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Relative virulence of isolates of *Sclerotinia homoeocarpa* with varying sensitivity to propiconazole

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Abstract

Isolates of *Sclerotinia homoeocarpa* were collected from across southern Ontario from areas where demethylation inhibiting fungicides reportedly had never been used. Forty of these isolates with propiconazole EC_{50} values ranging from 0.003 to 0.069 μg ml^{-1} were inoculated onto creeping bentgrass (*Agrostis palustris*) in summer 1995 and summer 1996 to assess virulence. Each isolate was grown on mixed cereal grains, dried, ground up and applied separately at a rate of 10 g m^{-2} to 0.25 m^2 plots with three replicates per isolate. For both years, no spots were visible on the plots at time of inoculation; however, by the end of each experiment, there were up to 180 spots per plot with significant differences between isolates. Growth rate in culture was not significantly related to fungicide sensitivity (log-EC_{50} values). Statistically significant negative relationships were found in both years between AUDPC and log-EC_{50} values. These significant relationships imply that in the absence of propiconazole use, isolates that are more sensitive to propiconazole may out-compete isolates that are less sensitive. However, further study is required to determine the time frame for this to occur, and whether DMI-resistance prevention strategies can feasibly be based on the existence of resistance-related fitness costs.

Fungicide resistance is an increasing problem as more taxa of fungi are discovered to have resistance to fungicides (Eckert, 1988). This problem is further exacerbated as fungicides, particularly older ones, become banned or withdrawn from the market (De Waard et al., 1993). Many of the more recently introduced fungicidal active ingredients are systemic and have specific biochemical target sites in fungal cells, and thus are more prone to resistance development (De Waard et al., 1993). With these types of fungicides, such as benzimidazoles and demethylation-inhibitors, economically significant resistance problems often have been observed in areas of intensive use (Staub, 1991).

The demethylation inhibitors (DMI) are a relatively new group of systemic fungicides that control a broad spectrum of pathogens from all major fungal groups, with the exception of Oomycota (Scheinpflug, 1988: Sisler, 1988). Among the DMI fungicides in Canada, only propiconazole is registered for use on turf and only since fall 1994 (Canadian Pest Management Regulatory Agency database, Ottawa, Canada). Myclobutanil and other DMI fungicides are in the later stages of development for use on turf in Canada, but because DMI fungicides may have the same mode of action and show cross-resistance (Scheinpflug, 1988), there are strong concerns that field resistance to one would render the others useless.

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. This disease is called dollar spot because it forms small brown patches on closely mown turf up to the size of a dollar coin. In southern Ontario, the disease can be found from mid-June through October. Numerous fungicide applications are made annually to control this disease, especially in preventive programs based on 2-week schedules throughout the growing season. This high frequency of use provides a selective advantage for isolates that have some decreased sensitivity to the fungicide. 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 resistance to DMI fungicides have been reported in Illinois, Kentucky and Michigan (Doney and Vincelli, 1993; Golembiewski et al., 1995; Vargas et al., 1992). Because fewer fungicides are registered for use on turf in Ontario (Anonymous, 1994) than in the nearby American States, and because a DMI fungicide for turfgrass disease control was just recently registered in Canada, there are heightened concerns about development of DMI fungicide resistance in Canada.

In a previous study (Hsiang et al., 1997), we found that there may have been non-labelled use of a DMI fungicide in Ontario in the recent past. The DMI fungicide sensitivity distribution of one population of *S. homoeocarpa* differed from seven other populations with a skewed tail toward less sensitivity. Some isolates of *S. homoeocarpa* in this one population had significantly decreased sensitivity to DMI fungicides approaching levels reported for economically significant field resistance (Golembiewski et al., 1995). Unfortunately, there are no verified accounts of when, where or how much DMI fungicide might have been used on the turfgrass.

Growers often ask how quickly economically significant fungicide resistance will develop after the introduction of a new fungicide. Predictions on the development of fungicide resistance have often proven wrong; the ease of producing fungicide-resistant mutants in the lab often is not correlated with the development of economically significant fungicide resistance in the field (De Waard et al., 1993). Growers often also ask if a fungicide can be re-introduced in later years after resistance to that fungicide has developed. There is controversy in the literature regarding the fitness and persistence of fungicide-resistant biotypes in the absence of the fungicide selection pressure, with great variations by pathogen and fungicide. For some pathogens, the mutations which impart fungicide resistance also lead to decreased vigour and persistence (e.g. Engels & De Waard, 1996); while for other pathogen-fungicide combinations, the fungicide resistant phenotypes have been found to be very stable and persistent even after many years (e.g. Johnson & Theiling, 1990). The issue of fitness and resistance has been discussed previously for DMI fungicides (Leroux et al., 1988, Peever & Milgroom, 1994).

The fitness and persistence of some benomyl-resistant biotypes of several fungi are notorious (e.g. Hsiang and Chastagner, 1992; Johnson and Theiling, 1990), but there is insufficient research on *S. homoeocarpa* to say whether there are high costs in fitness associated with decreased DMI fungicide sensitivity. Anti-resistance strategies, whether to prevent resistance development or to combat resistance problems, are often based on the premise that isolates with decreased fungicide sensitivity have a decreased fitness in the absence of the fungicide selective pressure compared to wild types. More data are needed to test this premise.

The purpose of this study was to sample naturally occurring isolates of *S. homoeocarpa* varying in sensitivity to propiconazole, and to test whether this variation was correlated with fitness as represented by growth rate in culture and field virulence on creeping bentgrass in the absence of the fungicide. Specific hypotheses tested include: 1) growth rate of *S. homoeocarpa* in culture is correlated with DMI fungicide sensitivity; 2) growth rate of *S. homoeocarpa* in culture can be used as an indicator of field virulence and hence fitness; and 3) field virulence of *S. homoeocarpa* as represented by disease progression is related to DMI fungicide sensitivity. Because of the selection of naturally occurring isolates of *S. homoeocarpa* that reportedly did not have exposure to DMI fungicides, this study is particularly relevant to the formulation of strategies that prevent DMI resistance development.

Materials and methods

In 1994, isolates of *S. homoeocarpa* were collected from diseased turfgrass across southern Ontario, Canada, where DMI fungicides reportedly had never been used. These isolates were used in a previous study (Hsiang et al., 1997), and a subset of these isolates were used for the current study. There were eight sampling locations: Cambridge, Guelph, Barrie, St. Catharines, London, Windsor, Toronto and Kingsville. This area is bounded by Windsor in the west, St. Catharines 385 km to the east and Barrie 185 km to the north. At least 50 samples from each location were collected systematically using a grid with at least 1 m between samples. Approximately 20 leaf blades from an infection center nearest each grid point were collected and placed into a 20-ml vial. Vials were returned to the lab the same day and stored at 4 °C. 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 at 4 °C. Since this

fungus does not produce sexual nor asexual spores, a series of subcultures were made to ensure purity. A single isolate was retained per grid point sample.

All isolates were tested for sensitivity to propiconazole using an agar plug assay described by Detweiler et al. (1983). Technical grade propiconazole (courtesy of Green Cross, Mississauga, Ontario) was dissolved in acetone, 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). 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. There were 3 replicates per combination of isolate by fungicide concentration.

Plates were incubated at 20 °C, and radial growth measurements were made after 48 h. A mean radial growth rate (mm growth / 2 days) was calculated for each isolate with data from unamended medium (containing no propiconazole). The fungicide sensitivity of each isolate was estimated by EC_{50} values (effective concentration to cause 50% inhibition). EC_{50} values were determined for each isolate by calculating the percent inhibition by each fungicide concentration (= 1 - (the mean colony diameter on propiconazole-amended medium divided by the mean colony diameter on medium with no propiconazole)) X 100% and subjecting these 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 to thsiang@uoguelph.ca. To correct for the log-normal distribution of the EC_{50} data, the values were log transformed for further analyses.

The test plots were laid out in a 0.5 m × 0.5 m grid pattern on a native sand putting green. There were three replicate plots per isolate in a fully randomized design. This research green had been established in 1994 with Penncross creeping bentgrass at the Guelph Turfgrass Institute, Guelph, Canada. The green was maintained similarly to intensively managed turfgrass on golf courses. Management included fertilization in spring and fall to a total of 1 kg N per 100 m^2, irrigation as needed to prevent water stress, and mowing 3 to 6 times a week during the growing season to a 7 mm height. Only for research purposes had pesticides been applied to these plots, and no DMI fungicides had been used previously on these plots.

Out of over 400 isolates (Hsiang et al., 1997), 40 isolates representing a range of propiconazole sensitivities were chosen for field testing by arranging isolates in order of fungicide sensitivity and systemically selecting every tenth isolate for further testing. Inocula were grown on autoclaved, water-saturated, mixed cereal grains for two weeks, air dried for two days, ground with a blender and passed through a 2-mm mesh to remove large particles. This inoculum was mixed 1:9 (w/w) with whole wheat flour for more uniform application, and the mixture was stored at 4 °C until used. The inoculum for each plot was weighed out separately into small envelopes just prior to application, and the inocula were carefully sprinkled by hand onto plots that were marked out with spray paint. Each isolate was applied at a rate of 10 g inoculum mixture per m^2 onto 3 replicate plots of 0.25 m^2.

To enhance disease establishment, plots were inoculated in the evening after they had been irrigated for 5 min with an automated sprinkler system. Plots were also irrigated the next day for at least 5 min at 10 am, 2 pm and 6 pm. Plots were rated weekly for 6 weeks by counting the number of dollar spot patches per plot. This field experiment was first conducted in summer 1995 and then repeated again in summer 1996. AUDPC (Area Under the Disease Progress Curve) was calculated to represent disease progression where AUDPC = sum of ($disease_{time\times+1}$ + $disease_{time\times}$)/($time_{\times+1}$ - $time_{\times}$), modified from Campbell & Madden (1990). Correlation coefficients were then calculated between all pair-wise combinations of means of AUDPC, radial growth rates, and log-EC_{50} values. In addition, analysis of variance was conducted on AUDPC and log-EC_{50} data, and when a significant treatment effect was found, the test of least significant difference (LSD) was used to separate means.

Results and discussion

Prior to fall 1994, there was no registered use of DMI fungicides to control turfgrass diseases in Canada, and according to records, no DMI fungicides had been used in the areas sampled before the time of collection in summer 1994. The sampling sites across southern Ontario were chosen to give a broad geographical distribution of the fungus. More intensive sampling at each site allowed us to look at the within- and between-site variability of this organism. Based on molecular

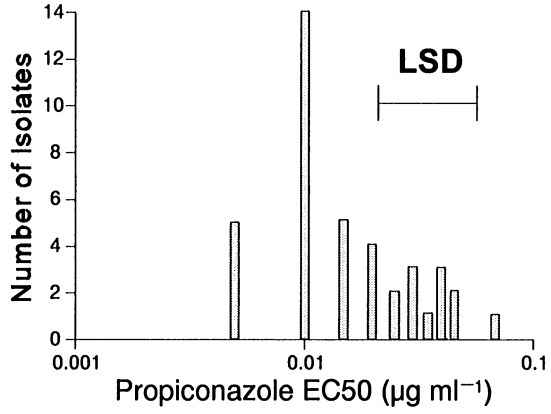

Figure 1. Distribution of *Sclerotinia homoeocarpa* isolates by degree of sensitivity to propiconazole. EC_{50} values were determined by growth on propiconazole-amended media at several concentrations compared to growth on unamended PDA media. LSD (p=0.05) was calculated after obtaining a significant isolate treatment effect in an analysis of variance of log-EC_{50} values.

markers, very little genetic variation between Ontario isolates has been found (manuscript in preparation). Raina et al. (1997) have also recently found that American isolates of *S. homoeocarpa* show very low levels of genetic diversity based on molecular markers. Because *S. homoeocarpa* does not appear to be differentiated across a region such as southern Ontario, this permitted us to pool all samples from southern Ontario, test for propiconazole sensitivity and then select a subsample of isolates that varied in their propiconazole sensitivity for use in virulence tests.

Golembiewski et al. (1995) found that the mean propiconazole EC_{50} values for isolates of *S. homoeocarpa* where DMI had never been used was 0.002 μg ml^{-1}, while field populations which were not adequately controlled by DMI had mean EC_{50} values of 0.103 μg ml^{-1}. EC_{50} values of Ontario isolates used in this study ranged from 0.003 μg ml^{-1} to 0.069 μg ml^{-1} (Figure 1) and encompassed most of the variation of that previous study. In past studies on resistance to DMI fungicides, resistance factors (defined as mean EC_{50} of resistant population / mean EC_{50} of sensitive population) of 1.8 up to 10 have been associated with unsatisfactory field control (Braun and McRae, 1992; Smith et al., 1991). For *S. homoeocarpa*, the data of Golembiewski et al. (1995) yield a mean resistance factor of 52. While none of the isolates in our study originated from areas of reported disease control failure with DMI fungicides, there was a 23-fold difference in EC_{50} between the most sensitive and the least sensitive isolates (Figure 1).

In 1995, the plots were inoculated on 27 Jul (day 206) at which time no symptoms of dollar spot were visible (Figure 2). Inspections on 31 Jul and 3 Aug also yielded no symptoms. By 9 Aug (day 220), symptoms were visible in every plot with an overall average of 48 spots per 0.25 m^2 plot. By the end of this field season, there were up to 180 spots per plot (Figure 2). In 1996, epidemics were much more difficult to induce. An inoculation in early July resulted in symptoms two weeks later which completely disappeared in the following week. Another round of inoculations on the same plots on 16 Aug (day 228) produced results 3 weeks later on 10 Sep (day 253). Disease continued to increase up to the last day of monitoring (day 281) but at a much lower rate than in 1995.

In 1995, in an experiment nearby that tested fungicide efficacy, disease progression on the control plots which were not inoculated with *S. homoeocarpa* showed that natural inoculum was able to cause disease, but at a level lower than that in our inoculated plots (Figure 2). Some plots may have been seriously contaminated by natural inoculum and this would have contributed to experimental error and reduced the strength of correlations between AUDPC and other variables. However, the higher disease severity in our test plots than in uninoculated controls and in areas just outside our plots indicated that the disease seen in these plots resulted at least partly from our inoculations. During both the 1995 and 1996 field seasons, some plots were heavily diseased while others had low levels of disease (Figure 2). This gave a very obvious checkerboard pattern among the plots which again indicated that at least some of the disease resulted from our inoculations, and that there could be differences in virulence between isolates.

AUDPC summarizes disease progression and is thus better than single point measurements of disease severity and simpler than complex mathematical models. Berger (1988) and Waggoner (1986) discuss AUDPC in detail. AUDPC can be regarded as a measure of virulence for plant pathogens. Virulence is probably a major component of fitness for plant pathogens, and can affect long-term persistence including that of fungicide-resistant isolates (Sanoamuang & Gaunt, 1995). Through analysis of variance, significant differences in virulence as represented by AUDPC (p = 0.05) were found between isolates in 1995 and 1996 (Figure 3). The large range of AUDPC in both years (Figure 3) demonstrates that variation in virulence was present

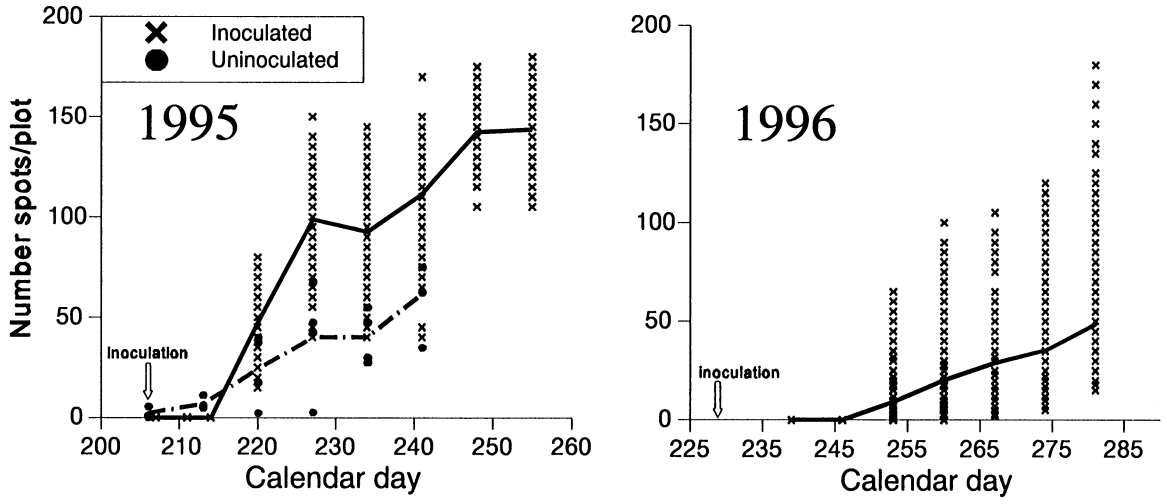

Figure 2. Disease progression of dollar spot on 0.25 m^2 creeping bentgrass plots during summer 1995 and 1996. The lines represent overall means for inoculated (1995, 1996) and control (1995) plots, and each point represents the mean of 3 replicate plots per isolate.

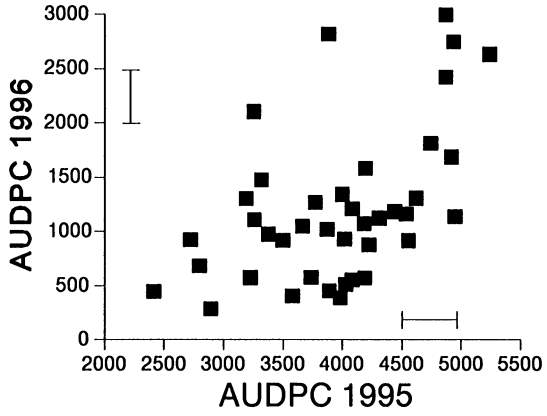

Figure 3. Relationship between virulence (AUDPC) of different *Sclerotinia homoeocarpa* isolates in 1995 vs. 1996. Each data point represents the mean of three replicate plots in both years. The bars near the axes are LSD values (p=0.05) obtained after significant isolate treatment effects in analyses of variance.

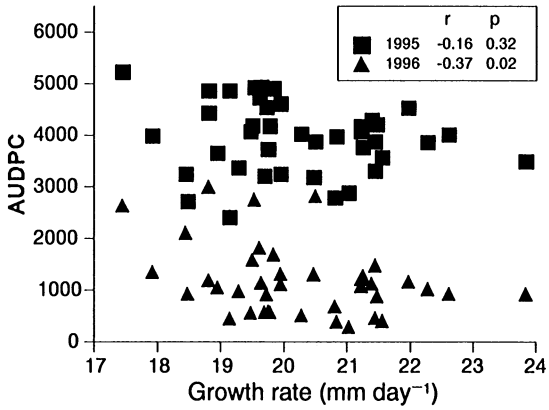

Figure 4. Relationship between virulence (AUDPC) and mycelial growth rate of *Sclerotinia homoeocarpa* on unamended media. Each data point represents the mean of 3 replicate plots (AUDPC) and 3 replicate plates (growth rate) per isolate.

in southern Ontario isolates of *S. homoeocarpa*. As far as we know, aside from hypovirulence caused by double-stranded RNA (Zhou & Boland, 1997), this is the first extensive report on variation in field virulence of *Sclerotinia homoeocarpa*.

The correlation between the AUDPC of the two years was highly significant (p=0.0006), but the coefficient was mid-range at r=0.52. The weather in 1996 was much less conducive to dollar spot disease development, and this may have contributed to some of the year-to-year variation as seen in this correlation (Figure 3) and correlations with other variables. Also, plot contamination by outside inoculum may have played a role.

Our 1995 data showed that isolate growth rate was not significantly correlated with AUDPC, however a significant negative relationship between these two variables was found in 1996 (Figure 4). There was no statistically significant relationship between log-EC_{50} value and isolate growth rate (Figure 5). This may be surprising because EC_{50} values incorporate growth rate as part of their calculation. The differences between isolates in growth on propiconazole-amended

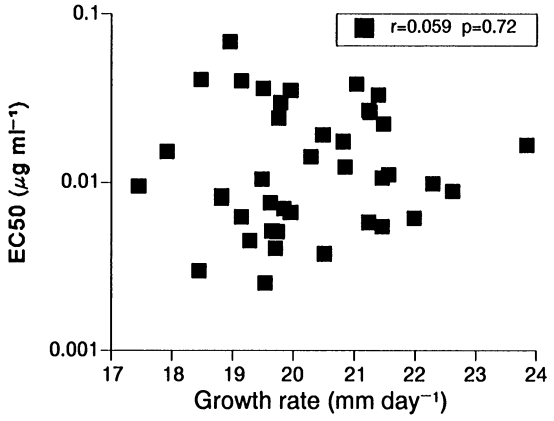

Figure 5. Relationship between *Sclerotinia homoeocarpa* sensitivity to propiconazole and mycelial growth rate on unamended media. Calculation of correlation coefficients (r) and probability values (p) used log-EC_{50} values. Each data point represents the mean of 3 replicates per isolate.

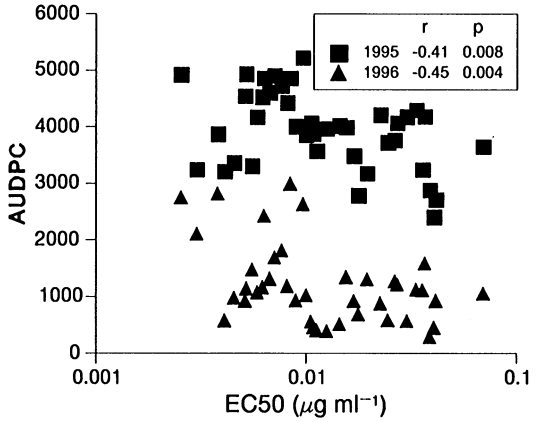

Figure 6. Relationship between virulence (AUDPC) and propiconazole sensitivity (EC50) of *Sclerotinia homoeocarpa* isolates. Calculation of correlation coefficients (r) and probability values (p) used log-EC_{50} values. Each data point represents the mean of 3 replicate plots (AUDPC) and 3 replicate sets of propiconazole-amended plates (EC_{50}) per isolate.

media may have been sufficient to mask this inherent relationship between growth rate and fungicide sensitivity calculations. Some previous studies on fungicide resistance have found that DMI-resistant isolates are slower growing in culture than their sensitive relatives (e.g. Leroux et al., 1988; Wellmann and Schauz, 1993). Growth rates in culture have been used as predictors of fitness (e.g. Dekker, 1988; Dekker and Gielink, 1979), but for *S. homoeocarpa*, this assessment must be approached with caution.

There were significant negative relationships between AUDPC and log-EC_{50} value in both years (Figure 6). The strength of these relationships ($p < 0.01$) allowed us to reject the hypothesis that there was no significant relationship between the two AUDPC and log EC_{50}. Because the EC_{50} value points were well distributed over the range of EC_{50} values, it was possible to derive a coefficient of determination (R^2 = 0.171 in 1995 and 0.200 in 1996) for the regression of AUDPC on log-EC_{50} values. This indicated that up to 20% of the variation in AUDPC (virulence) could be explained by log EC_{50} (sensitivity to propiconazole). The low amount of variation accounted for indicates that there were many other factors which contributed to variation in virulence. Such factors include climatic variation between the years, and inter-plot spread or natural contamination by the pathogen.

Two possible limitations of the current study can be remedied in future work. This study dealt only with naturally occurring isolates that presumably had not been exposed to a very strong DMI selection pressure; none of the isolates used came from areas where the fungicide was reported to fail to control disease. Inclusion of such isolates would allow stronger conclusions to be made about the fate of resistant isolates after the fungicide selective pressure is removed. The second limitation involves the potential for inter-plot spread of isolates and contamination by naturally occurring *S. homoeocarpa*. Although differences in isolate virulence showed up distinctly in a checkerboard plot pattern, and although disease was much more limited outside the test plots, we could not ensure that inter-plot contamination did not occur. The use of buffer zones with applications of non-DMI fungicides on these buffers could possibly ameliorate this situation for future work.

In summary, the results supported the hypothesis that isolates of *S. homoeocarpa* with reduced sensitivity to propiconazole showed a lower virulence. This relationship was statistically significant, but moderate as indicated by the moderately low correlation coefficient (Figure 6). The practical implication of this result is that in the absence of propiconazole use, isolates with less sensitivity to propiconazole could be outcompeted by isolates with more sensitivity to propiconazole but greater virulence. However, further study is required to determine the time frame for this to occur, and whether DMI-resistance prevention strategies can feasibly be based on the existence of resistance-related fitness costs.

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