Resistance to QoI Fungicides Is Widespread in Brazilian Populations of the Wheat Blast Pathogen *Magnaporthe oryzae*

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ABSTRACT

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Wheat blast, caused by *Magnaporthe oryzae*, is an important disease across central and southern Brazil. Control has relied mainly on strobilurin fungicides (quinone-oxidase inhibitors [QoIs]). Here, we report the widespread distribution of QoI resistance in *M. oryzae* populations

sampled from wheat fields and poaceous hosts across central and southern Brazil and the evolution of the cytochrome b (*cyt b*) gene. Sequence analysis of the *cyt b* gene distinguished nine haplotypes, with four haplotypes carrying the G143A mutation associated with QoI resistance and two haplotypes shared between isolates sampled from wheat and other poaceous hosts. The frequency of the G143A mutation in the wheat-infecting population increased from 36% in 2005 to 90% in 2012. The G143A mutation was found in many different nuclear genetic backgrounds of *M. oryzae*. Our findings indicate an urgent need to reexamine the use of strobilurins to manage fungal wheat diseases in Brazil.

Wheat blast is caused by the ascomycetous fungus *Magnaporthe oryzae* B. C. Couch (anamorph *Pyricularia oryzae* Cavara). Wheat blast was first detected in the 1980s in Paraná State (23) and has since spread to wheat-growing regions across central and southern Brazil (1,37) as well as to wheat fields in Bolivia, Argentina, and Paraguay (11) but has not been reported outside of South America (12). The major mechanisms of disease spread over short and long distances are airborne inoculum (42) and movement of infected seed (18). In the last decade, *M. oryzae* has also been associated with blast disease on barley, rye, and triticale in areas of central-western and southern Brazil (28). Because wheat blast has not yet been detected in North America, *M. oryzae* is considered a major quarantine pathogen and a possible threat to wheat crops in the United States (26,32,38).

M. oryzae is part of a species complex composed of fungal populations that show specialization toward different hosts in the Poaceae family, including rice, wheat, oat, barley, and millet, as well as more than 50 species of grasses (8,9,33,39,41). Recent multilocus phylogenetic studies by Zhan and collaborators (46) suggested that *Magnaporthe* is a polyphyletic genus and that *M. oryzae* occupies a basal clade separated from other species in this family. Kato and collaborators (24) reported that isolates of *M. oryzae* collected from *Eleusine*, *Panicum*, *Oryza*, *Setaria*, and *Triticum* spp. form a highly related group that is partially interfertile with the *Oryza* group that causes rice blast. However, the wheat- and the rice-adapted pathogens do not cross-infect (31,41). The *Oryza* group can be differentiated from the *Digitaria* group that is associated with *M. grisea*. The *Digitaria* group has been

consistently linked with blast disease on poaceous hosts other than rice (24). These findings, together with results from multigene phylogenies, established that *M. oryzae* and *M. grisea* are two distinct but closely related phylogenetic and biological species (8,9). However, Choi and collaborators (6) reported that, based on a multigene phylogeny, isolates recovered from *Digitaria* spp. in Korea grouped not only with the *M. grisea* clade but also with the *M. oryzae* clade. These authors also detected cross-infection of *Digitaria* and rice-derived isolates on both *Digitaria* spp. and rice. These observations challenge the prevailing dogma of a strict association between a *Digitaria* host and *M. grisea* (6).

Under field conditions, the physical proximity between cultivated plants and other poaceous hosts may facilitate genetic exchange among isolates of different *Magnaporthe* spp., leading to host jumps or host shifts. In fact, cross-infection events and interfertility are thought to have played a role in the emergence of wheat blast, though the origin of the *M. oryzae* populations adapted to wheat remains a mystery (5,31).

Control of wheat blast has been difficult due to highly favorable weather conditions in Brazil and the lack of durable resistance (1,26). Although some blast resistance has been reported in Brazilian wheat cultivars, it has not been durable, perhaps due to the high pathotype diversity found in *M. oryzae* populations (31). Currently, wheat blast control relies heavily on the application of foliar fungicides (30,31), especially using strobilurins formulated as the sole active ingredient or in mixtures with other fungicides. Strobilurins have been on the fungicide market since 1996 and belong to a group of fungicides known as quinone-oxidase inhibitors (QoIs) (4). QoI fungicides inhibit mitochondrial respiration by binding to the Qo site of the cytochrome bc₁ enzyme complex, blocking electron transfer and thereby disrupting mitochondrial respiration and ATP production (13). Due to their single-site mode of action, QoI fungicides pose a high risk for development of fungicide resistance. In fact, the development of QoI resistance was anticipated prior to their commercial introduction (4,21).

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Under field conditions, resistance to QoI fungicides was associated with nonsynonymous nucleotide substitutions in the cytochrome b (*cyt b*) gene that prevent fungicide binding (13). Two amino acid substitutions, from glycine to alanine at position 143 (G143A) and from phenylalanine to leucine at position 129 (F129L), are most often found in plant-pathogenic fungi with QoI resistance (16,21). Isolates with the F129L mutation express moderate resistance and are usually controlled by recommended field concentrations of QoI fungicides. On the other hand, isolates with the G143A mutation display a high level of resistance associated with failure of QoI fungicides to control disease (13). In several Basidiomyceteous species, the presence of an intron directly after the GGT triplet that codes for glycine at position 143 prevents other mutations at this position (21). Introns have not been detected in the *cyt b* sequence of *Magnaporthe* spp. (21,25). During the last 18 years, more than 30 plant pathogen species distributed across 20 genera were reported to show field resistance toward QoI fungicides (13,15). This list includes wheat pathogens such as *Blumeria graminis tritici*, *Microdochium nivale*, *Zymoseptoria tritici*, and *Pyrenophora tritici-repentis*, with populations from Europe and North America showing resistance levels ranging from an effective concentration that inhibited mycelial growth by 50% (EC_{50}) > 0.5 $\mu\text{g ml}^{-1}$ to EC_{50} > 2,000 $\mu\text{g ml}^{-1}$ (14,15,45). Resistance to QoIs was also reported in populations of *M. oryzae* associated with rice and populations of *M. grisea* associated with perennial ryegrass (*Lolium perenne*) (3,15,25, 27,29,44).

The use of QoI fungicides to manage wheat blast is of major concern because strobilurins were reported to be ineffective in the field during the past decade (26,30,34). The inability of QoI fungicides to control wheat blast was attributed to three factors: (i) significant gaps in our knowledge of epidemiological variables that influence disease development, (ii) incomplete understanding

of the pathogen's disease cycle, and (iii) technical difficulties that impede the complete protection of the wheat head during fungicide applications (19,20). Surprisingly, the possibility of field resistance to QoI fungicides due to mutations in *cyt b* was not considered a plausible explanation for the limited efficacy of strobilurin fungicides (17,20). Because QoI fungicides were used extensively to manage wheat diseases over the last 15 years (30), there would be strong selection on wheat pathogens, including *M. oryzae*, for mutations that confer QoI resistance. However, no scouting for detecting resistance to QoI fungicides in Brazilian populations of the wheat blast pathogen has been conducted thus far.

In these experiments, we tested the hypothesis that extensive use of QoI fungicides in Brazilian wheat fields led to the emergence and widespread distribution of *cyt b* mutations encoding QoI resistance in *M. oryzae* populations infecting wheat. We also considered whether *M. oryzae* populations infecting poaceous species adjacent to wheat fields could be a reservoir of QoI-resistant (QoI-R) strains. We sampled *M. oryzae* populations from both wheat fields and other poaceous species across central and southern Brazil and correlated mutations in the *cyt b* gene with sensitivity to azoxystrobin, a strobilurin fungicide widely used for managing wheat diseases in Brazil. We discovered a high frequency of the G143A mutation associated with high levels of QoI resistance in *M. oryzae* isolates sampled from both wheat and other poaceous host species across central and southern Brazil. The G143A mutation was found in different genetic backgrounds.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. In total, 325 monoklonal isolates of *M. oryzae* were included in this study (Table 1). In 2012, 289 isolates were obtained from seven states in Brazil (Distrito Federal, Goiás, Mato Grosso do Sul, Minas Gerais,

TABLE 1. Populations of the wheat blast pathogen *Magnaporthe oryzae* from wheat and weed species included in this analysis

Population	Year of sampling	Host	Number
Central-Southern Brazil Distrito Federal	2005	<i>Triticum aestivum</i>	36
	2012	<i>T. aestivum</i>	21
Goiás	2012	<i>Axonopus obtusifolius</i>	1
		<i>T. aestivum</i>	20
		<i>Cyperus rotundus</i>	1
		<i>T. aestivum</i>	18
Minas Gerais	2012	<i>Digitaria sanguinalis</i>	1
Mato Grosso do Sul	2012	<i>T. aestivum</i>	48
		Other poaceous hosts	50
		<i>Avena sativa</i>	12
		<i>Chloris distichophylla</i>	3
		<i>Cenchrus echinatus</i>	11
		<i>D. sanguinalis</i>	3
		<i>Elionurus candidus</i>	1
		<i>Echinochloa crusgalli</i>	5
		<i>Panicum maximum</i>	5
		<i>Urochloa</i> spp.	10
		<i>T. aestivum</i>	42
		Other poaceous hosts	37
		<i>A. sativa</i>	2
		<i>Chloris distichophylla</i>	6
		<i>Cenchrus echinatus</i>	2
		<i>Cynodon</i> spp.	1
		<i>D. sanguinalis</i>	6
Paraná	2012	<i>Eleusine indica</i>	3
		<i>Elionurus candidus</i>	1
		<i>Eragrostis plana</i>	2
		<i>P. maximum</i>	2
		<i>Rhynchelytrum repens</i>	4
		<i>Sorghum sudanense</i>	1
		<i>Urochloa</i> spp.	7
		<i>T. aestivum</i>	30
		<i>D. sanguinalis</i>	1
		<i>T. aestivum</i>	19
São Paulo	2012	<i>T. aestivum</i>	19
Total	...	...	325

Paraná, Rio Grande do Sul, and São Paulo). Wheat fields were sampled using a transect sampling strategy. In each field, blast-diseased wheat heads were sampled approximately every 3.5 to 12 m along six parallel transects (35 to 120 m long) separated by 10 m. Sixty symptomatic wheat heads were sampled per field with the goal of obtaining a single isolate from each head. Other poaceous hosts invading or adjacent to wheat fields were sampled mainly in Mato Grosso do Sul and Paraná. Infected leaves showing typical blast symptoms were collected from plants randomly distributed either within a field or around its perimeter. One single isolate was obtained from each plant sampled. In total, 198 isolates were recovered from wheat heads and 91 isolates originated from leaves of other poaceous species (Table 1). The remaining 36 *M. oryzae* isolates were sampled in 2005 as previously described (31) and consisted of representative multilocus simple sequence repeat (SSR) genotypes found in the major Brazilian wheat-growing areas at that time (31). Procedures used for fungal isolation and long-term storage of fungal cultures were described previously (31).

DNA of each isolate was extracted from freeze-dried mycelia with the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis) following the manufacturer's instructions.

Sequence analysis of *cyt b*. An internal fragment of *cyt b* was amplified from 325 isolates of *M. oryzae* by polymerase chain reaction (PCR) using the primers PgCytb-F1 (5'-AGTCCTAGT GTAATGGAAGC-3') and PgCytb-R1 (5'-ATCTTCAACGTG TTAGCACC-3') described earlier (25). PCR reactions were conducted in 25- μ l volumes containing ultrapure distilled water, $\approx$ 30 ng of DNA template, 0.1 μ M each primer, 80 μ M each dNTP, 2.5 mM $MgCl_2$, 2.5 μ l of 10 $\times$ PCR reaction buffer (Sigma-Aldrich), and 0.1 U of Taq Polymerase (Sigma-Aldrich). Amplification was performed in a Mastercycler Nexus PCR thermal cycler (Eppendorf, Hamburg, Germany) with cycling conditions as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s; with a final extension of 72°C for 8 min. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit in an ABI 3730xl automated sequencer (Applied Biosystems, Foster City, CA).

Sequences were visualized and aligned with Geneious R 6.7.1 (Biomatters, New Zealand). The partial *cyt b* sequences (510 bp long) were manually searched for the presence of mutations known to confer resistance to QoIs, including a G-to-C transition at position 428 of the reference sequence (mutation G143A) and a C-to-A transversion at position 387 of the reference sequence (mutation F129L) (25). DNA sequences AY245424 (*cyt b* QoI-S allele from *M. oryzae* isolate recovered from rice) (25), AY245426 (*cyt b* QoI-R with F129L mutation from *M. grisea* isolate recovered from perennial ryegrass) (25), and AY245427 (*cyt b* QoI-R with G143A mutation from *M. grisea* isolate recovered from perennial ryegrass) (25) were retrieved from GenBank and used as references for comparison. All the *cyt b* sequences obtained in this study were deposited at GenBank, National Center for Biotechnology Information (KM023775 to KM023783).

Distribution of QoI-R mutations in different strains and a test for multiple origins of the G143A mutation. To determine whether *cyt b* mutations encoding QoI resistance were limited to specific fungal lineages, we tested for the association of *cyt b* haplotypes with multilocus genotypes (MLGs) generated using 11 SSR markers (cnpt_mg-c013, -c047, -c060, -c065, -c108, -c129, -c147, -c168, -c233, -c248, and -p1e11), as previously described (36). The MLG data from the 2005 isolates were reported earlier (31). To determine the relationships among the *cyt b* haplotypes, we conducted reticulated phylogeny analyses using statistical parsimony and implemented by the program TCS 1.21 (7).

In vitro tests for QoI sensitivity. QoI sensitivity was assessed for 249 isolates with known *cyt b* sequences (156 isolates from wheat and 58 from other poaceous hosts sampled in 2012, and 35

from wheat sampled in 2005). QoI sensitivity was measured in vitro using a published protocol (40), with some modifications. To produce inoculum, isolates were grown on potato dextrose agar (PDA) (Himedia) containing both chloramphenicol and streptomycin at 50 μ g ml⁻¹ (PDA⁺) and incubated at 25°C with a photoperiod of 12 h of light and 12 h of darkness for 5 days. The fungicide Priori (azoxystrobin at 250 g liter⁻¹; Syngenta, Basel, Switzerland) was diluted 100-fold in deionized water to make a stock solution. The test medium was prepared by cooling PDA⁺ to 55°C and then adding 0.5 mM salicylhydroxamic acid (SHAM) (required to suppress the alternative oxidase pathway) and the azoxystrobin stock solution to 100 μ g ml⁻¹. Control plates were identical except that they lacked azoxystrobin. From each plate of fungal inoculum, 5-mm mycelial disks were removed from the edge of a 3-day-old colony and transferred onto PDA⁺ plates with or without azoxystrobin. Four isolates were tested on each petri plate and each plate was replicated six times. Colony growth was measured after incubation at 25°C for 5 days, subtracting the original mycelial disk diameter (5 mm) from each measurement. Colony growth was converted into relative growth (RG) for each isolate by comparing colony size in the presence of azoxystrobin with colony size on the control plates using the formula: $100 \times (\text{mean colony diameter on media amended with fungicide}) / (\text{mean colony diameter on media without fungicide})$. RG values were used for statistical analysis. The experiment was arranged in a randomized complete block design. A representative subgroup of 83 isolates (54 from wheat and 29 from other poaceous hosts) was selected to repeat the experiment. Isolates MO12.0.337 (sensitive to QoI [QoI-S]) and MO12.0.733 (resistant to QoI [QoI-R]) were included as controls in all assays. Analysis of variance (ANOVA) was performed to evaluate isolate and block effects and their interaction. ANOVA was performed with SAS statistical software ver. 9.2 (SAS Institute, Cary, NC). For pairwise comparisons between isolate means the Scott-Knott test was applied with Assistat version 7.7 beta (Federal University of Campina Grande, Paraíba, Brazil).

Determination of EC₅₀. EC₅₀ values were measured for eight sensitive isolates (MO12.1.129, MO12.1.132, MO12.1.130, MO12.1.150, MO12.1.237, MO12.1.239, MO12.1.240, and MO12.1.280) and seven resistant isolates (MO12.1.023, MO12.1.028, MO12.1.030, MO12.1.040, MO12.1.068, MO12.1.127, and MO12.1.146) following a procedure reported earlier (29), with some modifications. Preparation of media and test plates, plate inoculation, and incubation were as described above. Isolates were tested in PDA⁺ amended with SHAM (0.5 mM) and azoxystrobin to achieve final concentrations of 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5.0, 7.0, and 10.0 μ g ml⁻¹ of active ingredient (a.i.). Four isolates were arranged in each plate and four replicates were conducted for each treatment. This experiment was arranged in a completely randomized design. The experiment was conducted twice and results from both replicates were analyzed individually. Linear regressions for RG against the log of the fungicide concentration were determined and the EC₅₀ value was calculated by solving for the log of the fungicide concentration at RG = 0.50 using ED50plus v1.0 (43).

RESULTS

The G143A allele was widely distributed in many nuclear genotypes. A 510-bp *cyt b* fragment was amplified from 325 isolates of *M. oryzae*. In all, *cyt b* sequences distinguished nine haplotypes, four (H1 to H4) encoding the G143A mutation associated with QoI resistance. No haplotypes contained the F129A mutation (Fig. 1). The H1 haplotype was found in 70.4% of the tested isolates, including 191 isolates from wheat and 38 isolates from other poaceous hosts. The QoI-S H9 haplotype was found in 20.6% of the isolates, including 43 isolates from wheat and 24 isolates from poaceous species. The other seven haplo-

The wheat isolates were from seven Brazilian states located over a spatial scale of 948 km east to west and 1,409 km north to

Fig. 1. Polymorphic sites in the sequence of the cytochrome b (*cyt b*) gene from 325 isolates of *Magnaporthe oryzae* from Brazil. The first three sequences were downloaded from the National Center for Biotechnology Information database as references (25): rice wild-type quinone-outside inhibitor-sensitive (QoI-S) allele from *M. oryzae* isolates recovered from rice (AY245424); F129L QoI-resistant (QoI-R) reference sequence AY245426 showing the C-to-A transversion at codon 129; and G143L QoI-R reference sequence AY245427 showing the G-to-C transition codon 143. Numbers on the top of the alignment refer to the nucleotide position in the aligned polymerase chain reaction fragment (total length of 510 bp). Shaded rectangular boxes indicate the position F129L (base 66) and G143A (base 107) mutations of the *cyt b* sequence. Frequencies of each haplotype on *Triticum* and other Poaceae are also indicated. Sites and character types are t = transitions, v = transversions, and i = phylogenetically informative sites.

Populations and sampling year	<i>N</i>	GCT (A) ^w	<i>cyt b</i> -R haplotypes ^x	MLGs ^y	GGT (G) ^w	<i>cyt b</i> -S haplotypes ^x	MLGs ^y	Resistant isolates (%)
<i>Triticum aestivum</i> , 2005								
Central-Southern Brazil	36	13	H1	13	23	H9	23	36.1
<i>T. aestivum</i> , 2012								
Distrito Federal	21	20	H1	11	1	H9	1	95.2
Goiás	20	20	H1	10	0	...	...	100.0
Minas Gerais	18	17	H1	12	1	H9	1	94.4
Mato Grosso do Sul	48	31	H1	21	17	H9	12	64.6
Paraná	42	41	H1	35	1	H9	1	97.6
Rio Grande do Sul	30	30	H1	16	0	...	...	100.0
São Paulo	19	19	H1	17	0	...	...	100.0
Total	198	178		95 ^z	20	...	14	89.9
Other poaceous hosts, 2012								
Distrito Federal	1	1	H1	1	0	...	...	...
Goiás	1	0	...	...	1	H9	1	...
Minas Gerais	1	1	H2	1	0	...	...	...
Mato Grosso do Sul	50	9	H1 (7) ^z	7	41	H5 (1)	1	18.0
	...	...	H4 (2)	2	...	H6 (8)	8	...
	...	...	...	...	...	H7 (9)	7	...
	...	...	...	...	...	H8 (1)	1	...
	...	...	...	...	...	H9 (22)	13	...
Paraná	37	33	H1 (30)	17	4	H6 (2)	1	89.2
	...	...	H3 (1)	1	...	H7 (1)	1	...
	...	...	H4 (2)	1	...	H9 (1)	1	...
Rio Grande do Sul	1	0	...	...	1	H8	1	...
Total	91	44	...	28	47	...	30	48.4

south. In six states, the G143A mutation was found in more than 90% of the sampled isolates. In Mato Grosso do Sul, only 65% of the isolates were G143A (Table 2; Fig. 1). The weed isolates from Mato Grosso do Sul and Parana carried the G143A mutation at frequencies of 18 and 89%, respectively (Table 2; Fig. 2).

The QoI-R H1 haplotype was found in many different MLGs, indicating that the mitochondrial genome harboring the *cyt b* haplotype H1 with the G143A mutation has spread into many different nuclear genetic backgrounds. Among the 36 MLGs of *M. oryzae* included from 2005, 13 were QoI-R and 23 were QoI-S. Among the wheat isolates from 2012, 92 MLGs were QoI-R, 11 were QoI-S, and three had both QoI-R and QoI-S alleles. Among the isolates from other poaceous hosts, 19 MLGs were QoI-R, 22 were QoI-S, and 9 had both QoI-R and QoI-S alleles.

In total, 16 MLGs were isolated from both wheat and other poaceous hosts, of which 6 were QoI-R, 1 was QoI-S, and 9 were both QoI-R and QoI-S (Table 2).

Network of *cyt b* haplotypes. The H1 and H9 haplotype sequences were identical to the *cyt b* sequences obtained from *M. grisea* infecting *L. perenne* (25). All 325 *cyt b* sequences differed from the rice-derived *M. oryzae* isolate (25) by a T-to-A transition at position 356 of the alignment (position 949 in the original sequence) (25), which would produce an amino acid change from Phe to Tyr (Fig. 2).

Analysis of the nine *cyt b* haplotypes using statistical parsimony revealed three networks and one QoI-S haplotype (H7) from poaceous hosts that could not be connected to any other haplotype. Two of the networks contained only haplotypes found

Fig. 2. Geographical distribution of *Magnaporthe oryzae* isolates sampled from wheat and other poaceous hosts within and adjacent to wheat fields carrying either the GCT quinone-outside inhibitor (QoI)-resistant allele (light gray circle) or the GGT QoI-sensitive allele (dark gray circle) of the *cyt b* gene, associated with resistance and sensitivity to QoI fungicides, respectively. In Mato Grosso do Sul and Paraná States, T and P stands for *Triticum aestivum*-derived isolates and other poaceous host-derived isolates, respectively.

on poaceous hosts. The first network linked QoI-S haplotypes H5 and H6. The second network linked QoI-S haplotype H8 with QoI-R haplotype H4 and, subsequently, linked QoI-R haplotype H2 to H4 through seven undetected mutational steps. The third network contained haplotypes isolated from wheat and other poaceous species. The most frequently detected haplotypes, H1 (QoI-R) and H9 (QoI-S), were linked by one mutational step. Likewise, haplotype H9 was connected by one mutational step to the reference QoI-S *cyt b* haplotype from rice (25). In addition, H1 was linked to H3 (a QoI-R haplotype found only on poaceous hosts other than wheat) by two mutational steps (Fig. 3).

In vitro assay for QoI fungicide sensitivity. Isolates of *M. oryzae* showed differential as well as quantitative variation in response to exposure to azoxystrobin (Fig. 4). Growth of mycelia in the presence of azoxystrobin was interpreted as resistance to the fungicide (QoI-R), due to presence of the G143A mutation. The sensitivity test, including 249 *M. oryzae* isolates, showed a 100% correlation between the phenotype (growth or no growth) and presence or absence of the G143A mutation. We also detected quantitative differences in azoxystrobin resistance among the isolates carrying the G143A mutation (Table 3; Figs. 4 and 5).

Further examination of the variation for the level of azoxystrobin resistance (at 100 $\mu\text{g ml}^{-1}$ of a.i.) in a subgroup of 83 *M. oryzae* isolates (54 from wheat and 29 from other poaceous species) indicated that 31 wheat isolates and 16 other poaceous host isolates with the G143A mutation varied significantly in their level of resistance to azoxystrobin (Table 3). Among the QoI-R isolates, the average RG was 41 to 67% among wheat isolates sampled in 2005, 23 to 69% among wheat isolates sampled in 2012, and 49 to 62% among weed isolates. Correlation between the results from the two assays was positive and highly significant ($R = 0.86$, $P < 0.0001$ for wheat isolates and $R = 0.91$, $P < 0.0001$ for other poaceous isolates). The Scott-Knott test allowed us to identify isolates that showed significant quantitative differences and clearly separated QoI-R isolates from QoI-S isolates (Table 3).

EC₅₀ for azoxystrobin sensitivity and resistance. The EC₅₀ values of eight QoI-S isolates ranged from 0.014 to 0.076 $\mu\text{g ml}^{-1}$ of a.i. (Table 4), while the EC₅₀ for seven QoI-R isolates was higher than 10 $\mu\text{g ml}^{-1}$ (the highest dose tested in this assay, with RGs > 92%) (Table 4). ANOVA indicated nonsignificant $F_{\text{isolates-assays}}$ interaction between QoI-S isolates and assay replica-

Fig. 3. *Cyt b* gene haplotype network of *Magnaporthe oryzae* isolates from *Triticum* spp. and other poaceous hosts. The area of each circle is proportional to the number of isolates sampled from each haplotype. Haplotypes are connected by line segments representing a single mutational step. White squares represent unsampled haplotypes. *Cyt b* haplotypes are shown at the side of each node. Haplotypes H5–H6, H7, or H8–H4–H2 surpassed the 95% parsimony connection limit and were not connected to the major *Triticum* network.

tions ($P = 0.9471$), allowing for the pooling of the two assays. There were no significant differences among isolates in either category for EC_{50} ($P = 0.9436$).

DISCUSSION

This is the first research to reveal the occurrence of QoI resistance in populations of the wheat blast pathogen. We found

that QoI resistance is widespread and occurs at a high frequency in *M. oryzae* populations sampled from both wheat and other poaceous species growing adjacent to wheat fields. The H1 haplotype (QoI-R) was present at a high frequency across the major wheat-cropping areas in central and southern Brazil. The frequency of haplotypes carrying the G143A mutation increased from 36% in 2005 to 90% in 2012. Our dose-response assays showed that wild-type sensitive isolates had an average EC_{50} of

Fig. 4. Boxplot distribution of in vitro sensitivity of *Magnaporthe oryzae* isolates to the quinone-outer inhibitor (QoI) fungicide azoxystrobin. Relative growth was based on the ratio of the diametric growth of an isolate after 5 days of incubation on potato dextrose agar containing both chloramphenicol and streptomycin amended or not with azoxystrobin at $100 \mu\text{g ml}^{-1}$. Lane 2005 = Central-Southern Brazil sampled in 2005; lanes DF, GO, MG, MS, PR, RS, and SP contain *Triticum*-derived isolates sampled in 2012; lanes MS(P) and PR(P) contain isolates derived from other poaceous hosts. QoI-resistant (light gray circles) = isolates with QoI-resistant allele G143A at the *cyt b* gene and QoI-sensitive (dark gray circles) = isolates with QoI-sensitive allele (wild type).

Fig. 5. In vitro sensitivity of *Magnaporthe oryzae* isolates to the quinone-outer inhibitor (QoI) fungicide azoxystrobin. **A**, Isolates of *M. oryzae* growing in potato dextrose agar containing both chloramphenicol and streptomycin (PDA⁺) medium amended with azoxystrobin at $0 \mu\text{g ml}^{-1}$ and 0.5 mM salicylhydroxamic acid (SHAM). **B**, Isolates of *M. oryzae* growing in PDA⁺ medium amended with azoxystrobin at $100 \mu\text{g ml}^{-1}$ and 0.5 mM SHAM. Isolates 1 (Py70.4) and 3 (Py79.3) have *cyt b* haplotype H1 (QoI resistant), and isolates 2 (Py74.5) and 4 (Py86.1) have the *cyt b* haplotype H9 (QoI sensitive).

TABLE 3. Phenotypic characterization of *Magnaporthe oryzae* isolates for resistance to quinone-outside inhibitor (QoI) fungicides according to an in vitro sensitivity assay^w

Population ^x	Average relative growth (mm) ^y				<i>cyt b</i> Haplotype ^z
	Assay 1	SD	Assay 2	SD	
<i>Triticum aestivum</i> , 2005					
QoI-R isolates					
Py48.1	67.28 a	(± 6.18)	56.83 a	(± 3.19)	H1
Py41.4	63.89 a	(± 4.39)	57.05 a	(± 4.75)	H1
Py6025	62.50 a	(± 2.85)	56.30 a	(± 1.57)	H1
Py33.1	62.18 a	(± 4.49)	50.20 b	(± 4.05)	H1
Py53.1	61.31 a	(± 5.42)	53.08 b	(± 1.89)	H1
Py47.1	60.44 a	(± 6.80)	54.40 a	(± 3.19)	H1
Py79.3	59.34 b	(± 3.46)	54.13 a	(± 2.64)	H1
Py46.2	59.32 b	(± 8.01)	55.93 a	(± 5.51)	H1
Py91.1	58.74 b	(± 7.68)	55.61 a	(± 2.82)	H1
Py70.4	56.63 b	(± 7.11)	51.95 b	(± 2.82)	H1
Py30.2	56.16 b	(± 1.43)	59.20 a	(± 3.16)	H1
Py67.3	45.60 c	(± 11.00)	46.16 c	(± 3.29)	H1
Py53.4	41.09 c	(± 3.62)	41.26 d	(± 4.59)	H1
QoI-S isolates					
Py19.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py2.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py20.3	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py21.3	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py22.2	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py25.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py35.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py5003	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py5007	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py5038	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py5045	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py5046	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py6018	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py6038	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py75.4	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py86.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py96.2	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
Py97.1	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
<i>T. aestivum</i> , 2012					
QoI-R isolates					
MO.12.1.219	69.95 a	(± 5.15)	50.37 b	(± 1.70)	H1
MO.12.1.243	65.66 a	(± 8.22)	55.88 a	(± 4.67)	H1
MO.12.1.028	61.75 a	(± 8.57)	52.29 b	(± 3.78)	H1
MO.12.1.147	60.89 a	(± 4.37)	56.39 a	(± 3.29)	H1
MO.12.1.001i	57.04 b	(± 6.98)	55.51 a	(± 4.11)	H1
MO.12.1.148	54.90 b	(± 3.27)	51.58 b	(± 3.47)	H1
MO.12.1.134	51.67 b	(± 2.22)	48.74 c	(± 4.97)	H1
MO.12.1.020i	50.85 b	(± 10.55)	54.31 a	(± 3.93)	H1
MO.12.1.135	49.26 c	(± 3.67)	42.96 d	(± 8.63)	H1
MO.12.1.195	47.21 c	(± 7.24)	56.39 a	(± 2.65)	H1
MO.12.1.007	44.12 c	(± 3.11)	51.67 b	(± 9.13)	H1
MO.12.1.119	43.80 c	(± 8.48)	48.23 c	(± 8.51)	H1
MO.12.1.169	37.05 d	(± 3.87)	51.89 b	(± 6.05)	H1
MO.12.1.179	33.79 d	(± 4.51)	54.80 a	(± 3.72)	H1
MO.12.1.030	33.71 d	(± 4.93)	51.22 b	(± 4.87)	H1
MO.12.1.178	30.83 d	(± 2.22)	43.27 d	(± 8.23)	H1
MO.12.1.068	24.56 e	(± 6.57)	58.45 a	(± 1.58)	H1
MO.12.1.170	23.38 d	(± 15.58)	46.46 c	(± 4.72)	H1
QoI-S isolates					
MO.12.1.177	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
MO.12.1.183	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
MO.12.1.237	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
MO.12.1.239	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
MO.12.1.240	0.00 f	(± 0.00)	0.00 e	(± 0.00)	H9
<i>F</i> _{isolates}	96.27	<i>P</i> ≤ 0.0001	202.51	<i>P</i> ≤ 0.0001	...
<i>F</i> _{blocks}	0.87	NS	2.78	NS	...

(continued on next page)

^w In total, 83 isolates of *M. oryzae* were tested in vitro for resistance to QoI fungicides. Four isolates were tested per plate and each plate was replicated six times. Diametric growth was measured from colonies grown at 25°C for 5 days, and converted into relative growth (RG) = 100 × (mean colony diameter on medium amended with fungicide)/(mean colony diameter on medium without fungicide). The experiment was arranged as a randomized complete block design and it was conducted twice. QoI-R and QoI-S = QoI-resistant and -sensitive, respectively.

^x Population, sampling year, and isolates.

^y Relative growth in potato dextrose agar containing both chloramphenicol and streptomycin amended with azoxystrobin at 100 µg ml⁻¹. Averages for assays 1 or 2 followed by the same letter are not statistically different at α = 0.05 according to The Scott-Knott test conducted with Assisstat ver. 7.7 beta. SD = standard deviation. NS = not significant.

^z Data obtained from *cyt b* sequence analysis.

0.05 µg ml⁻¹ for azoxystrobin, whereas resistant isolates had an EC₅₀ > 10 µg ml⁻¹. The latter EC₅₀ is higher than the azoxystrobin concentration typically found in Brazilian wheat fields. According to information from the Brazilian Ministry of Agriculture (MAPA, available at http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons), typical azoxystrobin applications range from 2.5 to 10 µg ml⁻¹ of a.i., equivalent to 50 to 100 ml a.i./ha. Thus, the presence of the G143A mutation rendered *M. oryzae* isolates ≈200-fold more resistant to QoI fungicides than wild-type isolates. The high levels of QoI resistance, as well as the widespread distribution of the G143A mutation throughout the wheat-cropping areas in Brazil, likely result from the high selection pressure exerted by ≈15 consecutive years of strobilurin applications to manage wheat diseases (30,31). The high frequency of the G143A mutation is the best explanation for the lack of efficacy associated with using strobilurin fungicides to manage wheat blast (10,11). Similar findings have been reported for other pathosystems, including *Z. tritici* on wheat, *M. grisea* on ryegrass, and *Rhizoctonia solani* on rice (35,40,44).

In 48% of the isolates originating from poaceous species other than wheat, *cyt b* haplotypes with G143A were also detected, and at least one isolate from each of the poaceous species sampled from wheat fields carried the G143A haplotype H1. In Mato Grosso do Sul, the H1 haplotype predominated in isolates recovered from *Urochloa* spp.; whereas, in Paraná, it was predominantly recovered from *Urochloa* spp. and *Chloris distichophylla*. The *cyt b* haplotype network indicates that the QoI-R haplotype H3 (found exclusively on non-wheat poaceous hosts) likely originated from QoI-R haplotype H1, which was shared between wheat and other poaceous hosts. All of these findings suggest that the other poaceous species adjacent to wheat fields (many considered invasive weeds on wheat crops) may be an important reservoir for the pathogen that could contribute QoI-R inoculum during the early phases of a wheat blast epidemic, as suggested earlier (41).

The G143A mutation was found in many different MLG genotypes originating from both wheat and other poaceous hosts. The simplest explanation for this finding is uniparental inheritance of mitochondria carrying the G143A mutation during sexual reproduction between QoI-R and QoI-S or QoI-R and QoI-R isolates. In fact, a mixed reproductive mode, which includes both sexual recombination and clonal dispersal, was already reported for *M. oryzae* in Brazil (5,31). Another possible mechanism is anastomosis (i.e., hyphal fusion) between QoI-R and QoI-S isolates, as recently described by Avila-Adame in populations of *M. grisea* (now recognized as *M. oryzae*) (2). The haplotype network patterns

TABLE 4. Effective concentration that inhibited mycelial growth by 50% (EC₅₀) for a representative sample of quinone-outside inhibitor (QoI)-sensitive and QoI-resistant isolates of the wheat blast pathogen *Magnaporthe oryzae* from wheat^x

EC ₅₀ (µg azoxystrobin ml ⁻¹) ^y			
QoI-sensitive isolates		QoI-resistant isolates	
Isolate	Average (±SD) ^z	Isolate	Average ^z
MO12.1.237	0.0479 (± 0.0299)	MO12.1.127	>10
MO12.1.132	0.0762 (± 0.0526)	MO12.1.146	>10
MO12.1.240	0.0584 (± 0.0217)	MO12.1.023	>10
MO12.1.129	0.0142 (± 0.0171)	MO12.1.030	>10
MO12.1.130	0.0562 (± 0.0313)	MO12.1.028	>10
MO12.1.150	0.0517 (± 0.0345)	MO12.1.068	>10
MO12.1.239	0.0486 (± 0.0101)	MO12.1.040	>10
MO12.1.280	0.0663 (± 0.0271)	...	...
Mean EC ₅₀	0.0524 (± 0.0181)	...	>10

^x EC₅₀ assays were conducted following the same general procedures of the in vitro QoI-sensitive assay.

^y All QoI-sensitive isolates tested had *cyt b* haplotype H9, while the QoI-resistant isolates had *cyt b* haplotype H1.

^z Average of eight replicates (combining two independent assays with four replicates each). SD = standard deviation.

TABLE 3. (continued from preceding page)

Population ^x	Average relative growth (mm) ^y				cyt b Haplotype ^z
	Assay 1	SD	Assay 2	SD	
Other poaceous hosts, 2012					
QoI-R isolates					
MO12.0.594i	75.92 a	(± 19.41)	51.57 b	(± 4.28)	H1
MO12.0.572i	68.93 a	(± 10.55)	52.39 b	(± 2.44)	H1
MO12.0.613i	68.81 a	(± 11.20)	56.85 a	(± 9.00)	H3
MO12.0.625i	66.64 a	(± 10.27)	50.94 b	(± 2.50)	H1
MO12.0.534i	66.27 a	(± 5.61)	48.66 b	(± 7.78)	H1
MO12.0.601	65.30 a	(± 10.26)	54.65 a	(± 3.59)	H1
MO12.0.607i	62.66 a	(± 10.08)	51.38 b	(± 4.20)	H1
MO12.0.543i	62.33 a	(± 5.43)	55.11 a	(± 6.07)	H1
MO12.0.535i	62.25 a	(± 6.35)	53.55 a	(± 3.61)	H1
MO12.0.634i	62.14 a	(± 14.93)	61.68 a	(± 6.50)	H1
MO12.0.551i	57.95 b	(± 4.99)	58.55 a	(± 8.01)	H1
MO12.0.655i	57.18 b	(± 5.05)	56.62 a	(± 2.93)	H1
MO12.0.174	56.14 b	(± 7.17)	48.34 b	(± 0.97)	H1
MO12.0.056i	52.95 b	(± 6.54)	59.00 a	(± 4.70)	H1
MO12.0.578	52.47 b	(± 2.74)	43.42 b	(± 3.26)	H1
MO12.0.231	52.44 b	(± 8.73)	48.50 b	(± 3.37)	H1
QoI-S isolates					
MO12.0.146	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.144	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.212	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H6
MO12.0.222	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H6
MO12.0.232	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H8
MO12.0.321	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.322	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.337	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H7
MO12.0.347	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.368	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.555i	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H9
MO12.0.561i	0.00 c	(± 0.00)	0.00 c	(± 0.00)	H6
<i>F</i> _{isolates}	70.19	<i>P</i> ≤ 0.0001	165.00	<i>P</i> ≤ 0.0001	...
<i>F</i> _{blocks}	3.11	NS	2.15	<i>P</i> ≤ 0.0112	...

are consistent with a process of infraspecific evolution. However, our data are not conclusive on this possibility because we did not sequence several regions in each mtDNA genome to make a direct test of the hypothesis of many independent and parallel origins of the G143A mutation in the Brazilian population of *M. oryzae*.

Taken together, the results of this study clearly demonstrate that resistance to QoI fungicides is pervasive in populations of the wheat blast pathogen *M. oryzae* across the major wheat-cropping areas in central and southern Brazil. Because management of wheat blast disease relies strongly on fungicide applications, our study provides a strong justification to reexamine the use of strobilurins to manage major fungal diseases on wheat in Brazil. In fact, no other effective strategy is currently available for integrated management of wheat blast in Brazil (26,30).

The most appropriate action to manage the observed high levels of QoI resistance is to stop using QoI fungicides (13,16). If there is a high fitness cost associated with the G143A mutation, then populations of *M. oryzae* could return to their original sensitive levels (22). However, the G143A and G143S mutations reported earlier in *M. oryzae* infecting other grasses were stable and had no fitness cost (25). Under such an irreversible scenario, only anti-emergence strategies such as mixing a low-risk and a high-risk fungicide to delay the emergence of resistance to the high-risk fungicide would have been advisable; however, this strategy should have been implemented prior to the large-scale deployment of strobilurins for management of wheat diseases.

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