European Journal of Plant Pathology **101**: 93–99, 1995.

Genetic correlations in resistance to morpholine and piperidine fungicides in *Pyrenophora teres* populations

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Accepted 23 September 1994

Key words: fungicide resistance, evolution, quantitative genetics, cross resistance

Abstract

Highly significant genetic variation ($P < 0.001$) in resistance to the morpholine fungicides fenpropimorph, tridemorph and dodemorph and the piperidine fungicide, fenpropidin was found in different populations of *Pyrenophore teres* in North America and Europe which had not been previously exposed to these fungicides. Resistance phenotypes were continuously distributed for each fungicide in each population. Cross resistance relationships were determined by estimating genetic correlation coefficients in resistance to all pairwise combinations of fungicides. The majority of the correlation coefficients were highly positive for all fungicide combinations in all populations; eight of 36 (22%) coefficients were not significantly different from 1 ($P > 0.05$). This result is consistent with the hypothesis that many of the same genes, or genes in gametic disequilibrium, control resistance to more than one fungicide in most populations of *P. teres* and that these fungicides comprise a single cross resistance group. Three of 36 (8%) correlation coefficients were not significantly different from 0 ($P > 0.05$) indicating that, in these populations, independent genes controlled resistance to these fungicides. The results of this study indicate that although most of the same genes control resistance to morpholine and piperidine fungicides in *P. teres*, differences in frequencies of these genes among populations can result in different cross resistance relationships from one population to another.

Introduction

Cross resistance has important implications for the evolution of resistance to fungicides and the design of strategies to prevent or delay this evolution. Cross resistance has been defined as resistance to two or more fungicides conferred by the same genetic factors [Georgopoulos, 1977] and, therefore, can be thought of in terms of genetic correlations in resistance [Peever and Milgroom, 1993]. Genetic correlations are estimates of the degree to which two phenotypic characters, such as resistance to two fungicides, are controlled by the same genes. Genetic correlations are useful because they allow quantitative estimates of cross resistance to be made which can be tested statistically, and also allow inferences to be made about the genetic control of cross resistance in pathogen populations.

Genetic correlations in resistance to sterol biosynthesis inhibiting fungicides (SBIs) triadimenol, propiconazole, imazalil, fenarimol and fenpropimorph were estimated previously in populations of the barley net blotch pathogen, *Pyrenophora teres* Drechs. [Peever and Milgroom, 1993]. Correlation coefficients in resistance to the C_{14} demethylation-inhibiting (DMI) group of SBIs (triadimenol, propiconazole, imazalil, fenarimol) were generally high while coefficients between DMIs and one morpholine, fenpropimorph, were generally low, a result which was in agreement with previous cross resistance studies [Buchenauer et al., 1984; Girling et al., 1988; Hildebrand et al., 1988; Kendall, 1986; Thind et al., 1988]. Despite

these general patterns, correlation coefficients between certain pairs of DMIs were higher than others suggesting the existence of cross resistance 'subgroups' within the DMIs which were not related to chemical structure [Peever and Milgroom, 1993]. Additionally, some of the DMI/DMI correlation coefficients were not significantly different from 0, and some of the DMI/fenpropimorph correlation coefficients were not significantly different from 1 in certain populations. These differences in correlation coefficients were most likely the result of differences in frequencies of SBI-resistance genes among populations and/or gametic disequilibrium among resistance genes in these populations. The latter explanation is less likely because we have found no evidence of gametic disequilibrium in most of these populations [Peever and Milgroom, 1994]. The variation in correlation coefficients in resistance to SBIs observed among populations and among chemically similar SBIs suggest that a single model of cross resistance among SBIs and among all *P. teres* populations is not appropriate.

Morpholines and piperidines are SBIs which have a substantially different mode of action from the DMI group SBIs [Baloch et al., 1984; Berg et al., 1984; Girling et al., 1988]. The morpholine, fenpropimorph, and the piperidine, fenpropidin, both inhibit Δ^{14} reduction strongly and $\Delta^{8} \rightarrow \Delta^{7}$ isomerization weakly, while another morpholine, tridemorph, tridemorph, appeared to have the opposite pattern [Baloch et al., 1984; Berg et al., 1984; Girling et al., 1988; James et al., 1992]. Cross resistance studies with morpholine and piperidine fungicides in *Erysiphe graminis* f.sp. *hordei* revealed that resistances to fenpropimorph and fenpropidin were highly correlated while resistance between these two fungicides and tridemorph were not [Brown et al., 1991; Robertson et al., 1990]. These studies suggested that the same genes conferred resistance to fenpropimorph and fenpropidin but not to tridemorph. Tridemorph appeared to represent a distinct cross resistance group from fenpropimorph and fenpropidin which was related to mode of action rather than chemical structure.

The objective of this study was to quantify cross resistance relationships among morpholine and piperidine fungicides in *P. teres* populations that had not been previously exposed to these fungicides. This was accomplished by estimating genetic correlations in resistance to fenpropimorph, tridemorph, dodemorph and fenpropidin in six populations of *P. teres* from North America and Europe. Specifically, we tested the hypothesis that morpholine and piperidine fungicides do not constitute a single cross-resistance group in *P. teres*.

Materials and methods

Sampling P. teres *populations*. One hundred forty seven single-conidial isolates of *Pyrenophora teres* were randomly sampled from six barely fields growing in different parts of the world. Details of the samples are shown in Table 1. For the purposes of this study, isolates sampled from the same field were considered members of the same *P. teres* population.

Fungicides. One piperidine and three morpholine fungicides were obtained as technical-grade materials from their manufacturers. The morpholine fungicides fenpropimorph, tridemorph and dodemorph were obtained from BASF Corp., Research Triangle Park, NC, U.S.A. The piperidine fungi-

Table 1. Sources of *Pyrenophora teres* isolates

Population	*n*	Sampling date	Location	Barley cultivar	Collector
Alberta A	25	August 1991	Innisfail, Alberta, Canada	Harrington	S. Slopek
Alberta B	22	August 1991	Olds, Alberta, Canada	Harrington	S. Slopek
North Dakota A	23	July 1991	Stutsman Co., North Dakota, U.S.A	Robust	B. Steffenson
North Dakota B	27	July 1992	Towner Co., North Dakota, U.S.A.	unknown	B. Steffenson
New York	26	July 1990	Cattaraugus Co., New York, U.S.A.	unknown	D. Dewing
Germany	24	July 1991	Deggendorf, Bavaria, FRG	Trixi	G.M. Hoffmann

cide fenpropidin was obtained from Ciba-Geigy Corp., Greensboro, NC, U.S.A.

Resistance assays. Isolates of *P. teres* were stored on sterile filter papers at –20 °C and cultured on malt extract agar (MEA) containing 1.5% malt extract (William's Brewing, San Leandro, CA, U.S.A.) AND 2% agar (Difco, Detroit, MI, U.S.A) as described previously [Peever and Milgroom, 1992, 1993]. Resistance phenotypes were determined for each *P. teres* isolate using a radial growth assay on fungicide-amended MEA amended with 1% ethanol (controls) or with a single discriminatory concentration of one of the four fungicides solubilized in 1% ethanol [Peever and Milgroom, 1992, 1993]. Discriminatory concentrations of each fungicide were chosen by screening a small sample of isolates from each population on three to five concentrations of each SBI. The concentration which inhibited radial growth to a level of approximately 50% of the controls (EC_{50} value) was chosen for the assays [Peever and Milgroom, 1992, 1993]. For the Alberta A, North Dakota A and New York populations (Group A), fenpropimorph, tridemorph and fenpropidin were used at 1 µg/ml and dodemorph at 10 µg/ml. For the Alberta B, North Dakota B and German populations (Group B), fenpropimorph and fenpropidin were used at 5 µg/ml, tridemorph at 2 µg/ml and dodemorph at 10 µg/ml. Two replicates of each isolate were used in all assays to obtain an estimate of the environmental or assay variance. The resistance phenotypes of each population sample were determined in a singly resistance assay, each performed at a different time.

Data analysis

Partitioning variation in resistance phenotype. Variation in fungicide resistance phenotype was partitioned into genetic and environmental components for each fungicide using a nested ANOVA model similarly to that described previously [Peever and Milgroom, 1993]. Three sources of variation were identified (populations, isolates, replicates) with unequal numbers of isolates nested within each population. Since the resistance phenotypes of the Alberta A, North Dakota A and New York populations were determined on different concentrations of fungicides from the Alberta B, North Dakota B and German populations, two separate ANOVAs were performed, one for each group of 3 populations. Variance in resistance among populations and within populations (among isolates) was tested with an *F* test [Neter et al., 1985]. *F* values for variation among populations were obtained by dividing mean square for populations by mean square for isolates, and *F* values for variation among isolates were obtained by dividing mean square for isolates by mean square for replicates. Expected mean squares of the nested ANOVA model allowed estimation of the among-population, among-isolate and among-replicate components of variance [Lentner and Bishop, 1986]. The proportion of variation found among populations for each fungicide was calculated by dividing the among-population component of variance by the sum of the among-population, among-isolate and replicate variance components.

Estimation of correlation coefficients. Genetic correlation coefficients in resistance to each pair of fungicides were estimated using methods described previously [Peever and Milgroom, 1993]. The ANOVA and ANCOVA model in which variation or covariation in resistance phenotype was partitioned for each fungicide or pair of fungicides in each population is shown in Table 2. The ANOVA and ANCOVA models were identical and two sources of variation were identified (isolates and replicates). The expected mean squares of the model allowed estimation of the among-isolate component of variance ($\sigma_I^2(x)$ or $\sigma_I^2(y)$) and covariance ($\sigma_I(x,y)$) for each fungicide or pair of fungicides [Lentner and Bishop, 1986]. Correlation coefficients, r_G, were estimated using the appropriate variance and covariance components derived from the ANOVA or ANCOVA model as

$$r_G = \frac{\sigma_I(x,y)}{\sqrt{\sigma_I^2(x)\cdot\sigma_I^2(y)}}, \qquad (1)$$

where r_G is the among-isolate genetic correlation coefficient, $\sigma_I(x,y)$ represents the among-isolate genetic covariance in resistance to a given pair of fungicides (x and y) obtained from the ANCOVA table for each pair of fungicides. The terms $\sigma_I^2(x)$ and $\sigma_I^2(y)$ respectively, represent the among-isolate

Table 2. Analysis of variance (ANOVA) or analysis of covariance (ANCOVA) models for partitioning of variance or covariance in morpholine resistance phenotype into genetic and environmental components (Peever and Milgroom, 1993)

Source	df[a]	EMS[b]
Isolates	$n - 1$	$\sigma^2 + 2\sigma_I^2$
Replicates (Isolates)	$n\ (r - 1)$	σ^2

[a] degrees of freedom (n = population sample size, r = number of replicates)
[b] expected mean squares (variance or covariance components: σ_I^2 = isolates, σ^2 = replicates or assay)

genetic variances in resistance to fungicides *x* and *y* obtained from separate ANOVA tables for resistance to each fungicide. Ninety five percent confidence intervals for the correlation coefficients were estimated using the method of Ennos and Swales [1991] as described in Peever and Milgroom [1993].

Results and discussion

Phenotypic and genetic variation in resistance. Fungicide resistance phenotypes were distributed continuously for all fungicides in all populations. Representative frequency distributions of resistance phenotypes in the population from Bavaria, Germany are shown in Fig. 1. Similar distributions were obtained for the other populations. The continuous distributions suggest that resistance to these fungicides in *P. teres* may be under complex genetic control but crosses between isolates with extreme resistance phenotypes are necessary to test this hypothesis. Highly significant genetic variation ($P < 0.001$) was found among isolates for all fungicides in all populations (Table 3), indicating the potential for the evolution of higher levels of resistance to these fungicides within these populations.

Genetic differentiation among populations. Highly significant genetic variation in resistance ($P < 0.001$) was found among populations for all fungicides except for tridemorph among the Alberta B, North Dakota B and German populations (Table 3). High levels of differentiation in resistance to triadimenol, propiconazole, imazalil and fenarimol were also detected among these populations in a

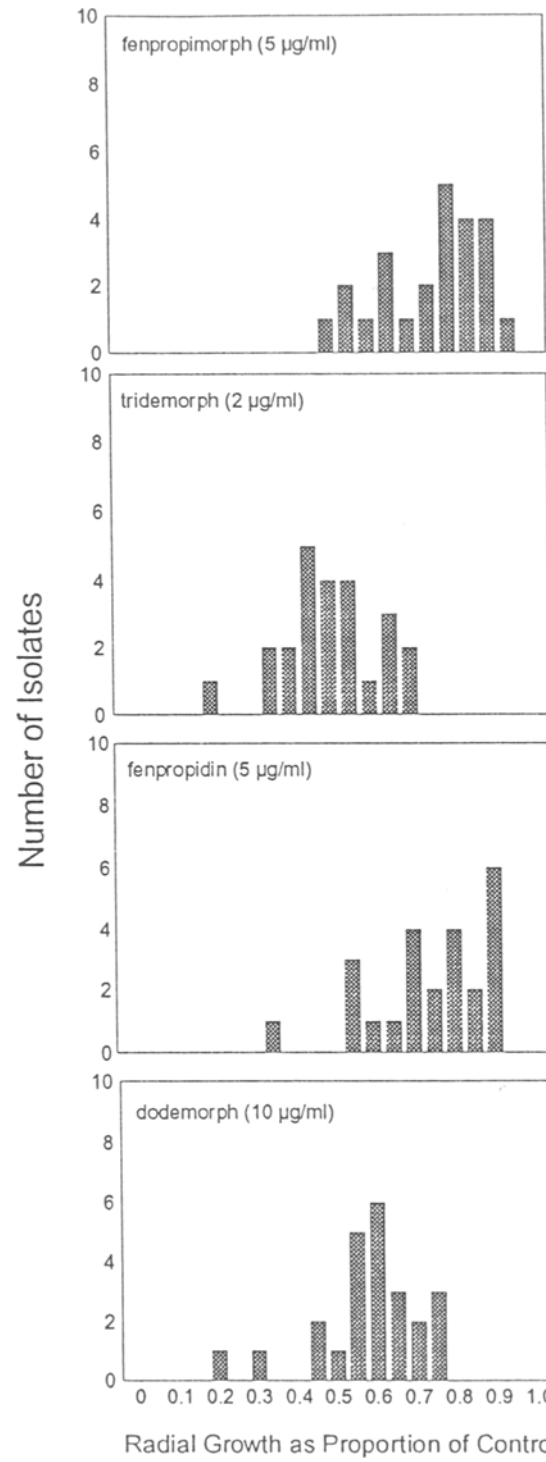

Fig. 1. Frequency distributions of resistance phenotypes in the *Pyrenophora teres* population from Bavaria, Germany. The resistance phenotype of each isolate represents the mean of the replicates determined on a singly discriminatory dose of fungicide (in parentheses).

Table 3. Analysis of variance (ANOVA) for resistance to morpholines and piperidines among and within *Pyrenophora teres* populations [a]

Source	df	Fungicide							
		Fenpropimorph		Tridemorph		Fenpropidin		Dodemorph	
		MS	*F*	MS	*F*	MS	*F*	MS	*F*
GROUP A[B]									
Population	2	1.78	63.6 ***	0.797	20.4 ***	0.794	34.5 ***	0.538	16.8 ***
Isolates (population)	71	0.028	10.0 ***	0.039	19.5 ***	0.023	23.0 ***	0.032	10.7 ***
Replicates (Isolates)(Replicates)	74	0.003		0.002		0.001		0.003	
GROUP B [C]									
Population	2	0.773	22.1 ***	0.010	0.33[NS]	1.461	39.5 ***	0.249	7.8 ***
Isolates (Population)	70	0.035	11.7 ***	0.003	10.0 ***	0.037	12.3 ***	0.032	16.0***
Replicates (Isolates)(Replicates)	73	0.003		0.003		0.003		0.002	

[a] MS = mean square. *F* is the *F* value for population (MS Population/ms Isolates) and for Isolates (MS Isolates/MS Replicates). Three asterisks indicate significance at $P < 0.001$. NS indicates lack of significance at $\alpha = 0.05$.
[b] Alberta A, North Dakota A and New York populations
[c] Alberta B, North Dakota B and German populations

previous study [Peever and Milgroom, 1993]. This high level of genetic differentiation observed among populations indicates that frequencies of genes controlling resistance to SBIs vary significantly among *P. teres* populations. Calculation of the among-population, among-isolate and replicate variance components revealed that an average of 42% of the total variation in resistance to fenpropimorph, tridemorph, fenpropidin and dodemorph was found among populations compared to 58% within populations. When variation in resistance to the SBIs triadimenol, propiconazole, imazalil and fenarimol and fenpropimorph was similarly partitioned in a previous study, 38% of the total variation in resistance was found among populations [Peever and Milgroom, 1993, 1994]. Both of these values are remarkably similar and in close agreement with an analogous statistic calculated for RAPD genetic markers in these same populations. In this study, 46% of the total variation was found among populations compared to 54% within populations [Peever and Milgroom, 1994]. All of the these studies have demonstrated a high level of genetic differentiation among *P. teres* populations, among the highest observed to date for plant-pathogenic fungi.

Correlation coefficients in resistance. Genetic correlation coefficients in resistance ranged form 0 to +1 but were generally high and positive for all combinations of fungicides and in all populations (Table 4). Twenty five of 36 correlation coefficients (70%) were significantly different from both 0 and 1 ($P < 0.05$). Eight of 36 coefficients (22%) were not significantly different from 1. No evidence for negative correlations in resistance was found in this study. Twenty two percent of the correlation coefficients were not significantly different from 1 which suggested that the same genes conferred resistance to these pairs of fungicides in these populations. Despite the high positive correlations observed between the majority of the fungicide pairs, 3 of 36 coefficients (8%) were not significantly different from 0 indicating that independent genes control resistance to these pairs in these populations. The fact that some of the correlation coefficients were not significantly different from 0 and that most of the coefficients were significantly different form both 0 and 1 was likely due to differences in frequencies of resistance genes among populations. Variation in correlation coefficients in resistance to DMI fungicides among populations was observed in a previous study [Peever and Milgroom, 1993]. These results have demonstrated that cross-resistance relationships can be quite variable among populations and among different combinations of SBI fungicides, even for fungicides with identical

Table 4. Estimates of genetic correlation coefficients for resistance to pairs of morpholines and piperidines using isolates sampled from different *Pyrenophora teres* populations

Population	*n*	Fungicide combination					
		Fenpropimorph Tridemorph	Fenpropimorph Fenpropidin	Fenpropimorph Dodemorph	Tridemorph Fenpropidin	Tridemorph Dodemorph	Fenpropidin Dodemorph
Alberta A	25	1.00 * (0.92,1.12)[a]	1.00 * (0.90,1.14)	0.69 (0.44,0.93)	0.98 * (0.88,1.09)	0.77 (0.54,0.99)	0.54 (0.23,0.85)
Alberta B	22	0.71 (0.52,0.90)	0.67 (0.48,0.86)	0.43 (0.24,0.62)	0.80 (0.61,0.99)	0.87 * (0.68,1.06)	0.56 (0.37,0.75)
North Dakota A	23	0.77 * (0.54,1.00)	$0.26^{n.s.}$ (–0.15,0.67)	0.49 (0.05,0.93)	0.55 (0.23,0.87)	0.70 * (0.37,1.03)	0.52 (0.11,0.93)
North Dakota B	27	0.73 (0.54,0.92)	0.47 (0.16,0.78)	0.51 (0.22,0.80)	$0.26^{n.s.}$ (–0.11,0.63)	0.67 (0.45,0.89)	$0.23^{n.s.}$ (–0.42,0.48)
New York	26	0.96 (0.93,0.99)	0.99 * (0.97,1.01)	0.81 (0.66,0.96)	0.96* (0.92,1.00)	0.81 (0.67,0.95)	0.87 (0.75,0.99)
Germany	24	0.75 (0.55,0.95)	0.84 (0.70,0.98)	0.68 (0.43,0.93)	0.67 (0.42,0.92)	0.53 (0.22,0.84)	0.58 (0.29,0.87)

[a] Confidence intervals for the coefficients (Ennos and Swales, 1991). Confidence intervals not including 0 and 1 are significantly different from 0 and 1 at the 5% probability level. Correlation coefficients not significantly different from 0 are indicated with the n.s. superscript and coefficients not significantly different from 1 are indicated by an asterix ($P < 0.05$).

mode of action and/or similar chemistry. These results also suggest that it is highly unlikely that a single model of cross resistance among SBI fungicides will be appropriate in all pathogen populations.

In this study, there was no evidence for higher mean correlation coefficients between any specific pairs of fungicides as was observed among some pairs of DMI fungicides in a previous study [Peever and Milgroom, 1993]. Brown et al. [1991] and Robertson et al. [1990] observed cross resistance between fenpropimorph and fenpropidin but not between these fungicides and tridemorph. No evidence of higher correlations in resistance to fenpropimorph and fenpropidin was found in the present study. The bimodal distribution of resistance to fenpropimorph and fenpropidin observed by Brown et al. [1993] suggested the existence of a single genetic locus controlling resistance to these fungicides and it is possible that this gene was largely responsible for the highly positive correlation in resistance observed to them. The intensive use of morpholines to control *E. graminis* f.sp. *hordei* on barley may have selected this resistance gene to high frequency in these populations. The *P. teres* populations sampled in this study have never been exposed to morpholine or piperidine fungicides and it is expected that the correlation coefficients estimated here may change if these populations are exposed to these fungicides and additional resistance genes are selected. From the present results, fenpropimorph, fenpropidin, tridemorph and dodemorph constitute a single cross resistance group in *P. teres* which precludes the independent evolution of resistance to any one of them. The practical implication of this is that once resistance evolves to any one of these fungicides, it cannot be replaced by any other fungicide in this group nor can these fungicides be combined in alternations or mixtures as a resistance management strategy.

Acknowledgements

Supported in part by Hatch Project NYC-15341, USDA-NRI Competitive Grant 91-37303-6429 to M.G. Milgroom and by an NSERC Postgraduate Scholarship (Canada) to T.L. Peever. The authors would like to thank D. Dewing, G.M. Hoffmann, H. Meier, S. Slopek and B. Steffenson for sampling *P. teres* populations, and BASF and Ciba-Geigy for supplying fungicides.

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