Sporulation by Races 0, 2, and 3 of *Cochliobolus carbonum* on Synthetic Medium and Sterilized Corn Leaves

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ABSTRACT

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Isolates of races 0, 2, and 3 of *Cochliobolus carbonum* collected from two locations in North Carolina were grown in vitro at 20, 24, or 28 C on potato-lactose agar (PLA) or on water agar with either autoclaved green corn leaves or senesced corn leaves. Races, temperatures, and media all significantly affected sporulation. Temperature × race and substrate × race interactions were not statistically significant. Therefore, it seems unlikely that differences in sporulation by races 2 and 3 on corn are influenced differentially by temperature or simple nutritional factors. Averaged over all factor levels, race 2 sporulated best, followed by race 3 and then race 0. The races ranked in the same order when compared for parasitic fitness in an earlier survey of race frequency changes over time in the two fields where these isolates were collected. In another experiment with 318 *C. carbonum* isolates from these two fields plus 14 from a field in Tennessee, race 2 mycelium grew significantly faster on PLA than mycelium of race 3 or race 0; any difference between race 3 and race 0 was not significant. Thus, mycelial growth rate may be a less reliable indicator of fitness than sporulation in vitro.

Additional keywords: *Bipolaris zeicola, Helminthosporium carbonum,* maize, *Zea mays*

Cochliobolus carbonum R.R. Nelson (anamorph: *Bipolaris zeicola* (G.L. Stout) Shoemaker) is a common necrotrophic leaf pathogen of corn (*Zea mays* L.) in many temperate zones of the world (10). Five pathogenic races of the fungus have been described. Race 0, which is nearly avirulent to corn and genetically quite distinct from races 1, 2, and 3, was reported recently by Welz and Leonard (12,13). Dodd and Hooker (2) found a fifth race in Illinois that is highly virulent on specific corn hybrids and inbreds. Race 1, which produces a host-specific toxin, has become rare in the United States because modern corn hybrids are not sensitive to its toxin (5).

Race 2 induces small round to oval lesions on corn leaves. It was the most frequently found race in past surveys (4,6,8,13), except in the Appalachian mountains, where race 3 prevailed for more than 30 yr (4,6,8). Race 3 is also reported as common in the northern Corn Belt, but no frequency data have been published (10). Because race 3 induces long, linear lesions that are larger than those of race 2, it was thought likely to have greater parasitic fitness. In the 1970s, race 3 began to spread from the mountains into eastern North Carolina, where it increased to about 20% of all isolates recovered by 1977. Thereafter, its frequency stabilized (6). In fact, a study of the 1987 epidemic revealed that race 3 had significantly lower parasitic fitness than race 2 in both of the field populations studied (13).

Leonard (4) suggested that the predominance of race 3 in the mountains might be due to specific adaptation to lower temperatures, but he did not find direct evidence for that. The optimal temperature for mycelial growth of both race 2 and race 3 was 28 C. In a subsequent study, Lodge and Leonard (8) detected a cline in the frequencies of races 2 and 3 along the eastern escarpment of the Blue Ridge Mountains of North Carolina, finding that the frequency of race 3 was correlated with elevation above sea level. Elevation was negatively correlated with mean summer temperature, which again raised the question of a temperature interaction with prevalence and fitness of race 3. Lodge and Leonard (8) attempted to compare sporulation by

Table 1. Description of isolates of *Cochliobolus carbonum* used in sporulation experiments

Race	Isolate[a]	Presence of indicated trait[b]						
		MAT-1	Psu+	Asc+	CyhR	CrbR	CadR	PDA+
0	12-1	+	−	−	+	−	+	−
0	12-9	+	+	+	+	+	+	−
0	12-16	+	+	−	+	+	+	−
0	12-20	+	−	−	+	+	+	−
0	12-24	−	+	+	+	−	+	−
0	12-35	−	+	−	+	−	+	−
	O/E[c]	4/4	4/4	2/2	6/6	3/3	6/6	0/0
2	12-59	+	+	−	+	+	−	+
2	15-54	−	−	−	+	+	−	+
2	19-47	−	−	−	−	+	−	+
2	13-16	+	+	+	−	+	−	+
2	13-47	−	+	+	+	+	−	+
2	16-50	+	+	+	−	+	−	+
	O/E	3/2	4/2	3/2	3/4	6/5	0/0	6/6
3	12-2	+	−	−	+	+	−	+
3	12-47	−	+	+	+	+	−	+
3	19-2	+	+	+	+	+	−	+
3	13-1	+	−	−	+	+	−	+
3	13-27	+	−	−	+	+	−	+
3	13-30	+	−	−	+	+	−	+
	O/E	5/3	2/1	2/0	6/6	6/6	0/0	6/6

[a] Isolates 12-, 15-, and 19- were from a field in Wilkes County, North Carolina. Isolates 13- and 16- were from a field in Yadkin County, North Carolina.

[b] MAT-1 indicates mating type 1; Psu+, ability to form pseudothecia; Asc+, ability to form ascospores; CyhR, tolerance of cycloheximide; CrbR, tolerance of carboxin; CadR, tolerance of cadmium; and PDA+, ability to sporulate on potato-dextrose agar.

[c] O/E are observed and expected numbers of isolates with the trait indicated. Expected numbers were calculated from character frequency data in race 0, 2, or 3 in the full set of isolates; they are rounded to full numbers.

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races 2 and 3 on intact corn seedlings inoculated in the greenhouse and exposed in the field in the mountains and eastern Piedmont of North Carolina. They reported that neither race 2 nor race 3 sporulated in isolated lesions on green leaves. Sporulation occurred only on senescent or necrotic tissues of the inoculated leaves. Spores usually were produced first at the margins of the leaves, on lower senesced leaves, and on leaf tips where the lesions had coalesced and induced necrosis. Lodge and Leonard were unable to quantify the relative numbers of spores produced by races 2 and 3 on intact corn plants in the field. From their results, measurement of sporulation in vitro seemed a more feasible approach.

The main objective of the present study was to test the hypothesis of adaptation of race 3 to lower temperatures by comparing sporulation of races 2 and 3 at different temperatures on sterilized corn leaves and on agar medium. The newly detected race 0 was included in the comparison. Furthermore, we addressed the question of whether in vitro sporulation and mycelial growth of *C. carbonum* were representative of corresponding fitness components in the field.

Table 2. Mycelial growth of *Cochliobolus carbonum* isolates[a] on potato-lactose agar after 5 days at room temperature

		Race					
		0		2		3	
Location	Sample	Mean[b] ± SD[c]	n[d]	Mean ± SD	n	Mean ± SD	n
Wilkes Co.	12	16.24 ± 5.99	20	20.29 ± 2.05	9	15.95 ± 3.42	11
	15	26.49 ± 2.34	6	20.02 ± 5.65	24	17.09 ± 1.75	12
	19	22.00	1	22.56 ± 3.07	25	18.00 ± 3.06	19
Yadkin Co.	13		0	19.09 ± 5.28	30	14.37 ± 3.83	16
	16		0	25.81 ± 6.11	33	19.85 ± 4.57	18
	20		0	23.92 ± 5.25	47	16.91 ± 1.67	11
Tennessee[e]	H4	25.32	1	17.59 ± 5.44	3	16.38 ± 3.60	46
Pooled mean[f]		18.97 ± 6.80 B	28	22.39 ± 5.63 A	171	16.91 ± 3.70 B	133

[a] The sample (332 isolates) included the 18 isolates used in the sporulation study.
[b] Means of millimeters of radial growth after 5 days (i.e., values standardized to 120 hr). Three colonies were measured per isolate.
[c] Standard deviation.
[d] Number of isolates.
[e] Mountain City, Johnson County, in the Blue Ridge Mountains.
[f] Means with the same letter are not significantly different at the 5% level as indicated by Scheffé's test.

Table 3. Analysis of variance of numbers of conidia produced by isolates of races 0, 2, and 3 of *Cochliobolus carbonum* in vitro on different substrates and at different temperatures

Source of variation[a]	df[b]	Mean square[c]	*F* value	*P* > *F*
Block[d]	2	150,638	101.67	0.0001
Substrate	2	1,052,953	87,18	0.0005
Temperature	2	224,160	24.44	0.0057
Race	2	216,656	34.23	0.0030
Isolate (race)	15	11,971	7.03	0.0001
Sub × temp	4	18,292	5.66	0.0184
Sub × race	4	3,430	1.34	0.3343
Sub × isol (race)	30	3,047	1.58	0.0656
Temp × race	4	3,859	0.45	0.7734
Temp × isol (race)	30	2,587	2.15	0.0059
Sub × temp × race	8	4,041	2.49	0.0570
Sub × temp × isol (race)	60	981	0.66	0.9615
Block × sub	4	12,077	...	...
Block × temp	4	9,171	...	...
Block × race	4	6,329	...	...
Block × isol (race)	30	1,702	...	...
Block × sub × temp	8	3,234	...	...
Block × sub × race	8	2,556	...	...
Block × sub × isol (race)	60	1,926	...	...
Block × temp × race	8	8,666	...	...
Block × temp × isol (race)	60	1,204	...	...
Block × sub × temp × race	16	1,621	...	...
Block × sub × temp × isol (race)	120	1,412	...	...

[a] Block is a random effect; substrate (sub), temperature (temp), race, and isolate (race) [isol (race)] are fixed effects.
[b] Degrees of freedom.
[c] Square root transformed numbers of conidia per square centimeter.
[d] The appropriate error mean squares for calculation of *F* statistics were determined from examination of expected mean squares.

MATERIALS AND METHODS

Experimental design. The experimental design was a randomized complete block (RCBD) with a 3 × 3 × 3 factorial arrangement of treatments (race, substrate, and temperature). Six isolates per race were used (Table 1). The treatment combinations were evaluated in three separate runs, with the runs considered as three blocks in time. All tests were made in 100-mm-diameter petri plates. The numbers of conidia produced per square centimeter of substrate represented the experimental observations. Altogether, the study comprised 486 observations (3 races × 6 isolates per race × 3 media × 3 temperatures × 3 blocks).

Fungal isolates. Isolates used in this study (Table 1) were chosen at random from a collection of 364 *C. carbonum* isolates obtained in 1987 from two corn fields, one in Wilkes County and the other in Yadkin County, North Carolina. These locations, both situated near the eastern Blue Ridge escarpment in western North Carolina, were known as common habitats for race 2 and race 3 of *C. carbonum* in previous surveys. The rather close fit of observed and expected frequencies of seven polymorphic traits (Table 1) suggests that the three experimental race populations, made up of six isolates each, can be considered representative of the true race populations. Until use, the isolates were stored as conidial suspensions in 30% (v/v) glycerol frozen at −70 C.

Substrates. Isolates were grown on potato-lactose agar (PLA) with 10 g of lactose per liter. Previous experiments have shown that isolates of *C. carbonum,* particularly those of race 0, conidiated substantially better on PLA than on potato-dextrose agar with 10 g of dextrose per liter (H. G. Welz and K. J. Leonard, *unpublished*). Mycelial plugs (5-mm-diameter) from the margins of 5–7-day-old cultures on PLA were transferred to plates containing the test substrates. Three different substrates were used: 25 ml of PLA, as described above; autoclaved "green" leaf disks (14 per plate, 1-cm-diameter) plated on 25 ml of 2% water agar which were cut from 2–3-wk-old hybrid corn plants (Pioneer Brand 3369A) grown in the greenhouse; and brown leaves similar to green leaves, except that leaf disks were cut from senesced leaves from mature field-grown corn plants.

Environment. Plates were left unsealed and incubated in growth cabinets at constant 20, 24, or 28 C. All temperature regimes were associated with the same light intensity of approximately 75

$\mu E \cdot m^{-2} \cdot s^{-1}$ (5,000 lx) supplied by cool-white fluorescent lamps for 16 hr per day.

Sporulation measurement. Ten days after inoculation, conidial suspensions were prepared by washing spores off the plates with 10–15 ml of deionized water. Suspensions were filtered through four layers of cheesecloth to remove mycelial fragments. One hour later, after the conidia had settled on the bottom of the test tubes, the volume of the suspensions was reduced to 4 ml. Each suspension was thoroughly shaken, and light transmission (T) of the suspension was measured immediately in a spectrophotometer at 520 nm. If T was less than 8%, suspensions were diluted to 8 ml and measured again. Numbers of conidia (C) per 4 ml were computed by the regression equation $C = 2{,}470{,}930 - 52{,}783 * T + 287.5 * T^2$ ($r^2 = 0.87$, $P = 0.0001$), which was determined by counting spores of a dilution series (57 entries) visually under a microscope with the aid of a hemacytometer.

Statistical analyses. An analysis of variance was performed on the data, based on the RCBD model (PROC ANOVA; 9). After square root transformation, the values of the dependent variable (numbers of conidia produced per square centimeter of substrate) were distributed normally (Shapiro-Wilk's $W = 0.97$, $P = 0.0001$; PROC UNIVARIATE; 9). Also, the variances of the means of transformed spore numbers, computed over isolates and experiments, were homogeneous, as indicated by a nonsignificant F_{MAX} value ($F_{MAX} = 3.94$, $P < 0.05$). F_{MAX} is the ratio of maximum to minimum variance (1). Significant main effects were compared by analyzing the confidence limits of the means by Scheffé's test based on appropriate error terms (11). This test is considered a conservative multiple range test.

Mycelial growth. During routine cultivation of *C. carbonum* isolates in the laboratory (room temperature, cool-white light of approximately 20 $\mu E \cdot m^{-2} \cdot s^{-1}$, PLA medium), photographs were taken of 332 isolates (Table 2) about 5 days after inoculation of the plates. A scale was included in each photograph, and the incubation time of each culture was recorded to the hour. Slides of the photographs were projected for measuring each colony radius. There were three colonies per plate, each started from a 5-mm-diameter mycelial plug. The mean radius for the three colonies per isolate was standardized to an incubation time of 120 hr. Based on expected mean squares, an ANOVA was run testing the effect of race vs. race × isolate error term. Means were compared by Scheffé's test. The sample of isolates tested for in vitro growth rate included 14 isolates collected in 1987 from a field near Mountain City, Tennessee, where race 3 was more frequent than race 2 (13).

Table 4. Treatment means and standard deviations of numbers of conidia produced by isolates of races 0, 2, and 3 of *Cochliobolus carbonum* in vitro on different substrates and at different temperatures

Treatment[a]	Level of treatment	Mean[b]		Std. dev.[b]
Block	1	49,929	C	40,512
	2	77,469	A	51,475
	3	69,231	B	37,533
Substrate[c]	PLA	24,699	B	17,950
	Green leaves	88,284	A	37,595
	Brown leaves	83,644	A	42,866
Temperature	20 C	46,890	B	38,945
	24 C	73,535	A	46,434
	28 C	76,202	A	43,591
Race	0	46,961	C	36,321
	2	79,728	A	45,696
	3	69,939	B	46,097

[a] There were 162 observations for each level of treatment.
[b] Conidia per square centimeter; means with the same letter are not significantly different at $P = 0.05$, as determined by Scheffé's test performed on square root transformed numbers of conidia.
[c] Potato-lactose agar (PLA), autoclaved green leaves on water agar, and autoclaved senescent leaves on water agar.

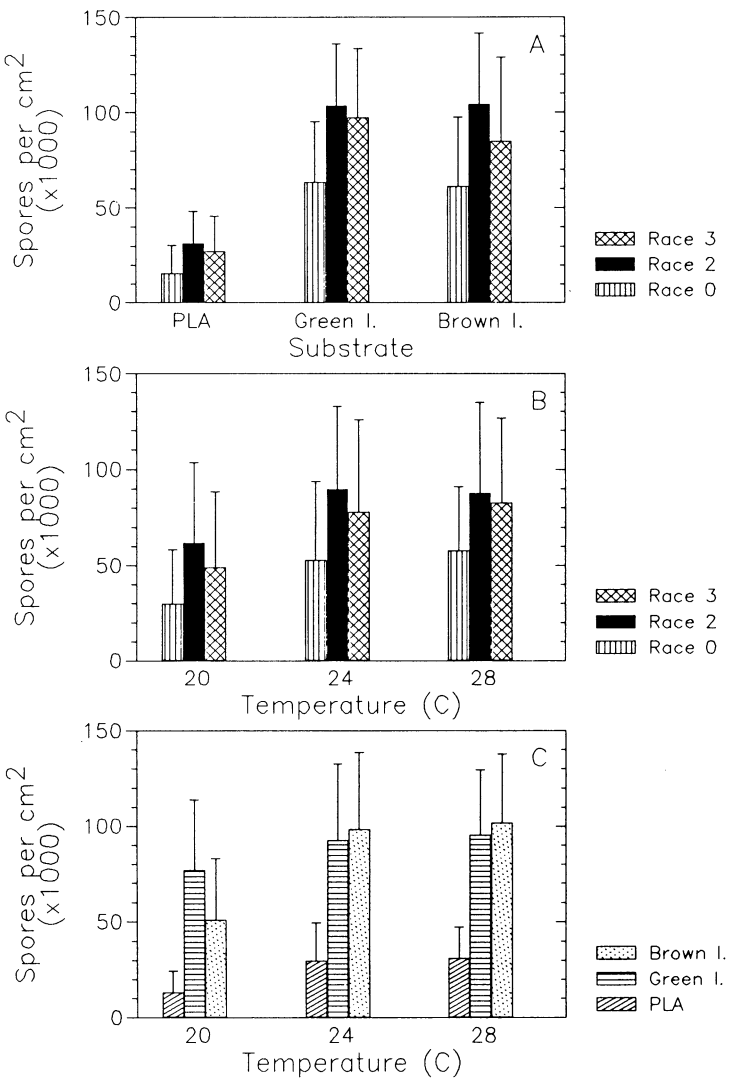

Fig. 1. Conidia production of races 0, 2, and 3 of *Cochliobolus carbonum* on three substrates and at three temperatures. Means are averaged over **(A)** temperatures, **(B)** substrates, and **(C)** races. Error bars indicate standard deviations of the means.

RESULTS

Substrates, temperatures, and races had significant effects on conidia production by *C. carbonum* (Table 3). The highly significant variation among isolates (nested within races) indicates that the differences among races seen here may be the result of the particular sample of isolates tested rather than of the races per se. Averaged over all treatments, race 2 produced significantly more conidia than did race 3, which produced significantly more than race 0 (Table 4). Significantly fewer conidia were produced on PLA than on green leaves or brown leaves, and significantly fewer were produced at 20 C than at 24 or 28 C (Table 4).

There were no significant interactions of substrate × race or of temperature × race (Table 3). The substrate × isolate (race) term ($P = 0.066$) indicates, however, that some isolates may show specific preferences among substrates. The green leaves were the superior substrate at 20 C, but brown leaves were better at 24 and 28 C (Fig. 1C), and this resulted in a significant interaction of substrate × temperature.

The three-way interaction among substrates, temperatures, and races also was important ($P = 0.057$), but it did not result in a clear reversal of ranking of races (Fig. 2). Sporulation by race 2 and race 3 was about equal on green leaves at 20 and 28 C; but under all other regimes, race 2 had increased spore production per unit area over race 3 or race 0 (Fig. 2). While green leaves were better than brown leaves as a substrate for race 3 on average over the three temperatures, this difference was due mainly to the poor sporulation by race 3 on brown leaves at 20 C (Figs. 2B and C).

Mycelial growth rates differed significantly among the three races ($F = 46.03$, df = 2, $P = 0.0001$). Scheffé's test indicated that race 2 grew significantly faster than race 0 or race 3, but the difference between race 0 and race 3 was not significant at the 5% level (Table 2).

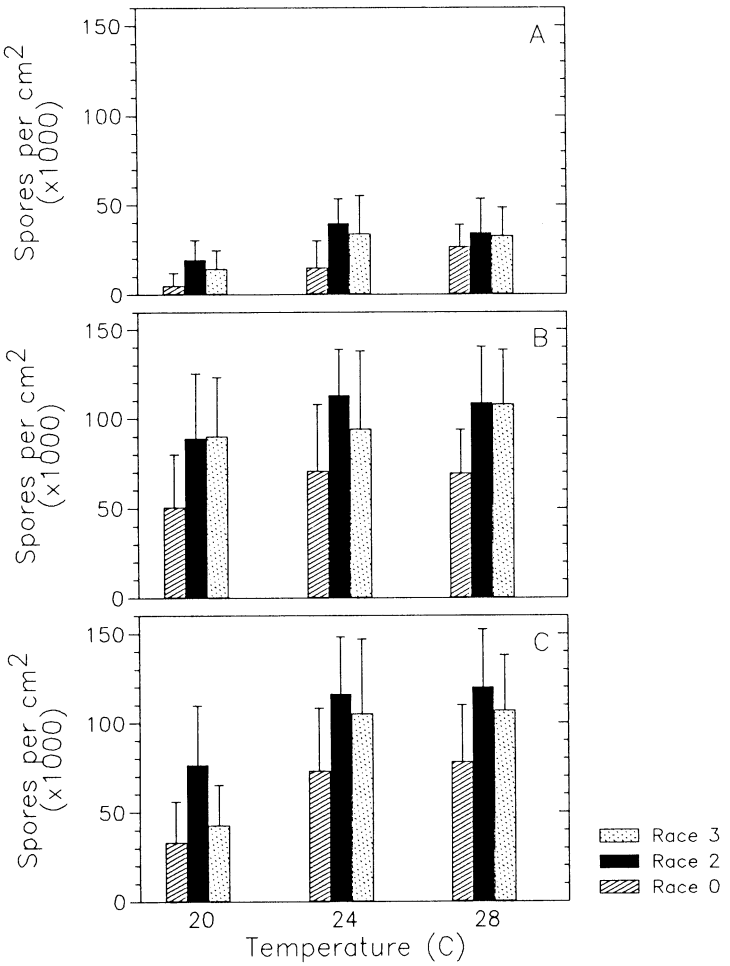

Fig. 2. Means and standard deviations of numbers of conidia produced in vitro by isolates of races 0, 2, and 3 of *Cochliobolus carbonum* per square centimeter of substrate at 20, 24, and 28 C on **(A)** potato-lactose agar, **(B)** sterilized green leaves, and **(C)** sterilized senescent leaves.

Table 5. Sporulation[a] and mycelial growth[b] of *Cochliobolus carbonum* race 0 and race 3 relative to race 2 in vitro on three substrates compared with parasitic fitness in the field

Race	Mycelium[c] on PLA	Sporulation[d]			Fitness[e]	
		PLA	Green lv	Brown lv	Wilkes	Yadkin
0	0.85	0.50	0.62	0.59	0.42	...[f]
2	1.00	1.00	1.00	1.00	1.00	1.00
3	0.76	0.87	0.94	0.82	0.82	0.84

[a] Isolates used in the sporulation study were a subsample (N = 18) of the samples in which field fitness was estimated (N = 364).
[b] Mycelial growth was measured in a sample (N = 332) which included most isolates of the sporulation study and the field study.
[c] Mycelial growth of colonies on potato-lactose agar (PLA) 5 days after inoculation.
[d] Sporulation measured per unit of substrate area: substrates were PLA, sterilized green leaves (green lv), and sterilized senescent leaves (brown lv).
[e] Data from Welz and Leonard (13), who estimated fitness from race frequency changes in the field, assuming a mean latent period of 7 days. Fields were in Wilkes and Yadkin Counties, North Carolina.
[f] Sample size too small for estimating fitness.

DISCUSSION

In vitro conidia production can be regarded as a measure of a fungal culture's laboratory fitness. "Relative laboratory fitness" can be calculated as the ratio of conidia production by isolates or races to that of a superior reference isolate or race. Calculated this way, races 0, 2, and 3 of *C. carbonum* in our experiment had relative laboratory fitnesses on all three substrates (Fig. 1A) that were similar to our estimates of parasitic fitness from field data (Table 5). This suggests that the qualities that determine in vitro sporulation may also influence competitiveness of *C. carbonum* phenotypes and represent an important component of their fitness in the field.

The in vitro mycelial growth rate was less correlated with fitness in the field than was sporulation. Although the mycelial growth of race 0 on PLA was at least as great as that of race 3, race 0 had very low fitness in the field. The significantly faster mycelium growth of race 2 than race 3 confirms earlier results obtained by Leonard (4), who found that race 2 isolates from the Piedmont grew faster than race 3 isolates from the mountains of North Carolina. It is interesting that mycelium growth is not correlated with lesion size, as race 3 induces significantly larger lesions than those of race 2 (8). This is not very surprising, but it

does reinforce the idea that differences in lesion types represent differences in the pathogen's response to resistance mechanisms in the host leaves. Xiao et al (14) found that race 3, but not race 2, produces compounds that induce susceptibility in corn leaves.

In vitro sporulation by race 3 on brown leaves, relative to that of race 2, fit the value for field fitness of race 3 more closely than did relative sporulation on green leaves. This seems biologically significant. Autoclaved brown leaf tissue is probably similar to necrotic or senescent leaf tissue on corn plants, which is the natural substrate for sporulation by *C. carbonum* in the field (7). Lodge and Leonard (8) did not find sporulation by either race 2 or race 3 on living green tissue. Undoubtedly, the fungus responds quite differently on autoclaved green leaf tissue than on living green leaf tissue. The difference probably results from an active defense mechanism in the living green leaves.

The lack of a significant differential interaction of race × temperature in the sporulation study (Table 3) confirms earlier results of Leonard (4). Therefore, the hypothesis that specific temperature adaptations caused the distribution pattern of race 2 and race 3 in North Carolina should be rejected. The absence of a significant race × substrate interaction suggests that a difference in a simple nutritional factor (e.g., sugar content of the substrate) is not the determining factor in the races' relative fitness. The significant substrate × temperature interaction was likely due to the greater sporulation at 20 C on green leaves than on the other substrates. However, these differences could not be detected based on standard deviations alone (Fig. 1) but are significant when compared with a more sensitive statistic (Table 1); but the biological significance of this is not clear.

In three surveys of *C. carbonum* populations in the western Piedmont of North Carolina conducted from 1971 until 1985 (4,6,8), the frequency of race 3 remained stable at close to 20%, suggesting that race 2 and race 3 should have nearly equal fitness in the Piedmont. The fact that race 3 continues to predominate in the Appalachian Mountains (4,8,13) suggests that race 3 is more fit than race 2 in the mountains. This is in contrast to our evidence of greater sporulation by race 2 and greater fitness of race 2 in the field during the 1987 epidemic in two locations of the western Piedmont (Table 5). When infection in the field is severe, the larger lesions of race 3 should cause more necrosis from coalescing lesions. This situation could lead to more sporulation by race 3 than by race 2, but it is rarely observed in the field (K. J. Leonard, *unpublished data*). It seems likely that other life cycle components, such as overwintering and between-season survival, may contribute to the composition of *C. carbonum* populations as well. In plant pathology, there is little evidence that ecological conditions contribute to the maintenance of polymorphisms. However, in plant population biology there are many examples of how environmental heterogeneity may stabilize diverse communities (3).

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