Influence of Naturally Occurring Marker Genes on the Ability of *Cochliobolus heterostrophus* to Induce Field Epidemics of Southern Corn Leaf Blight

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ABSTRACT

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Three loci in the plant pathogenic fungus *Cochliobolus heterostrophus* were evaluated in a common genetic background under field conditions: *Alb*1, which controls pigment production; *Cyh*1, which conditions relative sensitivity to cycloheximide; and *MAT*, which determines mating type. Albino isolates with no pigment (*alb*1) caused no measurable epidemic; $cyh1^R$ (resistant to cycloheximide) reduced the rate of epidemic development compared with $CYH1^S$ (sensitive to cycloheximide), but only in a *MAT*A background (there was no difference when alleles at *Cyh*1 were compared in a *MAT*a background); alleles at *MAT* had no consistent effect on epidemic development. The differences between alleles observed in the field were not readily apparent in growth chamber experiments.

The roles of specific genes in the population biology of plant pathogens are generally unknown, but are of considerable relevance to disease management. If a pathogen gene that confers high virulence (ability to overcome the effects of a specific host resistance gene) also has deleterious effects on the pathogen's fitness, then in the absence of conditions that select for virulence, the gene's frequency should decline in the pathogen population. The existence of this effect, which is referred to as "stabilizing selection" in the plant pathology literature (14), is fundamental to the success of disease management strategies such as regional gene deployment or multiline cultivars (1,9,12,15,16). However, there is no convincing evidence that any defined gene controlling virulence is subject to stabilizing selection in the field.

We have chosen *Cochliobolus heterostrophus* [anamorph: *Bipolaris maydis* (Nisikado and Miyake) Shoemaker = *Helminthosporium maydis* Nisikado and Miyake] as a model pathogen to study effects of single genes in the field. *C. heterostrophus* has several features favoring its adoption as a model: it can be cultured on defined medium, it can be genetically manipulated both sexually and asexually, mutations can be induced and recovered in culture, a stock of mutants is available, and it causes a single-season epidemic on its host, corn (*Zea mays* L.), in small field plots.

One approach to test critically the impact of a specific gene on pathogenic fitness is to construct strains of the pathogen that are genetically similar except for alternate alleles at the locus in question (11). Another approach is to use large heterogeneous populations in which the assessed gene is distributed randomly (10).

The purpose of the research reported here was to develop methods for quantifying the effects of mutant alleles on the ability of *C. heterostrophus* to induce epidemics in the field. We were interested in identifying alleles with two extremes of effect: alleles with minimum effect on epidemic development that can be used as neutral markers for future field studies and alleles that have inhibitory effect on epidemic development that can be used to render harmless individual isolates with recombinant DNA

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molecules. Both types of allele are needed to quantify with precision the epidemiological effects of genes that have been identified as pathologically significant in laboratory or greenhouse experiments.

MATERIALS AND METHODS

Isolates and production of inoculum. All isolates were genetically closely related to each other (Table 1), the essential differences among them being alternate alleles at single loci controlling pigment production (*Alb*1), mating type (*MAT*), and resistance to cycloheximide (*Cyh*1). Isolates were cultured on complete medium or potato juice agar, and stored on silica gel or in infected corn leaves (6). Inoculum for the 1979 field studies was produced by culturing the various isolates on autoclaved oat seeds in 500-ml flasks for 15–20 days. The colonized seeds were used to inoculate plants in the field. In 1980, inoculum was prepared by suspending in water containing 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) conidia from 10-day-old cultures growing on agar medium; the concentration was adjusted to 200 conidia per milliliter and plants in the field were inoculated with it. Each inoculated plant received 2 ml of inoculum. Inoculum for a competition experiment consisted of 2 ml of equal parts of conidial suspension from each of three isolates.

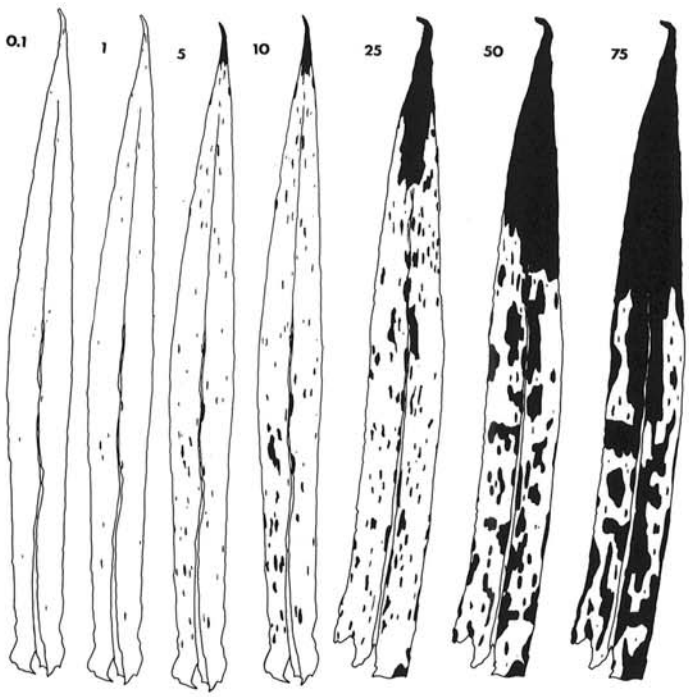

Fig. 1. Standard corn leaf diagrams illustrating various percentages infected by *Cochliobolus heterostrophus*. The diagrams were constructed as described in the text.

When conidial suspensions were inoculated onto corn plants in growth chambers (17), all isolates caused lesions that were indistinguishable from each other in terms of size, shape, color, and approximate number. However, we have observed that isolates with *alb*1 are killed much more readily by short wavelength UV light under laboratory conditions than are those with *ALB*1.

Crop husbandry. Corn seeds were planted in the field on 17 May 1979 and 22 May 1980; plants were thinned to a 15-cm spacing about 50 days after planting. Fertilizer (120 kg N, 100 kg K, and 52 kg P/ha) was applied at planting. Herbicides were atrazine (80 W, 0.56–0.84 kg [a.i.]/ha) and alachlor (4 E, 0.84 kg [a.i.]/ha) applied shortly after emergence. In 1979, carbofuran (10 G, 1.2 kg [a.i.]/ha) was applied at planting. Insecticide (diazinon 50 W at 0.25–0.50 kg [a.i.]/ha or carbaryl 50 W at 1.7 kg [a.i.]/ha) and fungicides (mancozeb 80 W at 1.8 kg [a.i.]/ha or benomyl 50 W at 2.24 kg [a.i.]/ha) were applied as needed to reduce the incidence of foliar insects and foliar fungal pathogens on seedlings. These pesticides were applied with a tractor-mounted, boom-type, hydraulic sprayer at a pressure of about 11.5 kg/cm^2 in a volume of 935 L/ha. Applications were halted at least 10 days prior to inoculation with *C. heterostrophus* so that inoculated tissue had been produced after the final fungicide spray.

After inoculation, plots were sprinkle-irrigated daily from 0730 to 0800 hr and from 1930 to 2000 hr at the rate of 2.5 mm/hr. Experimental units were plots in four randomized complete blocks. Plots were rectangular (2.7 × 1.8 m) and consisted of three rows, 0.9 m apart. Each plot of susceptible corn (inbred genotype B14A-Tms in 1979 and hybrid W64A-Tms × B14A in 1980) was surrounded by a fallow area (1.8 m wide) which was in turn surrounded by resistant corn (Agway 510 X with nonsterile cytoplasm). Distance between plots was 4.5 m along rows and 7.2 m perpendicular to rows.

Inoculation and disease assessment. In 1979, six colonized oat seeds were placed in the whorl of the center plant in each inoculated plot on 25 July. In 1980, 2 ml of conidial suspension was pipetted into the whorl of the center plant of each plot on 17 July; inoculation was repeated on 18 and 25 July. In both years, interplot

TABLE 1. Isolates of *Cochliobolus heterostrophus* used to study the development of epidemics in corn

Isolate	Genotype[a]	Description[b]	Year used
C4	*MAT*a	Progeny of a cross between isolates C2 (*alb*1;*MAT*A) and C3 (*MAT*a), each of which was recovered after six backcross generations to wild-type isolate C1 [b]	1979
C6	*alb*1; *MAT*A	Sibling of isolate C4	1979
C9	*MAT*A	Sibling of isolate C4	1979
C10	*cyh*1^R;*MAT*a	Cycloheximide-resistant mutant recovered after mutagenesis of isolate C2; it was backcrossed five generations to isolate C2 or C3.	1980
C11	*cyh*1^R;*MAT*A	Same cycloheximide resistance allele as described for isolate C10, except that it was backcrossed eight generations to isolate C2 or C3	1980
C12	*MAT*A	Sibling of isolate C11	1980
C13	*MAT*a	Sibling of isolate C11	1980

[a]The yeast convention (13) if followed for gene symbols. Loci are designated by three italicized letters; dominance and recessiveness are indicated by uppercase and lowercase letters, respectively; and drug resistance and sensitivity are denoted by the superscripts R and S, respectively. The mating type (*MAT*) locus is a special case where there are two alleles designated "A" and "a," both of which appear to be active. Both *alb*1 (albino) and *cyh*1^R (resistant to cycloheximide) were found to be recessive to the corresponding wild-type alleles by analyses of heterokaryons. All isolates produced T-toxin and therefore carried the *TOX* 1$^+$ allele at the previously defined *Tox* 1 locus (7). Isolates with the alternate allele, *tox* 1$^-$ (no detectable T-toxin produced), were not used in this study because we found in previous years that they cause very little disease under our experimental conditions.
[b]The derivation of isolates C1, C2, and C3 has been described (6,8).

interference (5) and/or contamination was detected by observing plots that had been left uninoculated.

Disease incidence was assessed every 7–14 days by using a set of standard diagrams (Fig. 1). The diagrams were constructed from leaves of corn plants naturally infected in the field. Leaves were photocopied and the areas of single and/or coalesced lesions were measured with a planimeter. Diagrams of leaves with 0.1, 1.0, 5.0, 25, 50, and 75% of the area affected (Fig. 1) were constructed. All leaves from the second below the first ear to the second below the tassel were assessed. Assessments were made on five plants: the inoculated plant and the two plants on each side of it in the row.

Analyses of variance were done on the arcsin transformation of the final proportion of tissue affected. Areas under the disease progress curve (AUDPC) (3) were analyzed after $\log_{10}$ transformation.

The composition of the pathogen population in experimental and control plots was analyzed shortly after the final disease assessment in each season. Leaves from several plants of the center row in each plot were removed in mid-September and pressed for storage. Isolations were made onto potato juice agar and mating types of isolates of *C. heterostrophus* were determined by pairing them with tester isolates (6); cycloheximide sensitivity was assessed on potato juice agar containing cycloheximide (100 μg/ml).

RESULTS

Disease induced by *alb*1 in the field in 1979 was barely detectable (Table 2); no lesions were found beyond the point of inoculation. The four *alb*1 isolates recovered from the plots at the end of the season (Table 3) were from the point of inoculation; all other isolates of *C. heterostrophus* recovered were *ALB*1, and therefore were presumably contaminants, since there was either interplot interference or contamination from outside the plots in 1979 (Table 2). Because of its inability to cause measurable disease in the field, *alb*1 was not assessed in 1980.

The relative effects of $CYH1^S$ and $cyh1^R$ on epidemic development were variable. When alleles at *Cyh*1 were compared in *MAT*a background (Table 2, 1979) there was no significant difference between $cyh1^R$ and $CYH1^S$ as measured by AUDPC. When the same comparison was made in *MAT*A background (Table 2, 1980), $CYH1^S$ caused more disease than $cyh1^R$, although $cyh1^R$ competed well with $CYH1^S$ when the two were together in a mixture of isolates (Table 3).

The effect of *MAT* on the epidemic-inducing potential of *C. heterostrophus* was inconsistent. In 1979, the epidemics induced by *MAT*a were less severe than those induced by *MAT*A (Table 2). In 1980, there was no significant difference between the epidemics induced by *MAT*a and *MAT*A (Table 2). However, in a plot inoculated with a mixture of equal numbers of three isolates, those with *MAT*a increased relative to those with *MAT*A (Table 3).

Interplot interference or background contamination was monitored each year. In the 1979 experiment, plants in uninoculated plots became measurably diseased (Table 2). Some inoculated plots were apparently contaminated, since certain isolates recovered at the end of the season carried markers different from those in the original inoculum (Table 3). In the 1980 experiment, uninoculated plots had no evidence of disease caused by *C. heterostrophus*, and at the end of the season inoculated plots contained only those genotypes composing the initial inoculum, with the possible exception of the plots that were inoculated with isolates with the $cyh1^R$ allele (Table 3). It is possible that the $CYH1^S$ isolates recovered from plots inoculated with $cyh1^R$ (Table 3) were not contaminants, but rather revertants, since we have observed occasional instability at the *Cyh*1 locus in culture (alleles at the *MAT* and *Alb*1 loci are very stable in culture).

The 1980 experiment included a plot inoculated with a mixture containing equal numbers of conidia from $CYH1^S$;*MAT*A, $CYH1^S$;*MAT*a, and $cyh1^R$;*MAT*A (200 conidia per milliliter, 2 ml per plot). Disease was assessed throughout the season and the composition of the pathogen population at the end of the season (9 September) was determined by sampling from lesions in the plot. The kinetics and magnitude of the epidemic induced by the mixture of isolates were similar to those for epidemics induced by either $CYH1^S$;*MAT*A or $CYH1^S$;*MAT*a alone, but were more severe than that induced by $cyh1^R$;*MAT*A (Table 2). The frequency of $cyh1^R$;*MAT*A and $CYH1^S$;*MAT*A each declined from 33% of the population at the beginning of the season to 19% at the end, whereas $CYH1^S$;*MAT*a increased from 33 to 62% (Table 3).

DISCUSSION

The small field plot technique permitted detection of differences not seen in growth chamber experiments. The most dramatic example of this is the *Alb*1 locus. Under artificial lights in a growth chamber, isolates bearing *alb*1 or *ALB*1 cause approximately equal numbers of similar-sized lesions. In the field, *alb*1 caused essentially no disease. Similarly, we observed no difference between lesions caused by $CYH1^S$ or $cyh1^R$ or between *MAT*A and *MAT*a in a growth chamber, but differences between alleles at the *Cyh*1 locus were sometimes detectable in the field. The sensitivity of the field assay, which involves several cycles of infection, may be greater than the single-cycle growth chamber assay. Alternatively, selection pressures in the field may differ from those in the growth chamber. The results of our field experiments are consistent with

TABLE 2. Severity of epidemics caused by isolates of *Cochliobolus heterostrophus* in small field plots of corn

	AUDPC[a]	
Genotype	1979	1980
*ALB*1;$CYH1^S$;*MAT*A	9.16 X[b]	4.89 A
*ALB*1;cyh^R;*MAT*A	···	2.04 B
*ALB*1;$CYH1^S$;*MAT*a	3.52 Y	4.70 A
*ALB*1;$cyh1^R$;*MAT*a	2.84 Y	···
*alb*1;$CYH1^S$;*MAT*A	0.71 Z	···
Mixture[c]	···	3.48
Uninoculated	0.33 Z	0

[a] Area under the disease progress curve (AUDPC) for the five center plants in the middle row. Units are proportion-days. Analyses were performed using the $\log_{10}$ transformation of the AUDPC.

[b] Numbers within a column followed by a letter in common are not significantly different ($P = 0.05$) according to Duncan's multiple range test. Standard errors were 0.166 and 0.144 in 1979 and 1980, respectively.

[c] The mixture inoculum consisted of equal numbers of $cyh1^R$;*MAT*A, $CYH1^S$;*MAT*A, and $CYH1^S$;*MAT*a (200 conidia per milliliter, 2 ml per plot). It was applied to a single plot only and the resulting AUDPC was not considered in the statistical analysis.

TABLE 3. Composition of *Cochliobolus heterostrophus* populations isolated from small plots of corn at the end of the growing season

Inoculum	*C. heterostrophus* isolates recovered (no.)		
1979[a]	*alb*1;$CYH1^S$	*ALB*1;$CYH1^S$	*ALB*1;$cyh1^R$
*alb*1;$CYH1^S$;*MAT*A	4	25	0
*ALB*1;$CYH1^S$;*MAT*a	0	14	3
*ALB*1;$cyh1^R$;*MAT*a	0	6	16
Uninoculated	0	28[b]	0
1980	cyh1^R;*MAT*A	$CYH1^S$;*MAT*A	$CYH1^S$;*MAT*a
$cyh1^R$;*MAT*A	38	4	0
$CYH1^S$;*MAT*A	0	26	0
$CYH1^S$;*MAT*a	0	0	17
Mixture[c]	13	13	41

[a] *MAT* was not determined for isolates recovered in 1979, except for those from uninoculated plots.

[b] Three isolates from uninoculated plots were $CYH1^S$ and produced little or no pigment in culture, thus initially appearing to be albino. However, tests for *MAT* showed that two were *MAT*a, clearly contaminants that produced so little pigment in culture that they appeared to be albino; this is not uncommon among field isolates of *C. heterostrophus*. It is not unlikely that the third isolate also was a contaminant with weak expression of *ALB*1 in culture.

[c] The mixture consisted of equal numbers of conidia from $cyh1^R$;*MAT*A, $CYH1^S$;*MAT*A, and $CYH1^S$;*MAT*a.

the observations that Leonard (11) made in the greenhouse. He found that *alb*1 reduced the competitive ability of *C. heterostrophus*, but that alleles at *MAT* either had a small effect or none at all.

We have reconfirmed the need to determine precisely the influence of putative marker genes on pathogenicity or fitness of an organism before deriving conclusions involving marked isolates. Mating type, albinism, and drug resistance have been used as markers in several previous studies. Mating type appears to be a neutral marker because neither *MAT*A nor *MAT*a had a consistent effect on epidemics produced by marked isolates. Because of the dramatically limiting effect of albinism on pathogenicity of *C. heterostrophus*, we urge caution in deriving conclusions from studies in which albino isolates are compared with pigmented ones (2). Resistance to cycloheximide (*cyh*1^R) tended to suppress pathogenicity of *C. heterostrophus* (once significantly) in our studies. Consequently, if this gene is used to mark isolates for field studies, the confounding influence of the gene would have to be considered in deriving conclusions.

To make the experimental system function properly, several factors are important. (i) The corn genotype chosen for the small plots should grow vigorously. The hybrid that we used in 1980 grew better than the inbred used in 1979. (ii) The corn genotype chosen for the border rows should be highly resistant to *C. heterostrophus*. (iii) Competing, naturally occurring pests must be suppressed to avoid confounding effects. (iv) Overhead irrigation is necessary to promote rapid secondary pathogen cycles. (v) Plots should be inoculated several times to obtain uniform disease among replicate plots.

We have established the technology to quantify effects of single defined alleles on single season epidemics in the field. Isolates with alternate alleles at a single locus were made genetically similar by backcrossing. This permitted normalization of the many uncontrollable variables encountered in field experiments. With appropriate modifications the system could be used to assess effects of specific host alleles or to monitor marked pathogen populations over several growing seasons. Development of innocuous markers would make it possible to monitor gene flow in field populations over time. Further, by using markers, such as auxotrophs, that permit conditional epidemics (4), it may be possible to test the effects of novel gene combinations under field conditions without hazard to the environment or agro-ecosystem. For laboratory and growth chamber experiments, *alb*1 provides a suitable first line of biological containment since it cannot survive at all in the field.

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