Vegetative Compatibility and Virulence of the Spinach Anthracnose Pathogen, *Colletotrichum dematium*

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ABSTRACT

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We characterized 215 isolates of *Colletotrichum dematium* collected from spinach in Arkansas, California, New Jersey, Oklahoma, Texas, and Ontario, Canada, for vegetative compatibility (using nitrate-nonutilizing mutants) and lesion type (primary or secondary). Isolates recovered from spinach leaves not previously damaged were identified as primary anthracnose isolates, whereas those recovered from leaves with white rust lesions (caused by *Albugo occidentalis*) were identified as secondary anthracnose isolates. Conidial size and colony color on acidified lima bean agar were recorded. Thirty-nine isolates representing all vegetative compatibility groups (VCGs), lesion types, and geographic origins were further compared in greenhouse virulence tests on the spinach cultivars Fall Green and Grandstand. Isolates of *C. dematium* from tomato and onion were also included in the virulence and vegetative compatibility tests. All isolates, regardless of host, geographic origin, VCG, or lesion type, produced slightly curved, hyaline conidia with an average size of 21.5–30.9 × 3.0–3.8 μm. Two VCGs (VCG1 and VCG2) were identified among the 215 spinach isolates examined. Isolates of both VCGs were recovered from Arkansas, New Jersey, and Oklahoma. All isolates from California (32) and Canada (4) belonged to VCG1, whereas all isolates from Texas (22) belonged to VCG2. One tomato and one onion isolate each represented unique VCGs. In general, *C. dematium* isolates from spinach were more virulent on spinach than the isolates from onion and tomato; thus, the forma specialis designation, *C. dematium* f. sp. *spinaciae*, appears warranted. Select isolates of *C. d. spinaciae* representing each of the two VCGs and lesion types from different geographic areas could not be differentiated on the basis of virulence in greenhouse pathogenicity tests.

Anthracnose of spinach, caused by *Colletotrichum dematium* (Pers.) Grove f. *spinaciae* (Ellis & Halst.) Arx (= *C. spinaciae* Ellis & Halst.), can cause severe damage to spinach under favorable environmental conditions (10,11,15,18, 24). Lesions on leaves initially appear as water-soaked areas and can expand, become necrotic, coalesce, and destroy the entire leaf. Acervuli with protruding black setae are often produced and can be useful in disease diagnosis.

Ellis and Halsted (12) first described the spinach anthracnose fungus from New Jersey in 1890 as *C. spinaciae*. Sherf and MacNab (23) described two species capable of causing spinach anthracnose, *C. spinaciae* Ellis & Halst. and *C. spinacicola* Nom. nov. (= *Gloeosporium spinaciae* (Ellis & Everhart)). *C. spinacicola* had small (2.0–2.5 × 5–10 μm), straight conidia and was more virulent than *C. spinaciae*, which had larger (2.5–4.0 × 13–29 μm), slightly curved conidia. Currently, neither species designation is considered valid (1,2,25, 26). Von Arx (25,26) described the spinach anthracnose pathogen as *C. dematium* but, recognizing its host specialization, designated it as *C. dematium* f. sp. *spinaciae*.

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In Arkansas, two anthracnose diseases of spinach have been described (11,13, 14): primary anthracnose, caused by *C. spinaciae*, a disease of otherwise healthy foliage; and secondary anthracnose, caused by an undescribed *Colletotrichum* sp., a disease of foliage previously attacked by other pathogens, such as the white rust pathogen (*Albugo occidentalis*) or *Cercospora* sp. Dechmani and Goode (11) reported that the primary and secondary anthracnose pathogens were "clearly distinguishable" but provided no criteria for distinguishing them apart from their differential virulence on spinach.

Nitrate-nonutilizing mutants have been used extensively to study vegetative compatibility and the relationships between vegetative compatibility groups (VCGs) and virulence in a number of fungi (5,16,28), particularly *Fusarium oxysporum* (see Correll [4] for a review). More recently, these techniques have been used to study the population biology of several species of *Colletotrichum* (3,9,27).

The objective of this study was to examine variation in the spinach anthracnose pathogen population in North America. Samples of infected foliage were collected from primary and secondary lesions at widely separated geographic locations. All isolates were characterized by vegetative compatibility and colony color on a test medium. Selected isolates representing all categories of lesion type, geographic origin, and VCG were further tested for virulence and conidial size. A preliminary report of this work has been published (6).

MATERIALS AND METHODS

Isolates. Infected tissue from numerous spinach cultivars was obtained during 1989–1992 at different times of the year (fall and spring) and from plants and leaves of various ages. Tissue was placed on lima bean agar (Difco) acidified with 3 ml of 85% lactic acid per liter of sterile medium (ALBA). All cultures were purified by recovering a single spore or a hyphal tip. The cultures were grown on ALBA twice before storage on green bean agar (GBA) (226 g of green bean baby food [Gerber Products, Fort Smith, AR] and 20 g of agar per liter of water). A sterile filter paper disk was placed on the agar surface before transfer of the culture. After the medium was colonized, the filter paper was removed, allowed to air-dry under sterile conditions, and stored desiccated at 4 C (8). All cultures were grown at room temperature (approximately 25 C) under four cool-white fluorescent lights with a 12-hr light-dark cycle.

Isolates recovered from otherwise healthy foliage with no other apparent lesions were considered primary anthracnose isolates. Isolates recovered from within the border of a white rust lesion (caused by *A. occidentalis*) were recorded as secondary anthracnose isolates. Isolates of *C. dematium* from onion (ATCC 26388, 34305, and 44202) and tomato (ATCC 58111 and 58112) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD).

Vegetative compatibility. Isolates were tested for vegetative compatibility as previously described (7,21). Briefly, a minimal agar medium containing 1.5% potassium chlorate was used to generate nitrate-nonutilizing (*nit*) mutants (7). The phenotype of the *nit* mutants was determined by growing them on media amended with nitrogen from one of several sources (7). To test isolates for vegetative compatibility, selected *nit* mutants (a *nit1* and a NitM) were paired on minimal agar medium with nitrate-N as the sole nitrogen source. The development of dense aerial mycelium where the two *nit* mutants came in contact was an indication of complementation. All pairing tests were repeated at least three times.

Cultural comparisons. Colony color of isolates grown on ALBA, as viewed from the bottom of the dish, was recorded after 3–4 days. Spore length and width were also measured for 31 isolates representing different hosts, VCGs, lesion types, and geographic origins. Cultures were grown on GBA as described above for 5–7 days, and 25 spores per isolate were measured at ×1000 magnification.

Virulence. Spinach plants (cultivars Fall Green and Grandstand) were grown under greenhouse conditions from pregerminated seed. A dilute fertilizer (Peters 20-20-20, with trace elements, 120-ppm concentration) was applied daily. Plants were at the two- to four-leaf stage (about 3 wk old) when inoculated.

Forty-two isolates, selected to represent all hosts, VCGs, lesion types, and geographic origins, were compared for virulence on each cultivar. Spores from cultures grown on GBA for 7–10 days were suspended in water, adjusted to 1×10^6 spores per milliliter, and sprayed onto plants until runoff. Plants were placed in a dew chamber for 36 hr, then returned to the greenhouse (temperature range 15–25 C). After 6 days, the first set of true leaves was scored for disease symptoms on a scale of 0–7, where 0 = healthy, no symptoms; 1 = 1–10% of leaf area damaged; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%; 5 = 76–90%; 6 = >90% of leaf area damaged; and 7 = completely collapsed leaf.

Experimental design. Inoculated plants for each isolate were arranged in a completely randomized block design with four replications. Each replication consisted of a single pot with three plants. Thus, a replication was the mean disease rating of six leaves. The inoculation experiment was conducted four times with a selected group of isolates in each test. Three tests were conducted in March–April 1991 and one in April 1992. Each experiment included a water control. Data were analyzed by analysis of variance, and the least significant difference (LSD) for each experiment is shown in Table 2.

RESULTS

Isolates. In all, 215 isolates of *C. dematium* were obtained from spinach: 130 from plants from the Arkansas River Valley (Arkansas and Oklahoma), 32 from California, 27 from New Jersey, 22 from Texas, and four from Ontario, Canada. Most (>90%) of the isolates were recovered from individual lesions on different leaves.

Of the 130 isolates from the Arkansas River Valley, 57 were primary lesion isolates (recovered from otherwise healthy spinach tissue), and the remaining 73 were secondary lesion isolates (recovered from white rust lesions). All isolates from outside the Arkansas River Valley were primary lesion isolates.

Vegetative compatibility. Two distinct VCGs (designated VCG1 and VCG2) were identified among the 215 spinach isolates of *C. dematium* examined. Both groups were recovered from both primary and secondary lesions (Table 1). Isolates of both groups were recovered from the Arkansas River Valley and from New Jersey: 64 isolates from the Arkansas River Valley were in VCG1, and 65 were in VCG2; 10 isolates from New Jersey belonged to VCG1 and 17 to VCG2 (Table 1). All 32 isolates from California and all four from Ontario belonged to VCG1, and all 22 isolates from Texas belonged to VCG2. Thirty-five of the 64 VCG1 isolates (53%) and 21 of the 65 VCG2 isolates (33%) from the Arkansas River Valley were from primary lesions.

The *nit* mutants of one isolate (JG24 from a primary lesion from the Arkansas River Valley) formed weak heterokaryons with *nit* mutant testers from both VCG1 and VCG2, indicating that this isolate was weakly vegetatively compatible with both VCGs. Five single-spore isolates were recovered from the parent culture of isolate JG24 and further examined; *nit* mutants of all of these single-spore isolates also formed weak heterokaryons with the *nit* mutant testers from both of the spinach VCGs.

We were unable to recover *nit* mutants from two of the three onion isolates. Complementary *nit* mutants (*nit1* and NitM) were recovered only from one tomato isolate (ATCC58112) and one onion isolate (ATCC34305). Each of these isolates represented a unique VCG that was vegetatively incompatible with the spinach isolates.

Cultural comparisons. All but two of the 104 isolates in VCG2 produced a distinct yellow pigment when cultured on ALBA. However, it was important to record the colony color after 4 days, because the yellow color often faded. The two isolates in VCG2 that did not produce a yellow pigment also were unusual in that they did not produce the dark pigment typical of *C. dematium* and appeared to be albino mutants. None of the 110 VCG1 isolates produced the yellow pigment on ALBA, and six appeared to be albino mutants.

Thirty-one isolates were compared for spore shape and size. All isolates examined, from both spinach VCGs and both lesion types, from all geographic origins and hosts, had slightly curved, hyaline conidia. Conidial dimensions of all isolates were similar, with mean length 21.5–30.9 μm and mean width 3.0–3.8 μm (*data not shown*).

Virulence. Most spinach isolates were highly virulent (disease rating greater than 4.0) in the greenhouse inoculation tests (Table 2). Isolates from the two VCGs and from the two lesion types were not statistically different in virulence ($P = 0.01$ according to Fisher's LSD). However, the disease ratings were variable, and some individual isolates in a particular inoculation experiment were rated as weakly virulent (disease rating less than 2.0) or moderately virulent (disease rating 2.0–4.0). For example, isolate RC2 appeared weakly virulent in experiment 4 on cultivar Fall Green. One unusual spinach isolate, JG30, was weakly virulent in all three inoculation tests in which it was included. In a test of spore germination of spinach isolates on water agar, isolate JG30 germinated much more slowly than the other isolates examined (J. C. Correll, *unpublished*). It is therefore possible that the slower spore germination of JG30 may have contributed to its overall lower disease rating.

In general, the one tomato and two onion isolates included in the inoculation test were weakly virulent on both spinach cultivars tested. However, one onion isolate (ATCC34305) was moderately virulent on both Grandstand and Fall Green in two of the four experiments, and the tomato isolate (ATCC58112) was moderately virulent on Fall Green in the third experiment and highly virulent (disease rating 4.1) on Fall Green in the fourth experiment.

DISCUSSION

All isolates of *Colletotrichum* recovered in this study from spinach in North America fit the general description of *C. dematium*. Isolates with small, straight spores fitting the description of *C. spinacicola* (23) were not recovered. Although we examined only a few isolates of *C. dematium* from plants other than spinach, the results from the virulence tests support the forma specialis designation, *C. dematium* f. sp. *spinaciae*, used by von Arx. The mean disease

Table 1. Lesion type, vegetative compatibility group (VCG), and geographic origin of isolates of *Colletotrichum dematium* from spinach

Lesion type[a]	VCG	Geographic origin[b]	Number of isolates[c]
Primary	1	AR/OK	35
		CA	32
		NJ	10
		Canada	4
	2	AR/OK	21
		NJ	17
		TX	22
Secondary	1	AR/OK	29
	2	AR/OK	44

[a] Primary lesion isolates were recovered from otherwise healthy spinach leaves; secondary lesion isolates were recovered from white rust lesions (caused by *Albugo occidentalis*).

[b] AR/OK isolates were recovered from the Arkansas River Valley, which includes both Arkansas and Oklahoma. The Canadian isolates were obtained from Ontario.

[c] One isolate, JG24, was a "bridge" isolate that was weakly vegetatively compatible with both VCGs and is not listed in the table.

ratings (mean of four tests) of the three nonspinach isolates were less than 2.0, and the isolates were considered weakly virulent on spinach. The spinach isolates all had mean disease ratings greater than 2.0 and were considered moderately or highly virulent on spinach.

The spinach anthracnose pathogen population in North America appears to be composed of two distinct VCGs. VCG1 was recovered in Arkansas, Oklahoma, California, New Jersey, and Ontario, and VCG2 was recovered from Arkansas, Oklahoma, New Jersey, and Texas. It is not clear why both groups were not recovered from all geographic locations; perhaps the sample size was too limited or VCG occurrence was restricted in a given field or geographic area.

Table 2. Vegetative compatibility group (VCG), lesion type, and virulence of isolates of *Colletotrichum dematium*

Isolate[a]	VCG	Lesion type[b]	Virulence[c]							
			Grandstand experiment				Fall Green experiment			
			1	2	3	4	1	2	3	4
Control			0.0	0.1	0.1	0.3	0.0	0.1	1.5	0.0
JG13	1	P	5.2	5.2	—	—	6.3	5.1	—	—
JG29	1	P	3.5	5.3	—	—	3.4	4.3	—	—
JG103	1	P	5.8	6.7	—	—	6.0	5.0	—	—
JG125W	1	P	4.1	5.9	—	—	5.6	5.2	—	—
JG165	1	P	6.1	5.9	—	—	6.4	5.2	—	—
JG31	1	S	3.9	6.5	—	—	6.2	4.4	—	—
JG9	1	S	5.0	5.9	—	4.7	5.4	5.0	—	3.0
JG68	1	S	4.6	6.6	—	—	6.0	4.7	—	—
JG113	1	S	6.0	5.3	5.8	—	5.5	4.5	5.4	—
JG131W	1	S	4.9	6.2	6.0	—	6.3	6.2	6.0	—
RC1	1	P	—	—	6.6	5.2	—	—	4.1	4.5
RC2	1	P	—	—	4.6	5.3	—	—	5.2	1.3
RC3	1	P	—	—	3.4	5.7	—	—	2.2	2.1
RC4	1	P	—	—	6.6	6.9	—	—	3.5	2.7
CA1	1	P	—	—	—	7.0	—	—	—	6.6
CA4	1	P	—	—	—	6.8	—	—	—	6.1
CA7	1	P	—	—	—	6.3	—	—	—	2.7
CA10	1	P	—	—	—	6.2	—	—	—	2.7
CA13	1	P	—	—	—	7.0	—	—	—	6.0
SJ16W	1	P	—	—	—	7.0	—	—	—	4.8
SJ17W	1	P	—	—	—	6.1	—	—	—	5.7
JG24[d]	1/2	P	5.7	6.9	6.7	—	6.3	6.7	6.3	—
JG10	2	P	5.5	5.4	6.2	—	6.3	5.7	4.5	—
JG55	2	P	4.4	5.8	—	6.6	6.5	5.4	—	6.4
JG125Y	2	P	5.1	6.5	—	—	6.3	6.5	—	—
JG128	2	P	2.5	6.5	—	—	6.1	6.4	—	—
JG145	2	P	4.9	6.0	—	—	6.4	5.4	—	—
MB1	2	P	—	—	—	5.8	—	—	—	4.2
MB2	2	P	—	—	—	7.0	—	—	—	7.0
MB4	2	P	—	—	—	6.8	—	—	—	7.0
MB6	2	P	—	—	—	6.6	—	—	—	6.1
MB8	2	P	—	—	—	7.0	—	—	—	6.7
SJ10Y	2	P	—	—	—	5.6	—	—	—	3.4
SJ14Y	2	P	—	—	—	6.3	—	—	—	5.9
LG2	2	S	5.4	5.9	—	—	6.0	6.4	—	—
JG6	2	S	4.5	6.9	—	6.0	6.2	5.4	—	5.9
JG22	2	S	4.8	6.3	—	—	6.2	4.9	—	—
JG30	2	S	0.0	1.5	1.0	—	0.0	1.0	1.1	—
JG1314	2	S	4.4	5.6	—	—	6.1	5.4	—	—
ATCC58112	3		0.1	1.7	1.8	1.9	0.0	0.6	2.1	4.1
ATCC34305	4		0.0	0.1	2.5	3.3	0.0	0.2	2.1	3.8
ATCC44202			0.0	0.4	0.2	0.0	0.0	0.2	0.5	0.2
$LSD_{(0.05)}$			1.1	1.1	1.5	1.5	0.9	1.2	1.4	2.2
$LSD_{(0.01)}$			1.5	1.4	2.0	2.0	1.1	1.6	1.9	3.0

[a] JG or LG = Arkansas River Valley; RC = Ontario, Canada; CA = Salinas, CA; SJ = New Jersey; MB = Uvalde, TX; ATCC = American Type Culture Collection (strains from plants other than spinach).

[b] P = primary anthracnose isolate; S = secondary anthracnose isolate recovered from a white rust lesion.

[c] Disease rating on each cultivar on a scale of 0–7, where 0 = healthy, no symptoms; 1 = 1–10% leaf necrosis; 2 = 11–25%; 3 = 26–50%; 4 = 51–75%; 5 = 76–90%; 6 = >90% leaf necrosis; and 7 = leaf completely collapsed.

[d] This isolate was a "bridge" isolate that was weakly vegetatively compatible with both VCG1 and VCG2.

The limited VCG diversity identified in the contemporary population indicates that this pathogen is reproducing asexually. If sexual reproduction were occurring, and assuming that vegetative compatibility is controlled at multiple loci (19,22), then a reassortment of alleles at the vegetative incompatibility (*vic*) loci would result in multiple VCGs. VCG diversity has also been shown to be relatively low in populations of the cucurbit anthracnose pathogen, *C. orbiculare* (27), in North America. In contrast, VCG diversity in *C. gloeosporioides* varied from very low to high, depending on the host from which isolates were obtained. For example, isolates of *C. gloeosporioides* from northern joint-vetch (9) belonged to a single VCG, whereas many isolates of the same pathogen from apple each represented a unique VCG. Consequently, VCG diversity may be a useful indicator of population diversity in *Colletotrichum*.

The presence of a "bridge" isolate that was weakly vegetatively compatible with both VCGs suggests that the two VCGs may be closely related. Isolates that bridge VCGs have also been identified in *F. oxysporum* (17,20).

No consistent differences in virulence on two spinach cultivars were detected in greenhouse tests of isolates from geographically diverse locations, from both VCGs, and from both primary and secondary lesions. In addition, both VCGs were recovered from primary and secondary lesions. Thus, neither the virulence data nor the VCG data support the hypothesis of two "distinct" *Colletotrichum* anthracnose pathogens of spinach in North America.

Colony color on ALBA was first observed during isolations from symptomatic spinach leaves. When lesions with evidence of *Colletotrichum* sporulation were streaked onto ALBA, some isolates developed a pale yellow color that was visible on the underside of the streaked colonies. Yellow colony color was closely correlated with VCG: 102 of 104 isolates in VCG2 produced the yellow pigment, whereas none of the 110 isolates in VCG1 produced the pigment. In a few of the streaked isolations, both yellow and white colonies developed. In these instances, we determined that isolates representing both VCGs were recovered from a single lesion.

The use of *nit* mutants to examine vegetative compatibility can expedite studies on population diversity of *Colletotrichum* as it has with several species of *Fusarium*. We did not encounter difficulty in obtaining *nit* mutants from an isolate of a given VCG to complement the *nit* mutant testers from that VCG, as reported by Brooker et al (3). However, selected *nit1*s and NitMs from a large group of isolates were used as tester

nit mutants in our pairing tests. Although it is possible that certain isolates from a given species may pose difficulties in the analysis of VCGs, this is unlikely to present a problem when populations with a large sample size are examined. It should be emphasized, however, that simply selecting phenotypically distinct *nit* mutants does not ensure that the mutants will necessarily be the best tester strains in complementation tests for a particular VCG.

The use of *nit* mutants to examine VCGs in populations of *F. oxysporum* has improved our understanding of the genetic relationship of virulence in this important plant pathogen (7). We anticipate that more extensive examination of VCGs in populations of various species of *Colletotrichum* will also yield useful information.

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