbarium resembled the necrotic spots formed on grapefruit and orange leaves after the March 1980 freeze; nevertheless, whether the necrosis on the dried herbarium material was induced by freeze or some other agent is not certain. It may be significant that only lemon leaves were deposited in the herbarium, because lemon leaves are more prone to freeze injury than those of grapefruit or sweet orange. A few detached conidia were found on the surface of the necrotic tissue on type specimens F 46452 near fasciculate conidiophores, from which they presumably originated. These conidia were verrucose, indistinctly septate, and similar in diameter to those of *M. citri*. Spermogonia and immature ascocarps also were present in the type material, and some of them bore remnants of conidiophores. These features were not mentioned in the original description of *C. citri-grisea* (1).

Contrary to earlier reports (3,5,6), it now seems reasonably certain that *C. citri-grisea* Fisher is the anamorph of *M. citri*. Missing in the original description of *C. citri-grisea* are some useful identifying features: conidia are verrucose and sometimes catenulate, and conidiophores are also produced singly, without a stroma, on extramatrical hyphae on the phylloplane and on agar media. Suggestions (3,5,6) that the anamorph of *M. citri*, and hence *C. citri-grisea*, might more appropriately be described under the genus *Stenella* still need consideration, because the production of catenulate conidia is not characteristic of the genus *Cercospora*.

Since finding the fasciculate conidiophores of *C. citri-grisea* on freeze-induced leaf spots, I have found them occasionally on leaf lesions caused by fertilizer burn or physical abrasion from grove traffic. Also, they sometimes occur in spots on rough lemon and Rangpur lime (*Citrus limonia* Osbeck) leaves caused by host-specific strains of *Alternaria citri* Ellis & Pierce. Interestingly, Fisher (1) specifically mentioned Rangpur lime as a species on which greasy spot symptoms developed into depressed necrotic spots. I have never observed such a phenomenon, even on Rangpur lime, and I believe that Fisher probably observed the fasciculate conidiophores of *C. citri-grisea* on preexisting Alternaria leaf spots. This view is further supported by the appearance of the symptoms in Fig. 3 of Fisher's publication (1), which seem identical to those of Alternaria leaf spot.

Tests were made in the greenhouse on artificially freeze-induced leaf spots to determine whether the fruiting structures of *M. citri* that developed on freeze-induced leaf lesions on grapefruit trees could have arisen from hyphae already present in the leaf. Three months after container-grown grapefruit plants were inoculated with isolates of *M. citri* but before greasy spot symptoms appeared, small pieces of dry ice were placed on the upper surface of leaves for 1–2 sec. The resulting necrotic spots became white in 3–4 days and resembled naturally freeze-induced spots. After another 3–4 days, subepidermal stromata of *M. citri* began to appear in the necrotic tissue (Fig. 1G) on both leaf surfaces. After the plants were placed in a damp chamber at near 100% relative humidity every night for 1 wk, some of the stromata produced fasciculate conidiophores with conidia that were occasionally catenulate. Later, these stromata produced spermogonia and ascocarps. However, most stromata developed into spermogonia and ascocarps without first producing conidiophores. No fruiting bodies of *M. citri* were observed on dry ice-induced spots on uninoculated leaves. Thus, the stromata that developed on the freeze-induced leaf spots after the 1980 freeze probably arose from latent infections of *M. citri* already present in the affected leaves.

Other tests were made in the greenhouse to determine whether airborne inoculum of *M. citri* could colonize existing necrotic leaf tissue and produce fruiting structures thereon. Leaves on container-grown grapefruit plants were injured by holding small pieces of dry ice on them to produce necrotic spots about 5 mm in diameter. One week later, the plants were sprayed with a suspension of fragmented hyphae from cultures of *M. citri* derived from ascospores formed in ascocarps on decomposing leaves. After inoculation, the plants were placed in a moist chamber for 3 days and thereafter maintained on the greenhouse bench during the day and transferred to the moist chamber only at night. After 4 wk, stromata of *M. citri* had developed on five of 60 freeze-induced spots. Fasciculate conidiophores with verrucose conidia were observed on about 5% of the stromata on these spots. The results indicate that *M. citri* can colonize existing necrotic leaf tissue, which might explain why fruiting structures of *M. citri* sometimes appear in the necrotic tissue of Alternaria leaf spots on Rangpur lime and rough lemon leaves. These spots are formed only on very young leaves, so it is unlikely that the slow-penetrating fungus *M. citri* could already be present in the leaf before the Alternaria-induced necrosis appears.

About 5% of leaves on the grapefruit trees in the grove where the spore trap was operated showed freeze-induced necrotic spots and patches. The numbers of ascospores relative to conidia of *M. citri* trapped per month per 0.17-mm width of spore trap tape were: April 40:0, May 726:1, June 1,358:2, July 309:0, August 210:1, and September 55:1. Some conidia could have arisen from conidiophores produced on the phylloplane. The spore-trapping data showed that even if the supply of conidia is supplemented by conidia produced on conidiophores on freeze-induced leaf lesions, the asexual state of *M. citri* probably has very little impact on greasy spot epidemiology.

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