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Resistance to triazole fungicides in *Pyricularia* species is associated with invasive plants from wheat fields in Brazil

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ABSTRACT. Triazole fungicides have not been effective for managing the wheat blast disease in Brazil. A broad analysis across six geographical populations of *Pyricularia graminis-tritici* in central-southern Brazil indicated a high level of resistance to triazole fungicides. Since *P. graminis-tritici* is also associated with others poaceous species, here, we analyzed whether triazole-resistant isolates of the blast pathogen could be recovered from other poaceous hosts that are invasive of sprayed wheat fields. In addition to *P. graminis-tritici (Pygt),* we also evaluated the levels of sensitivity of three other grass-associated blast pathogens, which included *P. grisea* (*Pg*), *P. pennisetigena* (*Pp*), and *P. urashimae* (*Pu*). Resistance to the triazole fungicides tebuconazole and epoxiconazole was assessed phenotypically based on EC_{50} values and molecularly by analysis of the presence of mutations in the *CYP51A* gene, which encodes for the target enzyme 14-alpha-demethylase. We detected triazole-resistant *Pyricularia* spp. (*Pg*, *Pp*, *Pu* and *Pygt*) that is associated with *Avena sativa*, *Cenchrus echinatus*, *Chloris distichophylla*, *Cynodon* sp., *Digitaria horizontalis*, *D. sanguinalis*, *Panicum maximum* or *Urochloa* spp. The major outcome from our study was the evidence that invasive poaceous species from wheat fields could be an important source of triazole resistant fungal inoculum for the initial phases of the wheat blast epidemics.

Keywords: blast disease; *CYP51A* gene; sterol demethylation inhibitor (DMI) fungicides; epoxiconazole; tebuconazole.

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Introduction

Since the late 1980's, wheat blast has been considered a major disease, causing high yield losses on crops from central-southern Brazil (Maciel, 2011; Maciel et al., 2014). After its first report in 1986 in northern Paraná State, Brazil (Igarashi, 1986), it has rapidly spread to all of the wheat cropping areas of the country as well as to Argentina, Bolivia, and Paraguay (Maciel, 2011). Initially restricted to South America, wheat blast was recently introduced in Bangladesh in Southeastern Asia (Callaway, 2016; Islam et al., 2016).

Management of wheat blast disease is considered particularly difficult due to the inexistence of durable varietal resistance and the lack of effective systemic fungicides (Maciel et al., 2014; Pagani, Dianese, & Café Filho, 2014; Castroagudin et al., 2015). Despite their low efficacy for controlling blast disease on wheat ears, systemic fungicides such as triazoles have been extensively and widely used in Brazilian wheat fields since the 1990's for managing other fungal diseases, including leaf rust, powdery mildew, leaf spots and gibberella diseases (Navarini & Balardin, 2012; Tormen et al., 2013; Debona, Favera, Corte, Domingues, & Balardin, 2009).

These triazole fungicides have three major active ingredients: epoxiconazole, prothioconazole and tebuconazole (Maciel, Paludo, Silva, Scheeren, & Caierão, 2008). Triazole fungicides belong to the sterol demethylation inhibitor (DMI) group, which is characterized by the inhibition of ergosterol biosynthesis, an important component of the fungal cell membrane (Snelders, Karawajczyk, Schaftenaar, Verweij, & Melchers, 2010). The target of these fungicides is the 14-alpha-demethylase enzyme, which is encoded by the *CYP51* gene, a member of the cytochrome P450 family (Zhan, Stefanato, & McDonald, 2006).

The emergence of fungicide resistance could be one of the main causes of the low efficacy of triazole (Ceresini et al., 2018). A strong selection pressure resulting from several years of extensive and frequent applications of triazoles for disease control may have triggered the emergence of resistant pathogen

populations (Lucas, Hawkins, & Fraaije, 2015). This scenario of intensive triazole usage leading to the emergence of resistance and reduced fungicide efficacy has been reported in Europe, South America, and Asia for many plant pathogens associated with cereal crops such as *Erysiphe graminis* on barley and wheat (Buchenauer & Hellwald, 1985) and *Mycosphaerella graminicola* (Brunner, Stefanato, & McDonald, 2008) and *Parastagonospora nodorum* on wheat (Pereira, McDonald, & Brunner, 2016).

Resistance to triazoles may be directly related to (1) mutations in the *CYP51* gene that encode the target protein resulting in decreased protein affinity for the inhibitors; (2) overexpression of the *CYP51* gene, and (3) increased efflux of toxic compounds out of the fungal cell due to overexpression of the gene encoding membrane transport proteins. A combination of these mechanisms is also possible (Cools & Fraaije, 2013).

Mutations in the paralog A of the *CYP51* gene (*CYP51A*) were considered the primary cause for the reduction in sensitivity to triazoles in fungi with multiple *CYP51* genes, such as the wheat head blight pathogen *Fusarium graminearum* (Jiang, Liu, Yin, & Ma, 2011; Fan et al., 2013), the rice blast pathogen *Pyricularia oryzae* (Yan et al., 2011), and the wheat blast pathogen *P. graminis-tritici* (Ceresini et al., 2018). In fact, widespread distribution of resistance to the triazole fungicides epoxiconazole and tebuconazole has been reported in populations of *P. graminis-tritici* from several wheat fields in central-southern Brazil (Ceresini et al., 2018).

Since *P. graminis-tritici* can also be associated with others poaceous species (Castroagudin et al., 2016), the main objective of our study was to test whether triazole-resistant isolates of the wheat blast pathogen could be recovered from other poaceous hosts that are invasive of triazole-sprayed wheat fields. Since three other blast pathogens (*P. grisea*, *P. pennisetigena*, and *P. urashimae*) were obtained from the sampling of these invasive poaceous hosts in our study, we tested the additional hypothesis that triazole spraying on wheat fields has contributed to and selected for resistance in non-target *Pyricularia* species.

Up until now, there has been no report of the occurrence of triazole resistance in populations of blast pathogens associated with other poaceous hosts that are invasive of wheat fields in Brazil. Thereby, we evaluated the levels of sensitivity of the blast pathogens *P. graminis tritici*, *P. grisea, P. pennisetigena*, and *P. urashimae* to the triazole fungicides epoxiconazole and tebuconazole. The levels of triazole sensitivity were determined based on the individual EC_{50} values (the effective concentration that inhibits 50% of mycelial growth). We also analyzed the *CYP51A* gene for the presence of particular mutations that could be correlated with triazole resistance. Finally, a reticulate phylogeny of the *CYP51A* gene was built and examined to describe the evolutionary relationships among haplotypes of the *Pyricularia* species.

Material and methods

Thirty-two isolates of *Pyricularia* ssp. were used in this study, of which 28 were from blast-diseased poaceous species that are invasive to wheat fields and four from wheat blast. These isolates comprised four *Pyricularia* species: *P. grisea* (*Pg*, N = 4), *P. pennisetigena* (*Pp*, N = 4), *P. urashimae* (*Pu*, N = 4) and *P. graminis-tritici* (*Pygt*, N = 20). These isolates were obtained in 2012 by sampling diseased plants using transect system in Paraná (PR) and Mato Grosso do Sul (MS) and later identified at the species level (Castroagudin et al., 2016; Crous et al., 2016; Reges et al., 2016). In addition, seven isolates of *P. oryzae* (*Po*), sampled from rice fields in Goiás (GO) and Tocantins (TO) in 2007, were included as sensitive standards (Table 1).

Two fungicides labeled for managing wheat blast disease in Brazil were selected to assess the resistance of *Pyricularia* spp. to triazoles: Folicur 200 EC (tebuconazole 200 g L^{-1}, Bayer S.A., Belford Roxo, Brasil) and Tango Cash (epoxiconazole 75 g L^{-1}, BASF S.A., Guaratinguetá, Brasil). For *Pg*, *Pp*, *Pu*, and *Pygt*, fungicide resistance screening was performed using a dose-response curve that contained the following seven final concentrations: 0.0, 0.0, 0.3, 0.75, 0.9, 1.8, 4.1, and 6.8 µg mL^{-1} tebuconazole and 0.0, 0.04, 0.10, 0.30, 0.675, 1.0, and 2.0 µg mL^{-1} epoxiconazole. Dose-response curves for the triazole-sensitive isolates from *Po* were determined using the following nine final concentrations: 0, 0.003, 0.005, 0,01, 0.02, 0.04, 0.075, 0.150, and 0.3 µg mL^{-1} tebuconazole and 0.0, 0.0015, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.075, and 0.1 µg mL^{-1} epoxiconazole.

Table 1. Description of isolates of *Pyricularia* species associated with blast disease on invasive poaceous species from wheat fields that were used for assessing DMI fungicide resistance [a].

Species, isolates	Host	Origin
Pyricularia grisea (Pg)		
12.0.082	Crabgrass (*Digitaria sanguinalis*)	Amambai, MS
12.0.264	Crabgrass (*D. sanguinalis*)	Aral M., MS
12.0.713	Jamaican Crabgrass (*Digitaria horizontalis*)	Londrina, PR
12.0.733	Jamaican Crabgrass (*D. horizontalis*)	Londrina, PR
Pyricularia pennisetigena (Pp)		
12.0.046	Sandbur (*Cenchrus echinatus*)	Aral M., MS
12.0.102	Sandbur (*C. echinatus*)	Amambai, MS
12.0.358	Guinea grass (*Panicum maximum*)	Aral M., MS
12.0.408	Sandbur (*C. echinatus*)	Aral M., MS
Pyricularia urashimae (Pu)		
12.0.212	Guinea grass (*P. maximum*)	Aral M., MS
12.0.224	Oat (*Avena sativa*)	Aral M., MS
12.0.561i	Guinea grass (*P. maximum*)	Londrina, PR
12.0.595i	Weeping finger grass (*Chloris distichophylla*)	Londrina, PR
Pyricularia graminis-tritici (Pygt)		
12.1.127	Wheat (*Triticum aestivum*)	Amambai, MS
12.1.130	Wheat (*T. aestivum*)	Amambai, MS
12.1.146	Wheat (*T. aestivum*)	Amambai, MS
12.1.150	Wheat (*T. aestivum*)	Amambai, MS
12.0.045	Barnyard grass (*Echinochloa crusgalli*)	Amambai, MS
12.0.046	Signal grass (*Urochloa brizantha)*	Amambai, MS
12.0.231	Signal grass (*U. brizantha*)	Aral Moreira, MS
12.0.232	Signal grass (*U. brizantha*)	Aral M., MS
12.0.322	Oats (*A. sativa*)	Aral M., MS
12.0.347	Oats (*A. sativa*)	Aral M., MS
12.0.368	Signal grass (*U. brizantha*)	Aral M., MS
12.0.534i	Indian goosegrass (*Eleusine indica*)	Londrina, PR
12.0.535i	Sandbur (*C. echinatus*)	Londrina, PR
12.0.555i	Jamaican Crabgrass (*D. horizontalis*)	Londrina, PR
12.0.572i	Guinea grass (*P. maximum*)	Londrina, PR
12.0.578i	Star grass (*Cynodon* spp.)	Londrina, PR
12.0.594i	Oat (*A. sativa*)	Londrina, PR
12.0.607i	Weeping finger grass (*C. distichophylla*)	Londrina, PR
12.0.613i	Weeping finger grass (*C. distichophylla*)	Londrina, PR
12.0.625i	Crabgrass (*D. sanguinalis*)	Londrina, PR
Pyricularia oryzae (Po)		
662	Rice (*Oryza sativa*)	Formoso, GO
364	Rice (*O. sativa*)	Lagoa Impacto, TO
623	Rice (*O. sativa*)	Lagoa Impacto, TO
630	Rice (*O. sativa*)	no data
656	Rice (*O. sativa*)	Formoso, GO
662	Rice (*O. sativa*)	Lagoa D. Carolina, TO
704	Rice (*O. sativa*)	Lagoa Arco Iris, TO

[a]Seven isolates of the rice blast pathogen (*Po*) were included as DMI-sensitive references, while four *Pygt* isolates from wheat blast were included as DMI-resistant isolates.

The fungal inoculum consisted of 5 mm discs obtained from 5-day-old cultures of *Pyricularia* spp. that were grown on potato-dextrose-agar (PDA) [18 g L^{-1} of potato-dextrose (Himedia, Mumbai, MA, India) and 15 g L^{-1} of agar (Himedia), supplemented with chloramphenicol and streptomycin (50 µg mL^{-1} each)]. The inoculum was transferred to PDA medium supplemented with different concentrations of tebuconazole and epoxiconazole. The fungal mycelial growth was determined after 5 days incubation at 25°C and 12 hours of light, measuring the colony diameters. The EC_{50} values (effective fungicide concentration, in µg mL^{-1}, capable of inhibiting 50% of mycelial growth) were determined with the program ED50plus v1.0 (Vargas, 2000), according to the procedures described by Ma, Yoshimura, Holtz, and Michailides (2005) with modifications and using the fungicide concentrations converted into log and fungal relative growth data. The experimental design was completely randomized with six repetitions per treatment, and the experiment was replicated once. Analysis of variance and the test for comparisons of means (Scott-Knott at $p \leqslant 0.05$) were performed using the software R and the statistical package Agricolae (R Development Core Team, 2011).

For fungal DNA extraction, the mycelial mass of thirty-nine isolates of *Pyricularia* spp. was obtained by cultivation in PD broth (potato-dextrose, Himedia, Mumbai, MA, India) for seven days at 24°C and 150 rpm. The DNA was extracted with the GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, USA) according to the manufacturer's recommendations and quantified using a spectrophotometer NanoDrop® 2000c (Thermo Fisher Scientific, USA).

Seven primers were designed to ensure amplification of the *CYP51A* gene for all five *Pyricularia* species that were included in our study (Table 2). For each species, three distinct primer combinations were used in PCR reactions for complete coverage of the *CYP51A* gene (Additional file 1, Table 2).

Table 2. Description of primers used for amplification and sequencing of *CYP51A* genes from *Pyricularia* species associated with invasive plants and rice in Brazil.

Primer	Sense	Sequence (5'- 3')	Species [a]
*CYP51A*_-278F	Forward	CTTTTGTCACTTGTTCTCTGCC	*Pg*, Pgt*, Pp*, Pu*, Po**
*CYP51A*_1F	Forward	ATGGCTTTCTTCTTCCCATC	*Pgt, Pp, Pu*
*CYP51A*_-242F	Forward	TAAATCCCTCTGGCTTAATCGC	*Pg, Po*
*CYP51A*_662F	Forward	GCCCCATCAACTTCCTAG	*Pg*, Pgt, Pu, Po*
*CYP51A*_757R	Reverse	TGAGGTCCATGTAAACATCG	*Pg, Pgt, Pp, Pu, Po*
*CYP51A*_1345R	Reverse	CAAAGGGCAGGTAAGGACTC	*Pg, Pgt*, Pp*, Pu*, Po**
*CYP51A*_1749R	Reverse	AGAGATATGCCTCATTGCTAAA	*Pg*, Pgt*, Pp*, Pu*, Po**

[a] * Indicates primer used for polymerase chain reaction amplification.

Polymerase chain reaction (PCR) was performed in a ProFlex thermal cycler (Applied Biosystems, USA) with a final volume of reaction of 25 µL containing ultrapure distilled water, 50 ng of total fungal DNA, 0.3 µM each primer, 0.2 mM dNTP, 2 mM MgCl2, 2.5 µL of 10X buffer and 1 U of Taq DNA polymerase (Sigma-Aldrich, USA). The following cycling conditions were used: initial denaturation at 95°C for 7 min.; 35 cycles of 95°C for 1 min., 52°C as annealing temperature for 1 min., and 72°C for 1 min.; and final extension at 72°C for 7 min. for *Pg, Pp*, *Pu*, and *Pygt*, while for *Po*, the annealing temperature was set at 55°C. Amplifications of the DNA fragments were checked on a 1% agarose gel. The sequencing reactions were performed at Macrogen Inc., Seoul, South Korea, using an ABI 3700 DNA analyzer. To obtain total coverage of the *CYP51A* gene (1551 bp), three sequencing reactions using primers described in Additional file 1 and Table 2 were performed for each isolate, and the fragments obtained were aligned to generate a consensus sequence. The consensus DNA sequences for each isolate were aligned and analyzed using the software Geneious R v. 9.0.5 (Biomatters, Auckland, New Zealand). The complete sequence of *CYP51A* for *Pp*, *Pu*, *Pygt*, and *Po* was 1551 bp in length, while for *Pg,* it was 1420 bp (~92% of gene coverage).

Haplotype frequencies were determined using the program DnaSP version 5.10.1 (Rozas, 2009). The *CYP51A* gene sequences were checked for synonymous and non-synonymous mutations using as reference the *CYP51A* gene sequence from *Po* isolate 622 (with sensitive phenotype for both tebuconazole and epoxiconazole).

The phylogenetic relationships among distinct *CYP51A* haplotypes were determined by reconstructing a reticulate phylogeny using the parsimonious statistical method implemented in the program TCS version 1.21 (Clement, Posada, & Crandall, 2000). We also build an UPGMA phylogenetic tree using the Geneious R tool Tree Builder, assuming the evolutionary model HKY. The internode support for the branches was tested by bootstrap with 1,000 data resampling.

Results and discussion

Increasing concentrations of the DMI fungicides tebuconazole and epoxiconazole resulted in mycelial growth reduction for all isolates of *Pyricularia* spp. from different poaceous species (Figure 1; Additional file 2). There were also differences among isolates within species, allowing for the discrimination between extreme DMI-resistant and DMI-sensitive phenotypes in four out of the five *Pyricularia* spp. examined (*Pg*, *Pp*, *Pu*, and *Pygt*; Additional file 2). The mean species effect was significant at $p \leqslant 0.001$ for both tebuconazole and epoxiconazole, and the EC_{50} values were significantly different among *Pyricularia* species (Scott-Knott at $p \leqslant 0.05$) (Figure 2).

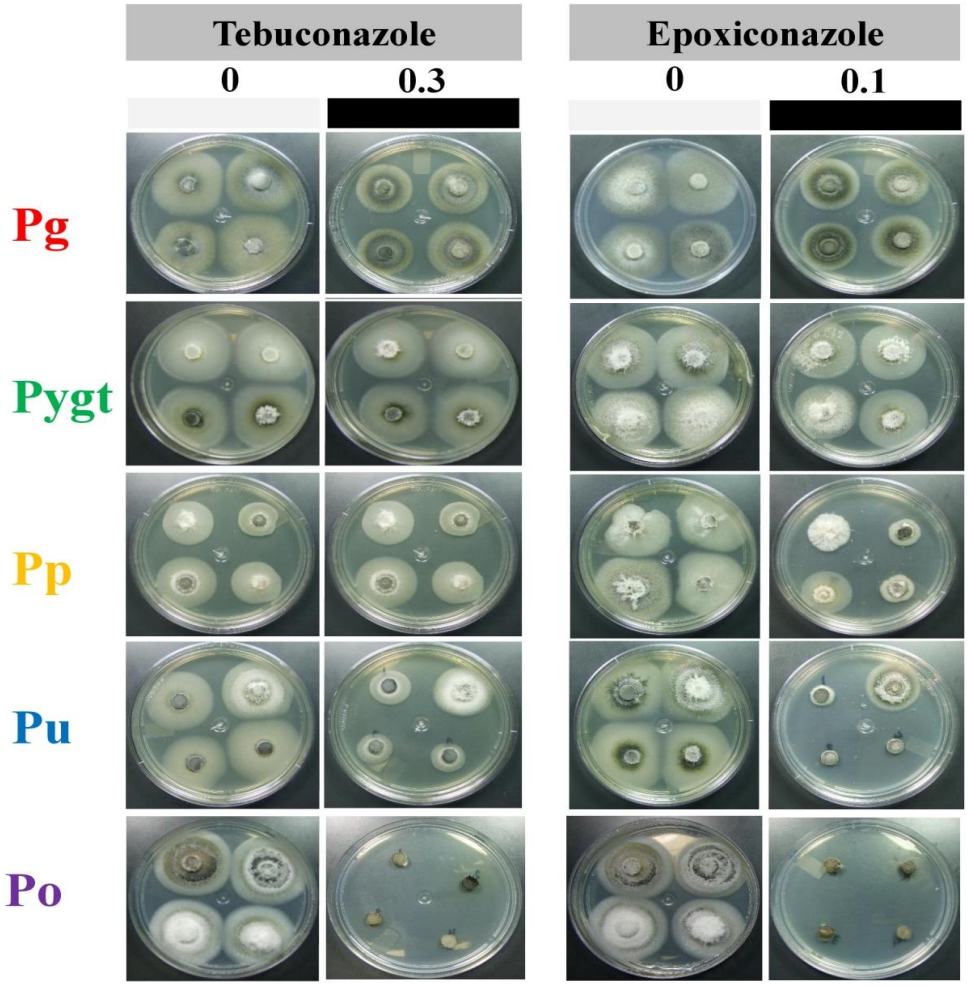

Figure 1. Mycelial growth of isolates from *Pyricularia grisea* (*Pg*), *P. pennisetigena* (*Pp*), *P. urashimae* (*Pu*), *P. graminis-tritici* (*Pygt*) and *P. oryzae* (*Po*) in potato-dextrose-agar medium (PDA) without fungicide or with 0.3 µg mL^{-1} tebuconazole or 0.1 µg mL^{-1} epoxiconazole.

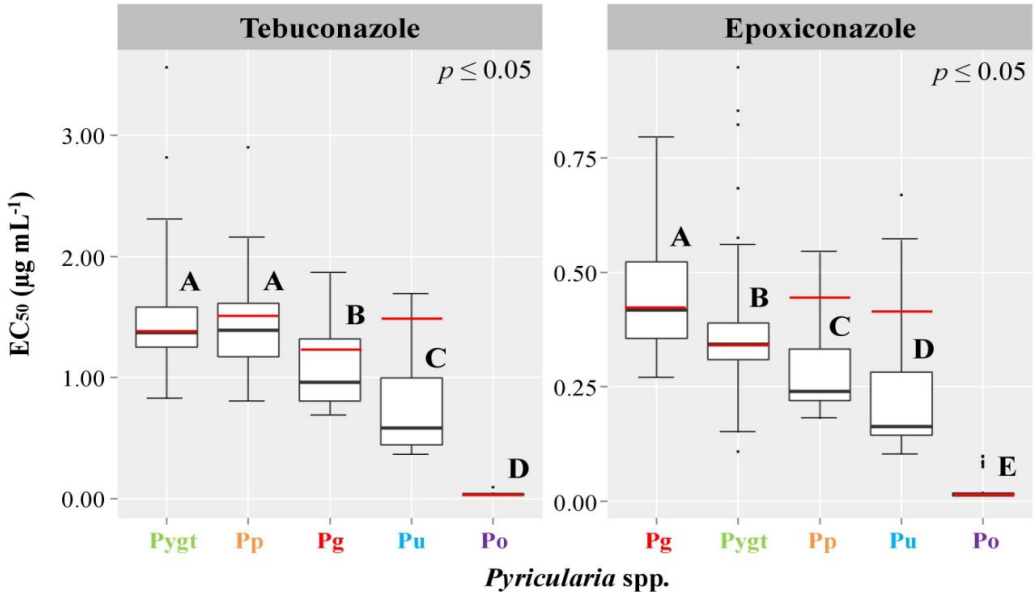

Figure 2. Boxplot representing the variation in EC50 values to DMI fungicides by isolates of *Pyricularia* spp. The mean EC_{50} values for isolates of each *Pyricularia* species with the *CYP51A* gene sequenced were indicated by a red line[a]. [a] A complete randomized experimental design with six repetitions per fungal isolate of each species was used. The experiment was replicated once. Data from the two experiments were combined for the variance analyses because there were no significant differences between experiments ($F_{tebuconazole}$ experiments = 1.945^{NS}, $p = 0.1639$; $F_{epoxiconazole}$ experiments = 3.166^{NS}, $p = 0.0759$) and the ranking of species based on their EC_{50} values was consistent across experiments, indicating no significant interaction. The species effect was significant for both fungicides ($F_{tebuconazole}$ species = 255.27^{***}, $p < 0.001$; $F_{epoxiconazole}$ species = 159.42^{***}, $p < 0.001$). Five *Pyricularia* species were compared: *P. grisea* (Pg, N = 4 isolates), *P. pennisetigena* (Pp, N = 4) *P. urashimae* (Pu, N =4), *P. graminis-tritici* (Pygt, N = 20) and *P. oryzae* (Po, N = 7). Boxplots followed by the same capital letters indicated no significant differences between species in EC_{50} values (Scott-Knott test at $p < 0.05$).

Pygt and *Pp* were highly resistant to tebuconazole, showing the highest EC_{50} values (EC_{50} = 1.438 and = 1.421 µg mL^{-1}, respectively), and significantly different from the other species ($p \leqslant 0.05$). *Pu* and *Pg* where also resistant to tebuconazole, but with intermediate EC_{50} values varying from 0.771 to 1.074 µg mL^{-1}, respectively. In contrast, *Po* was highly sensitive to tebuconazole, with a very low mean EC_{50} = 0.036 µg mL^{-1}, which was significantly lower than that of all the other *Pyricularia* species examined (Figure 2).

For epoxiconazole, *Pygt* and *Pg* were highly resistant, with EC_{50} values varying from 0.357 to 0.445 µg mL^{-1}, respectively, and significantly different from one another ($p \leqslant 0.05$). *Pp* and *Pu* showed intermediate EC_{50} values (= 0.284 and 0.233 µg mL^{-1}, respectively), whereas *Po* presented the lowest EC_{50} (= 0.026 µg mL^{-1}), significantly lower than all the other species ($p \leqslant 0.05$) (Figure 2).

From a total of 13 mutations detected along the *CYP51A* gene from *Pp*, *Pu* and *Pygt*, one was nonsynonymous, while 12 were synonymous. The single nonsynonymous mutation was found at residue 158 of the *CYP51A* gene with a resulting amino acid change from arginine to lysine R158K (Table 3).

Table 3. Description of mutations found in the sequences of the *CYP51A* gene of *Pyricularia* spp.

Haplotype	Species [a]	Frequency	Tebuconazole Mean EC_{50} (µg mL^{-1})	Epoxiconazole Mean EC_{50} (µg mL^{-1})	Position (bp) [b]	4 4 4 4 7 0 6 7 2 8 8 3	1 4 5 6 7 9 9 9 0 9 3 7 5 0 2 4 8 8 4 5 6 6 7 5 3
H6	*Po*	7	0.0357	0.0265	Reference	A A A C G	C A C C T T G G
					Nonsynonymous	*	
					Type of mutation [c]	t v t t t	V t t T v t T T
					Replacement of aa [d]	S I Q P R158K	S V F L L S A T
							Mutations
H1	*Pygt*	16	1.4381	0.3572		G C G T A	G G T □ G C A A
	Pp	1	1.4210	0.2849		G C G T A	G G T □ G C A A
	Pu	1	0.7716	0.2326		G C G T A	G G T □ G C A A
H7	*Pygt*	1	1.4381	0.3572		G C G T A	G G T T G C A A
H8[e]	*Pg*	1	1.0742	0.2849		G C G G A	□ G □ T G C A □

[a]Species analyzed in this study: *Pyricularia grisea* (*Pg*) (MF381155), *P. pennisetigena* (*Pp*) (MF381153), *P. oryzae* (*Po*) (MF381150), *P. urashimae* (*Pu*) (MF381152) and *P. graminis-tritici* (*Pygt*) H1: (MF381151) and H7 (MF381154). [b] Position according to the sequence deposited in the GenBank/NCBI used as reference for the alignment (from the isolate 622 of *P. oryzae* (*Po*) (MF381150) represented by the haplotype H6). [c] t = transitions and v = transversions [d] aa = amino acids [e] For simplification, only the most common mutations with other haplotypes were described for the isolate H8 of *P. grisea*. A total of 52 non-synonymous mutations were detected for H8.

For *Pg*, in contrast, in addition to the mutation resulting in the R158K substitution, another 51 nonsynonymous mutations were detected. The occurrence of these 52 mutations was possibly related to the phylogenetic distance between *Pg* and the group that includes the sister species *Pp*, *Pu*, and *Pygt* (Figure 3). In fact, the phylogenetic tree for the *CYP51A* gene evidenced that the species *Pp*, *Pu*, and *Pygt* shared the identical haplotype H1, which was closely related to H7 but distinct from *Po*, all with high bootstrap support. In contrast, the clade containing *Pp*, *Pu*, *Pygt*, and *Po* was only 80.9% similar to *Pg* (Additional file 3). The reconstruction of a reticulate phylogeny also allowed for the depiction of nucleotide variation within the *CYP51A* gene, the frequency of occurrence of the haplotypes detected, and their relationships (Figure 4). Four distinct haplotypes were detected: H6 – DMI sensitive, associated exclusively with *Po* (MF381150) (N = 7); H1 – DMI resistant, which was the most frequent among the haplotypes (N = 18), associated with *Pp* (MF381153), *Pu* (MF381152) and *Pygt* (MF381151), and distinct from H6 by eleven mutational steps; H7 – DMI resistant, which was found only once (i.e., a singleton) in the *Pygt* isolate 12.0.145 (MF381154); and the haplotype H8 – DMI resistant, also detected only once in *Pg* (MF381155), which was separated from H6 by 48 mutational steps (Figure 4). The most frequent haplotype, H1, and haplotype H7 were differentiated by only one synonymous mutation (C756T).

The major outcome of our study was the evidence that invasive poaceous species from wheat fields could be an important DMI-resistant fungal inoculum source for the initial phases of a wheat blast epidemic (Urashima & Kato, 1998). Additionally, the invasive poaceous species that is closer to the fungicide-sprayed wheat fields could have an important role as a DMI-resistant inoculum reservoir between cropping seasons.

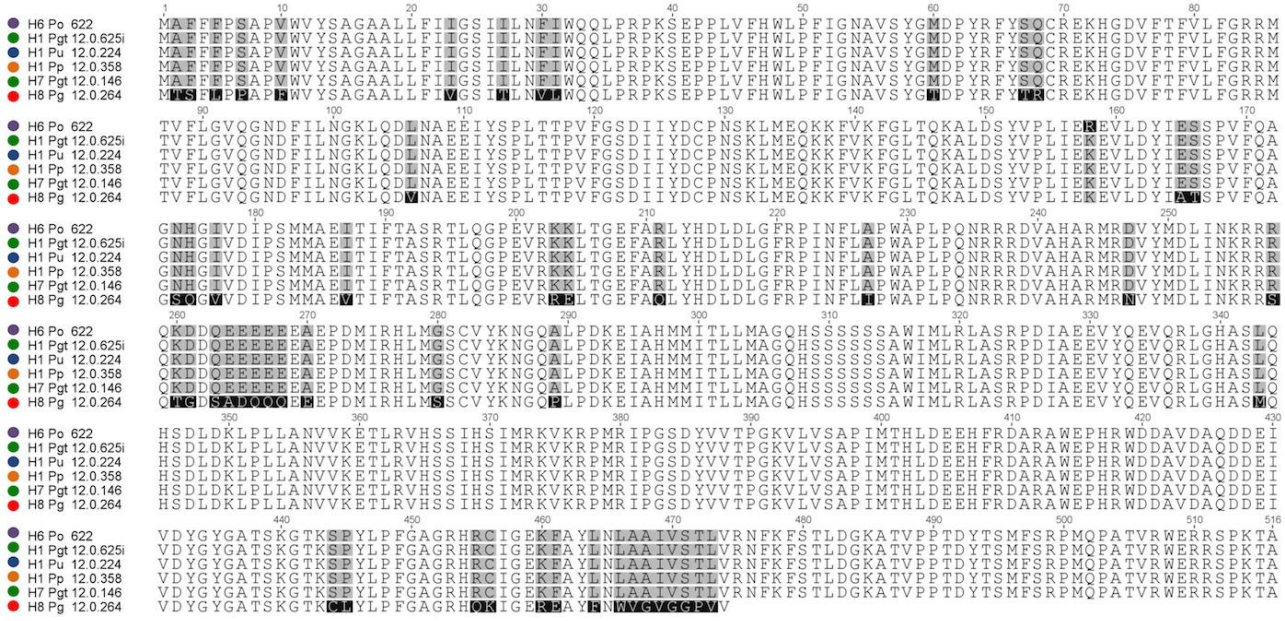

Figure 3. Alignment of amino acid sequences translated from the *CYP51A* gene nucleotide sequences obtained from the isolates 12.0.264 (*P. grisea*), 12.0.625i (*P. graminis-tritici*), 12.0.358 (*P. pennisetigena*) and 12.0.224 (*P. urashimae*), to identify non-synonymous mutations by comparing with the reference sequence of the sensitive isolate 622 (*P. oryzae*). Haplotypes (H) were represented for each species.

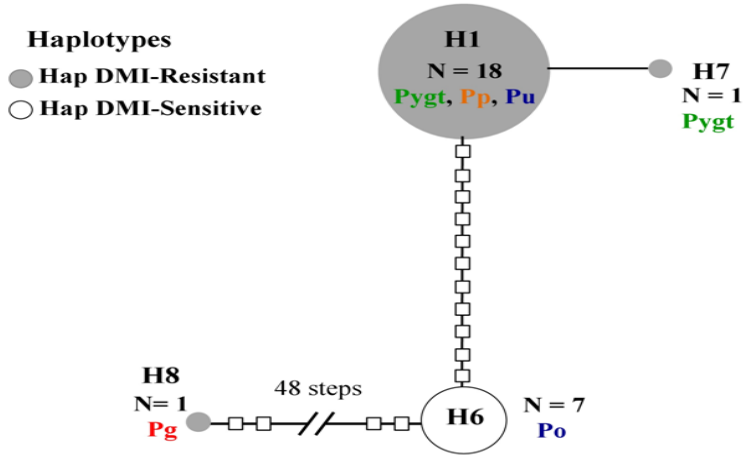

Figure 4. Network of haplotypes of the *CYP51A* gene from *Pyricularia* spp. The area of each circle is proportional to the number of isolates sampled from each haplotype. The lines between one circle and another represent the mutational steps between haplotypes.

Three *CYP51A* haplotypes (H1, H7 and H8) associated with resistance to DMI fungicides were detected among the *Pyricularia* species that were sampled from several invasive plant species in wheat fields. Castroagudin et al. (2015), who surveyed *Pyricularia* spp. associated with invasive plants from wheat fields, also detected two of the most common *cytB* haplotypes (H1 and H3) containing the G143A mutation that confers resistance to the QoI fungicide azoxystrobin. The H1-QoI resistant haplotype was detected in 48% of the isolates from invasive plant species, mostly from signal grass (*Urochloa* spp.) and weeping finger grass (*Chloris distichophylla*).

In our study, the number of host plant species harboring DMI-resistant isolates of *Pyricularia* spp. was high. We detected triazol fungicide-resistant *Pyricularia* spp. associated with oats (*Avena sativa*), sandbur (*Cenchrus echinatus*), weeping finger grass (*Chloris distichophylla*), star grass (*Cynodon* spp.), Jamaican crabgrass (*D. horizontalis*), crabgrass (*Digitaria sanguinalis*), barnyard grass (*Echinochloa crusgalli*), Indian goosegrass (*Eleusine indica*), Guinea grass (*Panicum maximum*), and signal grass (*Urochloa brizantha*).

In terms of levels of resistance, with the exception of the rice blast fungus *P. oryzae*, which was used as our sensitive standard, all of the other four *Pyricularia* species (*Pygt*, *Pg*, *Pp*, and *Pu*) associated with invasive *Poaceae* were resistant to DMI fungicides.

For tebuconazole, the mean EC_{50} for the four species was 1.18 μg mL^{-1} (ranging from a minimum of 0.69 to a maximum of 3.56 μg mL^{-1}). These EC_{50} values for tebuconazole were either similar or lower than the values described in the literature for resistant fungi associated with diseases on wheat or other cereals, which were heavily sprayed with DMI fungicides. Such examples included *Mycosphaerella graminicola* [maximum EC_{50} = 0.91 μg mL^{-1} (Stammler & Semar, 2011)], *Rhynchosporium commune* [maximum EC_{50} = 2.00 μg mL^{-1} (Hawkins et al., 2014) and *Zymoseptoria tritici* [maximum EC_{50} = 10.00 μg mL^{-1} (Cools & Fraaije, 2013).

For epoxiconazole, the mean EC_{50} for the four *Pyricularia* species was 0.33 μg mL^{-1} (with a minimum of 0.10 and a maximum of 0.95 μg mL^{-1}). In comparison with the same wheat or other cereals' pathogens, the following epoxiconazole EC_{50} were described: *Mycosphaerella graminicola* [maximum EC_{50} = 0.05 μg mL^{-1} (Stammler & Semar, 2011), *Rhynchosporium commune* [maximum EC_{50} = 0.23 μg mL^{-1} (Hawkins et al., 2014)] and *Zymoseptoria tritici* [maximum EC_{50} = 0.48 μg mL^{-1} (Cools & Fraaije, 2013). Based on the labeled doses of 75 g epoxiconazole ha^{-1} and 150 g tebuconazole ha^{-1} recommended for fungicide sprays in Brazilian wheat fields by the Brazilian Ministry of Agriculture, Livestock and Food Supply – MAPA (http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons), and using the method proposed by Castroagudin et al. (2015) considering the average wheat plant height at heading stage of approximately 0.82 meters, we estimated that epoxiconazole and tebuconazole are sprayed at the concentrations of 0.0055 μg mL^{-1} and 0.0180 μg mL^{-1} per hectare of wheat fields, respectively. Comparing these resulting field concentrations with the mean EC_{50} values that were estimated, we concluded that, on average, all four *Pyricularia* species from invasive grasses resisted at 60 to 65 times higher field doses of epoxiconazole or tebuconazole, respectively.

An R158K point mutation in the *CYP51A* gene that was conserved in four *Pyricularia* species (*Pg*, *Pp*, *Pu*, and *Pygt*) but absent in the reference species for DMI sensitivity (*Po*) may be related to resistance to tebuconazole and epoxiconazole.

In a countrywide population-based study in which 178 *Pygt* isolates were sampled from seven wheat fields in Brazil, Ceresini et al. (2018) also described the predominance of the R158K mutation in all four DMI-resistant haplotypes detected (H1, H2, H3, and H4). The predominant *CYP51A* haplotype associated with *Pp*, *Pu* and *Pygt* from invasive species was identical to the H1 haplotype described by (Ceresini et al. 2018), which was the most commonly found in all seven wheat blast populations (N = 175).

However, as reported for few other fungal plant pathogens, in addition to target site mutations found in the *CYP51A* gene of *Pg*, *Pp*, *Pu*, and *Pygt*, resistance to DMI fungicides could also be related to other mechanisms of quantitative nature, such as increase in ABC transporter efflux (Nakaune et al., 1998) and overexpression of the *CYP51A* gene (Ma et al., 2005; Coleman & Mylonakis, 2009; Abou Ammar et al., 2013).

Due to the quantitative and polygenic nature of the resistance attributed to DMI fungicides, the resistance to DMI fungicides found in all four *Pyricularia* spp. from Poaceae species that are invasive to wheat fields may be a result of slow and gradual selective pressure exerted on the pathogen populations due to long-term use of DMI fungicides at high dosages (Deising, Reimann, & Pascholati, 2008; Lucas et al., 2015).

To avoid the intensification of this scenario over the next few years, the adoption of anti-resistance management strategies is urgently needed. To decrease the selective pressure towards resistant pathogen populations, such strategies would include rotations of fungicides with different modes of action (Milgroom & Fry, 1988) and adoption of mixtures of single-target-site, high-risk fungicides with multiple-target-site, low-risk fungicides (Lucas et al., 2015).

Conclusion

All four *Pyricularia* species (*Pygt*, *Pg*, *Pp*, and *Pu*) associated with invasive *Poaceae* were resistant to DMI fungicides.

Several invasive poaceous species adjacent to sprayed wheat fields constitute an important inoculum reservoir of DMI-resistant *Pyricularia* spp. for the initial phases of the blast epidemic, especially for wheat blast.

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