Qualitative Inheritance of Fungicide Tolerance in a Natural Population of Cochliobolus carbonum

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ABSTRACT

Single gene tolerance to specific fungicides was detected in some isolates of a worldwide collection of *Cochliobolus carbonum*. Tolerance to Acti-dione [3-(2-[3,5-dimethyl-2-oxocyclohexyl]-2 hydroxyethyl) glutarimide] and Cadminate (cadmium succinate) enabled some isolates to tolerate much higher concentrations than others for both vegetative growth and spore germination. Genetic linkage between genes for Cadminate tolerance and Acti-dione tolerance or between mating type and race was not evident. An experimental model is now available for the evaluation of fungicide tolerance in a disease situation. Phytopathology 61:458-462.

Additional key words: genetics, maize, *Helminthosporium carbonum*.

Georgopoulos & Zaracovitis (3) recounted the observation by Theophrastes (INQUIRY INTO PLANTS IX, Chapter XVII) that "the virtues of all drugs become weaker to those who are accustomed to them, and in some cases become entirely ineffective . . ." The general acceptance of the principle of drug resistance in human and veterinary medicine as well as in insect control is seldom paralleled with plant disease control problems. Reports (2, 4, 5, 15, 16, 18, 19, and others) of suspected fungicide tolerance hint of such a principle. But as Georgopoulos & Zaracovitis (3) point out, "tolerance to fungicides by plant-pathogenic fungi is reported surprisingly rarely". The lack of the common occurrence of this phenomenon in pathogenic fungi has been attributed to several factors. Some explain that, until recently, most fungicides were broad-spectrum, nonspecific chemicals, and hence fungicide tolerance would not easily evolve in fungi. Another explanation offered is that fungicides are applied at concentrations that would mask potential problems. As the science of fungicide chemistry moves to more specific fungicides which are applied as lower dose, low-volume sprays over larger areas, fungicide tolerance problems may move from the subtle to the spectacular.

Failure of the chemical control of a plant disease is often dismissed as either improper formulation or application of the fungicide. It is possible that what has been attributed to the fungicide may indeed be fungicide tolerance. The results of improper fungicide formulation or application and fungicide tolerance would be identical. Fungicide tolerance could easily be overlooked.

Three criteria should be considered in demonstrating biologically significant fungicide tolerance: (i) the heritability of the trait; (ii) its potential for existing in a natural population; and (iii) a relative epidemiological advantage in the presence of the fungicide.

The heritability of fungicide tolerance has received intensive laboratory study. Most of this work has been accomplished with fungi not pathogenic to plants. Differences in response to fungicides have been observed in a few plant pathogens (1, 3, 11, 12, 13), but often the trait was unstable over time. The absence of a sexual stage limits the testing of the heritability of these differences by sexual crosses. Demonstration of the heritability of fungicide tolerance is desirable, since nonheritable tolerance may not play a role in most disease epidemics.

Investigations involving the genetics of fungicide tolerance frequently have utilized induced mutants. This approach, although productive, may not represent what occurs in nature. Laboratory demonstrations of fungicide tolerance do not imply any epidemiological advantage in the presence of a specific fungicide. Several observations of apparent fungicide failure have been correlated with laboratory tolerance of some isolates (5, 15), yet others report no correlation of fungicide use and tolerance (14, 17).

To intensively evaluate fungicide tolerance, a plant pathogen-crop model was sought which would allow determination of the heritability of fungicide tolerance in a natural population that could be tested in the field for its biological significance. With the criteria previously discussed in mind, we selected the *Cochliobolus carbonum* Nelson (*Helminthosporium carbonum* Ullstrup)—*Zea mays* L. disease model. The sexual stage of *C. carbonum* is readily produced in culture (6) for genetic analysis. A worldwide collection of isolates pathogenic to a variety of gramineous species had been assembled over the past 10 years by one of the authors.

The diversity of this collection has been demonstrated for pathogenicity (8, 9, 10). Included in this species are members that have previously been considered as separate species (e.g. *C. victoriae*), since (i) the described differences segregate as single genes; (ii) the isolates are similar morphologically; and (iii) the members show no demonstrable genetic isolation (8).

The choice to work with a natural population minimized disturbed Darwinian fitness and approached what might occur in nature. The experiments reported here involved the screening of this collection for differences in response to commercially available fungicides, genetic analyses of apparent qualitative differences, estimates of the frequency of the trait in the population, tests for gene linkage, stability, and specificity of the trait. The association of fungicide tolerance as vegetative growth and as the frequency of spore germination was also sought.

MATERIALS AND METHODS.—All fungicides were tested as known concentrations of active commercial chemical in potato-dextrose agar (PDA). The chemicals were added to previously autoclaved cooled water as suspensions or solutions, and added to molten medium cooled to approx 60 C. The supplemented medium was then poured into sterile, disposable plastic petri plates and used immediately.

Preliminary experiments were conducted with 10 selected isolates on 0, 10, and 100 ppm active Cadminate (cadmium succinate); Acti-dione [3-(2-[3,5-dimethyl-2-oxocyclohexyl]-2 hydroxyethyl) glutarimide]; Dyrene [2,4-dichloro-6-(*O*-chloroanilino)-*s*-triazine]; thiram tetramethyl thiuram disulfide); captan [*N*-(trichloromethyl) thio-4-cyclohexene-1, 2-dicarboximide]; Dithianon (2,3-dicyano-1,4-dithia-anthraquinone); 8-quinolinol (8-hydroxyquinoline); zineb (zinc ethylenebis dithiocarbamate); folpet [*N*-(trichloromethyl) thio phthalimide]; Demosan (1,4-dichloro-2,5-dimethoxybenzene); Difolatan [*N*-(1,1,2,2-tetra-chloroethyl) sulfenyl-cis-4-cyclohexene-1,2-dicarboximide]; Daconil (tetrachloroisophthalonitrile); Benlate [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] and Foote No. 471-38 (identity not available). The chemicals were evaluated by placing an inverted 3-mm diam mycelium-agar plug of an isolate from the periphery of an advancing colony on the previously prepared PDA plates. Each concentration was replicated 3 times. All cultures were incubated under constant fluorescent light. Linear growth measurements were recorded over several days before the colony reached the edge of the plates.

Information from this study was used to pursue differences among all available isolates. The concentration selected approached the tolerable limit of the most intolerant isolates and one of lesser concentrations approximating halfway between the upper limit and the check. Evaluation of several of the compounds was repeated to establish that differences did indeed exist.

The tolerable limit of isolates to Cadminate and Acti-dione was established by culturing 11 selected isolates on increasing increments of the fungicides. The selection of these isolates was based on their response to the two fungicides and their established capacity to mate well.

The specificity of the trait was determined by testing the selected isolates of known reaction to Cadminate and Acti-dione against compounds of analogous composition. Cadmium acetate, cadmium perborate, cadmium carbonate, cadmium chloride, cadmium sulfate, cadmium nitrate, chloride salts of copper, tin, nickel, manganese, zinc, mercury, iron, and the sulfate salt of silver were studied in addition to Cadminate. The Acti-dione investigation involved the structurally related antibiotics streptovitacin A and streptimidone.

Sexual crosses by a method previously described (6) (modified slightly using filter paper supplemented with zinc sulfate and chemically impure β-sitosterol) were attempted between tolerant × intolerant, intolerant × intolerant, and tolerant × tolerant isolates of compatible mating types. Random ascospore analyses of resulting progeny were conducted for the fungicides Cadminate and Acti-dione. Two methods were used to classify progeny. Progeny were first screened on Cadminate and Acti-dione-supplemented PDA by placing inverted mycelium-agar plugs (7 progeny/plate) on concentrations inhibitory to the intolerant parent but not to the tolerant parent. In an attempt to remove any effects resulting from the use of PDA transfer plugs, dilute spore suspensions were seeded onto concentrations of the test chemical inhibitory to the intolerant isolates. This method appeared satisfactory in classifying progeny into two types of response to the respective fungicides. To investigate the reaction uniformity of the quantal groups, all progeny were subjected to increasing concentrations of the test chemicals up to and including the intolerable limit of the two groups. For this study, however, cadmium chloride was substituted for Cadminate.

To verify further the specificity of the tolerance in *H. carbonum* to Acti-dione and related glutarimide antibiotics, progeny of selected crosses were used for further screening. Multiple plug transfers of the progeny to PDA supplemented with 10 ppm of these three glutarimides allowed comparisons of the effects of the antibiotics on vegetative growth.

The effects of prolonged vegetative growth in the presence of the fungicide were studied with four isolates, one tolerant and one intolerant of the two chemicals. These studies were designed to evaluate the stability of the phenotypic response to the fungicide in a selective environment. The Cadminate-tolerant isolate was maintained for 5 weekly vegetative transfers on PDA alone and PDA supplemented with 25 ppm Cadminate. These concentrations of fungicide were determined by ED_{50} studies and allowed sufficient growth for transfer. Directly following culture in the presence of the chemical, the cultures were transferred to PDA alone and to media containing 25 and 250 ppm of Cadminate. Vegetative growth rates in cm per day were compared to the original isolates that had been stored in water blanks to measure differences that could be attributed to selection. An identical experiment was conducted with a tolerant isolate and one intolerant to

Acti-dione for 5 weekly vegetative transfers in the presence of 7.5 ppm Acti-dione for the tolerant and 0.75 ppm for the intolerant isolate.

The possible association of fungicide tolerance as measured by vegetative growth with the capacity of spores to germinate at various concentrations of the two chemicals was studied. Four isolates were selected on the basis of differential reaction to fungicide and mating type. In addition, all four isolates were known to belong to Race I by reaction on specific corn inbreds. Spore germination studies were conducted with dilute (1% Tween 20 [polyoxyethylene sorbitan monolaurate] in water) spore suspensions on PDA supplemented with increasing concn of the fungicides. Spores with germtubes greater than one-half the length of the spore after 24-hr incubation were scored and expressed as the per cent of those viewed. Determinations were based on counts in excess of 100 spores. All tests were repeated to check the reproducibility of the results.

Identifications of mating type by tester matings (7) and progeny race (10) were made when applicable for linkage studies.

RESULTS.—Sixteen of the 82 isolates tested were tolerant of 1,800 ppm Cadminate without a corresponding decrease in growth. Sixty-six other isolates showed slight growth at 300 ppm and no growth at 400 ppm Cadminate. These isolates were designated intolerant. Since the response of tolerant isolates to inordinate concentrations of Cadminate might have been due to the insolubility of the compound, further tests were conducted with cadmium chloride, which has a solubility in water far in excess of the range studied. Similar response to cadmium chloride was observed, which indicated that isolate response to Cadminate was a valid measure of tolerance.

Fifty-two of 79 isolates tested were capable of growing in the presence of 10 ppm Acti-dione. Those tolerant isolates were able to grow slightly at 40 ppm but not at 50 ppm, with one noted exception. This one exception was capable of slight growth at 50 ppm Acti-dione. All of the intolerant isolates barely tolerated 1 ppm and showed no growth at 2 ppm.

Spore suspensions of the isolates showed, without exception, no involvement of the PDA transfer plug in the responses of the isolates to the chemical.

All isolates tolerant of Cadminate were also tolerant of all other cadmium compounds tested. Those intolerant of Cadminate were intolerant of all cadmium compounds. No differences among isolates were associated with tolerance for cadmium and the other metals, suggesting a specificity of the Cadminate tolerance for the element cadmium.

Isolates that were tolerant to Acti-dione were tolerant to the two other glutarimides, indicating that the mechanism(s) of tolerance to Acti-dione is probably the same as that of the two other antibiotics. These results for both chemicals were further substantiated by results obtained with progeny from sexual crosses.

Genetic analysis showed that tolerance to both Cadminate and Acti-dione in *C. carbonum* is inherited as single genes (Table 1). In crosses involving differences in both traits, the genes segregated independently. Moreover, no linkage was detected between mating type or race and tolerance to Cadminate or to Acti-dione. Crosses of intolerant × intolerant parents resulted in progency of one class, all intolerant of their respective chemicals. Only one cross between Acti-dione-tolerant parents was achieved. All but one of the progeny were tolerant to Acti-dione, demonstrating the allelism of the gene. The one intolerant exception may represent an intraallelic recombinant between pseudoalleles. Classification of the progeny by the tolerable limit to Acti-dione did not show the reciprocal recombinant, but this might not be expected since (i) a "double-dose" might not exhibit extra Acti-dione tolerance; and/or (ii) random ascospore isolation could easily miss such an infrequent event. Contamination, however, cannot be ruled out. Repeated tests of this cross gave the suggestion of a second modifying gene allowing very slight growth up to 60 ppm Acti-dione, but not at 70 ppm. This trait is characteristic of one of the parents but not of the other, and gave a segregation ratio of 67 extra-tolerant:61 tolerant.

Numerous attempts to cross tolerant × tolerant par-

TABLE 1. Segregation for fungicide tolerance of crosses of *Cochliobolus carbonum*

No.		Cadminate		Anti-dione	
Cross	Progeny	Type of cross	Tolerant: intolerant	Type of cross	Tolerant: intolerant
1 × 2	50	T × I[a]	26:24	T × I	24:26
1 × 3	26	T × I	13:13	T × I	12:14
4 × 2	111	T × I	62:49[b]	I × I	0:111
4 × 3	66	T × I	35:31	I × I	0:66
5 × 2	62	I × I	0:62	T × I	33:29
6 × 2	89	I × I	0:89	T × I	46:43
6 × 3	34	I × I	0:34	T × I	14:20[c]
7 × 3	26	I × I	0:26	T × I	12:14
8 × 2	50	I × I	0:50	T × I	24:26
8 × 3	59	I × I	0:59	T × I	29:30
8 × 9	129	I × I	0:129	T × T	128:1
10 × 11	47	I × I	0:47	I × I	0:47

[a] T = tolerant at specified levels relative to intolerant (I).
[b] Chi-square probability = 20+% (accept 1:1).
[c] Chi-square probability = 30% (accept 1:1).

ents were not successful, probably because of the low mating capacity of the isolates rather than an association with fungicide tolerance.

The response of isolates to prolonged vegetative growth in the presence of Cadminate or Acti-dione did not vary after five weekly vegetative transfers. This suggests that the isolate reactions to these fungicides is stable. Increased tolerance of isolates in the presence of the fungicide was not demonstrable. The only change measured was some limitation of colony establishment where small amounts of the chemical were transferred in the agar plug (Fig. 1-A, B).

Without exception, the per cent germination of spores of tolerant isolates exceeded those of intolerant isolates at corresponding concentrations of the test chemicals (Fig. 2-A, B).

The proportionately ineffective concentrations of Cadminate to the tolerant isolate were evident for the per cent germination as well as for the vegetative growth rate. Indeed, the nature of the response curves differ markedly from those for the intolerant isolate. For further removal of the effects of solubility as a factor influencing the response to Cadminate, growth rate and germination studies were conducted with increasing increments to 400 ppm cadmium chloride (Fig. 2-C). In addition, the range between 600 and 900 ppm Cadminate was repeated several times for the tolerant isolate in order to estimate better the relation between dose and response. These studies demonstrated that the response of the tolerant isolate is a reproducible response to cadmium and not an artifact, since

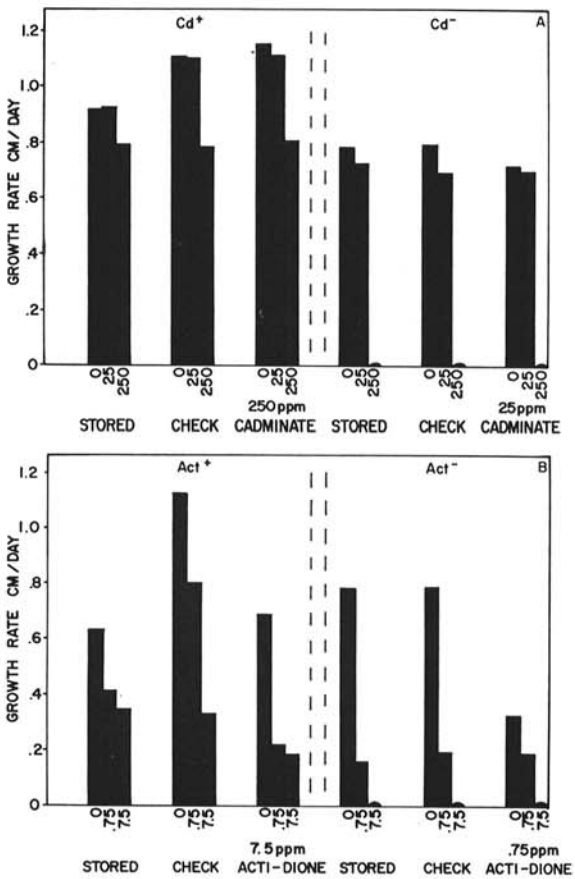

Fig. 1. Vegetative growth rate comparisons of *Cochliobolus carbonum* for **A)** a Cadminate-tolerant (Cd+) and intolerant (Cd−) isolate cultured for five vegetative generations in the presence and absence of tolerable concentrations of Cadminate relative to the checks stored in water blanks; **B)** an Acti-dione-tolerant (Act+) and intolerant (Act−) isolate cultured for five vegetative generations in the presence and absence of tolerable concentrations of Acti-dione relative to the checks stored in water blanks.

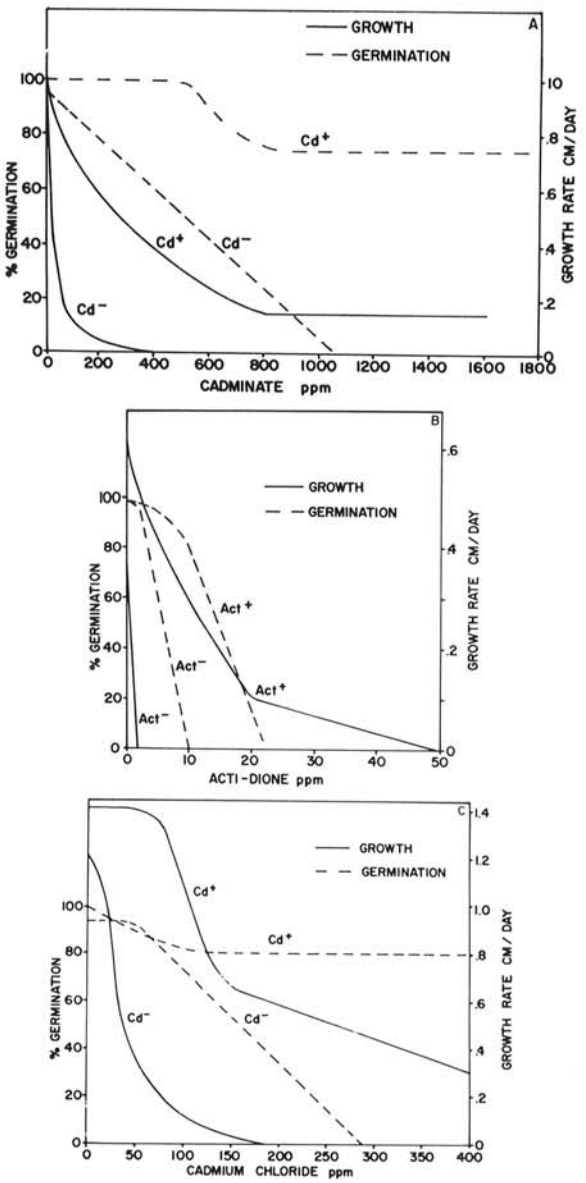

Fig. 2. Dosage-response as per cent germination and growth rate of *Cochliobolus carbonum* for **A)** a Cadminate-tolerant (Cd+) and an intolerant (Cd−) isolate; **B)** an Acti-dione-tolerant (Act+) and an intolerant (Act−) isolate; **C)** isolates Cd+ and Cd− to cadmium chloride.

comparable results of the dose-response relationship were repeatable for both cadmium compounds.

DISCUSSION.—The presence of specific-gene fungicide tolerance in a natural population of a plant pathogen permits an evaluation of the epidemiological importance of these genes in a disease situation. The advantages of testing this model are apparent. No attempt was made to secure fungicide tolerance through induced mutation. Hence, we have not knowingly disturbed the relative Darwinian fitness of these isolates. The advantage of these genes to a plant pathogen can be tested, since *C. carbonum* is a pathogen of maize.

The existence of these genes in a natural population of a plant pathogen raises several interesting points when considering the diversity of the species *C. carbonum*. Diseases incited by this organism include Victoria blight of oats, *Helminthosporium* leaf spot of corn, and a number of diseases of wild and cultivated grasses. The extensive application of fungicides, especially Cadminate and Acti-dione, has never been used to control these diseases. Moreover, the collection was worldwide. One isolate was obtained from infected sheep's fescue (*Festuca ovina* L.) in northern Alaska inside the Arctic Circle. This isolate was Cadminate-tolerant. Selection for this trait in the presence of the fungicide is simply not a justifiable explanation for the frequency of the traits in the sampled population. One possibility is that selection against these genes is minimal. Pleiotropism and/or tight linkage with other traits could also account for their presence.

Consideration should also be given to the interpretation of fungicide dose-response curves. Such curves are sometimes used to suggest the mode of action of a toxicant based on the slope and/or position of the curve. Isolate heterogeneity could cause large discrepancies in the nature of the curve. Moreover, it is common in toxicity studies to mention the test species and rarely, if ever, refer to the specific isolate employed.

We do not know the disease advantage of these two genes for fungicide tolerance in nature. Speculation on their importance would not lead to a better understanding of fungicide tolerance and therefore will not be attempted. We can, however, through this model now ask several questions. If we supply what we consider optimum conditions for expression of fungicide tolerance in the field, will an epidemiological advantage be expressed? If not, why not? Answers to these questions should provide insight into the principle of fungicide tolerance.

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