Occurrence of Race 2 of *Colletotrichum trifolii* in North Carolina and Resistance of Alfalfa Cultivars and Breeding Lines to Races 1 and 2

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ABSTRACT

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Moderate to severe damage from anthracnose (*Colletotrichum trifolii*) is widespread on alfalfa in the eastern and southeastern United States. During July 1979, anthracnose lesions were collected from eight fields of alfalfa in five counties in North Carolina. Sixty-three isolates of three species of *Colletotrichum* were recovered. Nineteen isolates with conidia nearest the average length and width of *C. trifolii* were selected for testing. Seedlings of Arc and Saranac AR were inoculated with conidia from single-spore cultures of each of the 19 isolates and with conidia from cultures known to be race 1 or 2. All 19 isolates were race 2; 17 had been obtained from plants in 1- to 3-yr-old stands of Arc alfalfa in Iredell, Rowan, and Davidson counties. Resistance to race 1 (isolate PA) and race 2 (isolate NC-4) in 31 alfalfa cultivars and breeding lines was evaluated. Resistance to race 2 was found in Saranac AR, Vangard, and breeding lines with Saranac AN 4 or Vernal AN 4 in their pedigree. Mycelial growth of race 1 (PA) was compared with that of three isolates representative of race 2 (NC-4, D-3-9, M-8) at 4–36 C in four-degree increments. Optimum temperature for growth for all isolates was between 20–28 C, and minimum and maximum temperatures for growth were 8 and 32 C, respectively.

Additional key words: disease resistance, *Medicago sativa*, pathogenic races, virulence

Anthracnose caused by *Colletotrichum trifolii* Bain is widespread on alfalfa (*Medicago sativa* L.) in the eastern and southeastern United States (1,7,12), where it causes moderate to severe damage (1,2,7). Control is based on resistance to *C. trifolii* obtained by recurrent selection (5,9). In field tests, resistant cultivars yield more than susceptible cultivars (4).

Recent reports from North Carolina (16) and Maryland (11) indicate that some isolates of *C. trifolii* are capable of inducing disease in resistant cultivars Arc and Liberty. These isolates were designated as race 2 (10). Our investigation sought to determine whether race 2 isolates occur in locations other than where the race was first found in North Carolina, to evaluate resistance to races 1 and 2 in alfalfa cultivars and germ plasm adapted to the mideastern states, and to determine the growth of race 1 and 2 isolates in culture at several temperatures.

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MATERIALS AND METHODS

Collection and testing of isolates. In July 1979, we visited eight field-plantings of alfalfa in Alamance, Davidson, Guilford, Iredell, and Rowan counties in North Carolina to collect stems with anthracnose lesions. The stems were surface sterilized in 1% sodium hypochlorite for 1 min, rinsed in sterile distilled water, and incubated at 24–26 C for 2 days in a chamber kept near 100% relative humidity with moistened filter paper. Sporulating lesions were rubbed on 3- to 5-wk-old Saranac seedlings, and plants were kept moist at 20–25 C for 72 hr in a chamber with intermittent misting. Plants were returned to a greenhouse bench for 4–11 days at 18–25 C. Plants that collapsed were surface sterilized and placed on cornmeal agar (CMA).

We recovered 63 isolates of three species of *Colletotrichum*. Conidia from each isolate were measured in water on a microscope slide and examined under × 430 magnification. Nineteen isolates with conidia that measured nearest to the average length and width of *C. trifolii* conidia as described by von Arx (15) were selected for testing. Other species of *Colletotrichum* were obtained but were not included in this study (6). These 19 isolates were tested for pathogenicity to alfalfa seedlings and compared with 8 other isolates—2 isolates of race 1 (PA and Re 1), 2 isolates of race 2 (NC-4 and Hay 5-1), and 4 isolates of unknown race identity (Ct-1, Ct-2, Ct-4 and Mn 1-4).

Race 1 isolate (PA) of *C. trifolii*, originally obtained from the U.S. Regional Pasture Laboratory, University Park, PA, has been the standard isolate used for increasing resistance in the North Carolina breeding lines (3). Race 2 isolate (NC-4) was originally obtained in Rowan County in 1977 (16). Other isolates of *C. trifolii* (Re 1 and Hay 5-1) were provided by S. A. Ostazeski, and isolates of unknown races (Ct-1, Ct-2, Ct-4, and Mn 1-4) were provided by F. I. Frosheiser. Isolates designated with the letters A, B, C, D, or F were from fields of Arc and Liberty alfalfa grown in Rowan County. Isolates K and L were from different fields of Arc in Davidson County, and isolate M came from an Arc planting in Iredell County. All of these stands were 1–3 yr old. The N isolates came from a 9-yr-old stand of alfalfa (unknown cultivar) in Iredell County. Single-spore cultures were prepared from each isolate and stored at 3–4 C on potato-dextrose agar until used for inoculum.

Seeds of Arc and Saranac AR alfalfa were scarified, inoculated with an appropriate strain of *Rhizobium meliloti* Dang (The Nitragin Co., Milwaukee, WI), and planted 6 mm deep in pressboard trays (14 × 19 × 7 cm) containing a 1:1 mixture (v/v) of peat-perlite, Metromix-200 (Florist Products, Inc., Des Plaines, IL), and fumigated sand. The seedlings were thinned to 25 per row, and after 3 wk the trays were sealed inside unvented plastic freezer bags. The plants were inoculated with a conidial suspension sprayed through a slit cut in the bag until they were dripping. After inoculation, the bags were removed and the trays were put into a mist chamber at 20–25 C for 3 days, during which intermittent misting kept the foliage wet. Seedlings were returned to the greenhouse to complete 3–4 wk of incubation at 18–24 C.

Inoculum from each single-spore isolate was prepared by flood-inoculating

lima bean agar in petri dishes and incubating the cultures for 7 days at 24–26 C. Conidia were washed from the agar surface with distilled water. The conidial suspensions were adjusted to 1×10^6 conidia per milliliter in a final volume of 1 L of distilled water supplemented with two drops of Tween 20 (14) and 30 ml of freshly squeezed and filtered orange juice (8). Control plants were sprayed with Tween 20 and orange juice without spores. To be sure that plants in sealed bags were not exposed to unwanted inoculum before being sprayed with the test isolate, we sprayed control 1 before and control 2 after the seedlings were inoculated.

Seedlings were rated for disease on a severity scale of 1 to 5 (5,9): 1 = no lesions or only hypersensitive flecking; 2 = small, nonsporulating lesions; 3 = typical diamond-shaped lesions not girdling the stem but with sporulation and setae in the acervuli; 4 = stem-girdling lesions with sporulation, but with new shoots originating from lower axillary buds; and 5 = dead plant. Resistance of plants to each isolate was evaluated on the basis of percentage of total number of plants scored 1 or 2.

The experiment, which was done twice, was arranged in a randomized complete block with two replicates. An analysis of variance and means separation with Duncan's new multiple range test for means comparison (13) were performed.

Evaluation of cultivars and breeding lines. The alfalfa cultivars evaluated are available to growers in the southeastern United States, and the breeding lines tested were from an ongoing alfalfa breeding program for increased yield and persistance of alfalfa for this region (Tables 1 and 2). Seeds were scarified and inoculated with *R. meliloti* as before and planted in wooden flats ($50 \times 38 \times 7$ cm) containing a 1:1 mixture (v/v) of fumigated loam soil and sand. Twenty-five seeds of each entry were placed in furrows 6 mm deep and 2 cm apart and covered with fumigated sand. A flat of seedlings was considered to be a replicate. Seedlings were watered and fertilized as needed to maintain vigorous growth. The number of germinated seedlings per row was counted before inoculation.

Inocula of race 1 (PA) and race 2 (NC-4) were prepared as before. For the mixed inoculum of races 1 and 2, equal concentrations of conidia of each isolate were combined to prepare a final concentration of 1×10^6 conidia per milliliter. Seedlings were scored for disease as before, and a disease severity index was calculated (6). Resistance of each entry was evaluated on the basis of percentage of total number of plants scored 1 or 2. Flats containing uninoculated seedlings or seedlings sprayed with Tween 20 and orange juice without spores were included in each test as controls.

The inoculation of the alfalfa entries was completed in three runs during two studies. The first run had six replicates and the second had four (first inoculation study; Table 1). Space in the mist chamber was limited, so only two replicates of the controls were included. The third run had six replicates (second inoculation study; Table 2). Because of space limitations, only one flat served as a control. An analysis of variance and means separation with Duncan's new multiple range test were performed on each study (13). Data for the control in the second study were not analyzed.

Table 1. Response of 10 alfalfa cultivars and breeding lines to inoculation in the greenhouse with race 1 (PA), race 2 (NC-4), and a mixture of races 1 and 2 of *Colletotrichum trifolii*[a]

Cultivar or line	Control		Race 1		Race 2		Races 1 and 2	
	DSI[b]	Resistant seedlings[c] (%)	DSI	Resistant seedlings (%)	DSI	Resistant seedlings (%)	DSI	Resistant seedlings (%)
Arc	1.54 b[d]	87 a	2.03 a	65 b	4.99 c	0 a	4.82 d	2 ab
Liberty	1.37 ab	91 ab	2.64 a	49 b	4.99 c	0 a	4.90 d	1 ab
Saranac	1.22 ab	94 ab	4.82 b	1 a	4.94 c	0 a	4.95 d	0 a
Saranac AR	1.00 a	100 b	2.48 a	55 b	3.25 a	38 d	3.33 ab	38 d
NCMP 1	1.26 ab	94 ab	2.56 a	55 b	4.54 bc	7 bc	4.19 bcd	16 c
NCMP 3	1.18 ab	95 ab	1.83 a	64 b	3.38 a	34 d	3.01 a	44 d
NCMP 4	1.18 ab	96 ab	2.67 a	51 b	4.98 c	0 a	4.69 cd	3 ab
NCMP 6	1.11 ab	97 ab	2.44 a	56 b	3.95 ab	18 c	3.87 abc	25 cd
NCMP 9	1.30 ab	92 ab	2.62 a	52 b	4.83 c	2 ab	4.51 cd	10 bc
NCMP 11	1.00 a	100 b	2.31 a	58 b	4.19 bc	16 c	3.36 ab	35 d

[a] Values are the means of 10 replicates (6 in the first run and 4 in the second).
[b] DSI = disease severity index, which is the calculated average of the disease severity scores. 1 = no lesions or only hypersensitive flecking; 2 = small, nonsporulating lesions; 3 = typical diamond-shaped lesions not girdling the stem, with sporulation and setae in the acervuli; 4 = stem girdling lesions with sporulation, but with new shoots originating from lower axillary buds; and 5 = dead plant.
[c] Percentage of all plants with disease severity scores of 1 or 2.
[d] Means in columns followed by different letters differ significantly ($P = 0.05$, Duncan's new multiple range test).

Table 2. Response of 24 alfalfa cultivars and breeding lines to inoculation in the greenhouse with races 1 and 2 of *Colletotrichum trifolii*[a]

Cultivar or line	Control		Race 1		Race 2	
	DSI[b]	Resistant seedlings (%)[c]	DSI	Resistant seedlings (%)	DSI	Resistant seedlings (%)
Apollo	1.00	100	4.34 ef[d]	8 a	4.61 cde	5 a
Arc	1.00	100	2.46 ab	63 def	4.88 defg	2 a
Cherokee	1.00	100	4.41 ef	4 a	4.85 cdefg	1 a
Cimarron	1.00	100	3.02 d	43 b	4.59 cd	9 ab
Classic	1.29	93	4.32 e	9 a	4.59 cd	7 ab
Gladiator	1.14	96	4.27 e	12 a	4.81 cdefg	0 a
Phytor	1.20	95	4.66 efg	4 a	4.74 cdefg	6 ab
Saranac	1.83	79	4.83 g	0 a	4.97 g	0 a
Saranac AR	1.20	95	2.55 bc	59 cde	3.21 ab	43 d
Thor	1.67	83	4.73 fg	5 a	4.92 efg	0 a
Vangard	1.30	93	2.36 ab	62 cde	3.83 bc	22 c
Williamsburg	1.00	100	4.47 efg	7 a	4.80 cdefg	4 a
WL 311	1.00	100	4.40 ef	9 a	4.68 cdefg	5 a
WL 312	1.00	100	4.51 efg	3 a	4.77 cdefg	3 a
WL 318	1.15	96	4.57 efg	4 a	4.64 cdef	5 a
NCMP 2	1.00	100	2.14 a	75 f	4.17 bc	18 bc
NCMP 5	1.00	100	2.46 ab	69 ef	4.78 cdefg	2 a
NCMP 7	1.00	100	2.89 cd	54 bcd	4.87 defg	2 a
NCMP 8	1.00	100	2.29 ab	64 def	3.06 a	42 d
NCMP 10	1.15	96	2.52 abc	63 def	3.48 b	36 d
NCMP 12	1.17	96	2.41 ab	65 def	4.92 efg	1 a
NCMP 13	1.00	100	2.43 ab	69 ef	4.77 cdefg	5 a
NCW 18	1.38	91	3.00 d	50 bc	4.55 c	9 ab
NCW 22	1.00	100	3.12 d	44 b	4.55 c	7 ab

[a] Values are the means of six replicates.
[b] DSI = disease severity index, which is the calculated average of the disease severity score. 1 = no lesions or only hypersensitive flecking; 2 = small, nonsporulating lesions; 3 = typical diamond-shaped lesions not girdling the stem, with sporulation and setae in the acervuli; 4 = stem girdling lesions with sporulation, but with new shoots originating from lower axillary buds; and 5 = dead plant.
[c] Percentage of all plants with disease severity scores of 1 or 2.
[d] Means in columns followed by different letters differ significantly ($P = 0.05$, Duncan's new multiple range test).

Isolate growth at various temperatures. We determined the effect of temperature on mycelial growth for isolates PA (race 1), D-3-9 (rare 2), M-8 (race 2), and NC-4 (race 2). For each isolate, plugs (9 mm diam) of actively growing mycelia on CMA were placed in the center of CMA plates and incubated at 4, 8, 12, 16, 20, 24, 28, 32, or 36 C. The diameter of the colony was measured in two directions and averaged. Measurements were recorded at 2-day intervals for 14 days. The distance each isolate grew in 2 days was used to compute the growth rate (millimeters per day), and the mean and standard deviations for four dishes for each isolate were computed at each temperature.

RESULTS AND DISCUSSION

Variations in virulence were detected among isolates when Saranac AR seedlings were inoculated with 27 isolates of *C. trifolii* (Fig. 1A). Except for Re 1, isolate PA was the least virulent (75% seedling survival); however, it was similar in virulence to 10 other isolates. Isolate L-5 was the most virulent (32% seedling survival), but it was similar in virulence to five other isolates. Among these isolates, groupings with insignificant statistical differences (ie, those with a common letter) included as few as six (letter i) and as many as 18 (letter c) isolates. Isolate Re 1 apparently lost virulence to alfalfa in storage, because when it was last tested (R. E. Welty, 1978, *unpublished data*), it was virulent on Saranac seedlings without resistance to *C. trifolii*. In the two checks, an insignificant number of seedlings died without showing symptoms or signs of anthracnose.

Arc seedlings were statistically separated into two groups based on their percentage of seedling survival (ie, plants scored 1 or 2) (Fig. 1B). Isolates Ct-1, Ct-2, Ct-4, and Mn 1-4 were not statistically different from PA (race 1). Nineteen isolates (those sharing the letter e) collected during the survey were not statistically different from the known isolates of race 2 (NC-4 and Hay 5-1) and were all considered to be race 2. Percentage of seedlings surviving after inoculation with known isolates of race 2 (NC-4 and Hay 5-1) were similar to each other (9 and 8%, respectively). These data support earlier conclusions based on Saranac AR and Arc that physiologic specialization for races 1 and 2 pathogenicity exists (11,16) and that virulence does vary (6).

Tests of the alfalfa seedlings inoculated in the greenhouse indicated that Arc, Liberty, and Saranac AR were resistant to race 1 isolates (Table 1). The North Carolina (NC) breeding lines (NCMP 1, 3, 4, 6, 9, and 11) previously selected for resistance to race 1 isolates were resistant, which was also consistent with results obtained in other experiments (R. E. Welty, *unpublished data*). When these same populations were inoculated with the race 2 isolate, the highest resistance was found in Saranac AR and NCMP 3, with lesser resistance to *C. trifolii* in NCMP 6 and 11. Other entries were all susceptible to the race 2 isolate. Reactions of the four cultivars inoculated with a mixture of conidia of races 1 and 2 isolates were similar to those reactions induced by the race 2 isolate, whereas the reaction of the six NCMP germ plasms varied.

This experiment suggests that disease escapes may occur when only one-half the number of conidia reach the target in a mixture, as opposed to the number reaching the target in a single-race inoculum. In view of this, seedlings in the next experiment were inoculated with single races.

Entries in a second greenhouse experiment included breeding lines not previously screened for resistance and cultivars recently released and available to growers in the southeastern United States (Table 2). The entire NC germ plasm (NCMP entries) had moderate (about 40–50%) to high (60% and higher) resistance to the race 1 isolate. Resistance to race 1 was also found in Arc, Cimarron, Saranac AR, and Vangard. The most resistant cultivars among the populations inoculated with race 2 were Saranac AR, NCMP 8, and NCMP 10. Vangard and NCMP 2 had some resistance to race 2. The remaining cultivars and breeding lines were considered susceptible to race 2.

Results from studies completed elsewhere indicate that *C. trifolii* isolates vary in virulence (6). Results from inoculating Saranac AR seedlings with the 26 isolates of *C. trifolii* in this study confirmed this finding. Our study also confirmed a wider distribution of race 2 than previously reported for North Carolina (16) and extended the known occurrence of race 2 to include two separate fields of Arc on the same farm in Davidson County. The 11 isolates from Rowan County were from a cultivar trial on the Piedmont Research Station in which the original culture of race 2 (NC-

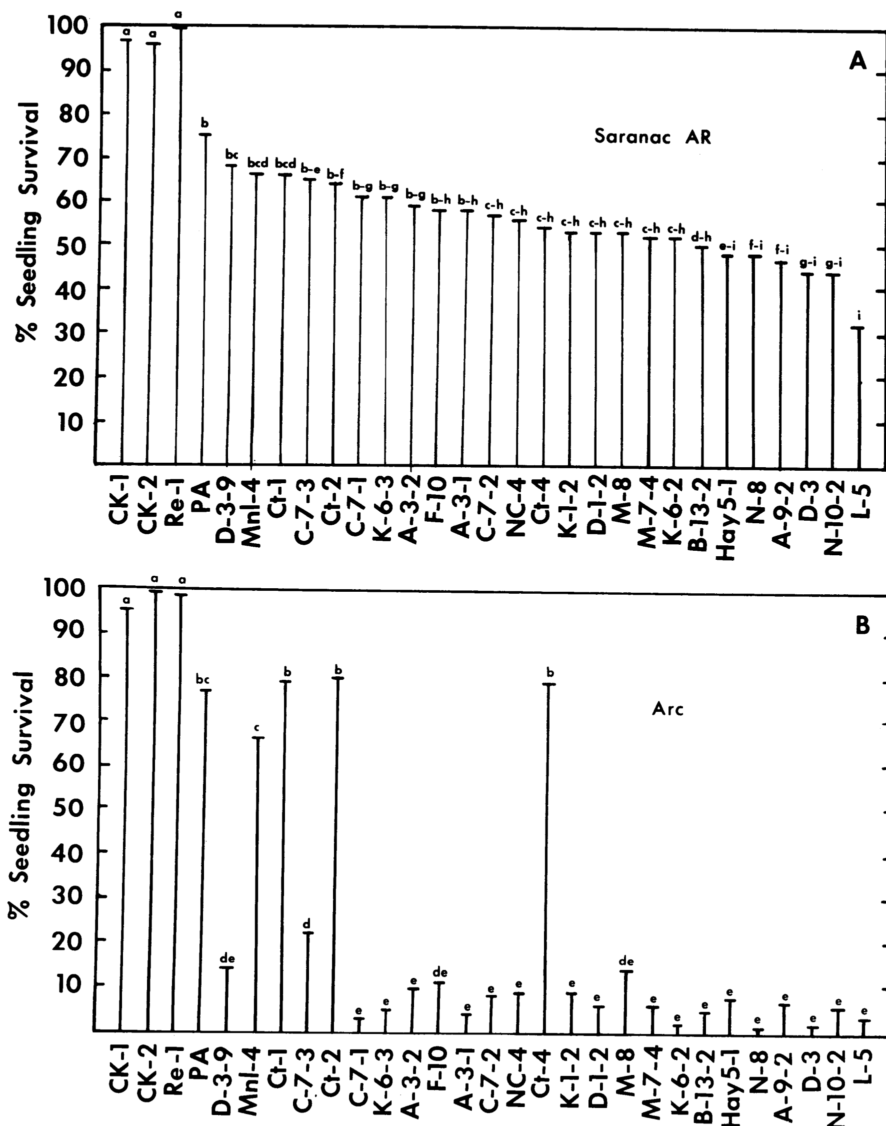

Fig. 1. Response of 3-wk-old seedlings of Saranac AR (**A**) and Arc (**B**) to inoculation in the greenhouse with 27 isolates of *Colletotrichum trifolii*. Columns with the same letters are not significantly different ($P = 0.05$) using Duncan's new multiple range test.

Table 3. Growth rate (mm/day) of race 1 (PA) and race 2 (NC-4, D-3-9, and M-8) of *Colletotrichum trifolii* in cornmeal agar at temperatures between 4 and 36 C

Degrees Celsius	Race 1	Race 2		
	PA	NC-4	D-3-9	M-8
4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	0.4 ± 1.0	0.5 ± 0.7	0.4 ± 1.1	0.5 ± 0.7
12	1.0 ± 1.1	1.0 ± 1.0	0.8 ± 0.8	1.0 ± 0.7
16	1.6 ± 1.3	1.5 ± 0.8	1.3 ± 1.5	1.8 ± 0.9
20	2.3 ± 1.3	2.4 ± 0.5	1.7 ± 0.6	2.5 ± 1.5
24	2.8 ± 1.0	2.6 ± 1.9	1.9 ± 0.3	2.6 ± 1.1
28	2.4 ± 1.2	2.4 ± 1.2	1.8 ± 1.3	2.8 ± 1.3
32	1.3 ± 1.5	1.6 ± 0.9	1.3 ± 1.0	1.4 ± 1.1
36	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

4) was obtained.

Five of the 15 NC breeding lines evaluated had 25% or more seedlings resistant to race 2. A review of their pedigrees indicated that Saranac AN 4 was predominant in development of NCMP 3 and NCMP 10 and of lesser importance in NCMP 6 and NCMP 11. One parent of NCMP 8 was Vernal AN 4. We believe that resistance to race 2 in these five breeding lines probably came from Saranac AN 4 and Vernal AN 4.

Resistance to *C. trifolii* in Arc, Vernal AN 4, and Saranac AN 4 has different derivations (5). Arc was developed by inoculating the cultivar Team with *C. trifolii* and saving plants with a disease severity score of 1. Vernal AN 4 was developed from Vernal plants that survived inoculation with a disease severity score of 1 and 2 in the first cycle of selection. Plants with a disease severity score of 2 were included because too few plants had a disease severity score of 1. For the same reason, Saranac plants with disease severity scores of 1 to 4 were saved in the first cycle of recurrent selection and with scores of 1 and 2 in the second cycle in the development of Saranac AN 4. Devine et al (5) suggested that resistance to *C. trifolii* in Arc may be controlled by a single dominant allele, whereas resistance in Vernal and Saranac is under different genetic control. Other work supports these hypotheses (3). Further work is needed, but resistance in Arc appears to be caused by vertical resistance to race 1, whereas resistance in Saranac AN 4 is more complex.

When the mycelial growth rates of three isolates of race 2 (NC-4, D-3-9, M-8) were compared with race 1 (isolate PA), optimum growth for all isolates occurred between 20 and 28 C, with minimum and maximum temperatures for growth at 8 and 32 C, respectively (Table 3).

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