Resistance to Q_oI (Strobilurin-like) Fungicides in Isolates of *Pyricularia grisea* from Perennial Ryegrass

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ABSTRACT

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In August 2000, azoxystrobin was ineffective in controlling gray leaf spot of perennial ryegrass at a golf course in Lexington, KY and at two golf courses in Illinois. Isolates suspected of being fungicide-resistant ("suspect isolates") were compared to "baseline" isolates obtained from sites with no known use of quinol-oxidizing inhibitor (Q_oI) fungicides. Conidial germination of *Pyricularia grisea* was tested in vitro with 100 µg of salicylhydroxamic acid per ml. For baseline isolates, 50% effective concentration (EC₅₀) values for azoxystrobin and trifloxystrobin were 0.015 to 0.064 and 0.013 to 0.078 µg/ml, respectively; EC₅₀ values for suspect isolates were 2.39 to 44.8 and 0.31 to 111, respectively. All suspect isolates exhibited significantly ($P = 0.05$) lower sensitivity to Q_oI fungicides than all baseline isolates. The mean EC₅₀ values for suspect isolates for azoxystrobin and trifloxystrobin were 690 and 827 times higher, respectively, than the means for baseline isolates. In the laboratory, azoxystrobin and trifloxystrobin provided essentially complete control of disease induced by nine baseline isolates in vivo. Azoxystrobin and trifloxystrobin provided poor to no control of disease induced by six of eight suspect isolates; control of disease induced by the remaining two isolates was partial for azoxystrobin and complete for trifloxystrobin. We conclude that one or more biotypes of perennial ryegrass-infecting strains of *P. grisea* with resistance to Q_oI fungicides have emerged. This is the first report of resistance to Q_oI fungicides in *P. grisea*. Furthermore, this is one of two Q_oI-resistant fungal pathogens collected in the United States during the 2000 growing season, the first instances reported for North America.

Pyricularia grisea (Cooke) Sacc. (teleomorph *Magnaporthe grisea* (Hebert) Barr) has long been known to cause disease on numerous grass hosts (15). Isolates of *P. grisea* unique to certain cool-season turfgrasses have emerged in recent years to cause gray leaf spot on perennial ryegrass (*Lolium perenne* L.), a highly destructive disease throughout much of the range of this turfgrass (16). While cultivars of perennial ryegrass exhibit slight differences in susceptibility (9), all currently available cultivars can become severely diseased under high disease pressure. Cultural practices, such as minimizing nitrogen fertility during summer, can contribute to disease management, but alone they do not provide adequate control under high disease pressure (16). Consequently, the judicious use of fungicides is critical for successful management of this disease (17).

Azoxystrobin and trifloxystrobin are among the most effective fungicides used to control gray leaf spot of perennial ryegrass (17). These antifungal strobilurins are members of a new class of fungicides,

the Q_o inhibitors (Q_oI). The Q_oI fungicides inhibit the respiratory cytochrome *bc*₁ complex by blocking electron transport at the quinol-oxidizing (Q_o) site (1,4,5,21). Because of the highly specific mode of action of the Q_oI fungicides, concern exists over the potential for the selection of pathogen subpopulations with reduced sensitivity to these fungicides. At least two mechanisms for the development of resistance are possible. Point mutations in the cytochrome *b* target site have been shown to substantially reduce sensitivity of several fungi to agents that inhibit electron transport at the Q_o site (2,22). In addition, induction of the alternative oxidase pathway, which is found in many fungi, can circumvent the target site of the Q_oI fungicides in vitro (7,8,11,13,23). However, alternative oxidase appears to play no significant role in pathogenesis on Q_oI-treated plants in natural populations of pathogens controlled by these fungicides (12,13,23).

During the summer of 2000, severe outbreaks of gray leaf spot were observed or reported in perennial ryegrass swards in three locations treated with labeled rates of azoxystrobin (described in Materials and Methods). The objective of this study was to test the hypothesis that subpopulations of *P. grisea* with resistance to Q_oI fungicides had been selected in field populations at these sites. A preliminary report of aspects of this work has been published (19).

MATERIALS AND METHODS

Collection sites and maintenance of isolates. During August 2000, natural outbreaks of gray leaf spot resulting in moderate to severe damage were observed on portions of five fairways at Griffin Gate Golf Resort in Lexington, KY (total area affected approximately 1.1 ha based on visual assessment), two fairways at Lincolnshire Golf Course in Champaign, IL (approximately 2.4 ha reportedly affected), and 18 fairways in Bloomington Golf Course in Bloomington, IL (approximately 9 ha reportedly affected). All fairways had been recently treated with labeled rates of azoxystrobin, and all disease outbreaks occurred well within the normal period of protection from that fungicide (17). Located on one of the diseased fairways at Griffin Gate was a field experiment evaluating several fungicide programs for gray leaf spot control. In that experiment, disease progress in plots treated with azoxystrobin was not significantly different from plots treated with water alone (18); this was in stark contrast to the excellent control provided by azoxystrobin at the same site in a previous test (20). Samples of diseased turfgrass were collected from representative sites from each location.

Mass-transfer isolates or single-conidium isolates were obtained from all samples collected using a variety of standard mycological culture procedures. Telomere fingerprinting was used to confirm that each isolate obtained by mass transfer was originally derived from a single individual (M. Farman, unpublished data). With the exception of isolates GG-5-2b-1 and GG-5-2b-2, isolates collected from the same fairway were collected from different locations within the fairway or on different dates. Isolates suspected of being resistant to Q_oI fungicides (hereafter referred to as "suspect isolates") were compared with "baseline isolates" collected from sites with no previous use of Q_oI fungicides (Table 1). The history of use of Q_oI fungicides at sites where baseline isolates were collected was determined by personal interview or correspondence. Fungicide use records were obtained for all sites where suspect isolates were collected.

Cultures were maintained on one-fifth or one-tenth strength Difco oatmeal agar. For storage, isolates were grown on sterile discs of Whatman No. 1 filter paper overlaid on one-tenth strength oatmeal agar. The colonized paper discs were air-dried, and stored aseptically at -20°C. For all

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tests, conidia and mycelium were spread onto one-tenth strength oatmeal agar in petri dishes to produce conidia; in later tests, several pieces of autoclaved leaf of perennial ryegrass were added to the molten medium to enhance sporulation. Cultures were maintained at room temperature under fluorescent lights (15 to $25 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 8 to 12 days, after which conidia were harvested into deionized water and quantified using a hemacytometer. All tests were conducted with cultures that had been subcultured from a stored paper disc no more than once.

In vitro tests. Initial tests were conducted to evaluate whether alternative oxidase affected the sensitivity of isolates to Q_oI fungicides. To test effects of fungicides on conidial germination, chemical solutions, fresh conidial suspension, and sterile deionized water were mixed in individual wells of a 24-well plate. Dilutions were appropriate to achieve uniform concentrations of conidia and solvents in all test wells. Technical-grade azoxystrobin (Zeneca Professional Products, Wilmington, DE) and trifloxystrobin (Novartis Crop Protection, Inc., Research Triangle Park, NC) were dissolved in acetone and diluted 100-fold in sterile deionized water to create stock solutions; the final concentration of acetone was 0.1% in all test wells, including controls without fungicide. Salicylhydroxamic acid (SHAM) was added to wells at 100 $\mu\text{g}/\text{ml}$ to inhibit alter-

native oxidase (11). A stock solution of SHAM (50 mg/ml) was prepared by dissolving in methanol and diluting 100-fold in sterile, deionized water; the final concentration of methanol was 0.1% in all test wells, including controls without SHAM. Arbitrarily selected baseline isolates and suspect isolates were tested in a series of 10-fold dilutions of azoxystrobin or trifloxystrobin with and without SHAM; the ranges used for both fungicides were 0.001 to 10 $\mu\text{g}/\text{ml}$ for baseline isolates and 0.1 to 100 $\mu\text{g}/\text{ml}$ for suspect isolates. After mixing chemicals and conidia in test wells (one well for each fungicide concentration tested), three 30- μl droplets of each chemical concentration were placed into an individual 100-mm polystyrene petri dish and each droplet was covered with a cover slip. The dishes were sealed with Parafilm and incubated in the dark for 18 to 24 h. After incubation, conidia were scored at 100 $\times$ as either ungerminated or germinated (germ tube at least the length of the conidium or with an appressorium). For sampling conidia, the cover slip in each replicate droplet was divided into quadrants and conidia were counted from microscope fields arbitrarily located within each quadrant (avoiding the center and edge of the cover slip) until 100 conidia were counted. In all tests, germination in fungicide-free controls was 85 to 95%. Relative germination (RG) was calculated for each replicate as 100 $\times$ percent germination in the presence of fungi-

cide divided by mean percent germination of that isolate in the absence of fungicide.

Conidial germination was considered to be the best indicator of sensitivity to Q_oI fungicides since, in a preliminary test, reductions in in-vitro mycelial growth of five baseline isolates in the presence of 5 $\mu\text{g}/\text{ml}$ azoxystrobin ranged from 22 to 56% and 61 to 78% in the absence and presence of SHAM, respectively (P. Vincelli and E. Dixon, *unpublished data*), indicating that mycelial growth of *P. grisea* was rather insensitive to azoxystrobin, even in the presence of SHAM.

To test the hypothesis that SHAM alone affected germination, eight arbitrarily selected baseline isolates and five suspect isolates were tested with and without 100 $\mu\text{g}/\text{ml}$ SHAM in the absence of fungicide. These data were analyzed by analysis of variance (ANOVA) following a completely randomized design (14).

In a separate series of experiments, 50% effective concentration (EC_{50}) values for azoxystrobin and trifloxystrobin were determined in the presence of 100 $\mu\text{g}/\text{ml}$ SHAM in all test wells, including fungicide-free controls. From preliminary evaluations using a 10-fold dilution series of the fungicides, ranges of concentrations appropriate for testing each group of isolates were identified. RG was determined for each isolate over a three-fold dilution series within those ranges. For isolates whose EC_{50} could not be determined from the first threefold dilution series, tests were repeated with threefold dilutions over a different range of concentrations. EC_{50} values for selected baseline isolates were determined several times; EC_{50} values for all suspect isolates were determined at least twice in completely separate tests. The linear regression equation for RG against $\log_{10}$ (fungicide concentration) was determined, and the EC_{50} was calculated by solving for $\log_{10}$ (fungicide concentration) at $\text{RG} = 50$. EC_{50} values were analyzed via ANOVA without blocking variables or other restrictions on randomization and compared using Tukey's studentized range test.

In vivo tests. A series of experiments was conducted to evaluate the level of disease control provided by Q_oI fungicides against selected baseline isolates and against all suspect isolates. Clean, used, 35-mm film canisters were filled with soil-less potting mix and seeded with 40 to 50 seed of perennial ryegrass (cultivar unknown). Watering was by subirrigation using a wick system. Seedlings were maintained at 28°C under fluorescent lights (40 to $60 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 2.5 weeks, during which they were trimmed to a height of approximately 4.5 to 5 cm 8 days after seeding and at 3 to 4 day intervals subsequently. Eighteen to 24 h before inoculation, plants were treated with commercial formulations of azoxystrobin, (1.2 g/liter Heritage 50WG, [Zeneca Professional

Table 1. Isolates of *Pyricularia grisea* from perennial ryegrass tested for in vitro sensitivity to quinol-oxidizing inhibitor (Q_oI) fungicides

Isolates	Source	History of use of Q _o I fungicides
KY-96	Lexington, KY	Collected prior to commercial Q _o I availability
KY-97	Lexington, KY	Collected prior to commercial Q _o I availability
MS-97	Griffin, GA	No known use
OH-97	Dayton, OH	Collected prior to commercial Q _o I availability
CHRF	Silver Spring, MD	No known use
RG-NJ	Lawrenceville, NJ	No known use
RS-NJ	Sparta, NJ	No known use
RHF2-NJ1	New Brunswick, NJ	No known use
RCM-NJ	Cape May Co., NJ	No known use
RGT-NJ	Atlantic Co., NJ	No known use
FH	Hagerstown, MD	Collected prior to commercial Q _o I availability
HO	Hanover, PA	Collected prior to commercial Q _o I availability
PRG-320	Manhattan, KS	Collected prior to commercial Q _o I availability
GG-5-2b-1	Lexington, KY	Research site: small plots treated with azoxystrobin one to two times in 1996, one to two times in 1998, and one time in 2000; the entire research site was treated with azoxystrobin by the superintendent twice in 1999. The rest of the fairway had been treated four times with azoxystrobin since 1998
GG-5-2b-2		
GG-5-4b		
HR-2000-5 ^z		
HR-2000-2-2 ^z		Fairway treated a total of five times with azoxystrobin since 1998 when control failure was recorded
GG-2b	Lexington, KY	
IL-00-1 ^z	Champaign, IL	
IL-BL-00 ^z	Bloomington, IL	Fairways treated a total of 10 times with azoxystrobin since 1998 when control failure was recorded

^z Single-conidium isolate.

Products)], trifloxystrobin, (0.75 g/liter Compass 50WG, [Novartis Crop Protection, Inc.]), or water. These concentrations were equivalent to field rates of 1.22 and 0.765 kg/ha, respectively (the highest labeled rates of these products, assuming a spray volume of 1,018 liters/ha). All canisters from the same fungicide treatment were sprayed as a group by applying the spray from two directions until runoff using a hand-held, hand-pump sprayer with the nozzle set to spray a fine mist. Inoculum was prepared by harvesting conidia grown as described above, counting spores with a hemacytometer, and diluting to achieve a concentration of 2×10^5 conidia/ml (first experiment) or 5×10^5 conidia/ml (second experiment). One isolate (HR2000-2-2) was tested at 8×10^4 and 2×10^5 conidia/ml in the first and second test, respectively, due to poor conidiation; in the third and fourth test, isolates GG-2b and GG-5-2b-1 were tested at 3×10^5 conidia/ml for the same reason. Four replicate canisters of plants were inoculated for each fungicide $\times$ isolate combination by spraying (Chromist Laboratory Spray Unit, Gelman Sciences, Ann Arbor, MI) a conidial suspension to runoff over all leaves; all units inoculated with the same isolate were treated simultaneously. Nine arbitrarily selected baseline isolates and all eight suspect isolates were tested. Inoculated units were placed into an airtight humidity chamber for incubation. In the first experiment, canisters with different fungicide treatments were arranged at random among chambers. In subsequent experiments, for each isolate, canisters treated with water were placed in separate chambers from those treated with fungicide; canisters treated with azoxystrobin or trifloxystrobin were sometimes placed into a common chamber. Chambers were incubated for 24 h in the dark at 25°C, after which chambers were transferred to light for 72 h at 28°C. Humidity chambers were then unsealed, moisture was allowed to evaporate, and the plants were grown for another 3 days while disease continued to develop. Plants were harvested 7 days after inoculation by cutting at soil level; 10 tillers were selected arbitrarily and assessed visually for foliar blighting. Tillers were scored as 0, 2, 5, and 10% foliar blighted, and thereafter at 10% increments up to 100%. Disease severity was averaged across the 10 tillers in a canister, and these data were analyzed by ANOVA following a completely randomized design and compared by Tukey's studentized range test. Each isolate was tested at least twice in separate experiments.

RESULTS

In vitro sensitivity. SHAM alone did not significantly affect conidial germination (Table 2). Without SHAM, all baseline isolates exhibited at least 40% RG at azoxystrobin concentrations as high as 10 μ g/ml (Fig. 1). RG in two isolates was partially

inhibited by trifloxystrobin at 10 μ g/ml and was completely inhibited in two others. When 100 μ g/ml SHAM was included, RG of baseline isolates was highly or com-

pletely inhibited by azoxystrobin or trifloxystrobin at concentrations of 0.1 μ g/ml. In the absence of SHAM, none of the suspect isolates were completely inhibited by ei-

Table 2. Conidial germination of *Pyricularia grisea* isolates in the presence and absence of salicylhydroxamic acid (SHAM) at 100 μ g/ml

Isolate	Germination (%) ^z	
	Without SHAM	With SHAM
Baseline isolates		
KY-96	93.0 (1.73)	90.3 (2.03)
CHRF	94.7 (1.20)	93.0 (0.58)
MS-97	92.0 (2.65)	92.7 (2.33)
KY-97-1A	93.0 (2.00)	93.0 (1.00)
OH-97	95.3 (1.20)	94.0 (2.08)
FH	95.0 (1.53)	94.7 (1.20)
HO	91.0 (1.15)	90.0 (1.53)
GG-98	93.3 (1.45)	93.7 (0.67)
Suspect isolates		
GG-2 b	86.0 (1.53)	90.7 (2.60)
GG-5-2 b	90.0 (1.73)	90.0 (2.08)
HR-2000-2-2	89.7 (2.96)	89.7 (2.03)
HR-2000-5	90.0 (1.15)	91.3 (3.76)
IL-00-1	85.3 (2.60)	86.3 (1.76)

^z No significant ANOVA effect ($P > 0.19$) was observed for SHAM in any isolate. Italicized values in parentheses are standard errors of the means.

Fig. 1. In vitro sensitivity of isolates of *Pyricularia grisea* to quinol-oxidizing inhibitor (Q_0 I) fungicides in the absence and presence of salicylhydroxamic acid (SHAM). Standard errors of the means for each experiment were $\leq 2.9\%$.

ther fungicide at concentrations as high as 100 µg/ml. Even in the presence of SHAM, RG at 1.0 µg/ml of azoxystrobin or trifloxystrobin exceeded 50% for all suspect isolates.

For baseline isolates, ranges for EC₅₀ values for in vitro conidial germination in 100 µg/ml SHAM were 0.015 to 0.064 and 0.013 to 0.078 µg/ml for azoxystrobin and trifloxystrobin, respectively (Table 3). The mean EC₅₀ values for baseline isolates were <0.030 µg/ml for both fungicides. For suspect isolates, EC₅₀ values were 2.39 to 44.8 and 0.31 to 111 µg/ml for azoxystrobin and trifloxystrobin, respectively; for the same fungicides, the mean EC₅₀ values for suspect isolates were 690 and 827 times higher, respectively, than the means of baseline isolates. All suspect isolates exhibited significantly less sensitivity

($P = 0.05$) to both Q_oI fungicides than all baseline isolates. Most suspect isolates were more than two orders of magnitude less sensitive to azoxystrobin and trifloxystrobin than the mean of baseline isolates (resistance factor >100).

In vivo sensitivity. Both Q_oI fungicides provided essentially complete control of disease caused by the nine baseline isolates tested (Tables 4 and 5). For six of the eight suspect isolates tested (GG-5-2b-2, GG-2b, HR2000-2-2, GG-5-2b-1, HR-2000-5, and IL-BL-00), azoxystrobin and trifloxystrobin provided poor disease control or no significant disease control ($P = 0.05$) compared to the water-treated control. Disease caused by isolates IL-00-1 and GG-5-4b was partially suppressed by azoxystrobin and essentially completely controlled by trifloxystrobin.

DISCUSSION

Ryegrass-infecting isolates of *P. grisea* with resistance to Q_oI fungicides were obtained from all three locations that experienced significant damage from gray leaf spot following applications of azoxystrobin in 2000, including a site where a complete loss of disease control with azoxystrobin was documented experimentally (18). Resistance to Q_oI fungicides was demonstrated both by in-vitro and in vivo tests. All of the resistant isolates exhibited cross-resistance to both fungicides in-vitro, and most resistant isolates exhibited resistance factors of >100 to azoxystrobin and trifloxystrobin. Azoxystrobin and trifloxystrobin provided poor to no in vivo control of six of the eight Q_oI-resistant isolates obtained, whereas two Q_oI-resistant isolates were partially controlled by azoxystrobin and completely controlled by trifloxystrobin. These two groups of isolates represent different genotypes, each of which was found to have a distinct single nucleotide substitution which results in an amino acid change in the target site of Q_oI fungicides: the former group possesses a G143A mutation in the cytochrome *b* gene, whereas the latter possesses an F129L mutation (3). These mutations have previously been associated with Q_oI resistance in *Saccharomyces cerevisiae* (2) or *Venturia inaequalis* (22). This is the first report of resistance to Q_oI fungicides in *P. grisea*. Furthermore, the isolates of *P. grisea* reported here, along with isolates of *Didymella bryoniae* also collected during the 2000 growing season (10), are the first plant pathogens reported to exhibit resistance to Q_oI fungicides in North America.

Q_oI-resistant isolates were collected from several locations at Griffin Gate Golf Resort where Q_oI fungicides had been used a total of only five to seven times over a 3-year period when the control failure was observed. Two of those applications were made in 1999 in order to minimize damage from summer patch in the dense populations of *Poa annua* in portions of the fairways; conditions prevailing in 1999 favored activity of that disease. Gray leaf spot was not detected on that golf course or on other golf courses in the region in 1999, most likely because of unfavorable weather conditions. It is probable that most if not all of the selection pressure for Q_oI resistance only occurred following the three to five remaining applications of azoxystrobin to these sites. Q_oI-resistant isolates of *P. grisea* were detected at golf courses in Bloomington and Champaign, IL where a total of 10 and 11 applications of Q_oI fungicides, respectively, had been made when the control failure occurred.

The rather rapid development of Q_oI-resistant subpopulations of *P. grisea* from perennial ryegrass suggests that the potential for Q_oI resistance in this pathogen is significant. Rapid development of a resistant subpopulation in the field does not

Table 3. Sensitivity of isolates of *Pyricularia grisea* from perennial ryegrass to quinol-oxidizing inhibitor (Q_oI) fungicides in vitro in salicylhydroxamic acid (SHAM) at 100 µg/ml

Isolate ^x	EC ₅₀ values (µg/ml)	
	Azoxystrobin ^y	Trifloxystrobin
Baseline isolates		
CHRF	0.015 r	0.014 m
CHRF	0.035 jkl	0.016 lm
RHF2-NJ1	0.017 qr	0.023 jkl
OH-97	0.020 pqr	0.029 jk
OH-97	0.023 n-q	0.032 j
OH-97	0.021 opq	0.023 jkl
KY-96	0.025 m-p	-
KY-96	0.026 m-p	-
KY-96	0.038 ij	0.028 jk
RCM-NJ	0.024 nop	0.019 klm
RGT-NJ	0.023 nop	-
FH	0.025 m-p	0.026 jk
HO	0.030 j-n	0.029 jk
PRG-320	0.028 j-o	-
RG-NJ	0.036 jk	0.078 i
RG-NJ	0.026 l-p	0.020 klm
RS-NJ	0.033 j-m	0.013 m
MS-97	0.031 j-n	0.028 jk
MS-97	0.027 k-o	0.034 j
MS-97	0.064 h	-
KY-97	0.036 hi	-
Mean	0.0290	0.0278
Suspect isolates		
GG5-4b	2.57 g	1.01 g
GG5-4b	3.55 f	1.10 g
GG5-4b	2.39 g	-
IL-00-01	3.61 f	0.44 h
IL-00-01	8.96 e	0.31 h
GG-5-2b-1	4.12 f	1.46 g
GG-5-2b-1	4.46 f	0.89 g
GG-5-2b-2	19.77 d	22.09 def
GG-5-2b-2	19.19 d	17.89 ef
IL-BL-00	23.09 cd	14.53 f
IL-BL-00	20.65 cd	14.77 f
GG-2b	27.00 bc	21.41 def
GG-2b	44.55 a	61.17 bc
HR2000-5	37.35 a	22.83 def
HR2000-5	34.64 ab	32.28 cd
HR2000-2-2	44.76 a	111.27 ^z a
HR2000-2-2	39.32 a	43.15 b
HR2000-2-2	-	25.14 de
Mean	20.0	23.0

^x Multiple entries for the same isolate represent separate EC₅₀ estimates based on separate tests.

^y Means within a column followed by the same letter are not significantly different, Tukey's studentized range test ($P = 0.05$). Arithmetic means are presented with statistical groupings based on analysis of log₁₀ transformed data.

^z Estimated via extrapolation of regression line.

necessarily preclude the effective use of the fungicide in other locations for a number of years. For example, resistance in strains of *Podosphaera xanthii* to benomyl and to DMI fungicides occurred 1 and 2 years after commercial introduction, respectively, and yet these fungicides remained important disease control tools in other areas for years until resistant strains became widespread (6). Parameters that might influence the speed of occurrence, buildup, and spread of Q_oI resistance in *P. grisea* are unknown. For example, it is not known whether Q_oI resistance in this pathogen imparts a fitness cost. Our field test at Griffin Gate Golf Resort suggests that resistant strains exhibit high parasitic fitness, as evidenced by identical rates of disease progress in plots treated with azoxystrobin or water (18). However, this hypothesis needs to be tested in a more rigorous way, including evaluation of the survival potential of resistant strains. Data from DNA fingerprinting studies with Q_oI-resistant isolates of *P. grisea* evaluated in the present study indicate that resistance occurs in several distinct genetic backgrounds in the Lexington site (3). It is unknown whether Q_oI resistance arose independently in several clones at that site, or whether a single strain was the source of a resistance gene(s) which was then recombined into several clones via parasexuality. If the former, this would raise concerns about the potential for rapid emergence of resistant isolates in other locations.

While our results point to a significant resistance risk in *P. grisea* isolates from

perennial ryegrass, Q_oI fungicides should continue to have a place in control programs for gray leaf spot. The Q_oI fungicides have been an important component of a gray leaf spot management program since 1998, when azoxystrobin received a federal registration for that disease. Avoiding the use of Q_oI fungicides in the many locations with no evidence of control failure would put more selection pressure on *P. grisea* towards resistance to the few remaining effective fungicides, thiophanate-methyl and certain DMI fungicides (17). The potential for rather rapid selection for resistance that we report here does, however, suggest, that users of Q_oI fungicides for gray leaf spot control should aggressively use strategies for reducing the risk of resistance. These include: the use of appropriate cultural practices to minimize disease pressure (17), thereby reducing the risk of occurrence of a fit mutant; strictly limiting the number of sprays per season with a Q_oI; avoiding sequential applications of Q_oI fungicides; and tank-mixing with fungicides having alternate modes of action (17). Our results also suggest that users should monitor Q_oI-treated swards closely for disease, in the event that application of an alternative product is necessary.

Our initial in vitro tests showed that, in the absence of SHAM, the sensitivity of conidial germination of resistant isolates could not be readily distinguished from that of baseline isolates. SHAM interferes with alternative oxidation (11), a metabolic pathway that can circumvent the target site

of the Q_oI fungicides in vitro and provides ATP in cells treated with a Q_oI fungicide (8, 11, 13, 23). Researchers have reported that isolates of *Venturia inaequalis* and *Septoria tritici* capable of alternative oxidation were less sensitive to the Q_oI fungicides in vitro but more sensitive in vivo (13, 23). Other studies have shown a poor correlation between in vitro sensitivity in the absence of SHAM and in vivo sensitivity to Q_oI fungicides (12). Alternative oxidase appears to play no significant role in pathogenesis on Q_oI-treated plants in natural populations of pathogens controlled by these fungicides, possibly because host flavones released during infection interfere with induction of this pathway (7). While it is important to verify key results in vivo, fungicide sensitivity tests conducted in vitro offer several advantages, including greater precision in estimating EC₅₀ values, lower requirements for space and labor, and quicker turn-around time. In order to screen isolates for sensitivity to Q_oI fungicides in vitro, however, it is necessary to include SHAM in the assay to inhibit alternative oxidation. Inclusion of SHAM would, of course, preclude detection of a mutant strain in which alternative oxidation was functional during host infection and colonization.

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Table 4. Sensitivity of isolates of *Pyricularia grisea* from perennial ryegrass to quinol-oxidizing inhibitor (Q_oI) fungicides in vivo

Treatment	% Foliar blighting ^z								
	Baseline isolates					Suspect isolates			
	KY-96	MS-97	OH-97	CHRF	RGNJ	IL-00-1	GG-5-2b-2	GG-2b	HR2000-2-2
Experiment 1									
Control	58.5 a	70.5 a	67.5 a	44 a	67.3 a	62.0 a	79.5 a	80.8 a	14.3 a
Azoxystrobin	0 b	1.4 b	0 b	0 b	0 b	18.5 b	62.5 a	57.8 b	5.5 b
Trifloxystrobin	0 b	0 b	0 b	0 b	0 b	0.3 c	69.5 a	72.5 ab	13.5 a
Experiment 2									
Control	65.8 a	71.3 a	70.3 a	47.0 a	68.3 a	55.5 a	74.5 a	63.0 a	13 a
Azoxystrobin	0.5 b	3.1 b	0.5 b	0.4 b	0.3 b	29.4 b	73.0 a	62.0 a	13 a
Trifloxystrobin	0 b	0 b	0 b	0 b	0 b	0 c	52.8 b	61.3 a	13 a

^z Within each experiment, means in a column followed by the same letter are not significantly different, Tukey's studentized range test, *P* = 0.05.

Table 5. Sensitivity of isolates of *Pyricularia grisea* from perennial ryegrass to quinol-oxidizing inhibitor (Q_oI) fungicides in vivo

Treatment	% Foliar blighting ^z									
	Baseline isolates					Suspect isolates				
	KY-96	HO	FH	RS-NJ	RHF2-NJ1	GG-2b	GG-5-2b-1	GG-5-4b	HR-2000-5	IL-BL-00
Experiment 3										
Control	50.8 a	24.3 a	58.0 a	57.0 a	64.8 a	60.8 a	66.8 a	62.3 a	69.8 a	65.0 a
Azoxystrobin	0 b	0.3 b	0 b	0 b	0 b	47.5 b	33.8 b	19.8 b	61.8 b	48.8 b
Trifloxystrobin	0 b	0 b	0 b	0 b	0 b	50.8 ab	39.5 b	1.3 c	60.8 b	51.3 b
Experiment 4										
Control	57.8 a	44.5 a	50.8 a	52.0	47.3 a	59.3 a	46.3 a	54.3 a	63.0 a	58.3 a
Azoxystrobin	0 b	0 b	0 b	0 b	0 b	55.3 a	29.8 b	23.8 b	56.3 a	50.8 a
Trifloxystrobin	0 b	0 b	0 b	0 b	0 b	54.0 a	28.0 b	1.0 c	47.3 a	48.3 a

^z Within each experiment, means in a column followed by the same letter are not significantly different, Tukey's studentized range test, *P* = 0.05.

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