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

D. R. MacKenzie, R. R. Nelson, and H. Cole

Former Research Assistant (present address, CIMMYT, Londres 40, Mexico 6, D.F., Mexico), Professor of Plant Pathology, and Associate Professor of Plant Pathology and Chemical Pesticides, The Pennsylvania State University, University Park 16802, respectively.

A joint contribution of the Department of Plant Pathology and the Pesticides Research Laboratory, The Pennsylvania State University, University Park 16802. Contribution No. 551 from the Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication 19 January 1970 as Journal Series Paper No. 3727.

The authors thank Robert F. Costantino, Department of Biology, The Pennsylvania State University, for statistical and interpretive advice.

Portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, The Pennsylvania State University.

Accepted for publication 15 November 1970.

ABSTRACT

A complex, heritable system for fungicide tolerance in a natural population of *Cochliobolus carbonum* was identified by sexual progeny response to selected concentrations of thiram as measured by vegetative growth rates on solid medium. The system is not associated with innate vegetative vigor, since fast growers on medium without fungicide were not necessarily tolerant to the fungicide. Tolerance to thiram was not associated with tolerance to captan or zineb. Random phenotypic responses to thiram for five vegetative transfers were suggestive of a cytoplasmic or nuclear-cytoplasmic system. The data suggest a re-evaluation of past interpretations of dose response curves, since phenotypic expression would vary with inheritance as well as with the environmental stress. The importance of this type of heritable system of fungicide tolerance under fungicidal spray programs in a natural disease situation is not known. Phytopathology 61:471-475.

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

It was reported previously that single gene tolerance to specific fungicides exists in a natural population of the plant pathogen *Cochliobolus carbonum* Nelson (*Helminthosporium carbonum* Ullstrup) (2). In the exploratory stages of that study, quantitative differences in tolerance to several fungicides were suspected. These differences were continuous, suggesting quantitative inheritance. To better understand the inheritance of tolerance in this species to these compounds, tetramethyl thiuram disulfide (thiram) was selected for intensive study. The choice of thiram was arbitrary, since similar differences in growth rates among isolates were observed in response to captan [N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide], Dyrene [2,4-dichloro-6-(*O*-chloroanilino)-*s*-triazine], Dithianon (2,3-dicyano-1,4-dithia-anthraquinone), and zineb (zinc ethylenebis dithiocarbamate).

MATERIALS AND METHODS.—Radial vegetative growth rate expressed as cm per day on solid medium was selected as the quantitative measurement most suitable for this study. In an attempt to provide conditions most conducive to vegetative growth, several studies were conducted to evaluate environmental variables. Constant light was known from preliminary experiments to enhance vegetative growth. In a replicated investigation with four isolates, the effects of the inoculum medium, growth medium, and the type of petri plate (pyrex and plastic) on vegetative growth were sought. Of the three media tested, no detectable influence of potato-dextrose agar (PDA), cornmeal agar (CMA), or Czapek's agar as inoculum plug was measured for vegetative growth of several isolates. Czapek's agar as a substrate was not as conducive to growth as were PDA and CMA. Pyrex and plastic plates did not differ measurably in their effects on vegetative growth. Based on these results, the exclusive use of PDA (prepared from fresh potatoes) in disposable plastic petri plates was continued. Medium preparation was identical to that described previously (2). This minimized the possibility of changes due to high temperatures of autoclaving and/or storage. Temperature control was not possible. For this reason, all experiments were self-contained in that all controls were included. Room temperatures were monitored throughout each experiment, and showed fluctuations of 2 to 3 C. However, data comparisons between experiments are not possible. Mean temperatures between some experiments differed by as much as 7 C. All experiments reported here were executed under constant incandescent lighting.

Evaluation of vegetative growth rates of several isolates was undertaken, to understand better the variable to be measured. In the absence of the fungicide, colony diam increases were near-linear for all isolates, with the plotted values originating from the X-axis at 1 day. In the presence of the fungicide the lag period was greater, and varied with the isolate tested. One alternative would have been to remove the lag period mathematically. This method of analysis was rejected because the lag period was considered to be an essential component of the response of isolates to thiram.

The second analytical alternative was to compute the growth rate as the total growth attained (before reaching the plate's edge) divided by the number of days

required to do so. This method was selected to (i) reduce the variable to a common number for statistical analysis; (ii) include the lag period in the computation; and (iii) avoid problems incurred by delayed plating techniques to equate time. We feel that the advantages of this analysis outweigh any disadvantages.

Eighty-two isolates were cultured on 0 (check), 10, and 100 ppm active thiram (65 WP) in three replications. Colony diam were recorded periodically and before reaching the plate's periphery. The results from these studies suggested that there were differences among isolates in response to thiram. Selected isolates were again tested to measure the reproducibility of the model. Based on these studies, four crosses involving parents of desired response to the fungicide were selected. Fifty-nine random ascospore progeny were obtained from one cross between parents of similar and relatively moderate tolerance. The remaining three crosses involved a common parent of moderate tolerance paired with a highly tolerant parent (Cross 2 [50 progeny]), a moderately tolerant parent (Cross 3 [80 progeny]) and one of lesser tolerance (Cross 4 [66 progeny]). The specific rates for these parents differed from 0.4 to 0.1 cm/day on 50 ppm thiram, with the specific values for each isolate varying with separate determinations. Hence the response is only a relative but repeatable measure.

Growth rates in cm per day were determined for the ascospore progenies by the procedure described for previous studies, except that the concentrations tested were 0 (check), 10, and 50 ppm thiram. The 10 ppm concentration was eliminated from the Cross 4 test based on information from the tests of the first three crosses. The choice of 50 ppm was to allow some growth of most progeny, since 100 ppm was found to be inhibitory to many isolates.

An investigation of the inheritance of tolerance to captan and zineb was conducted to see if tolerance to these chemicals is inherited concomitantly with thiram tolerance. All of the ascospore progeny from Cross 2 were compared by the procedures described for vegetative growth determinations at 0 (check) and 50 ppm thiram, captan, and zineb. Estimates of heritability and correlation coefficients were computed for all of the factors studied in order to elucidate the specificity of the tolerance systems.

Evaluation of the nature of the system conditioning thiram tolerance in *C. carbonum* was conducted on the assumption that chromosomal genes, through mitosis, would remain fixed over a short period of vegetative growth, whereas plasmagenes, without a fixed mechanism for equal division, would vary at random in the absence of the fungicide. Nine progeny from Cross 2 were selected at random and tested with the two parents for the stability of tolerance to 50 ppm thiram for five vegetative generations (i.e., mass transfers and subsequent growth of the colony). Inoculum for each vegetative generation was obtained from check plates of the previous generation to allow for the occurrence of random variation. Vegetative growth rates, computed as previously described, were used for statistical analysis.

Analysis and Results.—Quantitative (or polygenic) inheritance is characterized by a normal distribution of progeny types in which the extremes exceed that expected by random variation. Analysis of variance is one method of evaluating the extent of this transgressive segregation. Moreover, if heritability is demonstrated, the computed variances can be used to express the proportion of heritability (h^2) of that trait. Estimates of this nature are termed "broadsense heritability estimates", since dominance and epistasis would be included with additive genetic effects.

Statistical analysis of the progeny responses to thiram showed significant deviation from the population mean for all four crosses. Transgressive segregation was apparent in each case, since progeny were present that exceeded the limits expected with random variation. To illustrate progeny responses to 50 ppm thiram, the frequencies of individuals at intervals of 0.1 cm/day growth rate were plotted (Fig. 1). In addition, the probability curves (3) for each cross were computed as $Z = (x\text{-}\bar{x})/\sigma$ for the X-axis, where Z is the standard units of deviation, x is the variate response, $\bar{x}$ is the population mean, and σ is the standard deviation. The y-axis is the expression of the theoretical frequency density

$$Y_f = (N/\sigma\sqrt{2\pi})e^{-[(x-\bar{x})^2/2\sigma^2]}$$

where N is the total number of individuals, σ is the standard deviation, π and e are constants, x is the variate response, $\bar{x}$ is the population mean, and σ^2 is the population variance and is shown as the smooth distribution curve (Fig. 1). In all cases, the number of progeny more sensitive to 50 ppm thiram (i.e., plus or minus one standard deviation) was greater than the number of progeny showing significantly greater tolerance.

A statistically significant interaction between genotype (progeny) and thiram concentration for all crosses was evident. Correlation coefficients were computed to check the relationship between innately fast growers and tolerance to thiram. Coefficients between the check vegetative growth rate and 50 ppm were not significant, establishing the independence of the tolerance system from the vegetative growth system. Significant correlation between the check growth rates and 10 ppm were detected for Crosses 1 and 2, but not for Cross 3. There was no correlation between progeny responses at 10 and 50 ppm.

Organisms that can be clonally propagated offer a unique advantage in estimating heritability. The environmental influence on a system can be measured, through replication, as the within progeny variance (σ_e^2) in addition to any progeny X environment interactions. The between progeny variance is the sum of this environmental variance plus r (number of replications) times the genotypic variance (σ_g^2). Table 1 presents the one-way analysis of variance values of the

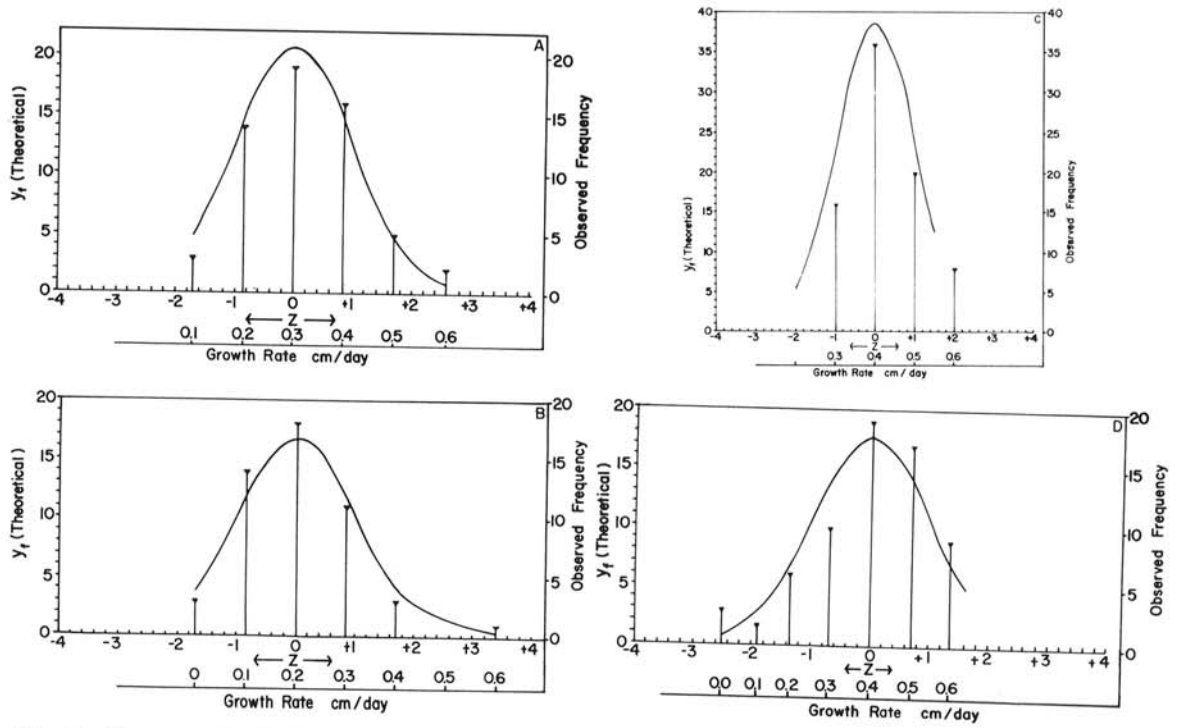

Fig. 1. Frequency distribution curves of progeny responses and the associated probability curves for vegetative growth rate on potato-dextrose agar supplemented with 50 ppm thiram for four crosses of *Cochliobolus carbonum*: **A)** Cross 1. **B)** Cross 2. **C)** Cross 3. **D)** Cross 4.

Cross 2 progeny response to 50 ppm thiram supplemented to PDA. The environmental variance (σ_e^2) is 0.005. The genotypic variance (σ_g^2) is the between progeny mean square minus σ_e^2 divided by the number of replications or $(0.033 - 0.005)/3 = 0.009$. The phenotypic variance (σ_p^2) is equal to $\sigma_e^2 + \sigma_g^2$ or 0.014. A broad sense estimate of heritability (h^2) is defined as the ratio of σ_g^2 to σ_p^2 or $0.009/0.014 = 0.64$. More precise estimates of the heritability of tolerance to thiram in *C. carbonum* may be computed. This has been done and these are presented with the computed variances in Table 2.

Estimates of the heritability of tolerance to 50 ppm captan and zineb indicated a heritable system of toler-

TABLE 1. Summary of a one-way analysis of variance for the responses of 50 progeny from a cross between two isolates of *Cochliobolus carbonum* (Cross 2) to 50 ppm thiram supplemented to potato-dextrose agar

Source of variation	Degrees of freedom	Sums of squares	Mean squares	Components of variance[a]
Between progeny	49	1.634	0.033	$\sigma_e^2 + r\sigma_g^2$
Within progeny	100	0.545	0.005	σ_e^2
Total	149	2.179		

[a] σ_e^2 = environmental variance; r = number of replications; σ_g^2 = genotypic variance.

TABLE 2. The phenotypic variance (σ_p^2) of the vegetative growth rate for the progeny of four crosses of *Cochliobolus carbonum* as influenced by thiram concentration and the partitioned components of the phenotypic variance as environment (σ_e^2), genotype (σ_g^2) and heritability of the trait (h^2)[a]

Cross	Thiram ppm	σ_p^2	σ_e^2	σ_g^2	h^2
1	0	0.016	0.005	0.010	0.661
	10	0.025	0.006	0.019	0.746
	50	0.018	0.007	0.011	0.606
2	0	0.095	0.013	0.082	0.863
	10	0.044	0.007	0.037	0.840
	50	0.015	0.005	0.009	0.631
3	0	0.016	0.005	0.011	0.679
	10	0.024	0.008	0.016	0.667
	50	0.011	0.005	0.006	0.530
4	0	0.021	0.004	0.016	0.790
	10				
	50	0.027	0.008	0.019	0.694

[a] $h^2 = \dfrac{\sigma_g^2}{\sigma_p^2}$ where $\sigma_p^2 = \sigma_g^2 + \sigma_e^2$.

ance to these fungicides. However, insignificant correlation coefficients between progeny response to thiram compared to captan and zineb suggest that concomitant fungicide tolerance did not exist. Highly significant correlations between the check growth rates and captan and zineb growth rates were detected. Tolerance to captan and zineb can be viewed as innate vegetative vigor in this instance. This conclusion is supported by the highly significant correlation between the phenotypic responses to captan and zineb.

The nine progeny from Cross 2 that were selected to test the stability of tolerance to thiram were found to be unstable. Isolates of little tolerance were consistently so. However, tolerant isolates showed large differences throughout the experiment in response to thiram as indicated by these linear growth rates. Statistical analysis of this observed variation of response to 50 ppm thiram for the five vegetative generations showed that a large portion of the total variance was attributed to an interaction between the genotype and the vegetative generation (Table 3). This random phenotypic response to thiram suggests the presence of either a heterocaryon or a cytoplasmic system for thiram tolerance. A truly nuclear-conditioned system in a homocaryon would not be expected to vary greatly over vegetative generations. We discount the heterocaryon explanation, since the progeny from this cross segregated 1:1 for three independent traits (2). Ascospores of *C. carbonum* arise from a single primary haploid nucleus (R. R. Nelson, *unpublished data*). Formation of a heterocaryon by inclusion of two non-sister tetrad nuclei in the ascus has been observed, but only rarely. Frequent heterocaryon formation by this means would be expected to interfere with single gene segregation ratios of 1:1.

A heritable cytoplasmic system appeared likely unless, through some fault of the experimental design, other unidentified factors (e.g., time measurement or media differences) had confounded the genotype x generation interaction. To test for confoundment, it was postulated that random variation of a cytoplasmic system would be as great within replications as it was over vegetative generations. The extent to which the vegetative generations variance exceeded the replication variance would be a measure of confoundment. Expressed mathematically, the assumption is:

$$\sigma^2_{vg} = \sigma^2_r + \sigma^2_x$$

where σ^2_{vg} is the variance associated with the vegetative generations, σ^2_r is the variance associated with replication, and σ^2_x is the extent of confoundment due to extraneous factors. Pooled variances from separate analyses of variances of all nine progeny gave values of $\sigma^2_{vg} = 0.0155$ and $\sigma^2_r = 0.016$. The difference between these two measures is considered insignificant (i.e., $\sigma^2_x = 0$). The variation observed over vegetative generations is equal to the variation encountered within replications. The variation observed in the vegetative generations is considered real and not an artifact of the design.

Other components of the total variance were identified with a genotypic response on 50 ppm thiram, but were independent of the vegetative generation (Table 3). The environmental component at 50 ppm thiram was nearly 4 times greater than the check relative to the total variances.

TABLE 3. Partitioned components of total variance for nine randomly selected progeny from a cross of *Cochliobolus carbonum* (Cross 2) grown for five vegetative generations on potato-dextrose agar alone and with 50 ppm thiram

Variance component	Check		Thiram 50 ppm	
	σ^2	% σ^2 total	σ^2	% σ^2 total
Total	0.066		0.047	
Genotype	0.008	11.7	0.021	44.1
Generation	0.028	42.8	0.002	3.6
Genotype × Generation	0.027	40.7	0.016	34.5
Environmental	0.003	4.8	0.008	18.0

DISCUSSION.—Tolerance to thiram in *C. carbonum* is heritable. The use of the term heritable fungicide tolerance need not imply nuclear genes. In fact, cytoplasmic inheritance of fungicide tolerance, previously reported by Jinks (1), would be identified in h^2 as all or part of this measure of heritability. Attempts to test the nuclear association of the trait through heterocaryon tests were unsuccessful because of our inability to force a heterocaryon between members of this species. We cannot at present distinguish between an independent cytoplasmic system or an interdependent cytoplasmic-nuclear system. The randomness of the phenotypic response to thiram is inconsistent with an independent nuclear-conditioned system of thiram tolerance.

The continuous nature of the data is suggestive of the complexity of the system. This system for thiram tolerance was shown to be independent of the vegetative growth system. Environmental stress on a complex system is generally thought to be greater than on a simple system. The addition of thiram increased this stress as indicated, by the 4-fold increase in the environmental variance. This source of variation in addition to the random variation of the heritable tolerance itself casts doubt on the universal use of dose response curves for predictive (e.g., ED_{50}) or interpretative (e.g., mode of action) usage with fungicides. Comparisons among fungicides may also be invalid, since the susceptible physiological system(s) to one fungicide may not be identical to other fungicides. If the susceptible systems differ, one might then conclude that so do the genetics of tolerance.

As plastic as the thiram tolerance system of *C. carbonum* appears it displays a measure of specificity, in that tolerance to one dithiocarbamate (thiram) does not impart tolerance to another (zineb) or to the unrelated compound captan.

Through the analyses used in these investigations, we feel competent to identify fungicide tolerant systems that are independent of vegetative growth rates of the checks. This distinction would appear to be quite necessary for proper interpretation of the response of fungi to specific fungicides. Much of the tolerance of isolates to captan and zineb that was identified as heritable cannot be considered distinct from differences in vegetative growth rates. One would then hesitate to call this type of response fungicide tolerance per se.

This distinction was possible for the thiram tolerance of *C. carbonum,* with the exception of significant correlations between check with no fungicide growth and 10 ppm thiram for Crosses 1 and 2. In these associations, about 16% of the variation obtained at 10 ppm was in common with check growth. This may well have been the "vegetative power" of some isolates at this relatively low concentration of thiram.

The universality and importance of this type of fungicide tolerance is questionable. Until one can design field experiments to cope with the problems involved with such a plastic system, an evaluation of its importance will remain disputable. The generality of such systems is suggested by the problems encountered by researchers, past and present, in delineating fungicide tolerance. The random variation of the described system could account for a part of this problem.

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