Genetic Analysis of Two Race 0 × Race 2 Crosses in *Cochliobolus carbonum*

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ABSTRACT

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Progeny from two crosses of race 0 × race 2 of *Cochliobolus carbonum* segregated in 1:1 ratios for loci controlling mating type, fungicide tolerance, and ability to sporulate on potato-dextrose agar. Segregations at single loci also were observed for three isozyme systems and for one of two bands in a fourth system. Progeny also segregated for four esterase bands, but the segregation patterns were complex. Pathogenicity to corn was inherited as two apparently complementary genes present in race 2 but lacking in race 0, which was avirulent on corn. Evidence was found for a new locus for production of pseudothecia and a new locus for production of asci and ascospores. These putative loci differ from the previously described single loci for pseudothecium and ascospore production in *C. carbonum*. Gene mapping analysis identified two linkage groups: one 11 cM (centiMorgans) long with two linked loci for esterase bands, and one 164 cM long with six loci including the mating type locus, one locus for cadmium tolerance, and two loci for esterase bands. A quantitative trait locus for conidium production was found within 2 cM of the *Est-B92* locus of the larger linkage group. The frequency distributions among race 0 × race 2 progeny for the numbers of conidia and pseudothecia produced were skewed toward poor production by most of the progeny relative to the parents.

Additional keywords: *Bipolaris zeicola, Helminthosporium carbonum,* linkage analysis, maize, QTL mapping, *Zea mays*.

Cochliobolus carbonum R.R. Nelson (anamorph: *Bipolaris zeicola* (G.L. Stout) Shoemaker = *Helminthosporium carbonum* Ullstrup) is a common, necrotrophic ascomycete causing a leaf spot disease on corn (*Zea mays* L.) and other grasses (28). Recently, race 3 of *C. carbonum* also was reported as a foliar path-

ogen of rice in Japan (36). Races of the fungus can be distinguished by their lesion types on corn. Avirulent race 0 induces only flecks or minute necrotic lesions on corn leaves (35). Race 1 produces a host-specific toxin and induces large necrotic lesions on susceptible genotypes, which are homozygous for the *hm1* allele, but only small round lesions on corn genotypes with *Hm1* (27,28). Race 2 induces similar small round to oval lesions on corn genotypes with either *hm1* or *Hm1*. Race 3 is characterized by long linear lesions on most corn lines and hybrids (28). A fifth pathotype that causes unique lesion types on certain corn genotypes was

described recently and designated race 4 (4).

Nelson (24) and Nelson and Kline (25,26) studied the genetics of pathogenicity in *C. carbonum* to more than 25 species of grasses. They found that pathogenicity on each host species was determined by either one or two genes. Nelson and Kline postulated as many as 35 pathogenicity genes, some linked, but they did not perform quantitative linkage analyses. Their isolates and host lines are no longer available for further mapping studies.

Evidence from race 2 × race 3 crosses indicated polygenic control of lesion length in *C. carbonum* (1). Even though the lesions induced by race 3 are larger than those of race 2, field experiments demonstrated that race 2 had greater parasitic fitness than race 3 (35). Evidently lesion size and parasitic fitness are controlled by different genes. On the other hand, in vitro sporulation on autoclaved corn leaves was positively correlated with parasitic fitness (34).

With sufficient genetic markers identified to specific linkage groups, it should be possible to distinguish genes that control lesion type, parasitic fitness, or sporulation capacity and to evaluate pleiotropic effects of specific genes for these traits. We initiated two crosses between race 0 and 2 isolates to study inheritance of several isozyme bands and to begin identifying linkage groups in *C. carbonum*. We chose race 0 as a parent, because it is avirulent to corn and genetically distinct from races 1, 2, and 3 (9,33,35). Thus, progeny from crosses of race 0 with 2 should segregate for a large number of genes, including genes for pathogenicity on corn.

Our objectives in the present study were to 1) determine inheritance of pathogenicity to corn, 2) determine inheritance of isozyme banding patterns, 3) identify genetic linkage groups among the loci involved, and 4) study the genetics of presumed quantitative traits such as sporulation and sexual fertility in *C. carbonum*.

MATERIALS AND METHODS

Crosses. For cross A, parental isolates were 12-12Bz (race 0) and yug y (race 2), and for cross B, 12-24Bz (race 0) and 13-16Bz (race 2). Yug y is a standard race 2 isolate collected by R. R. Nelson prior to 1965; records of its collection site (presumably in Yugoslavia) and date are unavailable. The other three isolates were collected in two North Carolina corn fields during 1987 (35). Previous experiments (35) showed that these parents differed in cultural morphology and fungicide tolerance as well as pathogenicity and mating type. They also have distinct isozyme phenotypes (33).

Matings were set up in polystyrene petri dishes on senescent corn leaf disks (1 cm in diameter) on modified Sachs agar (8). Mycelial plugs (5 mm in diameter) of the first parental isolate were placed on opposite sides of a pair of leaf disks, and a mycelial plug of the second parental isolate was placed between the two leaf disks. The plates were kept in the dark at room temperature. In fertile matings, pseudothecia were visible on leaf disks after 7 days. Asci and ascospores matured 20–25 days after mating. From leaf disks viewed under a dissecting microscope, single pseudothecia were transferred with a sterile needle to a thin (1 mm) layer of water agar and crushed in a drop of sterile water with a forceps. Single ascospores liberated from the pseudothecium were separated on the agar surface with a fine needle. Blocks of agar containing single random ascospores were transferred to plates containing 2% potato-lactose agar (PLA), 10 g of lactose per liter. After 3 days, mycelial plugs were cut from the margins of single-ascospore colonies and subcultured on fresh PLA plates. After another 5 days, isolates were subcultured for analysis and storage as conidial suspensions in 30% (v/v) glycerol at −70 C.

Pathogenicity tests. Ascospore isolates were tested for pathogenicity on corn hybrid Pioneer Brand 3369A. Seedlings were grown in vermiculite in 0.7-L pots fertilized weekly for 3–4 wk in a greenhouse. Plants were inoculated at the four- to six-leaf stage. Inoculum consisted of approximately 20-ml conidial suspensions of about 10^4 conidia per milliliter of tap water. Conidial suspensions were prepared from 7- to 10-day-old PLA cultures

and filtered through four layers of cheesecloth to remove mycelial fragments. Many isolates sporulated weakly, so conidia harvested from several plates were needed for sufficient inoculum concentration. The suspensions were sprayed onto seedlings with an atomizer (DeVilbiss Co., Somerset, PA) attached to an air pump (0.5 bar). Atomizers were rinsed with 70% ethanol and tap water between inoculations with different isolates. Inoculated plants were incubated in a moist chamber in the greenhouse overnight. Seven days after inoculation, lesion types were rated on a scale from 1 to 6: 1 = no reaction; 2 = flecks; 3 = flecks to small lesions; 4 = small lesions; 5 = small to average race 2-type lesions; and 6 = average race 2-type lesions. Pathogenicity tests were repeated for all isolates.

Mating type and fertility. All isolates were paired with fertile albino tester isolates of *C. carbonum* (14) of known compatibility (mating type MAT-1 (A) or MAT-2 (a)). Matings were set up similar to the crosses described above. Only white pseudothecia produced by albino tester isolates appeared in matings with isolates that lacked the ability to form pseudothecia, a trait commonly controlled by a single gene, *Psu–* (23). Matings with isolates capable of forming pseudothecia produced black, wild-type pseudothecia next to albino pseudothecia. Numbers of black pseudothecia per leaf disk were counted for quantitative assessment of pseudothecium production. For qualitative assessments of ascospore production, five to 10 mature black pseudothecia from each mating were crushed in a drop of water under a dissecting microscope. All mating tests were repeated. Production of ascospores could not be assessed in isolates that lacked the ability to form pseudothecia. For those isolates, ascospore production was treated as a missing value in the statistical analyses.

Fungicide tolerance. Ascospore isolates were tested for tolerance to 2 μg of cycloheximide [3-2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl glutarimide] per milliliter and 100 μg of cadmium acetate per milliliter. Cycloheximide and cadmium tolerances in *C. carbonum* are each controlled by a single gene (16,21). The fungicides were added from stock solutions to melted PLA in bottles immediately before pouring the media. Mycelial plugs from 5- to 7-day-old cultures were transferred to solidified media (including unamended PLA as a control). Diameters of colonies on amended and unamended media were measured after 5 days. Isolates were rated as tolerant to a fungicide if their colony diameters on the fungicidal medium were greater than 20% of that on unamended PLA (16,17). Tests for cadmium tolerance were repeated once or twice per isolate. Tests for cycloheximide tolerance were not repeated, because they yielded distinctly tolerant or sensitive responses typical of results from previous research (16,17). Cycloheximide-tolerant isolates were assigned the phenotype Cyh-R; sensitive isolates were designated Cyh-S. Likewise, reaction to cadmium was scored as either Cad-R or Cad-S.

Sporulation on potato-dextrose agar. Nearly all isolates of race 0, including isolates 12-12Bz and 12-24Bz, grow slowly and produce few, if any, conidia on potato-dextrose agar (PDA), whereas nearly all isolates of races 2 and 3 grow normally and sporulate abundantly on PDA (35). In this study, ascospore isolates forming cultures with clearly restricted growth, leathery colony appearance, and poor sporulation on PDA (less than 20% of a wild-type race 2 or 3 isolate, as estimated microscopically) were classified PDA–. Ascospore isolates with growth and sporulation typical of race 2 on PDA were classified PDA+. PDA– and PDA+ classifications were based on inspection of three colonies per isolate.

Sporulation on PLA. Ascospore isolates were assessed quantitatively for conidium production on PLA, a medium that supported sporulation by both the race 0 and the race 2 parents. Three mycelial plugs (5 mm in diameter) from 3- to 5-day-old cultures were transferred to 90-mm petri dishes containing 25 ml of PLA. Cultures were incubated at room temperature under cool white fluorescent lamps ($\sim$20 μE m^{-2} s^{-1}) with a 12-h photoperiod. Ten days after inoculation, the spores were washed off the plates in 10–15 ml of deionized water by means of a rubber policeman. Conidial suspensions were filtered through two layers of cheesecloth to remove mycelial fragments and then transferred

to test tubes. Conidia were allowed to settle to the bottom of the test tubes for 1 h, and the volume of each suspension was reduced to 4 ml.

Conidial concentrations of suspensions were determined by optical density. Each suspension was shaken thoroughly, and light transmission of the suspension was measured immediately in a spectrophotometer (Bausch & Lomb, Inc., Rochester, NY) at 520 nm as described by Welz et al (34). Measurements were repeated once for each isolate.

Isozyme analysis. Enzymes for isozyme analysis were extracted from germinated conidia as described by Welz et al (33). Briefly, conidia harvested from 14-day-old cultures on PLA ($>10^6$ conidia per isolate) were suspended in phosphate buffer, washed to remove germination inhibitors, allowed to germinate for 6 h, and stored at -80 C until use. Enzymes were extracted from homogenized conidia and germ tubes, and isozymes were identified by starch gel electrophoresis as described by Welz et al (33).

Eight enzyme systems that yielded high activity and well-separated bands in previous studies with *C. carbonum* (33) were selected: PGD (phosphogluconate dehydrogenase, EC 1.1.1.44), DIA (NADH diaphorase, EC 1.6.2.2), EST (α-esterase, EC 3.1.1.1), GPI (glucosephosphate isomerase, syn. PGI = phosphoglucose isomerase, EC 5.3.1.9), ACO (aconitase, EC 4.2.1.3), PGM (phosphoglucomutase, EC 2.7.5.1), MDH (malate dehydrogenase, EC 1.1.1.37), and GOT (glutamate oxaloacetate transaminase, syn. AAT = aspartate aminotransferase, EC 2.6.1.1). Each enzyme analysis for each isolate was done two or three times.

When the genetic basis of isozymes was clear from this or a previous study (33), enzyme alleles were coded according to the relative migration distance (R_f value) from the cathode. The most frequent allele (in natural populations) was represented, by definition, by $R_f = 100$. When the genetic basis of isozyme banding patterns was not fully understood, each band was considered to be due to a different locus, and the loci were tentatively designated with the prefix B for band and the migration distance, e.g., *Got-B62*.

Goodness-of-fit tests. The chi-square test with Yates correction for continuity (29) was used to test monogenic or digenic segregation patterns (1:1 and 3:1 ratios, respectively). Here, χ^2 has one degree of freedom and is computed as

$$\chi^2 = 2 \frac{(|observed - expected| - 1/2)^2}{expected}. \quad \text{(eq. 1)}$$

Gene mapping. The computer program MAPMAKER, version 1.9 (18) was run independently for crosses A and B to detect possible linkage groups in *C. carbonum*. A general description of the program was given by Lander et al (13). Because MAPMAKER was designed primarily for the analyses of diploid organisms, we treated our data as though they came from a (diploid) backcross (BC_1), which would segregate in the same way as the F_1 intercross of a haploid organism.

MAPMAKER first ran a two-point analysis to compute two-point recombination fractions and maximum LOD scores between all pairs of loci in the data file. The LOD score is the traditional measure of two-point linkage and is defined as "the $\log_{10}$ of the ratio of the likelihoods when the loci are taken to be at their maximum likelihood recombination fraction and when the loci are taken to be unlinked" (13). For example, if two loci have a LOD score of 3.0, it is 1,000 times more likely that the loci are linked rather than unlinked.

Next, MAPMAKER computed linkage groups from the two-point data. We selected 0.40 as the linkage threshold and 1.8 as the LOD-score threshold (i.e., loci were considered linked if the recombination fraction was <0.40 and the LOD score was >1.8). The choice of LOD-score threshold depends on the number of marker loci analyzed and the desired level of statistical significance (12). We then performed three-point analyses to confirm each of the three-point orders in the best map suggested from two-point analyses.

The linkage map with greatest statistical significance was saved,

and program MAPMAKER/QTL, version 0.9 (19) was run for sporulation and lesion-size data to identify and map any quantitative trait loci (QTLs). Assuming purely quantitatively inherited traits, MAPMAKER/QTL expects a normal distribution of the trait (complications arising from cosegregating major genes are discussed later). The data for sporulation and lesion size were skewed, so we applied square-root and log-transformation functions (30). The transformed data were approximately normally distributed. It was necessary to define another LOD threshold as a criterion for statistical significance of a QTL. Lander and Botstein (12) suggested two approaches to determine this value. The first applies to dense (e.g., restriction fragment length polymorphism [RFLP]) linkage maps and the second to the "sparse-map" case, which is dealt with here. According to Lander and Botstein (12), "To achieve an overall significance level of α when M intervals are tested, a nominal significance level of α/M should be required for each individual test, corresponding to a LOD threshold of $1/2$ ($\log_{10}e)(Z_{\alpha/M})^2$." Z^2 is approximately χ^2 distributed with 1 df. Here, M = 14 and α = 0.05, resulting in a LOD threshold of approximately 1.8. MAPMAKER/QTL scanned each linkage group and, in 2-cM (centiMorgans) intervals, calculated the log-likelihood for the presence of a QTL at that position (interval mapping). For example, a LOD score of 4.0 for a map position indicates that it is 10,000 times more likely that there is a QTL at that position than that there is none.

RESULTS

Inheritance of fungicide tolerance. Segregation data from cross B (Table 1) corroborate previous reports of monogenic control of sensitivity to cycloheximide in *C. carbonum* (16,21). Progeny from cross A segregated 1:1 for tolerance to cadmium as previously reported (16,21), but from cross B, there was a preponderance of sensitive progeny (Table 1), which caused a significant ($P < 0.05$) χ^2 value of 5.376 for the combined data of crosses A and B. This deviation might have been due to segregation distortion, low viability of Cad-R ascospores, or a second gene for cadmium sensitivity. It seems most likely, however, that there is a single locus with two alleles (*Cad-R* and *Cad-S*) controlling tolerance to cadmium as previously reported from the race 2 $\times$ race 2 crosses of MacKenzie et al (21) and Leonard (16).

Inheritance of isozymes. PGM, DIA, and MDH banding patterns clearly indicate segregation at single loci with two alleles (Table 1). The genetic interpretation of GOT and EST banding patterns is more difficult. The parental isolates in both cross A and cross B either expressed or did not express band GOT-B62 (Table 1), but all four had band GOT-B100. A single locus with two alleles cannot explain this situation. It is likely that there are two loci: one with a single allele for the GOT-B100 band and one with an allele for the GOT-B62 band. A second allele at the second locus could be either a null allele or an allele for another GOT-B100 band (33). However, for further analysis we ignored the fast band. Because the slow band (GOT-B62) segregated 1:1 (Table 1), we assumed two alleles at the second locus: one for GOT-B62 and the other with an unknown function.

We found seven different EST phenotypes among the progeny (Table 2). Their frequency distributions were not significantly different among the two crosses (χ^2 = 4.287, df = 6, P = 0.50) suggesting that sampling errors were limited. Bands EST-B100 and EST-B80 were expressed by all progeny and, therefore, were ignored in further analysis. If the other four bands are due to single, distinct loci, each band should have segregated 1:1. The data, however, support this interpretation only in the case of EST-B97, whereas for EST-B92, EST-B87, and EST-B73 the segregation pattern deviated significantly from 1:1 (Table 1). Therefore, our designations of the respective EST "loci" as *Est-B100* through *Est-B73* are tentative.

Inheritance of pathogenicity. The reported polygenic control of linear lesions typical of race 3 of *C. carbonum* (1,15) in race 2 $\times$ race 3 crosses might suggest a polygenic basis for the production of round or oval lesions in race 2. However, in both of our race 0 $\times$ race 2 crosses, we found a bimodal distribution

TABLE 1. Parental genotypes and segregation of genetic markers among progeny of two crosses between races 0 and 2 of *Cochliobolus carbonum*

	MAT		Cyh		Cad		Pda		Psu2		Asc2		Pgm		Dia		Mdh2		Got-B	Est-B			
Putative allele[a]	1	2	R	S	R	S	+	−	+	−	+	−	108	100	100	60	56	33	62	97	92	87	73
Parental genotype[b]																							
Cross A																							
12-12Bz	1	0	1	0	1	0	0	1	0	1	0	1	1	0	0	1	1	0	1	0	0	1	0
yug y	0	1	1	0	0	1	1	0	1	0	1	0	0	1	1	0	0	1	0	1	1	0	1
Cross B																							
12-24Bz	0	1	1	0	1	0	0	1	0	1	0	1	1	0	0	1	1	0	1	0	0	1	0
13-16Bz	1	0	0	1	0	1	1	0	1	0	1	0	0	1	1	0	0	1	0	1	1	0	1
Progeny																							
Cross A	9:19		...		17:17		13:20		15:13		7:8		13:18		16:15		18:14		13:14	14:16	24:6	18:12	17:13
Cross B	29:17		22:26		13:35		23:25		29:19		12:17		18:27		19:25		21:23		20:24	17:23	31:9	27:13	29:11
Total	38:36		22:26		30:52		36:45		44:32		19:25		31:45		35:40		39:37		33:38	31:39	55:15	45:25	46:24
χ^2 values[c]	0.014		0.188		5.376		0.790		1.592		0.568		1.420		1.080		0.013		0.225	0.700	21.73	5.157	6.300

[a] Allele designations given in text.
[b] Isolates 12-12Bz and 12-24Bz are race 0; yug y and 13-16Bz are race 2.
[c] Significant deviations from 1:1 ratios are indicated by $\chi^2 > 3.84$ (df = 1, $P = 0.05$) computed for the sums of progeny from crosses A and B.

TABLE 2. Esterase phenotypes among parents and progeny from two crosses between races 0 and 2 of *Cochliobolus carbonum*

R_f	Parent phenotypes I[b]	Parent phenotypes IV[c]	Progeny phenotypes[a] I	II	IV	VII	IX	X	XI
100	-	-	-	-	-	-	-	-	-
97	-		-			-			
92	-		-	-		-		-	-
87		-	-	-	-	-	-		
80	-	-	-	-	-	-	-	-	
73	-		-	-			-		
Frequency									
Cross A			11	11	2	4	7	3	2
Cross B			9	5	3	2	3	5	3

[a] Additional phenotypes occur among field isolates of *C. carbonum* (33).
[b] Expressed by race 2 parents yug y and 13-16Bz.
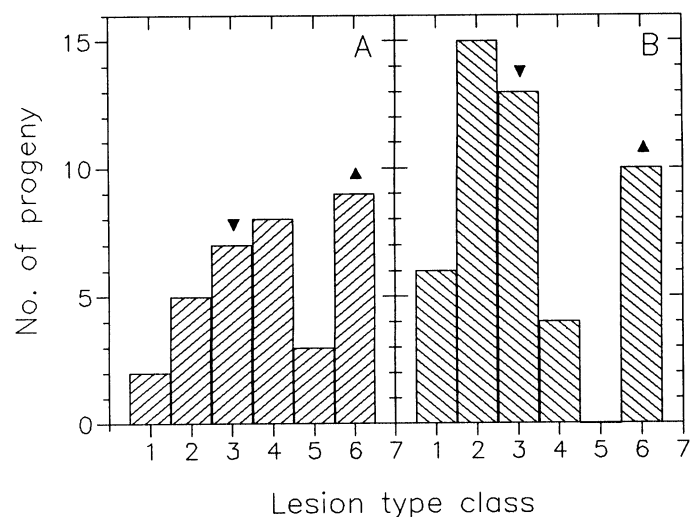[c] Expressed by race 0 parents 12-12Bz and 12-24Bz.

Fig. 1. Segregation for lesion type in two crosses of race 0 × race 2 of *Cochliobolus carbonum*: A, 12-12Bz × yug y and B, 12-24Bz × 13-16Bz. Triangles indicate types of lesions produced by race 0 parents (downward pointing triangles) and race 2 parents (upward pointing triangles). Lesion type was rated from 1 to 6: 1 = no reaction and 6 = average race 2-type lesions (i.e., round to oval necrotic lesions 1-2 mm in diameter).

of lesion sizes among progeny rather than a continuous distribution typical of polygenic inheritance (Fig. 1). In fact, our data suggest the action of two major genes in race 2 isolates yug y and 13-16Bz. When progeny that induced lesion types 1-5 were combined in an "avirulent class" and compared with progeny that induced lesion type 6 as the "virulent class," the segregation was 25:9 avirulent:virulent in cross A and 38:10 avirulent:virulent in cross B. These ratios fit a 3:1 segregation pattern ($\chi^2 = 0$, $P = 1.0$ in cross A; $\chi^2 = 0.250$, $P > 0.50$ in cross B). It appears that two major "virulence alleles" in isolates with lesion type 6 were complementary. The continuous distribution of progeny with lesion types 1-5 in both crosses could be due to minor genes. Most of the avirulent progeny induced either no lesions or lesions similar to the type-3 flecks/lesions of the race 0 parents that should have neither of the two major virulence alleles. If the two virulence alleles acted additively, intermediate lesion-type classes 4 and 5, induced by singular virulence alleles, should have been more frequent (i.e., as great as 50%) than they were (Fig. 1).

Inheritance of reproductive traits. Mating type (MAT-1 and MAT-2) and ability to sporulate on PDA (PDA+ and PDA−) were clearly inherited as single genes (Table 1). The ability to produce pseudothecia in races 2 and 3 was conditioned by a single gene (16,23), which we designated *Psu+*. All four parental isolates in our race 0 × race 2 crosses produced black pseudothecia (Psu+) in matings with albino tester isolates. Therefore, it was unexpected that the progeny in each cross segregated for pseudothecium production (Table 1; Fig. 2). Pseudothecium production was sharply skewed with half or more of the progeny producing few or no pseudothecia (Fig. 2), suggesting the presence of a second pseudothecial locus (*Psu2*) controlling reduced capacity for pseudothecium production. Apparently, race 0 isolates 12-12Bz and

12-24Bz (mean pseudothecium production 13.3 and 11.7 per mating) both have the *Psu2−* allele at this locus, whereas the race 2 parents, yug y and 13-16Bz (means 211.5 and 90.4), have *Psu2+*. With this assumption, the segregation pattern in both crosses supports the hypothesis of the second pseudothecial locus (Table 1). Progeny of cross A were scored as *Psu2+* if they produced >13.3 black pseudothecia in matings with albino tester isolates, whereas progeny of cross B that produced >11.7 black pseudothecia were rated *Psu2+*.

The ability of pseudothecial (Psu+) isolates to form asci and ascospores was controlled by a single locus (2), which we designated *Asc*. Although all four parental isolates were *Asc+*, the progeny of each cross segregated 1:1 for ascus production (Table 1), suggesting the presence of a second locus, *Asc2*. An *Asc2−* allele in race 0 at this putative locus may condition a reduced ability to produce asci and ascospores that is somehow accentuated to prevent ascospore production in race 0 × race 2 hybrids with *Asc2−*.

Although we interpreted the data from progeny mating tests as indicating a second major gene locus (*Psu2*) for pseudothecium production in *C. carbonum*, Figure 2 shows that part of the segregation for pseudothecium numbers may be due to genes with

small additive effects. The frequency distribution was clearly skewed; most of the presumed *Psu*+ progeny had low fertility (Fig. 2, filled bars), suggesting epistasis. Coadapted gene complexes in the more fertile race 2 parents may have been broken up in these "wide crosses" with race 0 isolates.

Conidium production also was skewed, with most progeny exhibiting sparse sporulation on both PDA and PLA (Figs. 3 and 4). This skewed distribution suggests epistasis in coadapted gene complexes for sporulation in the parental isolates. This interpretation seems reasonable even for sporulation on PDA where approximately half the progeny had the leathery cultural morphology and produced few or no conidia due to the *Pda—* allele (20 progeny in cross A and 25 progeny in cross B; Figs. 3 and 4). Lactose was a better carbohydrate source than was dextrose for sporulation by progeny as well as by both the race 0 and the race 2 parents (Figs. 3 and 4). The race 2 parents produced more conidia than did race 0, which is consistent with our report of the significantly superior sporulating ability of race 2 from field collections of races 2 and 0 (34).
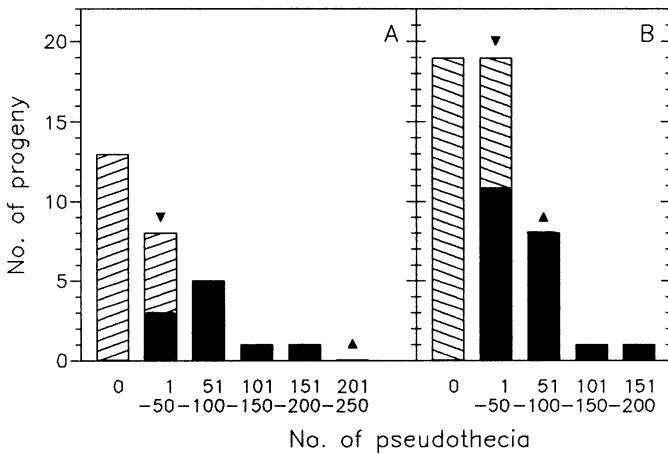
Mapping marker genes. Cross A produced 34 progeny with 13 segregating marker loci (Table 1). MAPMAKER found three small linkage groups comprising two loci each (Fig. 5). LOD scores for two linkage groups, designated Ia and II, were high, indicating very reliable inferences of linkage. Cross B produced 48 progeny with 14 segregating loci. MAPMAKER confirmed the linkage relationships found in cross A and showed a larger

Fig. 4. Segregation for conidium production in cross B of race 0 × race 2 of *Cochliobolus carbonum*. Triangles indicate numbers of conidia produced on potato-dextrose agar (PDA) (open triangles) or potato-lactose agar (PLA) (filled triangles) by the race 0 parent 12-24Bz (downward pointing triangles) and the race 2 parent 13-16Bz (upward pointing triangles). The solid bar segment indicates progeny carrying allele *Pda—* from race 0 for leathery colony morphology and inability to sporulate on PDA.

Fig. 2. Segregation for pseudothecium production in two crosses of race 0 × race 2 of *Cochliobolus carbonum*: **A,** 12-12Bz × yug y and **B,** 12-24Bz × 13-16Bz. Triangles indicate numbers of pseudothecia produced by race 0 parents (downward pointing triangles) and race 2 parents (upward pointing triangles). Filled bars represent progeny with putative allele *Psu2*+; hatched bars represent progeny with putative allele *Psu2—* causing the elimination or marked reduction of progeny's ability to produce pseudothecia.
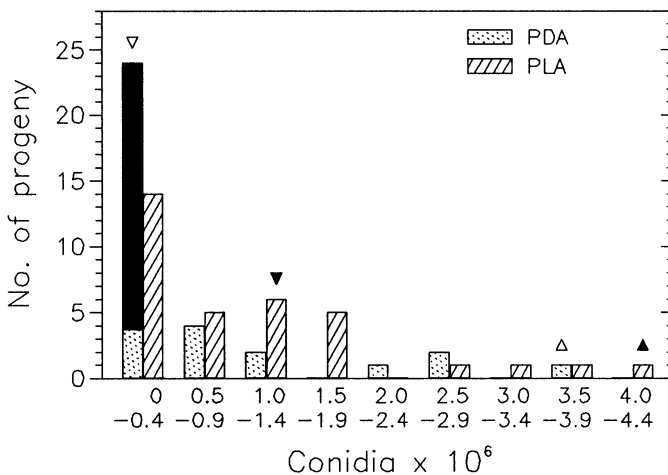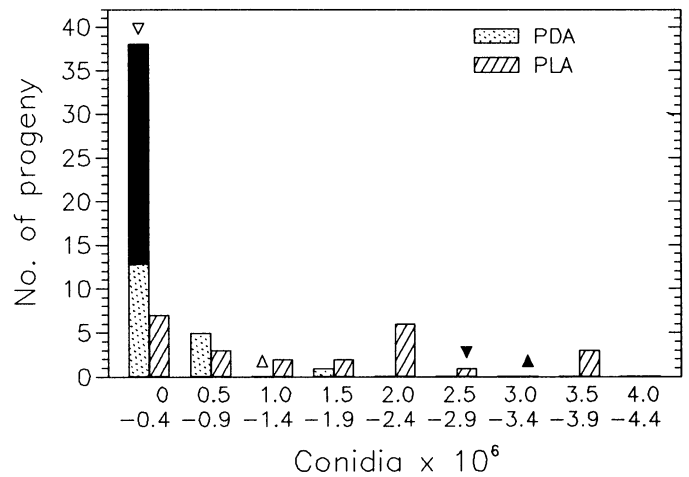
Fig. 5. Linkage groups identified by MAPMAKER for segregating loci of *Cochliobolus carbonum* in cross A (*n* = 34 progeny) and cross B (*n* = 48). Recombination fractions (RF) and LOD scores as determined by two-point analysis are indicated to the left between indicated loci. Loci were considered linked if their LOD scores ≥1.8 and RF < 0.40.

Fig. 3. Segregation for conidium production in cross A of race 0 × race 2 of *Cochliobolus carbonum*. Triangles indicate numbers of conidia produced on potato-dextrose agar (PDA) (open triangles) or potato-lactose agar (PLA) (filled triangles) by the race 0 parent 12-12Bz (downward pointing triangles) and the race 2 parent yug y (upward pointing triangles). The solid bar segment indicates progeny carrying allele *Pda—* from race 0 for leathery colony morphology and inability to sporulate on PDA.
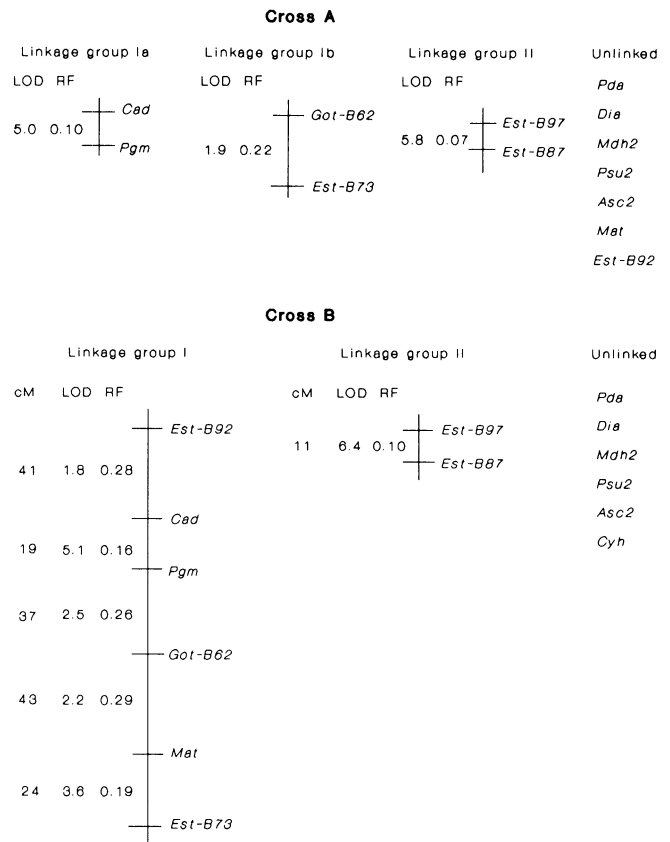
association of six loci in linkage group I. The sequence of loci in linkage group I (Fig. 5, cross B) had a log-likelihood of −50.06. The next best order attained a value of −50.99. Thus, the order suggested in Figure 5 was at least $10^{0.93} = 8.5$ times more likely than any other order. The greater number of linkages found in cross B than in cross A probably was due to the larger number of progeny analyzed and the smaller fraction of missing values in cross B (9.5 versus 14.0%). Six of 14 marker loci were unlinked. These included four established loci: *Pda, Dia, Mdh2,* and *Cyh*; and two putative loci: *Psu2* and *Asc2*.

Mapping QTLs. Conidium production on PLA and lesion size were analyzed for QTLs. Sporulation on PDA and pseudothecium production were not analyzed, because of the limited data sets. Cosegregation of major genes *Pda* and *Psu2* affecting these traits caused about 50% of the data to be lost from the already small number of progeny per cross. The distribution of raw sporulation data (PLA) was negatively skewed, i.e., to the left, for both crosses (Figs. 3 and 4). Log-transformation of data from cross A and square-root transformation of cross B data resulted in distribution parameter values considerably improved toward normality (cross A: skewness −0.31, kurtosis −1.10; cross B: skewness −0.13, kurtosis −1.11).

No significant QTL was found for the transformed conidium-production data from cross A when applied to the linkage map from cross B. The maximum LOD scores were 1.25 in linkage group I and 0.49 in linkage group II, respectively. With transformed sporulation data from cross B, however, a highly significant QTL was detected in linkage group II, with its most likely position 0–2 cM away from the presumed *Est-B97* locus. Its LOD score was 3.29 at that map interval, and 59.7% of the variance of sporulation could be explained by the presence of the QTL. However, over the full 11-cM interval between *Est-B97* and *Est-B87*, the LOD score in cross B was significant (LOD min. = 2.55). It is difficult, therefore, to state exactly where the QTL is located. A much denser genetic map and more progeny would be required to reveal this location. Values for cross A in the map interval 0–2 cM from the presumed *Est-B97* locus were only 0.35 and 5.1%.

Because the most likely position of the QTL mapped directly to *Est-B97*, a simple Student's *t* test provided another test of
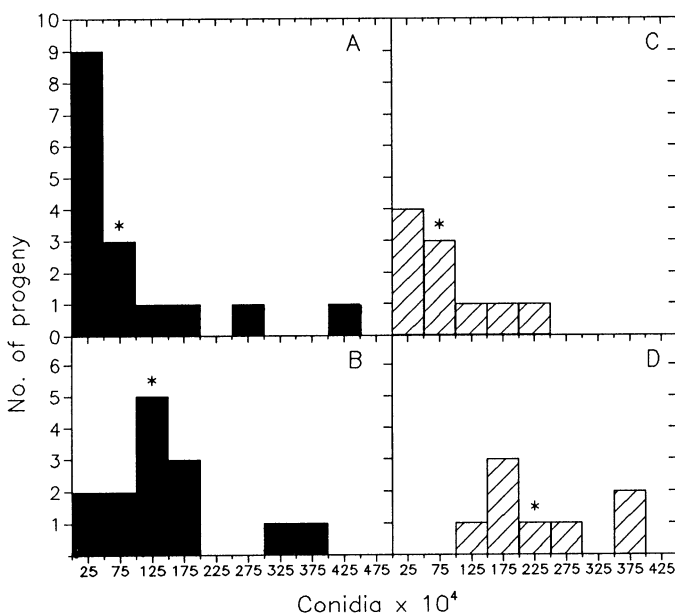
its significance by comparing the sporulation of phenotypes with and without the esterase band at $R_f = 97$. For cross A, this difference was not significant, and there was no obvious difference in the frequency distributions for progeny from cross A with and without the band (Fig. 6A and B). Progeny without the band (Fig. 6A) produced 0.87×10^6 conidia, whereas progeny with the band (Fig. 6B) produced 1.37×10^6 conidia (Fig. 6B) ($t = 1.203$, $P > 0.20$, df = 28). For cross B, the difference was highly significant ($P < 0.001$, $t = 4.456$, df = 16), and the frequency distribution was shifted toward greater sporulation by progeny with EST-B97 (Fig. 6D) than by progeny without it (Fig. 6C). Mean conidium production of progeny with EST-B97 from the race 2 parent was 2.47×10^6 versus 0.79×10^6 for progeny without the band.

As already described, segregation data from crosses A and B indicated the presence of two complementary loci for pathogenicity on corn (Fig. 2). When we eliminated all progeny carrying both pathogenicity alleles (i.e., lesion-type class 6 in Fig. 1) from the data files, we found an approximately normal distribution of lesion sizes in cross A (skewness −0.23, kurtosis −0.75) and cross B (skewness 0.09, kurtosis −0.69). Therefore, we did not transform the data. No significant QTL for lesion size was detected in these two linkage groups in either cross A (LOD max. = 0.59 and 0.08 in linkage groups Ia and II, respectively) or cross B (LOD max. = 0.83 and 0.38 in linkage groups I and II, respectively). Also, there was no association between presence of EST-B97 and the mean lesion size among progeny with lesion-type classes 1–5 in either cross A ($t = 1.403$, $P > 0.10$, df = 20) or cross B ($t = 0.810$, $P > 0.25$, df = 28).

Another way to infer linkage relationships or pleiotropic effects of genes is to identify positive correlations among traits. We found that lesion size, pseudothecium production, and sporulation on PDA and PLA were positively correlated (Table 3). Most of these correlations were in the intermediate range and were statistically significant. For progeny from both crosses, the highest correlation was between sporulation on PDA and PLA, and the weakest correlation was between sporulation on PDA and pseudothecium production. The greatest difference between the two crosses was in the correlation between lesion size and sporulation on PLA. This correlation was not significant for the cross B progeny ($r = 0.26$, $P = 0.226$) but was significant for the cross A progeny ($r = 0.46$, $P = 0.006$). Lesion size was significantly correlated with pseudothecium production in both crosses (Table 3).

DISCUSSION

Although race 0 appears to be distantly related to races 1, 2, and 3 of *C. carbonum* (9,33), the monogenic traits mating type (22) and cycloheximide tolerance (16,21) segregated in normal 1:1 ratios among progeny of race 0 × race 2 crosses. Cadmium tolerance, another monogenic trait (16,21), segregated in a 1:1 ratio in cross A, but the segregation appeared distorted in cross B (Table 1). The reason for this distortion is unknown, but it seems significant that for this and all the other markers in linkage group I (Fig. 5), cross B yielded more progeny with alleles from the race 2 parent than from the race 0 parent (Table 1).

Fig. 6. Frequency distribution of conidium production on potato-lactose agar among progeny from two crosses of races 0 and 2 of *Cochliobolus carbonum*: **A,** cross A, 12-12Bz × yug y progeny that did not express isozyme band EST-B97; **B,** cross A, 12-12Bz × yug y progeny that expressed isozyme band EST-B97; **C,** cross B, 12-24Bz × 13-16Bz progeny that did not express EST-B97; and **D,** cross B, 12-24Bz × 13-16Bz progeny that expressed EST-B97. Mean conidium production for each group of progeny is indicated by an asterisk.

TABLE 3. Coefficients of correlation between lesion size (LS), pseudothecium production (Psu), and conidium production on potato-dextrose agar (ConPDA) and potato-lactose agar (ConPLA) by progeny from two crosses[a] between races 0 and 2 of *Cochliobolus carbonum*[b]

	LS	Psu	ConPDA	ConPLA
LS		0.40*	0.31+	0.46**
Psu	0.45**		0.27ns	0.39*
ConPDA	0.31*	0.16ns		0.49**
ConPLA	0.26ns	0.26ns	0.63***	

[a] Correlation coefficients for cross A (12-12Bz × yug y; $n = 34$) are in the upper right half of the table; those for cross B (12-24Bz × 13-16Bz; $n = 23$) are in the lower left half of the table.

[b] +, *, **, and *** indicate significance at $P < 0.1$, 0.05, 0.01, or 0.001, respectively; ns indicates not significant.

Our previous observations of isozymes in field populations of *C. carbonum* (33) are consistent with the genetic data from the race 0 × race 2 crosses. Tests of field isolates suggested that PGM and DIA are controlled by single loci with two and three alleles, respectively. The inheritance of PGM and DIA isozymes in this study confirmed those conclusions. In the field study, expression of the apparently tetrameric MDH isozyme by most race 2 and 3 isolates suggested the presence of two loci, *Mdh1* and *Mdh2* (33). We were unable to test the inheritance of *Mdh1* in this study, because all four parental isolates had the same allele, *Mdh1-100*. However, the 1:1 segregation of bands at R_f = 56 and 33 supports the conclusion of a *Mdh2* locus with at least two alleles.

Two loci must be postulated for the observed GOT banding patterns. The isozyme patterns among field isolates (33) could be explained by a duplication of the *Got* locus for R_f = 100. The GOT-B100 band consistently stained more intensively when it occurred alone than when it occurred together with band GOT-B62. Such heavily stained single bands may result from a duplicated locus. The isolates with two bands may have a normal allele for GOT-B100 at the first locus, giving a weakly staining fast band and a mutated allele for GOT-B62 at the duplicated locus. The 1:1 segregation for GOT-B62 and the universal presence of GOT-B100 among the race 0 × race 2 progeny agrees with this interpretation. Another possibility is that all isolates had the *Got-B100* allele at one locus, whereas at the second locus race 2 parents yug y and 13-16Bz had a null allele and race 0 parents 12-12Bz and 12-24Bz had the *Got-B62* allele. This explanation, however, does not account for the darker staining fast band for those isolates that did not express GOT-B62.

The genetic basis of esterase banding patterns remains uncertain. Our data allow two conclusions. First, the four segregating bands (Table 2) were not the product of different alleles at the same locus or loci, because recombinant types occurred. Furthermore, they mapped to different loci (Fig. 5). For example, the two presumed loci, *Est-B97* and *Est-B87*, are closely linked (RF = 0.07 and 0.10; Fig. 5), but phenotype VII is a recombinant type (Table 2). Second, the highly significant χ^2 value for band EST-B92 suggests that it is not due to an independent allele at a single locus but may belong to an oligomeric EST isozyme. Several other esterase bands outside the R_f range from 100 to 73 were observed frequently, but they usually were weakly expressed (33). Esterase banding patterns are complex in many organisms (7), because esterases are nonspecific hydrolytic enzymes involved in many different biological pathways.

Pathogenicity of both race 2 isolates to corn was inherited by two apparently complementary genes, which the two race 0 parents lacked. This result is similar to those of Nelson (24) and Nelson and Kline (25,26) who found that pathogenicity of *C. carbonum* to some grass species was inherited as a single gene trait, but to other grass species, pathogenicity was controlled by two complementary genes. Nelson (24) crossed an isolate of *C. carbonum* pathogenic to corn, which he isolated from barley, with two *C. carbonum* isolates from *Avena* and *Ixophorus* spp. that were not pathogenic to corn. Progeny from each cross segregated in a 1:1 ratio, indicating a single gene for pathogenicity to corn in the barley isolate. Nelson's (24) putative gene for pathogenicity to corn differs from the virulence gene that controls production of race 1 toxin. The race 1 toxin gene confers a high level of virulence to toxin-sensitive corn lines, as demonstrated by Scheffer et al (27) for crosses of *C. victoriae* × race 1 of *C. carbonum*. Because Nelson's isolates are no longer available, we cannot determine whether either of our two complementary genes for pathogenicity to corn was present in Nelson's avirulent isolates or whether his barley isolate had both of our pathogenicity genes, with one being the pathogenicity gene he identified.

Inheritance of pathogenicity in race 0 × race 2 crosses differs from the inheritance of lesion type in crosses between races 2 and 3 of *C. carbonum*. In race 2 × race 3 crosses, Dalmacio et al (1) and Leonard (15) found a continuous distribution from the round or oval lesions typical of race 2 to the long linear lesions typical of race 3. They concluded that lesion type in race

3 is inherited as a quantitative trait. Race 3 likely has the same two major pathogenicity genes as race 2 in addition to polygenes that control the elongated shape of race 3 lesions. Previously we found much greater genetic similarity between races 2 and 3 than between race 0 and races 2 and 3 (33,35). The complementary pathogenicity gene action in race 2 has an interesting parallel to the complementary effects of toxic constituents in race 3. Xiao et al (36) recently reported that race 3 produces a toxin made up of at least three constituents. These constituents induce lesions in corn and rice leaves when applied together but not separately.

We found that the unique leathery colony morphology of nearly all race 0 isolates and their failure to sporulate on PDA is controlled by a single allele, *Pda−*, which is rare in race 2 and has not been found in race 3 of *C. carbonum* (35). We also found evidence of a second major locus controlling pseudothecium production. The new gene, which we tentatively designated *Psu2−*, appears to be hypostatic to a previously identified gene at the *Psu1* locus (our designation) required for pseudothecium formation (23). A cross of race 0 isolates 12-12Bz × 12-24Bz provides additional evidence for the *Psu2* locus. The cross yielded a few light-colored pseudothecia but no typical black pseudothecia, suggesting that *Psu2+* must be present in at least one parent to stimulate normal development and pigmentation of pseudothecia (H. G. Welz and K. J. Leonard, *unpublished data*). Both 12-12Bz and 12-24Bz produced fertile black pseudothecia in crosses with albino race 2 tester isolates.

The number of pseudothecia produced by isolates of *C. carbonum* is probably further affected by unknown polygenes. Kolmer and Leonard (10) demonstrated that the mean number of pseudothecia produced in matings among compatible isolates in the related species *C. heterostrophus* is under polygenic control. In six generations of selection, they increased the mean number of pseudothecia per mating by four times. Kolmer and Leonard (10) estimated a minimum of five to seven loci controlling pseudothecium numbers in addition to the major gene locus for pseudothecium production.

Evidence for a second locus, *Asc2*, controlling the production of asci and ascospores in *C. carbonum* is not as clear as that for the second pseudothecial locus, *Psu2*, because we did not quantitatively measure numbers of asci or ascospores produced in pseudothecia formed by parents and progeny from the race 0 × race 2 crosses. Nevertheless, we did find that approximately half of the progeny that formed black pseudothecia in test matings did not produce asci or ascospores in those pseudothecia. This is unusual, because all four parental isolates did form asci and ascospores in similar test matings. Apparently, the gene(s) that control ascus and ascospore production in races 0 and 2 do not function well in the mixed genomes of race 0 × race 2 hybrids. This might depend on matching *Asc1* and *Asc2* alleles. For example, *Asc1+,Asc2+* pseudothecia may produce many asci; *Asc1−,Asc2−* pseudothecia may produce few asci; and *Asc1+,Asc2−* or *Asc1−,Asc2+* pseudothecia may produce no asci. The *Asc2−* allele may be limited to race 0, because the *Asc2* locus has not been detected in any crosses of races 1, 2, or 3 of *C. carbonum* (1,2,16,22,23). We were not able to map either *Asc2* or *Psu2*, so their status is still tentative.

The skewed distribution of progeny toward low production of pseudothecia and conidia may be interpreted as "recombination loss" (3) resulting from epistasis in the parental isolates. Coadapted gene complexes in the race 2 parents may have been broken by recombination in "wide crosses" to race 0. A similar effect frequently limits the utilization of "wide crosses" in plant breeding where it counteracts heterosis (5). This explanation takes into consideration the genetic distance between races 0 and 2 observed in natural populations of *C. carbonum* (9,33,35). Evidence of effects of gene complexes in determining numbers of pseudothecia in the congeneric fungi *C. spiciferus* (32) and *C. heterostrophus* (11) has been reported.

We were able to construct a small linkage map from the segregating progeny of cross B that spanned eight loci and 175 cM. Fewer linkages were found for cross A than for cross B, probably

because of the smaller number of progeny and greater proportion of missing values in cross A. About half the loci we identified could not be placed in either of two linkage groups. This is not surprising, because recent studies indicate that the haploid number of chromosomes in the related species *C. heterostrophus* is probably 16 (31). To map all the chromosomes would require a much greater number of genetic markers, such as RFLP or other polymorphic DNA markers.

In previous experiments, Leonard (16) found that the *Cyh* locus for cycloheximide tolerance was not linked to loci for mating type (*Mat*), albinism, brown conidia, lavender pigment, or carboxin tolerance. The race 0 × race 2 crosses also gave no evidence of linkage between *Cyh* and *Mat*. Previously, Nelson (24) found evidence of linkage between *Mat* and a locus for pathogenicity to *Cynodon dactylon* in *C. carbonum* and linkage among loci for pathogenicity to *Eleusine indica, Oryza sativa,* and *Axonopus affinis*. Nelson and Kline (25,26) also found linkage among loci for pathogenicity to *Poa pratensis, Lolium multiflorum,* and *Festuca elatior* in crosses of *C. carbonum* × *C. victoriae*.

In our previously described survey of two natural populations of *C. carbonum*, we found no significant gametic phase disequilibria among the phenotypic markers for mating type, ability to sporulate on PDA, and tolerance to cycloheximide or cadmium (35). Results from the crosses of race 0 × race 2 indicate that loci for these traits are either unlinked or very weakly linked (e.g., *Cad* and *Mat*). Therefore, a low rate of sexual recombination each year should suffice to maintain these gametic phase equilibria.

With the MAPMAKER/QTL program, we identified a highly significant locus for ability to produce conidia on PLA within 2 cM of the esterase locus *Est-B97* in linkage group II. In the natural *C. carbonum* populations from which the race 0 parents and one race 2 parent were derived, 33% of race 0 isolates and 94% of race 2 isolates expressed band EST-B97 (33). Sporulation on PLA by race 0 isolates averaged 50% less than that of race 2 isolates from these populations (34). It seems likely that most race 0 isolates have the same detrimental allele at the sporulation QTL we found in the race 0 parents 12-12Bz and 12-24Bz. Among the race 3 isolates from these field populations, only 7% expressed esterase band EST-B97 (33), and sporulation on PLA by race 3 isolates averaged 13% lower than that of race 2 (34). Thus, this sporulation QTL may be lacking from most race 3 as well as race 0 isolates, or race 3 may have another allele less detrimental to sporulation than that of race 0. The apparent lack of genetic exchange between races 0, 2, and 3 (33,35) should maintain their differences in sporulation capacity due to the identified QTL if selection does not alter allele frequencies at the *Est-B97* locus and the associated QTL.

Although conidium production was significantly correlated with lesion size in progeny from cross A, we found no QTL for lesion size in the two linkage groups. Therefore, the QTL for sporulation could not account for the observed correlation. The positive correlations among race 0 × race 2 progeny for sporulation, lesion size, and pseudothecium production may reflect pleiotropism or a tendency for genes affecting these traits to remain associated. The race 0 parents rate low in all of these traits, and the race 2 parents rate high.

Hamid et al (6) measured lesion length and sporulation capacity per unit of lesion area for 20 race 3 isolates of *C. carbonum* on two cultivars of corn. From their data, we calculated that correlation coefficients between these traits were nonsignificant on both cultivars ($r = -0.21$, $P = 0.215$ for cv. Pa419P and $r = -0.35$, $P = 0.112$ for cv. Pa33). The lack of correlation between lesion length and sporulation is consistent with the observation of Lodge and Leonard (20) that sporulation does not occur in the lesions on intact plants until the leaves senesce or large areas of leaf tissue become necrotic. Then the fungus ramifies in the leaf tissue and sporulates outside the lesions. Welz et al (34) showed that race 2 isolates produced significantly more conidia on average than did race 3 isolates on autoclaved green or brown leaves of corn as well as on PLA. Thus, in field populations of *C. carbonum*, lesion size does not determine sporulating ability.

Welz et al and Welz and Leonard (34,35) showed that mean sporulation capacities of races 0, 2, and 3 of *C. carbonum* were significantly correlated with relative parasitic fitness as determined from changes in race frequencies in two field populations. Thus, the *Est-B97* allele, which is associated with increased sporulation capacity, also may be associated with increased parasitic fitness. Other things being equal, any close association with increased parasitic fitness should lead to an increase in frequency of the *Est-B97* allele. However, race 3 has persisted in North Carolina for many years in the presence of race 2 in spite of its lower parasitic fitness. Welz and Leonard (35) speculated that races 3 and 2 may occupy different niches during saprophytic survival in the absence of living corn plants. Similarly, the QTL for increased sporulation capacity may have pleiotropic affects on saprophytic fitness that prevent it from increasing its frequency in races 0 and 3. The possible association of *Est-B97* with fitness can be tested by sequentially sampling living corn leaves or crop debris in fields and testing the isolates for expression of *Est-B97*. Among the 24 isolates of race 0 collected on the first two sampling dates from a single field in North Carolina during 1987 (35), 18% from 9 July and 57% from 28 July expressed *Est-B97*, which supports the postulated association of *Est-B97* with parasitic fitness. Similar sampling of crop debris after crop maturity to test for a negative association of *Est-B97* with saprophytic fitness has not been done.

The results of this research show that races 0 and 2 of *C. carbonum* differ not only in pathogenicity to corn but also in their alleles at a number of major gene loci and in expression of polygenic traits. The skewed distributions for sporulation and pseudothecium production suggest that race 0 × race 2 hybrids may suffer reduced fitness from a breakup of coadapted gene complexes. Decreased fitness of hybrids could be one mechanism preventing gene flow between races 0 and 2 if crosses between these races occur in the field.

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