Effects of Inoculum Concentration and Temperature on Anthracnose Severity in Alfalfa

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ABSTRACT

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Three-week-old seedlings of eight cultivars of alfalfa susceptible or resistant to anthracnose were inoculated with four concentrations of conidia of race 1 or 2 of *Colletotrichum trifolii*. The severity of disease increased as inoculum increased; maximum severity was generally reached at 10^5 conidia per milliliter. In a second study, eight cultivars of alfalfa were inoculated with race 1 or 2 and incubated at 12, 16, 20, or 24 C. Disease severity was generally higher in seedlings inoculated with race 2, and in seedlings incubated at the highest temperatures. There were significant interactions among races, temperatures, and cultivars on disease severity. Disease severity increased in cultivars susceptible or resistant to race 1 as temperature increased. Disease severity among cultivars susceptible to race 2 was similar between 12 and 24 C; for cultivars resistant to race 2, disease severity increased as temperature increased. In a factorial study (races, temperatures, inoculum concentration, and cultivars) the level of resistance to race 1 or 2 in cultivars Saranac AN 4, Glacier AN 4, and Vernal AN 4 was influenced by temperature when the plants were inoculated with 10^6 conidia per milliliter. It is concluded that inoculum concentration and temperature should be controlled when managing a screening program for anthracnose resistance in alfalfa and screening and evaluation should be done at the most relevant temperature for field conditions in the area of adaptation. It is also suggested that the different genetic mechanisms controlling resistance in alfalfa to *C. trifolii* may be influenced by temperature.

Soon after Flemish alfalfa (*Medicago sativa* L.) germplasm was introduced into the United States, it was found to be highly susceptible to anthracnose caused by *Colletotrichum trifolii* Bain (15). Control of the disease was achieved by developing resistant cultivars (5,12), but resistance was not durable, and within 10 yr pathogenic races had developed (14,21). The new race capable of inducing disease in the previously anthracnose-resistant cultivar Arc was designated race 2 (13).

Plants within an alfalfa cultivar vary for many characteristics, including disease resistance, because the species is an autotetraploid and is naturally cross-pollinating. Variability is preserved during selection and hybridization, so that alfalfa cultivars are mixtures of genotypes. Thus, the level of disease resistance in a cultivar is characterized according to the frequency of resistant plants in a cultivar (9). Improvement in disease resistance includes both increasing the frequency of disease-resistant plants in a population and the degree of resistance of individual plants (9). Cultivars with 0–5% resistant plants are considered susceptible, 6–14% have low resistance, 15–30% are moderately resistant, 31–50% are resistant, and 51% or more have high resistance (11).

Inheritance of resistance to race 1 of *C. trifolii* in alfalfa has been studied (2,4,5). In germplasm accession MSHp6F AN 4 (later released as cultivar Arc), it was suggested that resistance is controlled by a single dominant allele, whereas, resistance in Saranac AN 4, Vernal AN 4, and Glacier AN 4 is under a different type of genetic control. These studies were done before the discovery of race 2.

Currently, 2- to 3-wk-old alfalfa seedlings are screened for resistance to *C. trifolii* with concentrations of conidia ranging from 5×10^5 to 1×10^6/ml (7,10,20,21). However, the effect of inoculum concentration on disease severity has not been studied for this pathosystem.

Environmental factors influence host-pathogen interactions (3), and a cultivar resistant at one temperature may be susceptible at another (19). Although the growth rates in culture of race 1 (isolate PA) and race 2 (isolate NC 4) are similar between 4 and 36 C (20), influences of temperatures on races and cultivars on disease severity are not known.

The present work was done to evaluate certain variables that are considered important during screening and evaluating cultivars for anthracnose resistance, and during studies on inheritance of resistance. These variables include the effect of inoculum concentration and temperature on severity of disease among cultivars inoculated with race 1 or 2 of *C. trifolii*.

MATERIALS AND METHODS

Cultures of *C. trifolii* race 1 (isolate PA, ATCC #42874) and race 2 (isolate NC 4, ATCC #42041) were used in these studies and their origin, storage, and increase for inoculum have been previously described (20). The cultivars included Saranac, Saranac AN 4, Vernal, Vernal AN 4, Glacier, Glacier AN 4, MSHp6F (released as Team), and MSHp6F-AN 4 (released as Arc). In the four preceding cultivar pairs, the first cultivar is susceptible and the second is resistant to race 1 of *C. trifolii* (5). Disease resistance was increased by three cycles of recurrent phenotypic selection in the laboratory and greenhouse. In the third study, cultivars WL 311 (1) and Riley(16), described as resistant to anthracnose, were included. Seeds of each cultivar were obtained from the alfalfa seed collection, U. S. Dept. Agriculture, ARS, Oxford Research Laboratory, Forage Research Unit, Oxford, NC 27565. Seeds were scarified, inoculated with an appropriate strain of *Rhizobium meliloti* Dang (The Nitragin Co., Milwaukee, WI), and planted in flats ($50 \times 38 \times 7$ cm) for the inoculum concentration studies and in Styrofoam® cups (180 ml) with drainage holes for the controlled-environment chamber studies. In all studies, seedlings were inoculated when the first trifoliolate leaves had expanded (about 3 wk). A conidial suspension in deionized water containing two

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drops of Tween-20 per liter was sprayed on the leaves and stems until they were wet and dripping (about 0.3 ml per plant), and the inoculated seedlings were kept moist for 3 days either in a mist chamber or enclosed in plastic bags. Plants were observed at 1- to 3-day intervals and disease severity was scored on a 1–5 scale about 3 wk after inoculation (8,22; and Table 3). The scores were averaged and used to determine a disease severity index (DSI) or a percentage of resistant plants (scores of 1 or 2) or susceptible plants (scores of 3, 4, or 5). Control seedlings in experiments 1 and 2 were sprayed with deionized water containing two drops of Tween-20 per liter but without conidia. Each experiment was repeated and the values presented are the averages of two runs. Data from each experiment were subjected to an analysis of variance test appropriate for the experimental design (17).

Inoculum concentration. Seeds of the eight cultivars were planted in flats containing a mixture of peat and vermiculite (Redi-earth, W. R. Grace and Co., Cambridge, MA 02140). Twenty-five seeds of each cultivar were placed in single furrows (one cultivar per row) 6 mm deep and 2 cm apart and covered. Seedlings were watered and fertilized as needed to maintain vigorous growth. The number of emerged seedlings per row was counted before inoculation and this number was used in computing the DSI and the percent resistant plants.

Inocula of races 1 and 2 were prepared from 7-day-old cultures growing on lima bean agar as previously described (20). The numbers of conidia per milliliter were determined with a hemacytometer and inoculum was diluted to nominal concentrations of 10^3, 10^4, 10^5, and 10^6 conidia per milliliter. Following the dilutions, conidial concentrations were verified by counting a portion of suspensions at 10^6 and 10^5 with a hemacytometer (averaged for 10–18 fields at each dilution). Concentrations below 10^5 could not be counted with a hemacytometer, so 10 μl of the suspensions containing 10^4 and 10^3 conidia per milliliter were placed on water agar (averaged for four petri dishes at each dilution) and incubated at room temperature (about 22 C) for 24–72 hr. Conidial concentrations were based on germination and counts were adjusted for viability.

Concentrations of conidia per milliliter in the dilutions of inoculum, verified by hemacytometer counts or culturing on water agar, showed the actual concentrations of conidia (average for both runs) at 1×10^6, 1×10^5, 1×10^4, and 1×10^3 for race 1 to be 1.1×10^6, 1.1×10^5, 0.6×10^4, and 1×10^3, respectively, and for race 2 to be 1.1×10^6, 1.2×10^5, 0.8×10^4, and 1.5×10^3, respectively. For convenience in this paper, the nominal concentrations are reported.

The experiment was arranged in a split-plot design with three replications in each of two runs with race-dilution treatment combinations being the whole plot treatments and with rows of cultivars in flats being the subplot treatments. The temperature in the room containing the mist cabinets was 16 C ± 1 C. Temperatures in the greenhouse ranged from 20 to 25 C and from 20 to 27 C for runs 1 and 2, respectively. Means were compared by using a protected LSD ($P = 0.05$).

Temperature and anthracnose development. The study was done in controlled-environment chambers and greenhouses (6) in the Southeastern Plant Environmental Laboratories (SEPEL) at North Carolina State University, Raleigh. Seeds of the eight cultivars were sown in a standard potting mixture of one-third peat moss-perlite (1:1, v/v) and two-thirds gravel in 180-ml Styrofoam® cups with drainage holes. Cups were placed in a wire rack designed to hold nine cups. As a control, a cup containing seedlings of susceptible cultivar Saranac was included in each rack. Cultivars were assigned locations within the rack and the racks were assigned to and within growth chambers by random numbers. Seedlings were thinned to five per cup and irrigated with a complete nutrient solution each morning and evening to maintain vigorous growth. The plants were grown in a greenhouse in natural daylengths, and nights were interrupted for 3 hr (from 2300 hours to 0200 hours) by incandescent lamps to provide long days. The greenhouse was programmed for a day temperature of 22 C (from 0700 to 1900 hours) and a night temperature of 18 C (from 1900 to 0700 hours).

Before inoculation, a cup of Saranac seedlings (control) was covered with a 1-L polyethylene bag and placed in the rack. Deionized water containing two drops of Tween-20 per liter without conidia was sprayed on the plants through a slit cut in the bag. The rack was filled with cups of seedlings (about 3 wk old) of each of eight cultivars and the rack was placed inside a large polyethylene bag and sealed. For inoculation, a suspension containing two drops of Tween-20 per liter and 10^6 conidia per milliliter was sprayed through a slit cut in the bag until all the foliage was wet and covered with inoculum. The slit was sealed with cellophane tape and the rack was placed in an environment chamber at 12, 16, 20, or 24 C in the dark for 3 days. Temperatures were recorded and varied ±0.5 C. Previous work found that light intensity during the infection period did not critically affect disease

TABLE 1. Percent survival of alfalfa seedlings of eight cultivars inoculated with four concentrations of conidia of *Colletotrichum trifolii*, race 1[w]

Cultivar	Inoculum level (conidia/ml) at: 10^3	10^4	10^5	10^6	Mean[x]
Team	85[y]	47	36	25	48
Saranac	69	21	16	14	30
Vernal	77	35	26	20	40
Glacier	71	36	25	16	37
Arc	96	89	88	79	88
Saranac AN 4	91	75	80	84	82
Vernal AN 4	99	97	88	78	91
Glacier AN 4	97	95	93	90	94
Mean[z]	85	62	56	51	

Source of variation	df	Mean square
Run	1	1,615.88
Concentration	3	11,180.69**
error (a)	19	251.23
Cultivar	7	17,900.65**
Interaction	21	784.29**
error (b)	140	92.16

[w] Values are the means of two runs, three replicates, and 12–25 plants per replicate depending on emergence.
[x] Cultivar means that exceed LSD 5.4 are different ($P = 0.05$).
[y] Cultivar means within each column that exceed LSD 5.4 are different, ($P = 0.05$).
[z] Concentration means that exceed LSD 6.8 are different ($P = 0.05$).

TABLE 2. Percent survival of alfalfa seedlings of eight cultivars inoculated with four concentrations of conidia of *Colletotrichum trifolii*, race 2[w]

Cultivar	Inoculum level (conidia/ml) 10^3	10^4	10^5	10^6	Mean[x]
Team	72[y]	26	18	14	32
Saranac	53	16	11	14	23
Vernal	63	25	18	20	31
Glacier	59	17	16	13	26
Arc	66	21	9	8	26
Saranac AN 4	92	80	73	69	78
Vernal AN 4	88	72	65	66	73
Glacier AN 4	83	61	68	62	68
Mean[z]	72	40	35	33	

Source of variation	df	Mean square
Run	1	9.63
Concentration	3	15,814.98**
error (a)	19	353.84
Cultivar	7	13,577.87**
Interaction	21	366.78**
error (b)	140	95.90

[w] Values are the means of two runs, three replicates, and 12–25 plants per replicate depending on emergence.
[x] Cultivar means that exceed LSD 5.5 are different ($P = 0.05$).
[y] Cultivar means within each column that exceed LSD 5.5 are different ($P = 0.05$).
[z] Concentration means that exceed LSD 8.0 are different ($P = 0.05$).

TABLE 3. Disease severity in eight alfalfa cultivars inoculated with conidia (10^6/ml) of two races of *Colletotrichum trifolii* and incubated at four temperatures[a]

		Disease severity index[b]				Resistant response (%)[c]			
C. trifolii	Cultivar	12 C[d]	16 C	20 C	24 C	12 C	16 C	30 C	24 C
Race 1	Team	1.2[e]	1.9	2.4	3.2	100	82	65	38
	Saranac	1.4	2.2	3.8	4.0	98	68	15	17
	Vernal	1.3	2.1	3.8	4.1	100	75	42	17
	Glacier	1.5	2.4	3.5	4.0	97	64	28	17
	Arc	1.2	1.5	2.1	2.3	100	98	77	75
	Saranac AN 4	1.0	1.2	1.7	2.0	100	97	85	77
	Vernal AN 4	1.0	1.4	1.9	2.4	100	97	78	67
	Glacier AN 4	1.1	1.6	2.0	1.9	98	95	73	85
Race 2	Team	4.9	4.9	5.0	5.0	0	2	0	0
	Saranac	4.9	4.9	5.0	5.0	2	2	0	0
	Vernal	4.5	4.9	5.0	5.0	10	2	0	0
	Glacier	4.8	4.9	4.9	4.7	3	3	0	7
	Arc	4.9	5.0	5.0	5.0	2	0	0	0
	Saranac AN 4	2.7	2.9	3.6	4.6	58	53	32	10
	Vernal AN 4	3.0	3.8	5.0	5.0	52	25	0	0
	Glacier AN 4	2.7	3.3	4.0	4.3	53	38	38	15

[a] Values are the means of two runs, six replicates, and five plants per replicate.
[b] Disease severity index (DSI) is the calculated average of disease scores 1-5:(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] Percent of all plants with disease scores of 1 or 2.
[d] Plants incubated at the indicated temperature (C) for 3 days.
[e] Comparing DSI for cultivars within a given whole plot (temperature and race) (LSD = 0.31, $P = 0.05$).

TABLE 4. Disease severity in alfalfa cultivars resistant and susceptible to anthracnose after inoculation with race 1 or 2 of *Colletotrichum trifolii* at varying temperatures and concentrations of conidia[a]

			Disease severity[b]					Resistant response (%)[c]				
C. trifolii	Concentration of conidia	Cultivar	12 C[d]	16 C	20 C	24 C	Avg.	12 C	16 C	20 C	24 C	Avg.
Race 1	10^3	Saranac	3.9[e]	3.1	3.9	2.8	3.4	7[f]	31	12	46	22
		Saranac AN 4	1.4	1.6	1.6	1.5	1.5	93	79	86	86	87
		Arc	1.7	2.0	1.6	2.0	1.8	86	75	87	73	81
		WL 311	2.9	2.8	2.9	2.7	2.8	42	37	40	42	40
		Riley	2.5	2.6	3.1	2.5	2.7	46	43	26	51	41
	10^4	Saranac	4.3	4.5	4.8	4.6	4.6	2	1	0	3	1
		Saranac AN 4	1.9	1.8	2.3	2.4	2.1	69	76	66	62	68
		Arc	2.0	1.7	2.2	2.1	2.0	77	84	74	72	77
		WL 311	3.7	3.9	4.3	4.2	4.0	14	5	5	7	7
		Riley	3.4	3.6	4.2	3.8	3.8	17	9	7	18	12
	10^6	Saranac	4.8	4.8	5.0	4.9	4.8	0	0	0	0	0
		Saranac AN 4	2.4	2.4	2.9	3.5	2.8	67	63	46	36	53
		Arc	2.1	2.4	2.7	2.4	2.4	76	75	74	75	75
		WL 311	4.1	4.3	4.5	4.9	4.4	5	2	5	0	2
		Riley	4.2	4.2	4.9	4.9	4.5	2	3	0	0	1
Race 2	10^3	Saranac	4.1	3.9	3.9	4.3	4.1	11	14	18	10	13
		Saranac AN 4	1.7	1.9	2.0	2.0	1.9	76	70	70	72	72
		Arc	3.6	3.9	3.8	4.3	3.9	16	13	19	13	15
		WL 311	3.1	3.1	3.4	4.1	3.4	37	39	21	12	26
		Riley	2.9	2.7	3.3	3.3	3.1	36	43	21	34	34
	10^4	Saranac	4.6	4.9	4.9	4.9	4.8	3	0	0	0	0
		Saranac AN 4	2.6	2.4	3.0	2.9	2.7	52	55	40	43	47
		Arc	4.5	4.5	4.6	4.8	4.6	1	3	3	0	1
		WL 311	4.1	3.8	4.2	4.6	4.2	7	21	7	3	8
		Riley	3.8	4.1	4.4	4.0	4.0	11	10	5	17	10
	10^6	Saranac	4.8	4.9	4.9	5.0	4.9	0	0	0	0	0
		Saranac AN 4	2.5	2.8	2.8	3.9	3.0	62	53	47	18	44
		Arc	4.8	4.8	4.9	5.0	4.8	0	0	0	0	0
		WL 311	4.5	4.6	4.7	4.9	4.7	2	1	0	0	1
		Riley	4.0	4.2	4.8	4.7	4.43	10	5	0	0	2

[a] Values are the means of two runs, three replicates, and 10 plants per replicate.
[b] Disease severity 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] Percent of all plants with disease scores of 1 or 2.
[d] Plants incubated at the indicated temperature (C) for 3 days.
[e] Comparing DSI for cultivars within a temperature and concentration of conidia (LSD = 0.5, $P = 0.05$).
[f] Comparing percent resistant seedlings with a temperature and concentration of conidia (LSD = 4.3%, $P = 0.05$).

development (22). After incubation, the bags were removed and the rack was returned to the greenhouse. The study was arranged as a 2-race × 4-temperature × 8-cultivar factorial in a split-plot design with the race-temperature combinations being the whole plot treatments and cultivars being the subplot treatment. The experiment was repeated (two runs) using six replications each time. Disease scores (range 1–5) were averaged to produce a DSI for each treatment variable (Table 3). Percent resistant plant data were transformed to arcsin values for the analysis of variance. DSIs were separated by using a protected LSD ($P = 0.05$).

To verify viability of the inoculum, the surface of 2% water agar in petri dishes was flooded with 1–2 ml of the conidial suspension. The dishes were incubated at 12, 16, 20, or 24 C and after 24, 48, and 72 hr, 200 conidia were examined at ×100 and conidia germination and the percent of germ tubes terminating in appressoria were determined.

Effect of race, concentration of conidia, temperature, and cultivar on anthracnose development. Seedlings were grown in SEPEL, inoculum was prepared and diluted, and seedlings were inoculated and incubated as previously described. The study was arranged in a 2-race × 3-conidia concentrations × 4-temperature × 5-cultivar factorial in a split-split-plot design with race-conidia concentration combination assigned to whole plots, temperatures to split-plots, and cultivars to split-split plots. Due to limited space in the environmental chambers, the number of replications per run was reduced to three and the number of seedlings per cup was increased to 10. The study was repeated once (two runs). The number of seedlings of each treatment variable was similar to experiment 2 (30 per run). Disease scores for runs were combined in the analysis of variance. Due to limited space, an uninoculated control was omitted from this experiment. Disease scores (range 1-5) were averaged to produce a disease severity index (DSI) for treatment variables and the percent resistant (scores 1 or 2) were transformed to arcsine values for the analysis of variance. Means for DSI and percent resistant were separated using a protected LSD ($P = 0.05$).

To verify germinability of the conidia, leaflets were removed from inoculated Saranac seedlings incubated for 72 hr at 12, 16, 20, or 24 C and air dried. They were stained with lactophenol containing 0.1% acid fuchsin and examined at ×100 and ×400 with transmitted light. Stained spores, appressoria, and germ tubes were well contrasted from the green uncleared tissue. Uninjured plant and fungus material could be examined for about 1 hr before the tissue became distorted.

RESULTS

Anthracnose developed in all experiments. The controls, included in experiments 1 and 2, remained free of anthracnose and were excluded from the statistical analysis. Throughout the study, inoculated plants with anthracnose symptoms were collected and incubated in moist chambers. Spores and setae typical of *C. trifolii* (18) were produced in the lesions.

TABLE 5. Germination of conidia of *Colletotrichum trifolii* and formation of appressoria and germ tubes on alfalfa leaflets after 3 days at 12, 16, 20, and 24 C

C. trifolii	State of development of conidia[a]	Germination (%) at: 12 C	16 C	20 C	24 C
Race 1	Germinated	80[b]	65	73	89
	With appressoria	70	43	39	52
	With germ tubes	3	4	8	33
	With appressoria and germ tubes	7	17	26	4
Race 2	Germinated	63	57	65	64
	With appressoria	16	24	9	10
	With germ tubes	47	27	47	49
	With appressoria and germ tubes	0	6	9	5

[a]Leaflets of Saranac were stained with lactophenol containing 0.1% acid fuchsin and examined at × 10 to × 400 with transmitted light.
[b]Values are based on observation of 200 conidia at each temperature.

Inoculum concentration experiment. Seedling emergence for the eight cultivars ranged from 12 to 25 per row. The total seedlings inoculated were 1,053–1,283 per cultivar. Seedling emergence for Saranac was 85%; Vernal, 83%; Glacier, 70%; Team, 72%; Saranac AN 4, 83%; Vernal AN 4, 71%; Glacier AN 4, 76%; and Arc, 70%. The number of seedlings inoculated with race 1 or 2 at any given conidial concentration ranged from 90 to 140 for each cultivar. When the data from run 1 and 2 for race 1 were combined and analyzed in a 2 × 4 × 8 factorial, runs were not a significant source of variation ($P = 0.05$), and results were combined and analyzed as a split-plot design with six replicates (Table 1). Sums of squares for nonsignificant interactions were combined into error b in the analysis of variance.

The main effects (inoculum concentration), the split-plot (cultivars), and the interaction were all sources of significant variation ($P = 0.01$). Seedling survival was generally the lowest at the highest levels of inoculum. At 10^3 conidia per milliliter, seedling survival was significantly higher than 10^4, 10^5, or 10^6.

Seedling survival varied for each cultivar, but Glacier AN 4 and Vernal AN 4 were the most resistant (as measured by percent seedlings scored 1 or 2) and Saranac was the most susceptible (percent scored 3, 4, or 5) of the cultivars tested. The interactions among cultivars and inoculum concentration were indicated when seedling survival of Vernal AN 4 inoculated with 10^6 conidia per milliliter was significantly less than that of Glacier AN 4, whereas at the three lower concentrations survival was similar. Also, seedling survival of Saranac AN 4 was significantly less than that of Vernal AN 4 at the three lower concentrations, whereas at 10^6 seedling survival for both cultivars was similar.

When the data from run 1 and 2 for race 2 were separated into a 2-run × 4-concentration × 8-cultivar factorial and analyzed, runs were not a source of variation, and the results were combined and analyzed as a split-plot design with six replicates. Sums of squares for nonsignificant interactions were combined into the source of variation for error b. The main plot effect (inoculum concentration), the split-plot effect (cultivar), and the interaction were all significant sources of variation ($P = 0.01$). Seedling survival was generally lowest at the highest concentration of inoculum, although there was no significant difference for the three highest concentrations (Table 2). Seedling survival of the three resistant cultivars were clearly different from the five susceptible cultivars. The interaction occurred among susceptible cultivars. At 10^4 conidia per milliliter, resistance of Arc was similar to those of Saranac and Glacier and also to those of Team and Vernal; at 10^5 conidia per milliliter, resistance of Arc was significantly less than those of Glacier, Vernal, and Team; at 10^6 conidia per milliliter, resistance of Arc was similar to that of Glacier, but significantly less than those of Saranac, Team, or Vernal. Race 2 was highly virulent on Arc.

Temperature and anthracnose development experiment. This study was done to evaluate how temperature influenced disease reaction. The main effects of the factorial design (race, temperature, and cultivar) and the 2- and 3-way interactions were all significant sources of variation in DSI and percent resistant plants ($P = 0.001$). Treatment means averaged for replicates and runs are given in Table 3. The interaction of cultivars within a whole plot (temperature and race) were compared by using a protected LSD = 0.31 ($P = 0.05$).

Cultivars that possessed the highest level of resistance to race 1 or 2 were, on the average, more susceptible at the higher than at the lower temperatures. Cultivars susceptible to race 1 had larger DSIs at higher temperatures whereas the DSIs of cultivars susceptible to race 2 were similar regardless of temperature.

A significant interaction occurred for Glacer AN 4 inoculated with race 1. At 24 C, the DSI of Glacier AN 4 was significantly less than the DSIs for Arc and Vernal AN 4, whereas at the three lower temperatures, the DSIs for these three cultivars were not significantly different. A similar interaction occurred with race 2 for Glacier AN 4. At 24 C, the DSI of Glacier AN 4 was statistically similar to that of Saranac AN 4, whereas at 16 and 20 C, their DSIs were statistically different.

Germination of conidia of race 1 on water agar at 12, 16, 20, and

24 C ranged 64–97%, 80–100%, and 81–100% after incubation for 24, 48, and 72 hr, respectively, and appressoria formed on 67–99% of the germ tubes. Germination of conidia of race 2 on water agar at 12, 16, 20, and 24 C ranged 7–17%, 51–59%, and 69–87% after incubation for 24, 48, and 72 hr, respectively, and appressoria formed on 67–99% of the germ tubes. The germination and appressorium formation for conidia after 3 days of incubation over the range of temperature tested was considered satisfactory for infection.

Four-way interaction experiment. Elements in the first two experiments were combined, and two cultivars (WL-311 and Riley) previously released as being resistant to anthracnose, were added. The main effects of this study (temperature, race, concentration of conidia, and cultivar) were all significant sources of variation ($P = 0.05$), for the DSI and percent resistant plants, as were the two-way interactions for cultivar × temperature, cultivar × race, cultivar × concentration of conidia ($P = 0.05$), and the three-way interaction for cultivar × concentration of conidia × race ($P = 0.05$, Table 4). These interactions were attributed to the cultivar effects because cultivars had various levels of resistance to race 1 or 2 of *C. trifolii*. When inoculum of either race contained 10^3 conidia per milliliter, the disease reactions were variable and less severe, and the differences were attributed to disease escape rather than disease resistance. When inoculum of race 1 contained 10^4 or 10^6 conidia per milliliter, Saranac AR and Arc were resistant and Riley, WL 311, and Saranac were susceptible. For race 2 at these concentrations of conidia, Saranac AR was resistant and WL 311, Riley, Saranac, and Arc were susceptible.

When disease severity for temperatures and cultivars inoculated with the same inoculum concentration (10^6 conidia per milliliter) in the last two studies are compared (Tables 3 and 4), DSIs for Saranac and Arc inoculated with race 2 were similar. However, the DSI for Saranac AN 4 was different at 20 and 24 C. For race 1, DSIs for Saranac, Saranac AN 4, and Arc were consistently larger in the third study (Table 4) than in the second study (Table 3). Obviously, factors other than just temperature and inoculum concentration are involved in the disease reactions of these cultivars.

Germination of conidia of race 1 or 2 on leaflets of alfalfa was not influenced by temperatures between 12 and 24 C (Table 5). However, the mode of germination was different for races 1 and 2. Germ tubes of conidia of race 1 tended to terminate in appressoria more often than germ tubes of conidia of race 2. Also, germ tubes of conidia of race 1 tended to form appressoria more frequently at 12 C than at 16, 20, or 24 C. Disease scores for susceptible cultivars with DSIs that range from 4 to 5 indicate that infection occurs at each of the four temperatures, whatever the mode of penetration.

DISCUSSION

In earlier studies (5,12) with race 1-type isolates, inocula containing unspecified concentrations of conidia were used, and progress was made in increasing resistance to anthracnose. In later studies (7,10,20,21), inoculum levels were specified (all were within the range tested here) that were sufficient for effective disease selection and cultivar evaluations. Also in earlier studies, incubation temperatures were either unspecified or ranged 10–30 C (7,10,20–22). Again, progress was made in increasing disease resistance.

With the discovery of race 2 (14,21) and the availability of race 1-resistant germplasm (5), an opportunity was provided to evaluate whether host reaction to races was influenced by either inoculum concentration or temperature. The results of these studies indicate that inoculum concentration and temperature influence the disease reaction of some cultivars. Also, temperature influences disease reactions and is different for races and cultivars. Consequently, inoculum concentration and temperature should be controlled when managing a screening program for anthracnose resistance in alfalfa. These data show, for example, that as incubation temperature decreases, race 2-type resistance decreases in race 1-resistant cultivars Saranac An 4, Glacier AN 4, and Vernal AN 4. As the change is always in the same direction, screening adapted germplasm at the most relevant temperature for field conditions for a specific region of the country would provide the most effective screening and evaluation method. When screening germplasm for the humid southeastern USA where anthracnose pressure is high, higher incubation temperatures should be included; when screening germplasm adapted to the cooler upper Midwest, lower temperatures could be used to prevent overwhelming resistance in an otherwise adapted cultivar (eg, race 2 and Vernal AN 4).

Results from this study confirm earlier reports (7,20) that Riley and WL 311 are susceptible to race 1 of *C. trifolii*. Reasons for this difference are not known, but it does not appear to be caused by temperature during infection. When seedlings of both cultivars were inoculated with a lower concentration of conidia (10^4/ml), the disease was slightly less severe and more plants were scored resistant than when they were inoculated with the higher concentration of conidia (10^6/ml). WL 311 was selected following three natural epidemics of anthracnose in the field (1) and Riley was selected for anthracnose resistance in the field (16). Perhaps during selection for resistance, inoculum concentration in the field was not sufficient to select resistant plants. Alternatively, they may express resistance in field selections that is not expressed in greenhouse selections.

Variations in disease severity at different temperatures support further the hypothesis that resistance to race 1 may be controlled by alleles different from those controlling resistance to race 2 (4,5). It also suggests that the expression of these alleles may be temperature-dependent. These experiments were not designed to determine if temperature alters resistance in the host or virulence in the pathogen. However, since race 1 and 2 originated from single-spore cultures and the cultivars are composed of heterogeneous individuals, it seems more likely that temperature influences the expression of resistance or susceptibility in the host more than virulence in the pathogen.

LITERATURE CITED

1. Beard, D. F., and Kawaguchi, I. I. 1978. Registration of WL 311 and WL 318 alfalfa cultivars. Crop Sci. 18:523.
2. Campbell, T. A., Schillinger, J. A., and Hanson, C. H. 1974. Inheritance of resistance to anthracnose in alfalfa. Crop Sci. 14:667-668.
3. Colhoun, J. 1973. Effects of environmental factors on plant disease. Annu. Rev. Phytopathol. 11:343-364.
4. Collins, H. B. 1974. The nature and inheritance of anthracnose [*Colletotrichum trifolii*(Bain and Essary)] resistance in alfalfa *Medicago sativa* L. Ph.D. thesis. North Carolina State University, Raleigh. 105 pp.
5. Devine, T. E., Hanson, C. H., Ostazeski, S. A., and Campbell, T. A. 1971. Selection for resistance to anthracnose (*Colletotrichum trifolii*) in four alfalfa populations. Crop Sci. 11:854-855.
6. Downs, R. J., and Bonaminio, V. P. 1976. Phytotron Procedural Manual. N.C. Agric. Exp. Stn. Tech. Bull. 244. 36 pp.
7. Elgin, J. H., Jr., and Ostazeski, S. A. 1982. Evaluation of selected alfalfa cultivars and related *Medicago* species for resistance to race 1 and race 2 anthracnose. Crop Sci. 22:39-42.
8. Graham, J. H., Devine, T. E., and Hanson, C. H. 1976. Occurrence and interaction of three species of *Colletotrichum* on alfalfa in mid-Atlantic United States. Phytopathology 66:538-541.
9. Kehr, W. R., Frosheiser, F. I., Wilcoxson, R. D., and Barnes, D. K. 1972. Breeding for disease resistance. Pages 335-354 in: Alfalfa Science and Technology. C. H. Hanson, ed. American Society of Agronomy, Madison, WI. 812 pp.
10. Morrison, R. P. 1977. A seedling-box test for evaluating alfalfa for resistance to anthracnose. Plant Dis. Rep. 61:35-37.
11. National Certified Alfalfa Variety Review Board. 1981. Report to members of the National Alfalfa Improvement Conference for 1981. (Available from J. H. Elgin, III, Executive Secretary, USDA-ARS, Beltsville Agricultural Research Center, Beltsville, MD 20705.
12. Ostazesky, S. A., Barnes, D. K., and Hanson, C. H. 1969. Laboratory selection of alfalfa for resistance to anthracnose, *Colletotrichum trifolii*. Crop Sci. 9:351-354.
13. Ostazeski, S. A., and Elgin, J. H., Jr. 1980. Physiologic races of *Colletotrichum trifolii*. (Abstr.) Phytopathology 70:691.
14. Ostazeski, S. A., Elgin, J. H., Jr., and McMurtray, J. E. III. 1979. Occurrence of anthracnose on formerly anthracnose-resistant 'Arc' alfalfa. Plant Dis. Rep. 63:734-736.
15. Roberts, D. A., Ford, R. E., Ward, C. H., and Smith, D. T. 1959.

Colletotrichum anthracnose of alfalfa in New York. Plant Dis. Rep. 43:352.
16. Sorensen, E. L., Stuteville, D. L., and Horber, E. L. 1978. Registration of Riley alfalfa. Crop Sci. 18:911.
17. Steel, R. G. D., and Torrie, J. H. 1969. Principles and Procedures of Statistics. McGraw-Hill, New York. 481 pp.
18. von Arx, J. A. 1957. Die Arten der Gattung *Colletotrichum* Cda. Phytopathol. Z. 29:431-468.
19. Walker, J. C. 1965. Use of environmental factors in screening for disease resistance. Annu. Rev. Phytopathol. 3:197-208.
20. Welty, R. E, Gurgis, R. Y., and Rowe, D. E. 1982. Occurrence of race 2 of *Colletotrichum trifolii* in North Carolina and resistance of alfalfa cultivars and breeding lines to race 1 and 2. Plant Dis. 66:48-51.
21. Welty, R. E., and Mueller, J. P. 1979. Occurrence of a highly virulent isolate of *Colletotrichum trifolii* on alfalfa in North Carolina. Plant Dis. Rep. 63:666-670.
22. Welty, R. E., and Rawlings, J. O. 1980. Effects of temperature and light on development of anthracnose on alfalfa. Plant Dis. 64:476-478.