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Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides in Ontario isolates of *Sclerotinia homoeocarpa*

T. Hsiang, L. Yang and W. Barton
Dept. Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (Fax: (519) 837-0442)

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Abstract

Four hundred and thirty-five isolates of *Sclerotinia homoeocarpa* from eight populations in southern Ontario were tested for sensitivity to the demethylation-inhibiting (DMI) fungicides, propiconazole, myclobutanil, fenarimol and tebuconazole. The isolates were collected in summer 1994 just prior to legal DMI fungicide use on turfgrass in Ontario. There were wide variations in sensitivities, and seven of the eight populations were very sensitive to the fungicides. Based on mean EC_{50} and the distribution of DMI sensitivity, one population near the U.S. border was suspected of having been previously exposed to DMI fungicide. Pairwise comparisons of EC_{50} values for the different fungicides showed low to moderate correlations between fungicides. EC_{50} values of myclobutanil and propiconazole had the best correlation, followed by the pair of tebuconazole and fenarimol. Other pairwise comparisons were not statistically significant except for a barely significant relationship between EC_{50} values of myclobutanil and tebuconazole. For field populations of plant pathogens, cross-resistance to different DMI fungicides may not be as strong as conventionally thought. The data collected here will allow comparison to subsequent years to look for detectable shifts in *S. homoeocarpa* sensitivity to DMI fungicides as they become more frequently used in Ontario.

Introduction

Dollar spot disease caused by *Sclerotinia homoeocarpa* F. T. Bennett is the most common disease problem of high maintenance turf in the Great Lakes region. In Ontario, the disease can be found from June through October. Many fungicide applications are made annually to control this disease, especially in preventive programs based on 2-week schedules. This high frequency of use provides a selective advantage for isolates that have some decreased sensitivity to the fungicide. Because few fungicides are registered for use on turf in Ontario (Anonymous, 1993a), there are strong concerns about development of economically significant field resistance and the consequent loss of fungicides.

The demethylation inhibitors (DMI) are a relatively new group of systemic fungicides that control a broad spectrum of pathogens from all major fungal groups, except Oomycota (Scheinpflug, 1988; Sisler, 1988). They inhibit the biosynthesis of ergosterol which is important for fungal membranes (Köller, 1988). DMI fungicides have been used for the control of dollar spot and other turfgrass diseases in the United States for more than ten years. Recently, several cases of dollar spot field resistance to DMI fungicides have been reported in Illinois, Kentucky and Michigan (Doney and Vincelli 1993; Golembiewski et al., 1995; Vargas et al., 1992).

Among the DMI fungicides, propiconazole has been registered in Canada since 1986, but for use on turf only since fall 1994. Myclobutanil has been registered in Canada for use on apples and grapes (Anonymous, 1993b) since 1992 but is not presently approved for turf. Fenarimol has never been registered in Canada. Triadimefon, while first registered in Canada in 1985, has never been registered for turf, and its Canadian registration was withdrawn in 1995. Myclobutanil and several other DMI fungicides are in various stages of registration for use on turf in Canada,

but because DMI fungicides have the same mode of action and show cross-resistance (Scheinpflug, 1988), there are strong fears that field resistance to one would render the others useless.

Until recently, no DMI fungicide was registered for use on turfgrass in Canada. This provided an opportunity to study baseline sensitivity of *S. homoeocarpa* to DMI fungicides prior to DMI fungicide use. Monitoring for fungicide sensitivity before widespread use of a fungicide group could provide valuable information on the normal variation in fungicide sensitivity of a population (Brent, 1995). Furthermore, this objective of monitoring the dollar spot pathogen for DMI sensitivity has been specifically identified by the Fungicide Resistance Action Committee as a major goal (Wade and Delp, 1990). The purpose of this work was to examine baseline sensitivity to DMI fungicides in *S. homoeocarpa* populations of Ontario. A second objective was to investigate the sensitivity and cross-resistance of *S. homoeocarpa* populations to several DMI fungicides which were in the process of registration for turfgrass diseases in Canada. A third objective was to look at the extent of variation in natural populations of *S. homoeocarpa* since this species is not known to produce sexual or asexual spores in North America, and very little is known about its variability.

Methods

Isolates of *S. homoeocarpa* were collected from diseased turfgrass in eight locations across southern Ontario in summer 1994 prior to DMI fungicide use on turfgrass. At least 50 samples from each location were collected systematically using a grid with at least 1 m between samples. Approximately 20 leaf blades were collected from an infection centre nearest each grid point and placed into a 20 ml vial. Vials were brought back to the lab the same day and placed into a 4 °C chamber. Fungi were isolated from each sample according to the method described by Cole et al. (1967). Each isolate was grown on potato dextrose agar (PDA), confirmed as *S. homoeocarpa* by comparison with known isolates, and stored on PDA at 4 °C until testing for fungicide sensitivity. A single isolate was retained per sample.

All isolates were tested for sensitivity to four DMI fungicides using an agar plug assay described by Detweiler et al. (1983). Each fungicide was diluted to target concentrations, and added to molten PDA (60 °C) while maintaining an equal final concentration of acetone (0.10% v/v). Acetone at this concentration did not inhibit growth (data not shown), while it was used to initially dissolve the fungicide and allow even distribution through the medium. A 5-mm-diam PDA plug was taken from the growing edge of an active mycelium and placed onto a PDA plate amended with 0, 0.001, 0.01, 0.10 or 1.00 μg ml^{-1} of propiconazole or myclobutanil or fenarimol. Tebuconazole was added to PDA to make concentrations of 0, 0.01, 0.1, 1.0 and 10.0 μg ml^{-1}. Technical grade propiconazole was provided by Green Cross, Mississauga, Ontario; myclobutanil by Rohm and Haas, Mississauga, Ontario; fenarimol by Dow Elanco, Toronto, Ontario; and tebuconazole by Chemagro, Mississauga, Ontario.

Each isolate was replicated three times per concentration for each fungicide. The plates were incubated at 22 °C, and diameter measurements made after 48 h. EC_{50} values (effective concentration to cause 50% inhibition) were determined for each isolate by calculating the inhibition (= 1 –(the mean colony diameter on amended media divided by the mean colony diameter on unamended media)) in percent and subjecting the data to probit analysis (SAS® PROC PROBIT). Probit transformation serves to straighten out the dosage-response curve and allows more accurate estimation of EC_{50} values compared to untransformed data (Sokal and Rohlf, 1981). A copy of the SAS® program statements for probit analysis can be obtained upon request via email from thsiang@uoguelph.ca. To correct for the log-normal distribution of the data, distribution and scatter plots were drawn up using the log EC_{50} values. Correlation analysis was conducted between the log EC_{50} values of the isolates to measure cross-resistance.

Results and discussion

The origins and number of isolates from each sample site are given in Table 1. The eight sample sites were distributed over a 40,000 km^2 area bounded by Windsor in the west, St. Catharines 385 km to the east and Barrie 185 km to the north (Figure 1). Out of an original 494 samples, 435 isolates were obtained and used in fungicide sensitivity testing. Although more than 50 samples were collected from each site, not all samples yielded an isolate and some isolates did not survive until testing. This was most notable for population SH6, which only had 21 representatives at time of fungicide testing (Table 1).

Table 1. Origin, host species, and number of isolates of *Sclerotinia homoeocarpa* collected in 1994

Population	Origin	Host species	Number of Isolates
SH1	Cambridge, Ontario	*Agrostis palustris* Huds.	53
SH2	Guelph, Ontario	*Poa annua* L./*Agrostis* spp.	58
SH3	Barrie Ontario	*Poa annua*/*Agrostis* spp.	59
SH4	St. Catherines, Ontario	*Poa annua*/*Agrostis* spp.	57
SH5	London, Ontario	*Agrostis palustris*	60
SH6	Windsor, Ontario	*Agrostis palustris*/*Poa annua*	21
SH7	Downsview, Ontario	*Agrostis palustris*/*Poa annua*	47
SH8	Kingsville, Ontario	*Poa annua*/*Agrostis* spp.	80

Within Ontario populations of *S. homoeocarpa*, there was a wide range in DMI fungicide sensitivity (Table 2). Fenarimol sensitivity had the greatest range for all populations, up to a 174-fold difference between the lowest and the highest EC_{50} value for SH2. The average ratio of maximum EC_{50} over minimum EC_{50} in each population was 30.7 for all four fungicides. This indicates that at the time of sampling, populations contained a mixture of biotypes varying in sensitivity, and this is supported by the sensitivity distribution graphs (Figure 2). This is the first study to quantify variation in a large collection of *S. homoeocarpa* isolates. This organism has not been observed to produce sexual or asexual spores in North America (Smiley et al., 1992), yet there is substantial variation between isolates.

Golembiewski et al. (1995) found that isolates of *S. homoeocarpa* from areas which had not been sprayed with DMI fungicides had ED_{50} values of 0.002 for propiconazole, and 0.03 μg ml^{-1} for fenarimol. Except for population SH6, these values are very similar to the means for most of our sampled populations in Ontario (Table 2). In our annual tests on fungicide control of dollar spot disease (e.g. Hsiang and Cook, 1993), we used a mixture of *S. homoeocarpa* isolates to inoculate plots. The mean propiconazole EC_{50} value of these isolates was 0.005 μg ml^{-1} (data not shown), and propiconazole has always been found to be extremely efficacious. The mean propiconazole EC_{50} value of the Ontario isolates in the current test excluding population SH6 was 0.007 μg ml^{-1} (Table 2) which indicated that our base populations had high sensitivity to propiconazole. This provided confirmation that at time of sampling, our base populations, aside from SH6, had received little, if any, exposure to DMI fungicides such as propiconazole.

Population SH6 showed the highest mean EC_{50} value for three of the four fungicides and was the second highest for tebuconazole (Table 2). In this population, the EC_{50} values were 0.026 (propiconazole) and 0.078 μg ml^{-1} (fenarimol), which were greater than the EC_{50} values of the other populations, but considerably less than 0.103 (propiconazole) and 0.26 μg ml^{-1} (fenarimol) found by Golembiewski et al. (1995) for isolates from areas of disease control failure with DMI fungicides. These results suggest that either a DMI fungicide may have been used on population SH6 in the past, or that ingress had occured from areas where DMI fungicides had been previously used. Population SH6 exists very close to the U.S. border (Figure 1), and there is a possibility that isolates with reduced DMI sensitivity were carried into Canada by wind or introduced by traffic. However, the population structure of SH6 does not support such an introduction since there is an absence of highly sensitive isolates and a visible shift toward reduced sensitivity compared to the other populations (Table 2, Figure 2). In addition, we have found that there is a slight fitness cost for decreased sensitivity to propiconazole (manuscript submitted), and hence introduced isolates with decreased sensitivity would not likely out-compete the existing sensitive isolates in the absence of a DMI fungicide selection pressure. Perhaps a combination of introduced isolates with reduced DMI-sensitivity coupled with minor, but non-labelled use, of a DMI fungicide has led to the observed population structure of SH6.

For DMI studies with other plant pathogens, resistance factors (mean EC_{50} of resistant population / mean EC_{50} of sensitive population) of 1.8 up to 10 or more have been found leading to unsatisfactory field control (Braun and McCrae 1992; Smith et al., 1991). Golembiewski et al. (1995) found a mean resistance factor for propiconazole of 51.5 for three populations of *S.*

Table 2. Mean and range of EC_{50} (μg ml^{-1}) for each fungicide by population of *Sclerotinia homoeocarpa* from Ontario

Population[a]	Propiconazole		Myclobutanil		Fenarimol		Tebuconazole	
	mean	range	mean	range	mean	range	mean	range
SH1	0.006	0.0004	0.136	0.036	0.029	0.001	0.020	0.007
		–0.018		–0.591		–0.154		–0.045
SH2	0.005	0.002	0.183	0.091	0.057	0.003	0.032	0.005
		–0.010		–0.818		–0.522		–0.094
SH3	0.008	0.003	0.218	0.025	0.030	0.002	0.016	0.004
		–0.003		–0.565		–0.113		–0.082
SH4	0.006	0.002	0.116	0.039	0.029	0.002	0.015	0.004
		–0.014		–0.315		–0.172		–0.033
SH5	0.009	0.002	0.320	0.095	0.022	0.002	0.013	0.003
		–0.028		–0.798		–0.089		–0.027
SH6	0.026	0.005	0.945	0.316	0.078	0.014	0.025	0.008
		–0.069		–3.134		–0.246		–0.053
SH7	0.008	0.004	0.324	0.087	0.025	0.003	0.017	0.004
		–0.012		–0.834		–0.115		–0.043
SH8	0.009	0.003	0.229	0.039	0.021	0.002	0.013	0.002
		–0.046		–1.562		–0.102		–0.039
LSD (p = 0.05)[b]	0.002		0.107		0.017		0.005	

[a] Populations of *Sclerotinia homoeocarpa* are described in Table 1.
[b] LSD is the least significant difference between arithmetic means within a column.

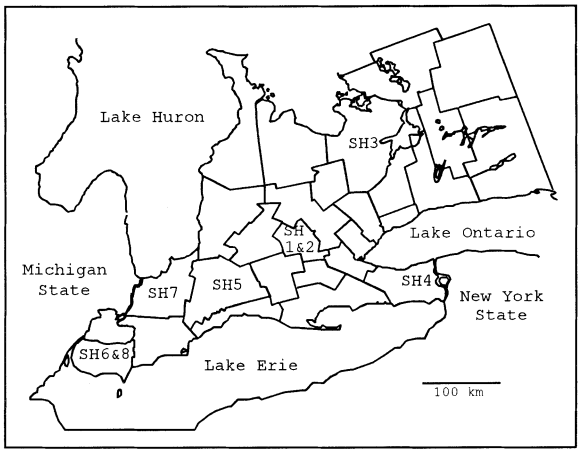

Figure 1. Map of southern Ontario, Canada, showing the counties where collections of *Sclerotinia homoeocarpa* (SH) were made.

homoeocarpa that had been exposed to DMI compared to three which had not. If we were to use SH6 as the 'resistant' population among our Ontario isolates, the resistance factors would range from 2.0 to 5.1 with a mean of 3.7 for the different fungicides and populations. This indicates that SH6 was not a resistant population in terms of economically significant field resistance.

Cross-resistance between DMI fungicides has been observed in many previous studies. Köller et al. (1991) for *Venturia inaequalis* found a very high correlation between ED_{50} values of fenarimol vs. myclobutanil. The data of Golembiewski et al. (1995), testing *S. homoeocarpa* from areas sprayed and not sprayed with DMI, suggested that ED_{50} values of triadimefon, fenarimol and propiconazole were highly correlated. Hermann and Gisi (1994) found positive cross-resistance in *Septoria tritici* between tebuconazole, propiconazole and other DMI fungicides. Kendall et al. (1993) found for *Rhynchosporium secalis* that some cross-resistance occurred between triadimenol, propiconazole and tebuconazole, although the change in sensitivity to tebuconazole was less than that for the two other fungicides. Similarly, we observed that population SH6 did not show a dramatic sensitivity shift for tebuconazole as it did for the other three fungicides (Figure 2).

Peever and Milgroom (1993) found that fenarimol sensitivity was highly correlated with sensitivity to triadimenol and imazalil (another DMI fungicide), although they found no significant genetic correlation between sensitivity to fenarimol vs. propiconazole for *Pyrenophora teres* in one of their two experiments. Kendall (1986) similarly found a lack of

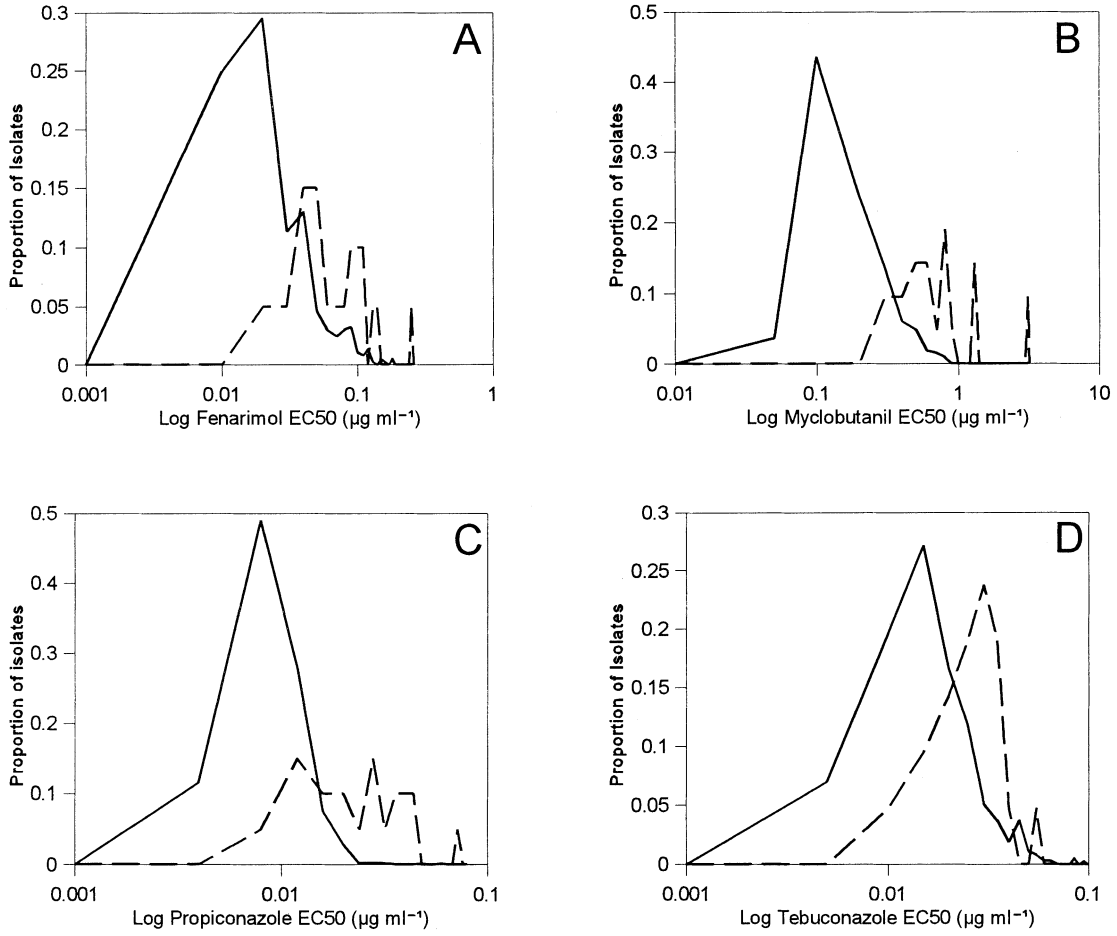

Figure 2. Distribution of DMI fungicide sensitivity in 435 Ontario isolates of *Sclerotinia homoeocarpa* separated into sensitive (solid line) and reduced sensitive (dotted line, SH6) populations. EC_{50} values were determined by agar plate assay: (A) fenarimol, (B) myclobutanil, (C) propiconazole and (D) tebuconazole.

cross-resistance between fenarimol and propiconazole for *P. teres*; however in her test of 13 fungicides, she found *P. teres* to differ from *Cladosporium cucumerinum* and *Sphaerotheca fuliginea* in having a more restricted range of cross-resistance. She observed significant cross-resistance for the latter two fungi to fenarimol and propiconazole. Our results show a low correlation between EC_{50} values of fenarimol vs. propiconazole (Figure 3) which was just not significant. Similarly, there was a low and not significant correlation between sensitivity to myclobutanil vs. fenarimol. The poorest relationship was found EC_{50} values of tebuconazole and propiconazole in terms of both correlation coefficient and statistical probability (Figure 3). This agrees with Robbertse et al. (1996) who found that some isolates of *Ramulispora herpotrichoides* sensitive to propiconazole were resistant to tebuconazole.

Köller and Wudden (1989) in their comprehensive research on cross-resistance in*Ustilago avenae* toward 20 DMI fungicides, found very high correlations (calculated from their data as 0.85 to 0.99) between the four fungicides used in the current study. Their work was based on one wild-type sensitive and four lab-generated resistant isolates, and thus might not be representative of wild-type or field-resistant isolates. Furthermore, there is still uncertainty as to the mechanism of DMI resistance under field conditions (Shirane et al., 1996; Stehmann and De Waard, 1996).

The best correlation was found between EC_{50} values of myclobutanil and propiconazole ($r = 0.438$, $p = 0.0001$). The second highest correlation, which

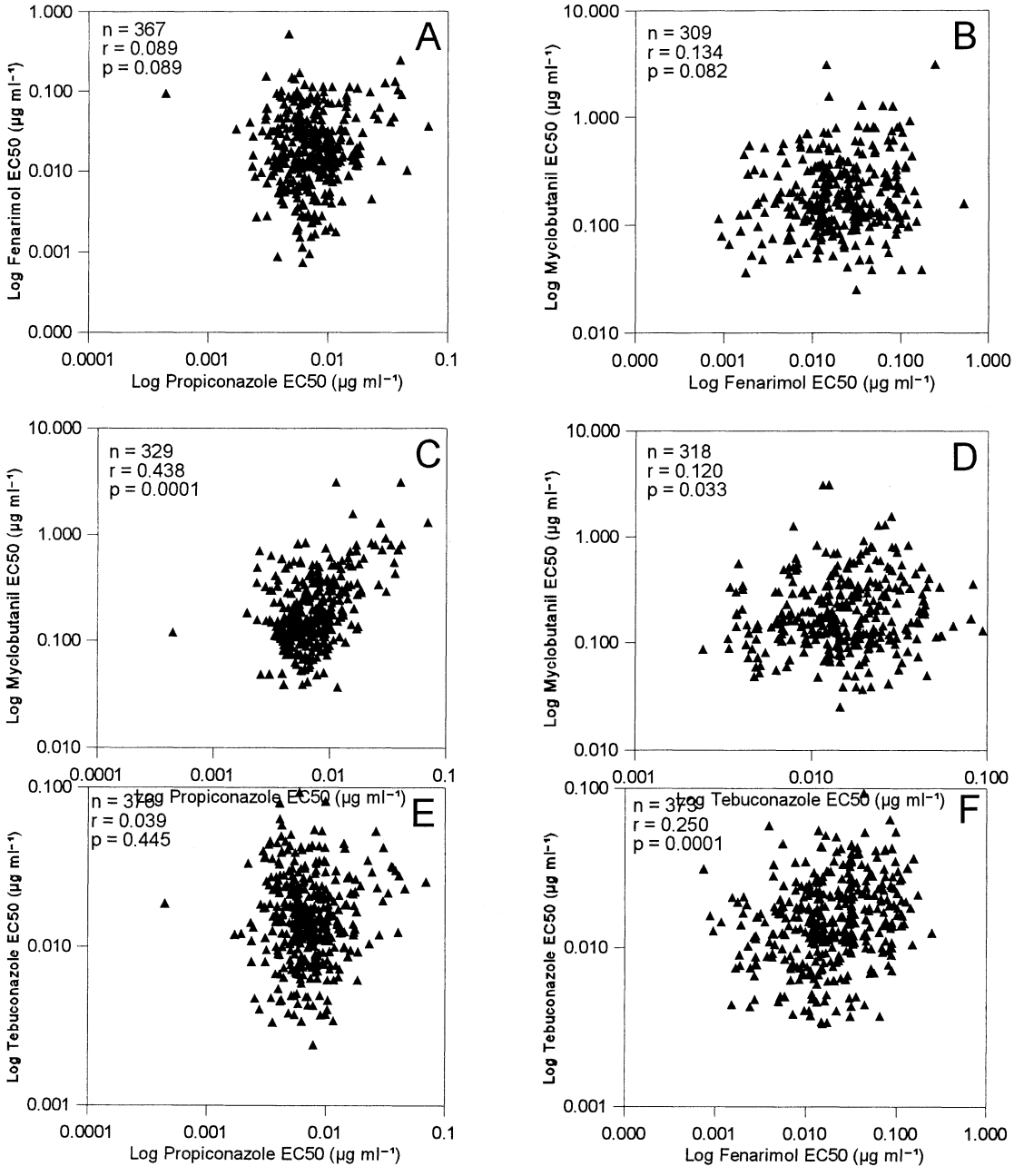

Figure 3. Cross-resistance patterns between DMI fungicides in *Sclerotinia homoeocarpa* isolates from Ontario. EC_{50} values were determined by agar plate assay: (A) fenarimol vs. propiconazole, (B) myclobutanil vs. fenarimol, (C) myclobutanil vs. propiconazole, (D) myclobutanil vs. tebuconazole, (E) tebuconazole vs. propiconazole, and (F) tebuconazole vs. fenarimol.

was also highly significant, was found between EC50 values of tebuconazole and fenarimol (r = 0.250, p = 0.0001). Thus, among the four DMI fungicides in this study, there appeared to be two well-correlated groups in terms of cross-resistance: propiconazole + myclobutanil, and tebuconazole + fenarimol. Köller and Wudden (1989) have discussed the occurrence of second modes of action of fenarimol and tebuconazole on sterol biosynthesis. Although the second modes of action are different, and although the second modes of

action are not thought to be primary determinants of the resistance factors (Köller and Wudden, 1989), there may be some similarities between these two fungicides that allow for a higher sensitivity correlation. These similarities may also allow for lower correlations with the two other fungicides in this study.

Perhaps, if we had tested isolates of *S. homoeocarpa* from areas of DMI-fungicide disease control failure, the cross-resistance relationships would have been stronger. Golembiewski et al. (1995) stated that 'DMI fungicides had not adequately controlled dollarspot on courses 4 through 6 since 1989', which probably indicated cross-resistance between available DMI fungicides. In any case, cross resistance to DMI fungicides shows variability by fungicide and fungal species, and is not as absolute as conventionally thought. Further research is needed to elucidate the basis of these different correlations in sensitivity between certain DMI fungicides.

Several studies in various pathosystems have measured baseline sensitivity before large-scale DMI fungicide use (Al-Mughrabi and Gray, 1995; Carisse and Pelletier, 1994; Köller et al., 1991; Penrose and Senn 1995; Robbertse et al., 1996; Smith et al., 1991). This information can then be used to evaluate population shifts in fungicide sensitivity in subsequent years (Brent 1995). Our study found that most *S. homoeocarpa* populations in Ontario were sensitive to DMI fungicides, but they all contained members which have reduced sensitivity. One of the eight populations sampled had probably been exposed to DMI fungicides. We have been unable to confirm prior DMI use at this site, but the population structure indicated such an exposure. We intend to continue sampling over the next few years to see whether there are detectable shifts in *S. homoeocarpa* sensitivity to DMI fungicides as they become more frequently used in Ontario.

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