Isolation, Pathogenicity, and Partial Host Range of *Alternaria limicola*, Causal Agent of *Mancha Foliar de los Citricos* in Mexico

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ABSTRACT

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Alternaria limicola is confirmed as the cause of *mancha foliar de los citricos* (citrus leaf spot), a leaf and twig disease of citrus in Mexico, formerly called "citrus bacteriosis." The fungus was isolated from 15 of 16 samples of symptomatic leaves of six species of *Citrus* collected during 1989 and 1990 in the Pacific coast state of Colima and from symptomatic leaves of an additional species of *Citrus* collected during 1990 in Guerrero, Mexico. *Xanthomonas campestris* was not isolated from any of the leaf lesions. Greenhouse-grown Mexican lime (*C. aurantiifolia*) and Duncan grapefruit (*C.* × *paradisi*) seedlings were inoculated by spraying young, actively growing, terminal foliage with aqueous suspensions containing 200–400 conidia per milliliter of either *A. limicola* or *A. citri*. Lesions similar to those observed on naturally infected plants developed only on those plants inoculated with *A. limicola*. The fungus was reisolated from all inoculated plants. This work confirms previous reports that the primary cause of *mancha foliar de los citricos* is a species of *Alternaria* and extends previous studies by fulfilling Koch's postulates for *A. limicola*. Additionally, it was determined that isolates of *A. limicola* from six other species of *Citrus* were identical in morphology to isolates from Mexican lime. Koch's postulates for isolates from these six additional host species were fulfilled. Lesion age and part of the lesion excised affected the isolation frequency of *A. limicola*. The highest isolation frequencies were from the part of the maturing lesions that included a high percentage of the necrotic area and from old lesions.

A foliar and twig disease of Mexican (Key) lime (*Citrus aurantiifolia* (Christm.) Swingle) was detected in Tecoman, Colima, Mexico, in December 1981. A pathotype of *Xanthomonas campestris* was irregularly associated with this leaf spot and was originally thought to be a variant of *X. c. citri* (Hasse) Dye, the causal agent of citrus canker. This new disease was referred to as "citrus bacteriosis" (7). Because of its apparent similarity to citrus canker, the disease was considered of regulatory importance by the United States, resulting in restrictions on internal movement of fruit and vegetative material of Mexican lime and Persian lime (*C. latifolia* Tanaka) and on export of certain citrus fruits from Mexico to the United States (7 Code of Federal Regulations part 319.27. "Citrus Canker—Mexico." 1983).

Rodríguez et al (7) reported infrequent and inconsistent isolation of bacteria from citrus bacteriosis lesions and difficulty in precisely reproducing the symptoms observed on naturally infected plants when plants were inoculated with the bacterial isolates. They suggested the possible involvement of other microorganisms as one explanation for these inconsistencies. Garza López (3) isolated an unidentified species of *Alternaria* from leaf lesions of Mexican lime and reproduced the symptoms on artificially inoculated seedlings of that host species. He therefore suggested that *mancha foliar de los citricos* (MFC), or citrus leaf spot, is a more appropriate name for this disease.

Simmons (8) described the fungus that causes MFC as *Alternaria limicola* Simmons & Palm based on isolates obtained in this study in 1989 and on an isolate from Colima that was sent originally to the International Mycological Institute (at that time the Commonwealth Mycological Institute) in 1979 by an unidentified person. That isolate was recognized at the institute as an undescribed species and was forwarded to Simmons in 1980.

MFC has been found in Mexican lime-producing states on the Pacific coast of Mexico, including Colima, Guerrero, Jalisco, Michoacan, Nayarit, and Oaxaca (7). Intensive cooperative surveys conducted by the United States and Mexico from 1983 to 1990 did not find the disease in any other citrus-growing region of Mexico. This disease has not been reported elsewhere in the world. Additional information on many aspects of MFC can be found in the review by Medina Urrutia et al (6).

This study was undertaken to: 1) confirm that a species of *Alternaria* is the primary cause of MFC, 2) extend previous work by fulfilling Koch's postulates for *A. limicola*, and 3) determine whether isolates from symptomatic leaves of other species of *Citrus* were the same as or different from isolates of *A. limicola* from Mexican lime.

MATERIALS AND METHODS

Effect of lesion part on recovery of *A. limicola* from leaf lesions of different ages. A preliminary experiment was conducted in order to determine how to isolate most successfully from leaf lesions. Eleven samples of symptomatic leaves of five species of *Citrus* were collected from a total of five orchards. Leaves were surface-disinfested for 1 min by washing with either sterile distilled water or 1% NaOCl. From each sample one lesion of each of three different maturity levels, designated as young (a water-soaked pustule), maturing (pustule center beginning to collapse), and old (center collapsed and necrotic), was cut sequentially into three parts. Part 1 was cut at the margin of the diseased and healthy tissue on the left-hand side of the lesion and included the margin of the lesion and the adjacent healthy tissue; part 2 was cut near the margin on the right-hand side of the lesion and included three-quarters of the lesion; and part 3 was cut 1 mm from the right-hand margin and included the remaining one-quarter of the lesion and adjacent healthy tissue. Lesion parts were then placed on potato-dextrose agar (PDA) or V8 juice agar, and plates were maintained in the dark at 22 C.

Isolation from naturally infected leaves. Sixteen samples from six symptomatic species of *Citrus*—Mexican lime, Persian lime, *C. aurantium* L. subsp. *aurantium* (sour orange), *C. macrophylla* P.J. Wester, *C.* × *paradisi* Macfady. (grapefruit), and *C. sinensis* (L.) Osbeck 'Valencia' (sweet orange)—were collected in five orchards in Tecoman, Colima, Mexico, in April 1989 and February 1990. A sample of *C. limettoides* Tanaka (sweet lime) was collected in Guerrero, Mexico, in February 1990. Leaves were surface-disinfested in 1% NaOCl for 1 min, then rinsed twice in sterile distilled water. Lesions were aseptically excised and placed on PDA; young lesions were left intact and maturing and old lesions were cut in half. Petri dishes were maintained at 22 C. Single-conidial isolates were derived by removing conidia from the initial isolation dishes, placing them on the surface

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of water agar, and later transferring single, germinating conidia to PDA. Attempts to isolate bacteria were carried out as previously described (7). Isolates obtained and their sources are listed in Table 1.

Inoculation in greenhouse. Two or four Mexican lime and two Duncan grapefruit seedlings were inoculated by spraying the terminal foliage with an aqueous spore suspension (including one drop of Tween per 100 ml of inoculum) containing 200–400 conidia per milliliter of *A. limicola*. Mexican lime was inoculated with 12 representative isolates (Table 1) and Duncan grapefruit with 10 isolates. Mexican lime and *C. jambhiri* Lush. (rough lemon) seedlings were inoculated with *A. citri* Ellis & N. Pierce in N. Pierce in the same manner as for *A. limicola*. The isolate of *A. citri* (ATCC 38962) was obtained originally in Florida from rough lemon and was reported to produce a host-specific toxin (5). Control plants were sprayed with sterile distilled water (including one drop of Tween per 100 ml of inoculum). For all treatments, both surfaces of the leaves were sprayed to runoff, without water-soaking the leaves. The surface of PDA in a petri dish was sprayed with the spore suspension in order to test for uniformity of spray deposit, the viability of conidia based on their germination, and contamination. Plants were covered with plastic bags for 24 hr. Inoculations were repeated at least once. All inoculations were performed in a quarantine greenhouse at the Beltsville Agricultural Research Center, Beltsville, Maryland.

In 1989, symptomatic leaves of Mexican lime and Duncan grapefruit were collected 10 and 12 days postinoculation, respectively. Symptomatic Mexican lime leaves inoculated with isolate E were collected again at 67 days. In 1990, Mexican lime and Duncan grapefruit leaves were collected 31 and 57 days postinoculation, respectively. Three or more lesions from each of two leaves from each isolate-host combination were used for reisolation of *A. limicola*, which was done in the same manner as described above for isolation from naturally infected leaves.

RESULTS

Isolation of *A. limicola* from lesion parts of different ages. *A. limicola* grew from 0–9% of young lesions placed on PDA or V8 (Table 2). Maturing lesions placed on PDA yielded *A. limicola* in all cases except when part 1 was disinfested with NaOCl. When maturing lesions were placed on V8, only parts 2 (27%) and 3 (9%) washed with sterile distilled water yielded *A. limicola*. When old lesions were placed on PDA, *A. limicola* was isolated from all lesion parts treated with either disinfestation procedure but was isolated more frequently from parts 2 and 3 than from part 1 (Table 2).

Isolation from naturally infected leaves. Foliar and twig lesions were observed on Mexican lime trees in the field (Fig. 1), and *A. limicola* was isolated with a combined frequency of 50% from all Mexican lime leaf samples (Table 3). Symptoms similar to those on Mexican lime were observed on leaves of six additional species of *Citrus*, and *A. limicola* was isolated from nine of the 10 samples of those six species (Table 3). The fungus was not isolated from the sweet orange sample collected in 1989 but was isolated from symptomatic leaves of that host species in 1990. The colony characteristics and conidium morphology of the isolates from these additional species of *Citrus* were identical to those of *A. limicola* from Mexican lime. The dried holotype culture has been deposited in the National Fungus Collections (US 1107559) (8). A living culture derived from the type has been deposited in the American Type Culture Collection (ATCC 66980). *X. campestris* was never isolated from any of the leaf lesions.

Inoculation in greenhouse. All Mexican lime and Duncan grapefruit seedlings inoculated with isolates of *A. limicola*, originally derived from all seven species of *Citrus*, developed symptoms identical to those observed on naturally infected plants (Fig. 2). Young lesions were pustules with water-soaked margins. As the lesions matured, the pustule center became somewhat necrotic, began to collapse, and had a water-soaked margin. The centers of old lesions were completely collapsed, resulting in flat lesions with necrotic centers and well-defined margins. In addition to leaf lesions, extensive puckering and cupping of leaves (Fig. 2) and defoliation were observed. Some old lesions on leaves inoculated with isolates E and J were atypical and were flat with

Table 1. Sources of *Alternaria limicola* isolates cited in this paper

Organism Isolate	Source *Citrus* species	Date isolated	Location
A. limicola			
A	*C. aurantiifolia*	Apr. 1989	Colima, Mexico
B	*C.* × *paradisi*	Apr. 1989	Colima, Mexico
C	*C. aurantiifolia*	Apr. 1989	Colima, Mexico
D	*C. aurantiifolia*	Apr. 1989	Colima, Mexico
E	*C.* × *paradisi*	Apr. 1989	Colima, Mexico
G	*C. aurantiifolia*	Apr. 1989	Colima, Mexico
H1	*C. aurantium* subsp. *aurantium*	Apr. 1989	Colima, Mexico
H2[a]	*C. a. aurantium*	Apr. 1989	Colima, Mexico
I	*C. latifolia*	Apr. 1989	Colima, Mexico
J	*C. aurantiifolia*	Apr. 1989	Colima, Mexico
A90[a]	*C. aurantiifolia*	Feb. 1990	Colima, Mexico
B90	*C. sinensis* cv. Valencia	Feb. 1990	Colima, Mexico
C90[a]	*C. a. aurantium*	Feb. 1990	Colima, Mexico
D90	*C. macrophylla*	Feb. 1990	Colima, Mexico
E90[a]	*C. aurantiifolia*	Feb. 1990	Colima, Mexico
Guer 47	*C. limettoides*	Feb. 1990	Guerrero, Mexico
"A. citri"[b]			
ATCC 38962	*C. jambhiri*	Before 1979	Florida

[a]Not used in greenhouse inoculations.
[b]Quotation marks signify uncertainty about appropriate name for isolate.

Table 2. Isolation of *Alternaria limicola* from leaf lesions of different levels of maturity using different disinfestation procedures and different agar media

Leaf lesion part[a]	Disinfestation[b]	Frequency of isolation[c] (%) Young lesions PDA	Young lesions V8	Maturing lesions PDA	Maturing lesions V8	Old lesions PDA	Old lesions V8
1	Cl	0	0	0	0	18	0
1	H_2O	9	0	9	0	18	9
2	Cl	9	0	18	0	36	0
2	H_2O	0	9	18	27	73	64
3	Cl	0	0	18	0	45	0
3	H_2O	9	0	9	9	64	36

[a]Part 1 = margin of diseased and healthy tissue on left-hand side of lesion, part 2 = three-quarters of lesion, part 3 = one-quarter of lesion and adjacent healthy tissue on right-hand side of lesion.
[b]Cl = surface disinfestation in 1% NaOCl for 1 min, H_2O = washing in sterile distilled water for 1 min.
[c]Number of isolates recovered/number of lesion parts plated × 100. Young lesion = water-soaked pustule, maturing lesion = pustule center beginning to collapse, old lesion = center collapsed and necrotic.

thin, translucent centers and irregular, flat margins. However, younger lesions were typical of MFC. No symptoms were observed on any of the plants inoculated with the "*A. citri*" isolate or on any control plants. (We place "*A. citri*" in quotation marks because of uncertainty about the appropriate name to apply to that isolate. Most workers have used *A. citri* for any *Alternaria* isolated from citrus. Simmons [8], in his extensive study of species of *Alternaria* on Rutaceae, found that "... from scores of citricolous specimens labeled *A. citri* that I have examined from worldwide sources, perhaps 90% bear *Alternaria* species morphologically quite unlike *A. citri*. ... a tiny minority can be equated with *A. citri* with some degree of confidence.")

All isolates of *A. limicola* except Guer 47 were reisolated from leaves of inoculated Mexican lime seedlings (Fig. 3) at frequencies ranging from 16.6 to 100% (Table 4). Isolate Guer 47 was never reisolated from inoculated Mexican lime. Isolates of *A. limicola* were reisolated from leaves of inoculated Duncan grapefruit seedlings with frequencies ranging from 16.6 to 50% (Table 4). In 1989, four of the seven isolates of *A. limicola* inoculated onto Duncan grapefruit seedlings were not reisolated 12 days postinoculation. No attempt was made to reisolate at a later date. In 1990, however, all isolates inoculated on Duncan grapefruit, including Guer 47, were reisolated. Attempts to reisolate from leaves inoculated with "*A. citri*" or from leaves of control plants were unsuccessful.

DISCUSSION

This work: 1) confirms that a species of *Alternaria* (2,3) is the primary cause of MFC; 2) extends previous work by fulfilling Koch's postulates for *A. limicola* on Mexican lime and Duncan grapefruit; 3) demonstrates that isolates from other citrus hosts are identical morphologically to isolates of *A. limicola* from Mexican lime, the primary host; 4) confirms reports of natural infection of species of *Citrus* by fulfilling Koch's postulates for isolates from six additional *Citrus* species on Mexican lime and Duncan grapefruit; and 5) demonstrates the effect of lesion age and part on isolation of *A. limicola*.

Garza López (4) and Stapleton and Garza López (12) reported natural infection of 15 *Citrus* cultivars, including the six *Citrus* species in this study, by a species of *Alternaria* isolated from Mexican lime. This study substantiates those reports by isolating *A. limicola* from six of those species of *Citrus* and fulfilling Koch's postulates for isolates

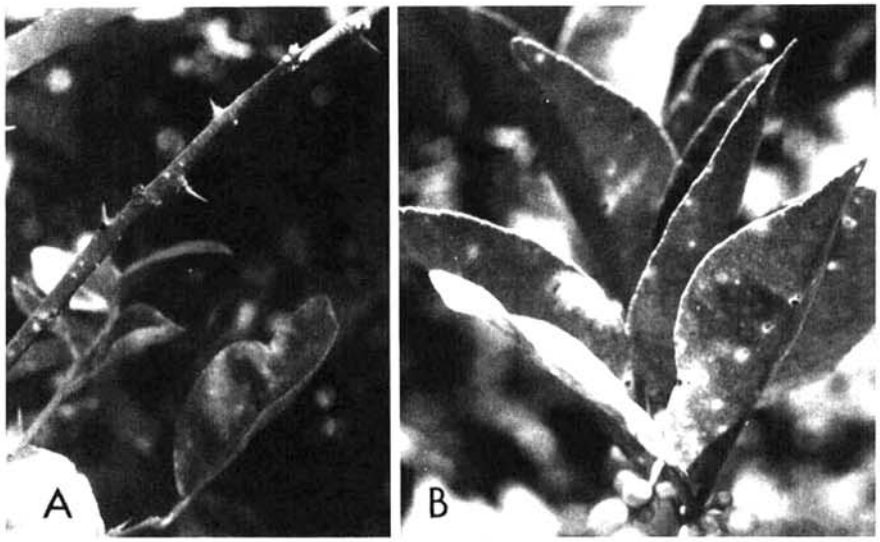

Fig. 1. Mexican lime naturally infected with *Alternaria limicola:* **(A)** Typical leaf and twig lesions and defoliation and **(B)** typical old leaf lesions with chlorotic margins and collapsed, necrotic centers.

Table 3. Isolation of *Alternaria limicola* from leaf lesions of naturally infected species of *Citrus*

Citrus species	Frequency (total no. samples)[a] 1989	1990	Combined frequency (%)
C. aurantiifolia	5/16 (5)	9/12 (2)	50.0
C. aurantium subsp. *aurantium*	3/5 (2)	2/5 (1)	50.0
C. latifolia	1/3 (1)	(0)	33.3
C. limettoides	(0)	3/9 (1)	33.3
C. macrophylla	(0)	1/3 (1)	33.3
C. × *paradisi*	2/6 (2)	(0)	16.6
C. sinensis cv. Valencia	0/3 (1)	1/5 (1)	12.5

[a]Number of lesions from which isolates were obtained/number of lesions plated. Number in parentheses indicates number of groves from which samples were collected.

Fig. 2. Mexican lime seedlings inoculated with *Alternaria limicola:* **(A)** Typical chlorotic, pustulate leaf lesions and cupping due to severe infection, 8 days after inoculation; **(B)** typical young pustulate leaf lesions and maturing lesions with centers beginning to collapse, 8 days after inoculation; and **(C)** typical young pustulate leaf lesions, old collapsed, necrotic lesions, and cupped and puckered upper leaf, 5 days after inoculation.

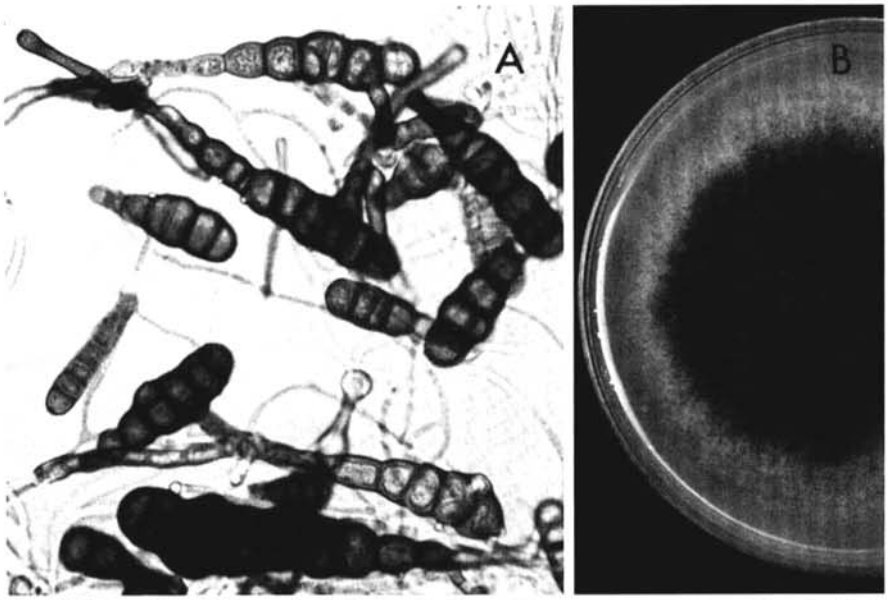

Fig. 3. (A) Conidia of *Alternaria limicola* with five to 10 transverse septa, several longitudinal septa, elongate beaks that often function as conidiophores or conidiogenous cells, and a monilioid appearance due to constriction at the septa. (×475) **(B)** Four-day-old culture of *A. limicola* reisolated on PDA from inoculated Mexican lime leaves.

Table 4. Reisolation of *Alternaria limicola* from leaf lesions of Mexican lime (ML) and Duncan grapefruit (DG) seedlings inoculated with isolates from a total of seven species of *Citrus*

Host	Year	Days after inoculation	No. of isolates used for inoculation	No. of isolates reisolated	Frequency[a] (%)
ML	1989	10	9	8	16.6–30
		67		1	100[b]
	1990	31	3	2[c]	30–35
DG	1989	12	7	3	16.6–50
	1990	57	3	3	28–33

[a] Number of lesions from which isolates were recovered/number of lesions plated × 100.
[b] Isolate E (from *C.* × *paradisi*, April 1989, Colima, Mexico).
[c] Nothing isolated from symptomatic leaves inoculated with isolate Guer 47 (from *C. limettoides*, February 1990, Guerrero, Mexico).

from those additional hosts. The only exception was the sweet lime isolate Guer 47 on Mexican lime, which was successfully isolated, however, from inoculated Duncan grapefruit.

Isolation frequencies both from material naturally infected in the field (Tables 2 and 3) and from plants inoculated in the greenhouse (Table 4) were variable. Isolation frequencies from naturally infected material were highest from all parts of old lesions and from part 2 of maturing lesions, the lesion part with the most necrotic tissue. *A. limicola* was rarely isolated from young lesions. This variability probably is due to the manner in which this fungus causes disease and the apparent limited amount of growth of the fungus in the lesion. Initial symptom development due to toxin production, with limited or later invasion of lesions by *A. limicola*, may explain the difficulty in isolating this fungus from young lesions. Cárdenas Soriano and Garza López (1) reported that the species of *Alternaria* they had isolated from symptomatic Mexican lime produced an extracellular substance that caused host protoplast condensation, hypertrophy, and cell collapse. J. G. Garza López, M. Kodama, and K. Kohmoto (*unpublished*) isolated four phytotoxic substances from culture and germinating spore filtrates of *A. limicola*. Those phytotoxic substances or others produced by *A. limicola* may affect cells prior to invasion by the fungus. Other workers (5,9,10,13) have reported the production of host-specific toxins by other phytopathogenic *Alternaria* species, including "*A. citri*," that play a role in development of other diseases of citrus.

Stapleton (11) reported fungal structures on external leaf surfaces and in subepidermal leaf tissues, especially in older "bacteriosis" lesions on Mexican lime leaves. Cárdenas Soriano and Garza López (1) reported that the *Alternaria* sp. was found mainly in the epidermal cells and the two subepidermal cell layers, and not in the hypertrophied spongy mesophyll cells. The variation in isolation frequency between lesions of different ages and from different parts of lesions can be explained in part by the results of these histological studies in that the fungus grows mainly into older, necrotic portions of lesions and only in limited cells in those lesions.

In 1989, only three of the seven isolates inoculated on Duncan grapefruit were reisolated (Table 4). This may be because lesions on Duncan grapefruit tended to remain pustular, as in young lesions on Mexican lime. It is likely that the fungus had not colonized the lesions on Duncan grapefruit to any extent, thus making reisolation difficult. Attempts to reisolate in 1989 after a longer postinoculation period may have been successful. All isolates were reisolated 57 days postinoculation in 1990. It is also possible that the Duncan grapefruit leaves inoculated in 1989 were somewhat older and therefore possibly less susceptible to colonization by *A. limicola*.

The water-soaked pustules typical of young lesions incited by *A. limicola* resemble lesions caused by some bacteria. If isolations were attempted mainly from young lesions and at the margin of healthy and diseased tissue, as is often done when attempting to isolate a bacterium and many fungi, then *A. limicola* likely would not be isolated. Therefore, the resemblance of young MFC lesions to those characteristic of bacterial infections may explain why *A. limicola* was not routinely isolated in initial attempts to determine the causal agent of the disease.

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