Mating and Pseudothecial Development in *Mycosphaerella citri*, the Cause of Citrus Greasy Spot

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ABSTRACT

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Greasy spot, caused by *Mycosphaerella citri*, is a serious disease of citrus in the Caribbean basin. *M. citri* is a loculoascomycete and produces pseudothecia in decomposing leaves after intermittent wetting and drying. A new in vitro mating technique was developed for production of pseudothecia on sterilized leaf disks in petri dishes. Of the single-ascospore cultures that were recovered from individual asci, four were one mating type and four were a second mating type (tentatively designated *mat+* and

mat-), indicating that *M. citri* probably is heterothallic and bipolar like most other loculoascomycetes. Most populations of ascospores recovered from individual leaves or from leaves from groves of different citrus species and various locations had a 1:1 ratio of mating types consistent with random mating. Cytological studies demonstrated that the ontogeny of pseudothecial development was similar to other loculoascomycetes. The formation of mature pseudothecia required 30 to 45 cycles of wetting and drying of infected, dead leaves which required ≈ 60 to 90 days. The in vitro system for pseudothecial production and the knowledge of the mating system in *M. citri* will facilitate genetic studies of this important pathogen.

Greasy spot, caused by *Mycosphaerella citri* Whiteside, produces oily lesions on the underside of citrus leaves that result in premature defoliation (16). Leaf loss eventually reduces tree vigor, yield, and fruit size. *M. citri* also infects fruit, causing greasy spot rind blotch, which reduces the acceptability of fruit for the fresh market. The disease affects all citrus, but is most severe on grapefruit and its hybrids. Greasy spot is economically important on all of the 320,000 ha of citrus in Florida as well as in eastern Mexico, Central America, and the Caribbean islands. Similar diseases occur in Argentina, Australia, and Japan (16).

M. citri, a loculoascomycete, produces ascospores in leaf litter on the grove floor (10,11,17). In Florida, most of the leaf drop occurs in January to February and peak ascospore release occurs in April to May (10,17). Whiteside (22) demonstrated that alternate wetting and drying was needed for pseudothecial development in dead, infected leaves. Mondal and Timmer (11) found that wetting of diseased leaves three times per week for 30 to 60 min maximized pseudothecial production. Most ascospores are released in a few hours after leaves are wetted once the pseudothecia are mature (10).

The mating systems and pseudothecial development of several loculoascomycete plant pathogens have been investigated. *Venturia inaequalis* was found to be heterothallic with a bipolar mating system many years ago (6) and a technique was developed later to produce pseudothecia under artificial conditions (14). The cytology of pseudothecial development and the conditions under which pseudothecia develop have been extensively studied (5,8,18,25). *Didymella rabiei*, the cause of Ascochyta blight of chickpea, was found to be heterothallic and bipolar, and an in vitro system has been developed to produce pseudothecia (18). *M. graminicola* was found to be heterothallic and bipolar, but pseudothecia cannot be produced in vitro, complicating genetic studies (7). The

causal agents of black leaf streak and Sigatoka disease of banana, *M. fijiensis* and *M. musicola*, respectively, have been well studied (9), and an in vitro system for production of pseudothecia has been developed for *M. fijiensis* (3). This species also was found to be heterothallic and bipolar (3,12).

The conditions required for pseudothecial development and ascospore release of *M. citri* have been elucidated in recent studies (10,11,22). However, no technique for the in vitro production of pseudothecia has been developed and the mating system has never been investigated. Previous histological studies have been conducted on the infection process and on pseudothecial structure (23), but detailed studies of the ontogeny of pseudothecial development are lacking.

The purpose of this study was to develop a technique for pseudothecial production in vitro and determine whether *M. citri* was homothallic or heterothallic. The distribution of mating types within asci, within individual leaves, and among groves was investigated to ascertain the segregation and distribution of mating types. Cytological studies were conducted to follow the development of ascocarps in relation to wetting and drying cycles.

MATERIALS AND METHODS

Isolation and maintenance of single-ascospore cultures. Cultures from single ascospores were obtained by collecting leaves with mature pseudothecia, soaking them in water for 10 to 15 min, blotting them dry, and affixing them to the lid of a petri dish using petroleum jelly. Ascospores were ejected onto 5% water agar when leaves were incubated overnight at room temperature (23 to 25°C). Individual germinated ascospores were transferred with the aid of a stereo microscope to dishes of potato dextrose agar (PDA) containing 0.1% tartaric acid. After the colonies had grown out, each isolate was transferred to a dish of PDA. A sterile 3-by-3-cm piece of filter paper was placed on the surface of the agar and pieces of mycelium were deposited at the edge of a filter paper (13). The dishes were incubated at 27°C until the culture had colonized the filter paper. Then the filters

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were lifted from the agar surface with a sterile forceps, placed in a sterile coin envelope, and dried overnight in a laminar flow hood. Envelopes were placed in a closed plastic container with CaSO_4 as a drying agent (Hammond Drierite, Xenia, OH) and stored at -20°C until needed.

In vitro mating technique. A method was developed to produce pseudothecia in vitro to determine the mating behavior and the mating type for individual isolates. Healthy, mature grapefruit leaves were collected and leaf disks, 1 cm in diameter, were cut using a cork borer and autoclaved for 15 min. Two sterile filter papers were placed in a petri dish and moistened with sterile water. Five autoclaved leaf disks were placed in each petri dish. Plugs (2 mm in diameter) were cut from PDA cultures of single-ascospore isolates of *M. citri*. The plug from one single-ascospore culture was placed 5 mm distant from the other on the upper surface of the leaf disk. These mating cultures were incubated at 23 to 25°C in the laboratory for 7 to 10 days, after which time the lids were removed and the leaf disks were allowed to dry. Subsequently, the cultures were moistened with 2 to 3 ml of sterile water every other day and allowed to dry each time at 23 to 25°C on the laboratory bench. Pseudothecial development typically was observed in compatible matings after 30 to 40 days.

Mating type distribution. The mating type of field-collected isolates was determined using the in vitro system and by pairing single-ascospore isolates with standard testers. Single-ascospore isolates GFO2 and GFO26, which produced abundant mature pseudothecia when mated using the in vitro system, were tentatively designated as *mat+* and *mat-*, respectively. All matings were performed twice to confirm the mating type of the culture.

To determine if mating type segregated within asci, ascospores were ejected onto water agar as indicated above. Clusters of eight ascospores were located on the water agar and presumed to have arisen from a single ascus. Single ascospores were separated with a fine needle, transferred to individual PDA dishes, and grown into colonies. In three cases, cultures were obtained from all eight ascospores, and these cultures were mated with the standard isolates in the in vitro system to determine mating type.

To determine the mating type distribution within individual leaves, three symptomatic leaves of grapefruit trees in Lake Alfred, FL were subjected to alternate wetting and drying to produce mature pseudothecia (11). Single-ascospore isolates were obtained from these leaves as described above. A total of 230, 315, and 49 isolates were obtained from leaves 1, 2, and 3, respectively, and the mating type of each determined using the mating technique.

Single-ascospore isolates were collected from leaves of different citrus species and different citrus areas within the state. From 39 to 105 isolates were collected from each source and all were tested for mating type using the standard isolates in the in vitro system.

Pseudothecial development. Mature grapefruit leaves with severe greasy spot were collected and air dried at ambient temperature. Leaves were soaked for 2 h and then air dried at 28°C for 2 days (11). Wetting and drying cycles were continued for 90 days. Periodically throughout the 90-day study, five- to seven-leaf disks, 3 mm in diameter, were cut from the leaves. This tissue was examined with a dissecting microscope and crushed, stained with cotton blue-lactophenol, and examined under a compound microscope at $\times 100$ to determine the stage of development of pseudothecia. As each stage was reached, the number of days and wetting-drying cycles for that stage was recorded. Not all pseudothecia were in the same stage of development at any given time. The experiment was repeated three times.

For photographic documentation of the developmental stages, leaf tissues were fixed in 3% glutaraldehyde in 0.066 M potassium phosphate buffer, pH 7.2, for 4 to 8 h at 26°C . The samples then were washed in the same buffer and post fixed in 2% OsO_4 in the same buffer for 4 h at 26°C or overnight at 4°C (1). The samples were dehydrated in an acetone series and embedded in

Spurr's resin. Sections ($0.75\ \mu\text{m}$ thick) were cut from samples representing each time period on an ultramicrotome (Reichert Optische Werke AG, Vienna, Austria) with glass knives and stained with methylene blue-azure A and basic fuchsin (15). Sections were observed using an Olympus AH-2 Vanox microscope and photographed using a Nikon Coolpix 995 digital camera.

RESULTS

In vitro mating technique. Mature fertile pseudothecia were produced on autoclaved leaf disks when some single-ascospore cultures were mated (Fig. 1A). The size and shape of the pseudothecia, asci, and ascospores corresponded well with the original description of *M. citri* (23). Incompatible crosses produced barren pseudothecia that contained no asci or ascospores (Fig. 1B). Ascospores were ejected from mature pseudothecia produced by this system as readily as from naturally infected material.

Distribution of mating types. Ascospores from three asci were successfully separated and grown. When mated in the in vitro system, all three had a ratio of 4:4 *mat+*:*mat-*, indicating that mating type segregated within each ascus. Thus, *M. citri* apparently is heterothallic with a bipolar mating system.

When single ascospores were recovered from individual field-collected grapefruit leaves, the ratio was not significantly different from 1:1 at $P \leq 0.05$ in two of three leaves (Table 1). The ratio of mating types was significantly different from 1:1 in one leaf, but fewer ascospores were tested from that leaf. Matings produced mature pseudothecia with one tester or the other in 78 to 100% of the crosses made.

Fig. 1. Pseudothecia of *Mycosphaerella citri* produced using the in vitro mating technique. **A**, Mature pseudothecia produced by mating of compatible types. **B**, Barren pseudothecia produced following pairings of incompatible types. Bar equals $200\ \mu\text{m}$ in **A** and **B**.

The ratio of mating types of single-ascospore isolates collected from different citrus species in various areas of Florida did not differ significantly from 1:1 at $P \leq 0.05$ in eight of the nine locations (Table 2). The percentage of the isolates that mated successfully with one of the two testers ranged from 71 to 98%.

Development of pseudothecia. After the dried symptomatic leaves were exposed to the wetting–drying cycles, stromatic initials (Fig. 2A) were formed quickly after 1 to 2 wet–dry cycles (2 to 4 days) (Fig. 3). Ascogonium formation and lumen initials (Fig. 2B and C) followed relatively quickly after 9 to 12 wet–dry cycles (18 to 24 days) (Fig. 3). The formation of pseudo-paraphyses and asci (Fig. 2D) required more time and there was greater variability among pseudothecia in reaching this stage. A total of 17 to 24 wet–dry cycles (34 to 48 days) were required to form asci (Fig. 3). From 10 to 15 additional cycles (54 to 78 days total) were required for differentiation, development, and maturation of asci (Fig. 2E to G). After ≈ 30 to 45 wet–dry cycles (60 to 90 days), pseudothecia had ejected their ascospores and were empty (Fig. 2H).

DISCUSSION

A new technique was developed that permitted production of pseudothecia and ascospores in vitro as has been done with *V. inaequalis* (6,14), *D. rabiei* (18), and *M. fijiensis* (3,12). We believe that alternate wetting and drying was a key factor in the success of the technique. In nature, it is important because fallen leaves that remain wet continuously are colonized by other fungi and decay before they produce pseudothecia (11). The other systems developed (3,7,12,14,18) do not utilize this procedure but, in some cases (7,18), inoculated plant tissues were placed under field conditions where they would be exposed to such conditions. We found that incompatible matings produced barren pseudothecia. Such structures have been reported with *V. inaequalis* (6) but are not mentioned in reports with other loculoascomycetes (3,7,12,14,18).

The in vitro mating technique allowed us to investigate the mating system of *M. citri* as has been done with other loculoascomycetes (3,7,12,18). All of our results are consistent with the hypothesis that *M. citri* is heterothallic and bipolar. We found two mating types that we have tentatively designated as *mat+* and *mat-*. The mating type locus from *M. citri* has not been cloned and sequenced; therefore, we cannot identify which mating-type idiomorph is homologous to *MAT-1* or *MAT-2* in other asco-

mycetes (20). Our preliminary attempts to amplify *MAT* genes from *M. citri* using primers designed from *M. graminicola* have been unsuccessful.

Our studies of ascospores from putatively single asci suggest that mating type segregates within a single ascus. Tetrad analysis will be needed to confirm these results. Only two mating types have been identified from the many matings that we have conducted with single-ascospore cultures from a wide geographical range in Florida. Reciprocal crosses among three single-ascospore cultures and numerous matings of arbitrarily selected cultures have not identified other mating types (*unpublished data*), suggesting that only two alleles occur at the *MAT* locus, as has been found in other heterothallic loculoascomycetes (24). Large numbers of reciprocal crosses with single-ascospore cultures are needed to confirm the absence of other mating types. Our findings are consistent with those of *V. inaequalis* (6,14,24), *D. rabiei* (18), *M. fijiensis* (3,12), and *M. graminicola* (6,20), all of which are heterothallic and bipolar. In these species, one individual carries either the *MAT-1* or *MAT-2* idiomorph at the *MAT* locus, but never both (19). In contrast, homothallic *Cochliobolus* spp. carry both idiomorphs in the same nucleus, but the structural organization of the *MAT* locus is different. In *Cochliobolus luttrelli* and *C. homomorphous*, the idiomorphs are fused, in *C. kusanoi* they are tightly linked, and in *C. cymbopogonis* they are unlinked (18). In the one known homothallic species of *Mycosphaerella*, *M. zeae-maydis*, the idiomorphs are linked but separated by ≈ 1 kb of noncoding DNA (19). In our studies, not all of the matings were fertile with either tester and, occasionally, the mating type ratio did not fit a 1:1 distribution. Thus, there may be other genes in *M. citri* that affect fertility of the fungus, and random mating may not be occurring in all populations.

From our matings of single-ascospore cultures obtained from field samples, mating type ratios were consistent with random mating. However, χ^2 tests of mating type ratios lack statistical power, and other approaches with additional unlinked molecular markers and large numbers of isolates from single populations will be required to rigorously test the random mating hypothesis in *M. citri*. Development of a *MAT*-specific polymerase chain reaction assay in *M. citri* would facilitate examination of larger numbers of isolates to determine whether mating is random, as has been done with *M. graminicola* (21). Characterization of the *MAT* locus also may be useful for phylogenetic studies of *M. citri* and other *Mycosphaerella* spp. affecting citrus (16), as has been done with other loculoascomycetes (2).

TABLE 1. Fertility and frequency of mating types among single-ascospore isolates of *Mycosphaerella citri* collected from individual grapefruit leaves

Leaf	<i>n</i>	Successful mating (%)	Mating type ^a		χ^2	<i>P</i>
			<i>mat+</i>	<i>mat-</i>		
GF L-1	230	83	95	105	0.50	0.45
GF L-2	315	100	160	155	0.078	0.90
GF L-3	49	78	26	12	5.16	0.05

^a Determined with standard tester isolates GFO2 and GFO26 using the in vitro mating technique.

TABLE 2. Fertility and frequency of mating types among single-spore isolates of *Mycosphaerella citri* collected from different citrus species and locations in Florida

Citrus species	Grove location	Total cross	Successful matings (%)	Mating type (<i>mat+</i> : <i>mat-</i>) ^a	χ^2	<i>P</i>
Grapefruit 2B	Lake Alfred	58	82	20:28	1.35	0.25
Grapefruit 19	Lake Alfred	39	77	17:13	0.56	0.45
Lime	Lake Alfred	60	85	31:20	2.39	0.20
Lemon	Lake Alfred	61	98	29:31	0.04	0.90
Sweet orange	Lake Alfred	42	95	20:20	0.00	1.0
Sweet orange	Immokalee	105	92	59:38	4.55	0.05
Murcott	Bereah	42	73	19:10	2.74	0.10
Grapefruit	Fort Pierce	83	85	35:36	0.01	0.90
Tangelo	Polk City	79	71	33:23	1.61	0.25

^a Determined with standard isolates GF02 and GF026 in the in vitro mating system.

Fig. 2. Ontogeny of pseudothecial development of *Mycosphaerella citri* in decomposing grapefruit leaves. **A**, Stromatic initial, **B**, ascogonial initial, **C**, lumen initial, **D**, formation of pseudoparaphyses and asci, **E and F**, asci mature but not ready for release, **G**, mature asci and ascospores, and **H**, exhausted pseudothecium. All bars equal 25 μ m.

Fig. 3. Number and range of wet–dry cycles required for each stage of development of pseudothecia of *Mycosphaerella citri*; 1 = stromatic initials, 2 = ascogonium formation, 3 = lumen initials, 4 = formation of pseudoparaphyses and asci, 5 = asci developed, contents not yet differentiated, 6 = asci developed, but ascospores not ready to release, 7 = mature asci, and 8 = pseudothecia exhaustion.

The stages of development of pseudothecia of *M. citri* are similar to those in other loculoascomycetes (5,18,24). Many of these fungi are pathogens of temperate crops; thus, pseudothecial development occurs at cold, winter temperatures. Ascospore release in those fungi is timed with the phenology of the host as temperatures rise in the spring. With *M. citri*, temperature affects the rate of pseudothecial development (11), but ascospore production is unrelated to the phenology of citrus, a subtropical evergreen. Even under optimal conditions of wetting–drying and temperature, a minimum of 60 days is required for ascospore maturity. Under natural conditions, development is much slower (9). The time required for pseudothecial development is similar to that of *V. inaequalis* (4,8,25) and *D. rabiei* (18), even though *M. citri* pseudothecia generally develop under warmer conditions. Pseudothecial maturation is not synchronous even under controlled conditions. This is consistent with our previous finding that, once the first ascospores in a leaf mature, ascospores continue to be released for three to four more cycles of wetting and drying (10).

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