Lack of Correlation Between Fitness and Resistance to Sterol Biosynthesis-Inhibiting Fungicides in *Pyrenophora teres*

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ABSTRACT

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Fitness costs associated with resistance to sterol biosynthesis-inhibiting fungicides (SBIs) were investigated by calculating correlations between SBI-resistance phenotypes and fitness phenotypes in *Pyrenophora teres*. Correlations between resistance to the SBIs triadimenol and propiconazole and two components of fitness were estimated with *P. teres* isolates randomly sampled from populations in North Dakota and Bavaria, Germany. The fitness components, latent period (the time from inoculation to the first appearance of a conidium) and sporulation (total sporulation per lesion), were determined quantitatively with detached barley leaf sections inoculated with *P. teres* conidia in the greenhouse. Resistances to triadimenol and propiconazole were measured as radial growth (proportion of the control) on a single discriminatory dose of each fungicide. The latent period varied from 5 to 11 days after inoculation and sporulation from 0 to 50,000 conidia per lesion. Significant genetic variation ($P < 0.05$) in fitness components was detected among *P. teres* isolates from both populations in three of four separate experiments. Significant genetic variation in resistance to triadimenol and propiconazole ($P < 0.001$) was detected among *P. teres* isolates from both populations. However, no significant correlation between fitness and resistance was obtained in any of the experiments. Therefore, we could not detect any fitness costs associated with resistance to triadimenol or propiconazole in these populations and conclude that SBI-resistance management strategies cannot depend upon the existence of fitness costs.

Additional keywords: DMIs, fungal resistance.

The evolution of resistance to fungicides is a serious problem in agriculture. If the appropriate level of variation in resistance exists in a plant-pathogen population, the use of fungicides places selective pressure on the population because resistant isolates have higher fitness in a fungicide-treated environment relative to sensitive isolates. Resistant isolates will be selected and increase in frequency in the pathogen population in subsequent generations, and the effectiveness of the fungicide applications may decline. It has been speculated with the sterol biosynthesis-inhibiting fungicides (SBIs) that fungicide-resistance genes that confer greater fitness in the presence of the fungicide have some associated fitness costs in the absence of the fungicide (6,7,13,28). Fitness costs associated with resistance genes are important from an evolutionary perspective because they allow selection against resistance in the absence of the fungicide leading to a decrease in the frequency of resistance genes in the pathogen population. SBIs constitute one of the most important groups of fungicides used in agriculture today, and much interest has been expressed in preventing the evolution of resistance to them as has occurred with other systemic fungicides (13). This will require detailed knowledge about the genetics of fitness and resistance and the relationships between these characters.

Several methods have been proposed to manage the evolution of resistance to fungicides in pathogen populations, and the theoretical aspects of these proposals have been reviewed (19). Fungicide mixtures and alternations of fungicides in time and space may delay the evolution of resistance (13,28). For these strategies to be effective, resistance to the fungicides used in the mixture or in alternation must not be correlated (no cross-resistance). The alternation strategy can delay the evolution of resistance to fungicides if fitness costs are associated with resistance to each fungicide used in the alternation. Most models of the evolution of resistance to fungicides have assumed fitness costs associated with resistance (2,19,25). Although there has been much speculation in the literature about fitness costs associated with fungicide-resistance genes, the available evidence is meager and rather contradictory. For example, some studies have demonstrated fitness costs associated with resistance (4,5,9,20,21,33) while others have not (3,11,15,29,32). One study found that isolates of *Phytophthora infestans* (Mont.) de Bary resistant to metalaxyl had greater fitness than the sensitive isolates, regardless of the presence of the fungicide (10). Variation in experimental results among studies may be explained by the use of different measures of fitness, fungicides, pathogens, or sources of isolates. Another possible explanation is that most of these studies involved few pathogen isolates, and fitness differences between resistant and sensitive isolates may have been due to differences in the genetic backgrounds of the isolates rather than to fitness costs associated with resistance genes.

Most fitness studies with SBIs have involved fungicide-resistant isolates selected in the laboratory on fungicide-amended media (4–6,11,15,21,32). The rationale for using laboratory-generated resistant mutants appears to be that such mutants represent the range of variability in resistance that is expected in the field. This assumption is not necessarily valid, because it has been found that benomyl-resistant isolates of *Venturia inaequalis* (Cooke) G. Wint. found in the field represent only a small subset of the total number of mutations to resistance that are possible in the laboratory (12). This suggests that many resistant mutants selected in the laboratory may not survive in the field, presumably because of lower competitive fitness. Another potential difference between laboratory and field-selected resistant isolates is the selection of fitness-modifying genes in the field. Genetic analysis of fitness costs associated with resistance to insecticides in the sheep blowfly, *Lucilia cuprina*, revealed the presence of fitness-modifying genes

that were selected in blowfly populations and increased the fitness of resistant strains to a level similar to that of the sensitive strains (18). Determining the fitness of laboratory-selected resistant isolates may therefore have little relevance to the fitness of resistant field isolates. Relatively few fitness studies have involved naturally occurring isolates sampled from field populations, and most of these studies have used very small sample sizes and isolates sampled from different populations (3,9,10,12,29). The potential problem of pooling isolates from different populations was revealed by a study of fitness costs associated with resistance to benomyl in two populations of *V. inaequalis* in which fitness costs were found in one population but not in the other (14). This suggests that fitness costs can be variable between populations because of different fitness costs associated with different resistance genes or differences in genetic backgrounds between populations. Only one study has examined fitness costs associated with resistance to SBIs in field-collected fungi (W. Köller, *personal communication*). In that study, however, fitness was determined by measuring spore production, spore germination, and mycelial growth in vitro, and it was not demonstrated that these measures of fitness were correlated to fitness in vivo.

In order to make inferences about the fitness of fungicide-resistant isolates in populations of pathogens, sampling must be representative of a particular population at a particular time and large enough to make meaningful inferences. Consideration must also be given to the reproductive biology of the pathogen and how the mode of reproduction may affect fitness and resistance to fungicides. Pathogens reproducing sexually undergo recombination, and fungicide-resistance genes are randomized into different genetic backgrounds with every generation. In asexually reproducing pathogens, however, resistance genes are effectively linked to all other genes in each clone present in the population. With pathogens that reproduce asexually or predominantly asexually, we have shown that the most appropriate correlations to measure fitness or resistance to fungicides are "among-isolate" correlations (23). These correlations do not indicate specifically whether fitness costs are associated with particular resistance genes but do show the relationship between fitness and resistance among clones of the pathogen in the population of interest.

Pyrenophora teres Drechs. causes net blotch of barley (*Hordeum vulgare* L.), which is an important disease of barley in most parts of the world. SBIs have been applied as either foliar or seed treatments to control this disease. Resistance to the SBI triadimenol evolved quickly in *P. teres*, and triadimenol was ineffective against this disease after only 3 yr of use in New Zealand (26). We demonstrated that resistance to triadimenol in isolates from several different populations was conferred by a major genetic locus as well as by several minor loci (22). High frequencies of triadimenol-resistant isolates have been found in many *P. teres* populations, even in populations where triadimenol has never been used (23), suggesting that significant fitness costs may not be associated with triadimenol resistance in *P. teres*.

The purpose of this study was to test the hypothesis that fitness costs are associated with resistance to the SBIs triadimenol and propiconazole in *P. teres*. We approached the question of fitness costs associated with resistance to SBIs from a population perspective by randomly sampling *P. teres* isolates from two different populations. The relationship between fitness and resistance to SBIs in these populations was determined by estimating correlation coefficients between triadimenol- and propiconazole-resistance phenotypes and the two fitness components, latent period and sporulation.

MATERIALS AND METHODS

Barley plants. Seed of barley cultivar Risø 5678-R was obtained from J. R. Aist, Department of Plant Pathology, Cornell University. Risø 5678-R is known to be susceptible to *P. teres*. Seeds were planted in 24-cell plastic flats consisting of 5- × 5-cm cells filled with potting mixture. Plants were maintained at 18–25 C in the greenhouse under natural lighting and thinned to one plant per cell after 2 wk of growth. Five-week-old plants (growth stage 22, Zadoks scale) were used for all experiments.

***P. teres* isolates.** *P. teres* isolates were randomly sampled from a commercial barley field (cultivar Robust) in Stutsman County, North Dakota, by B. Steffenson, North Dakota State University, Fargo, and from a research plot (cultivar Trixi) near Deggendorf, Bavaria, Germany, by G. M. Hoffmann and H. Meier, Technical University of Munich. For the purposes of this study, isolates of *P. teres* from the same barley field were considered to be members of the same *P. teres* population. Infected leaves were sampled from each population during July 1991 during the asexual growth phase of the pathogen, and 25 single-conidial isolates were obtained from each population (one isolate per infected leaf) and stored at −20 C on sterile filter paper (22). Isolates were grown on malt extract agar containing 1.5% (w/v) malt extract (William's Brewing Supplies, San Leandro, CA) and 2% (w/v) agar (Difco, Detroit, MI). Mycelial plugs were taken from the edges of the colonies after 5 days of growth at 25 C in the dark and transferred to V8-juice agar consisting of 17% (v/v) V8 juice, 3% (w/v) $CaCO_3$, and 1.5% (w/v) agar. Plates were placed under near-ultraviolet light (GE black light #F40BL), and a 10-h photoperiod (10 h light and 14 h dark) was used for 14 days to induce production of conidia.

Inoculation of plants. The surface of a plate containing a sporulating isolate of *P. teres* was flooded with 10 ml of sterile distilled water and then scraped with a metal spatula to dislodge conidia. The resulting conidial suspension was poured through two layers of sterile cheesecloth into a sterile 15-ml tube. Tubes were spun in a Beckman GP centrifuge (Beckman Instruments, Palo Alto, CA) at 1,500 *g* for 10 min to sediment the conidia. The supernatant was poured off, 300 μl of sterile distilled water was added, and the tubes were kept on ice to prevent conidial germination. Conidial suspensions of each isolate were counted and adjusted to 1×10^6 conidia per milliliter. One drop of a 10% (v/v) solution of Tween 20 was added to each tube as a surfactant. Barley plants were inoculated in the greenhouse by a variation of a technique developed previously to study lignification in wheat leaves (24). The oldest leaf on each plant was selected, laid out horizontally, and secured at each end to the plastic flat with pieces of tape. Inoculation involved placing 20-μl drops of conidial suspension from each isolate on the upper surfaces of the leaves; there were two inoculation sites per leaf. Isolates were randomized to leaves, and six replicates of each isolate were used in each experiment. Following inoculation, plants were covered with clear polyethylene bags to maintain high humidity and promote infection. Bags were removed after 48 h, and the plants were allowed to dry for a further 24 h, at which time lesions were visible on the leaves. Sections of leaves with lesions approximately 8 cm long were cut from the plants and placed in plastic petri plates containing wet filter papers. The plates were placed under near-ultraviolet light in the laboratory. Four independent experiments were performed at different times consisting of two repeated experiments (experiments A and B) for each population. Twenty-five North Dakota isolates and 21 German isolates were used.

Fitness components. Fitness of each isolate was determined by measuring latent period and total sporulation per lesion. Each lesion (six lesions per isolate per experiment) was examined daily under a dissecting microscope to determine the time from inoculation to appearance of the first conidium (latent period). When new conidia were no longer produced (approximately 11 days after inoculation), leaf pieces containing lesions were cut out and placed in 1 ml of 0.05 M $CuSO_4$ in Eppendorf tubes. The tubes were vortexed to dislodge conidia from the lesions, and the leaf sections were removed. The tubes were spun at high speed in a microcentrifuge, and the supernatant was decanted. One hundred microliters of 0.05 M $CuSO_4$ was added to each tube, and the tubes were vortexed again to resuspend the conidia. Drops (2-μl) were removed from each tube and placed on a microscope slide under a dissecting microscope (24×), and the conidia in each drop were counted. Three replicate drops were counted for each lesion.

SBI-resistance assays. Resistance of the isolates to the SBIs

triadimenol (Mobay Chemical Corp., Kansas City, MO) and propiconazole (Ciba-Geigy Corp., Greensboro, NC) was determined by radial growth assays on SBI-amended malt extract agar (22). Triadimenol and propiconazole were chosen because resistance to these two SBIs has been shown to be uncorrelated (22,23). Single discriminatory doses of triadimenol (10 μg/ml, 1% ethanol) and propiconazole (0.3 μg/ml, 1% ethanol) were selected for the assays. These doses closely approximated the population EC_{50} (50% effective concentration) values for each fungicide and have been shown in previous studies to be appropriate for determining SBI-resistance phenotypes of *P. teres* (22,23). Two replicates of each isolate were measured in each assay. Each replicate had its own control consisting of malt extract agar amended with 1% ethanol only. Resistance phenotype was expressed as the radial growth of each replicate as a proportion of the control. Mean SBI-resistance phenotypes of each isolate were determined by taking an average of the two replicates. Independent resistance assays were performed to determine resistance to triadimenol and propiconazole in each population.

Data analysis. Mean latent period and mean sporulation were calculated for each isolate. Variation in resistance to the SBIs and in the fitness components was partitioned by a nested analysis of variance (ANOVA) with replicates nested within isolates (23). Degrees of freedom for the ANOVAs differed among experiments because a different number of isolates was used in each experiment and because SBI-resistance phenotypes were not determined for all isolates. The significance of genetic variation among isolates in resistance to SBIs and fitness components was tested by an F statistic with mean squares for isolates in the numerator and mean squares for replicates in the denominator. Correlation coefficients between mean fitness components (latent period and sporulation) and mean resistance phenotypes to triadimenol or propiconazole were estimated with Pearson's standard product moment method (30). Correlation coefficients obtained by using the mean response for each variable have been shown to closely approximate the correlation coefficient obtained using "component" correlations, in which among-replicate and among-isolate sources of variance and covariance were partitioned (23). Therefore, we have used standard product moment correlations to estimate among-isolate correlations in this study. Correlation coefficients were estimated with Minitab 8 statistical software (Minitab Inc., State College, PA), and confidence intervals for the estimates were obtained with Fisher's Z transform method (30). Each experiment was analyzed independently.

RESULTS

Latent period. Four or five isolates used in each experiment did not produce any detectable lesions, and these isolates were not included in the analyses. This inability to cause disease symptoms was not correlated with SBI-resistance phenotype. Several isolates had a few replicates that produced lesions but did not sporulate; these replicates also were not included in the analyses. The shortest latent periods observed were approximately 5 days (115 h) after inoculation, and production of conidia continued until approximately 11 days (260 h) after inoculation in all experiments. Latent periods varied significantly ($P < 0.05$) among isolates in all experiments (Tables 1 and 2). These results indicated that there was significant genetic variation in latent periods in both the North Dakota and German populations. Latent period was continuously distributed in both populations, and results from the North Dakota population are shown in Figure 1A.

Sporulation. Total sporulation per lesion was 0–100 conidia per 2-μl drop, representing 0–50,000 conidia produced per lesion. Sporulation varied significantly ($P < 0.05$) among isolates in three of four experiments, indicating significant genetic variation in sporulation (Tables 1 and 2); sporulation was not significantly different among isolates from the North Dakota population in experiment B (Table 1). Sporulation was continuously distributed in both populations, and results from the North Dakota population are shown in Figure 1B.

SBI-resistance phenotypes. Triadimenol- and propiconazole-resistance phenotypes varied significantly ($P < 0.001$) among isolates in each population, indicating significant genetic variation in resistance to each SBI (Tables 1 and 2). These results agree with previous results from different samples from the same populations (23). Resistance to triadimenol was bimodally distributed in both populations, and results from the North Dakota population are shown in Figure 1A.

Correlations between fitness and resistance. Correlation coefficients between resistances to the SBIs and the components of fitness were estimated in each population. Representative cor-

TABLE 1. Analysis of variance (ANOVA) in resistance to sterol biosynthesis-inhibiting fungicides and in fitness components among *Pyrenophora teres* isolates sampled from the North Dakota population

	Resistance to triadimenol			Resistance to propiconazole			Latent period			Sporulation		
Source[a]	df	MS[b]	P[c]	df	MS	P	df	MS	P	df	MS	P
Experiment A												
Isolates	19	0.1026	< 0.001	11	0.0254	< 0.001	19	2,566	< 0.001	19	1,366	0.003
Replicates	20	0.0002		12	0.0003		64	400		64	543	
Experiment B												
Isolates	20	0.0996	< 0.001	13	0.0183	< 0.001	20	4,640	0.012	20	162	0.445
Replicates	21	0.0002		14	0.0004		46	2,074		46	156	

[a] Replicate experiments conducted at different times.
[b] Mean squares-nested ANOVA model (replicates nested within isolates).
[c] Probability of a greater F value under the null hypothesis of equal variances.

TABLE 2. Analysis of variance (ANOVA) in resistance to sterol biosynthesis-inhibiting fungicides and in fitness components among *Pyrenophora teres* isolates sampled from the German population

	Resistance to triadimenol			Resistance to propiconazole			Latent period			Sporulation		
Source[a]	df	MS[b]	P[c]	df	MS	P	df	MS	P	df	MS	P
Experiment A												
Isolates	15	0.0376	< 0.001	15	0.0156	< 0.001	15	3,861	0.001	15	4,860	< 0.001
Replicates	16	0.0009		16	0.0013		48	1,211		48	1,013	
Experiment B												
Isolates	16	0.0280	< 0.001	16	0.0173	< 0.001	16	7,077	0.002	16	1,374	< 0.001
Replicates	17	0.0018		17	0.0013		34	2,219		34	112	

[a] Replicate experiments conducted at different times.
[b] Mean squares-nested ANOVA model (replicates nested within isolates).
[c] Probability of a greater F value under the null hypothesis of equal variances.

relations between latent period and resistance to triadimenol and between sporulation and resistance to propiconazole in the North Dakota population are shown in Figure 1. None of the correlation coefficients between resistance to SBIs and fitness estimated in each population was significantly different from 0 (Table 3). This indicates that no relationship exists between resistance to triadimenol or propiconazole and latent period or sporulation in either the North Dakota or the German populations.

DISCUSSION

No evidence for fitness costs associated with resistance to the SBIs triadimenol or propiconazole in *P. teres* was obtained in this study. Fitness, as measured by latent period and sporulation, was not significantly correlated to resistance to SBIs in either population of *P. teres*, even though there was significant genetic variation in resistance in both populations. In a previous study (W. Köller, *personal communication*), no relationship was found between spore production, spore germination, or mycelial growth in vitro and resistance to fenarimol, a pyrimidine SBI, among isolates of *V. inaequalis* sampled from New York apple orchards.

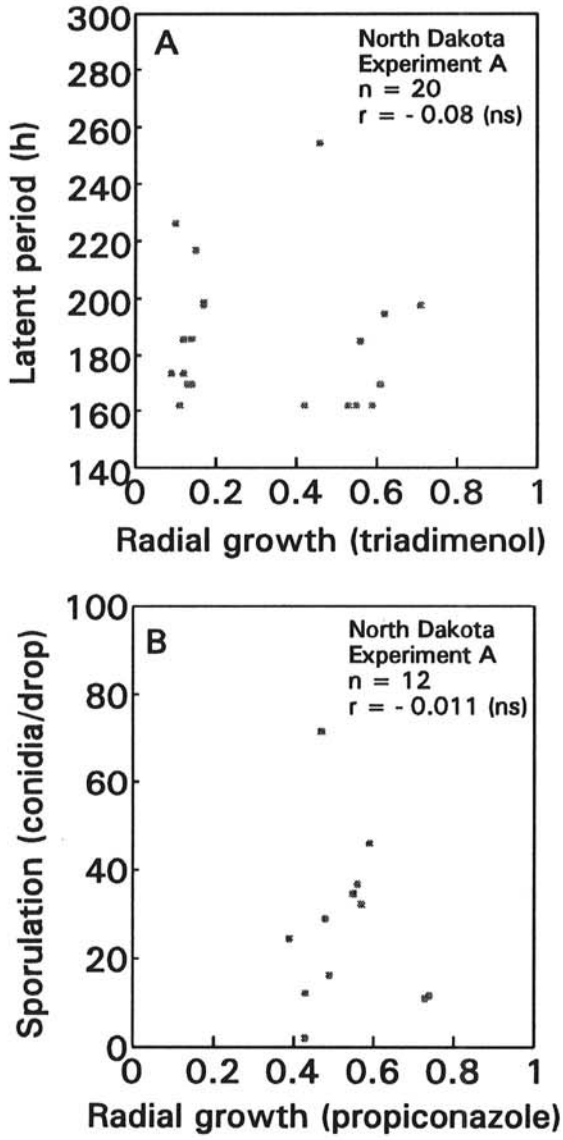

Fig. 1. A, Latent period (hours after inoculation) vs. resistance to triadimenol (radial growth as proportion of control) and **B,** sporulation (conidia per 2-μl drop) vs. resistance to propiconazole (radial growth as proportion of control) for isolates sampled from the North Dakota population of *Pyrenophora teres* in experiment A. Each data point represents a single isolate and is the mean of two replicates for resistance to sterol biosynthesis-inhibiting fungicides and six replicates for fitness components.

This study represents an attempt to estimate fitness costs associated with resistance to fungicides by using a correlative approach. In a previous study (23), correlations among resistances to five different SBIs were estimated with "component correlations," which involved partitioning variation and covariation in resistance to SBIs into "among-isolate" and "within-isolate" components by analyses of variance and covariance. It was also found that correlation coefficients calculated with component correlations could be closely approximated by determining a mean SBI-resistance phenotype for each SBI and isolate and calculating a Pearson product moment correlation with these means. In the present study, we estimated correlations between resistance to SBIs and fitness by determining mean SBI-resistance phenotypes and mean fitness components and calculating Pearson product moment correlations. These correlation coefficients should provide good estimates of the relationship between fitness and resistance to triadimenol and propiconazole among *P. teres* isolates in these populations.

P. teres undergoes one annual sexual generation followed by several asexual generations during the barley growing season (27). The genetic structures of the North Dakota and German *P. teres* populations were determined in a previous study in which we examined associations among random amplified polymorphic DNA markers (*unpublished*). The results obtained were consistent with the hypothesis that random sexual reproduction occurred in these populations. Therefore, isolates sampled from these populations should contain SBI-resistance genes randomized into different genetic backgrounds. Recombination may also increase the fitness of resistant isolates by recombining resistance genes into a more fit background (8,18). This phenomenon has been documented for resistance to insecticides in *L. cuprina* in Australia (18). Genotypes of *L. cuprina* resistant and susceptible to diazinon had similar fitness in the absence of the insecticide, but fitness of the resistant type declined when they were crossed and the resistant progeny were backcrossed to the susceptible parent. This result suggests that resistant isolates evolved smaller fitness costs through the selection of fitness-modifying genes in the field and also illustrates why it is important to consider genetic background effects in studies of fitness costs associated with resistance to pesticides. In the present study, we measured the fitness of isolates that were randomly sampled from *P. teres* populations during the asexual phase. For the purposes of this study, we have assumed that selection against resistance to SBIs occurs primarily during the asexual phase of *P. teres* and that correlations between fitness and resistance to SBIs are most appropriately determined with isolates sampled from the populations during asexual growth.

Most experiments to measure fitness costs associated with resistance to fungicides have measured fitness in terms of fitness "components," such as infection frequency, latent period, lesion size, and sporulation. This has been termed *predicted fitness* methodology as opposed to *realized fitness,* in which the growth rates of a small number of isolates are compared over several generations (1). We chose to measure the fitness components latent period and sporulation because latent period has been shown to be an important component of fitness in simulation models (17,34) and sporulation has been found to be highly correlated to fitness in the field in diverse fungi (16,31). Measuring fitness components also allowed us to compare many isolates in a single experiment, which was essential to obtain accurate estimates of the correlation coefficients in each population. The possibility exists that we did not measure the appropriate fitness components in order to detect fitness costs associated with resistance in this system. Components such as infection frequency, spore germination, and saprophytic survival ability were not measured in this study, and these components may have revealed significant correlations between resistance to SBIs and fitness.

Resistance to SBIs has evolved more slowly than resistance to other systemic fungicides with specific modes of action (13,28). It has been postulated that this may be due to fitness costs associated with SBI-resistance genes, which have prevented the evolution of significant levels of resistance in pathogen populations (6,7,13). To date, there is no experimental evidence for

TABLE 3. Correlation coefficients between resistance to sterol biosynthesis-inhibiting fungicides (SBIs) and fitness components in the North Dakota and German populations of *Pyrenophora teres*

Population Experiment	Number of isolates	Resistance to SBI	Latent period	Sporulation
North Dakota				
A	20	Triadimenol	−0.08[a] (−0.52,0.36)[b]	−0.08 (−0.52,0.36)
	12	Propiconazole	+0.08 (−0.50,0.66)	−0.11 (−0.69,0.47)
B	21	Triadimenol	−0.15 (−0.58,0.28)	+0.06 (−0.37,0.49)
	14	Propiconazole	−0.33 (−0.86,0.20)	−0.13 (−0.66,0.40)
Germany				
A	16	Triadimenol	+0.24 (−0.26,0.74)	−0.13 (−0.63,0.37)
	16	Propiconazole	+0.32 (−0.18,0.82)	−0.26 (−0.76,0.24)
B	17	Triadimenol	+0.22 (−0.26,0.70)	−0.41 (−0.89,0.07)
	17	Propiconazole	+0.17 (−0.31,0.65)	−0.39 (−0.87,0.09)

[a] Calculated by Pearson's product moment method (30).
[b] Confidence intervals (95%) calculated by Fisher's *Z* transform method (30).

such costs, and the results of this study also failed to find a significant correlation between resistance to SBIs and fitness. We speculate that the slower evolution of resistance to SBIs compared with the evolution of resistance to other systemic fungicides is primarily due to the lower levels of variation in SBI-resistance phenotype that have been found in pathogen populations. The lack of evidence for fitness costs associated with SBI-resistance genes in this and other studies may mean that fitness costs are not generally associated with resistance to SBIs in field-collected isolates and that SBI-resistance management strategies cannot depend upon the existence of fitness costs.

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