ADRIANO FRANCIS DORIGAN

FUNGICIDES RESISTANCE: IMPLICATIONS ON BIOLOGICAL PROCESS OF PATHOGENS POPULATIONS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutor.

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ADRIANO FRANCIS DORIGAN

FUNGICIDES RESISTANCE: IMPLICATIONS ON BIOLOGICAL PROCESS OF PATHOGENS POPULATIONS

RESISTÊNCIA À FUNGICIDAS: IMPLICAÇÕES NOS PROCESSOS BIOLÓGICOS DAS POPULAÇÕES DE PATÓGENOS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutor.

APROVADA em 8 de Dezembro de 2023.

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RESUMO GERAL

Brusone do trigo, causada por *Pyricularia oryzae* linhagem *Triticum* (PoTl), é uma das doenças fúngicas mais importantes e devastadoras que afetam os campos de trigo na América do Sul, Sudeste Asiático, África Austral, e agora na Alemanha e na Europa Central. No Brasil, populações de PoTl foram associadas à resistência a três grupos químicos diferentes, incluindo fungicidas inibidores da quinona oxidase (QoIs), fungicidas inibidores de desmetilação (DMIs) e fungicidas inibidores da succinato desidrogenase (SDHIs). No primeiro capítulo, nos discutimos uma revisão dos mecanismos subjacentes à resistência a fungicidas em fitopatógenos, com ênfase específica em mutações pontuais e mecanismos de sítios não-alvos associados a diferentes classes de fungicidas. Com o aumento do uso de fungicidas na agricultura, o rápido surgimento e evolução da resistência aos fungicidas tornou-se uma questão crítica, colocando desafios significativos à produção agrícola sustentável. Nossa revisão esclarece sobre mutações não-sinônimas nos genes alvo dos fungicidas, superexpressão dos genes alvo e regulação da bomba de efluxo, e como esses mecanismos afetam os fungicidas QoI, metil-benzimidazol (MBCs), DMI e SDHI. No segundo capítulo, apresentamos o resultado do isolado QoI-R PoTl mantendo uma vantagem adaptativa na severidade da doença em folhas e espigas ao longo do tempo. Para o isolado QoI-R PoTl, o modelo não linear logístico apresentou melhor ajuste às curvas de progresso da brusone do trigo ao longo de cinco ciclos sucessivos de infecção. No terceiro capítulo, os resultados deste estudo demonstram que o isolado QoI-R PoTl mantém maior aptidão e vantagens competitivas ao longo do tempo. Com base em estudos moleculares, a mutação G143A foi encontrada em 100% dos isolados resistentes aos QoI, indicando que a mutação G143A dos isolados QoI-R PoTl permaneceu estável. Essas descobertas podem ter implicações significativas para o manejo integrado no patossistema da brusone do trigo, uma vez que as vantagens de aptidão e a estabilidade podem contribuir para fixar e aumentar a proporção de isolados resistentes nas populações do patógeno no campo. Assim, os genótipos resistentes das populações PoTl com vantagens de aptidão podem prevalecer nos campos de trigo, mesmo após sucessivos ciclos de infecção na ausência de pressão de seleção, e os QoIs podem perder a sua eficácia permanentemente. Discutimos a importância ecológica de populações de patógenos que apresentam vantagens evolutivas, maior aptidão e resistência estável a QoIs. Esperamos que nossos resultados possam ajudar a compreender o manejo integrado da brusone do trigo nos campos de trigo brasileiros.

Palavras-chave: Resistência á fungicidas. Brusone do trigo. Vantagem adaptativa. Fungicidas sistêmicos. Ecologia e epidemiologia. Controle de doenças e manejo de pragas.

GENERAL ABSTRACT

Wheat blast, caused by *Pyricularia oryzae* pathotype *Triticum* (PoTl), is one of the most important and devastating fungal diseases affecting wheat crops in South America, Southeast Asia, Southern Africa and now in Germany and in central Europe. In Brazil, PoTl populations have been associated with resistance to three different chemical groups, including quinone outside inhibitor fungicides (QoIs), demethylation inhibitor fungicides (DMIs), and succinate dehydrogenase inhibitors fungicides (SDHIs). In the first chapter, we discuss a review of the mechanisms underlying fungicide resistance in crop pathogens, with a specific emphasis on point mutations and non-target site mechanisms associated with different fungicide classes. With the increasing use of fungicides in agriculture, the rapid emergence and evolution of fungicide resistance have become a critical issue, posing significant challenges to sustainable crop production. Our review sheds light on non-synonymous mutations in the target genes of fungicides, target gene overexpression, and efflux pump upregulation, and how these mechanisms affect the QoI fungicides, methyl-benzimidazole (MBCs), DMI and SDHI fungicides. In the second chapter, we present the result of that QoI-R PoTl isolate maintaining an adaptive advantage in disease severity on leaves and heads over time. For the QoI-R PoTl isolate, the nonlinear logistic model exhibits a better fit to the progress curves of wheat blast over the course of five successive infection cycles. In the third chapter, the results of this study demonstrate that the QoI-R PoTl isolate maintains higher fitness and competitive advantages over time. Based on molecular studies, the G143A mutation was found in 100% of the QoI-resistant isolates, indicating that the G143A mutation of the QoI-R PoTl isolates remains stable. These findings can have significant implications for the integrated management of the wheat blast pathosystem since fitness advantages stability can contribute to fixing and increasing the proportion of resistant isolates in field populations of the pathogen. Hence, resistant genotypes of the PoTl populations with fitness advantages may prevail on wheat fields, even after successive infection cycles in the absence of selection pressure, and QoIs may lose their efficacy permanently. We discuss the ecological importance of populations of pathogens that have evolutionary advantages, higher fitness, and resistance to QoIs stable. We hope our results can help insights into the integrated management of wheat blast in Brazilian wheat fields.

Keywords: Fungicide resistance. Wheat blast. Fitness advantage. Systemic fungicides. Ecology and epidemiology. Disease control and pest management.

SUMMARY

PART 1

GENERAL INTRODUCTION

Wheat (*Triticum aestivum*) is one of the most important cereal staple crops produced worldwide, contributing to global food security (ACEVEDO et al., 2018). Brazil harvested 6.6 million tons of wheat during the 2018/2019 season, representing 0.9% of the 735 million tons produced globally (COLUSSI et al., 2022; FAOSTAT, 2022). In Brazil, yield losses are due to many diseases, a lack of quality Brazilian wheat, and low internal production and supply. Thus, it is estimated that around 4 million tons of wheat grain annually are still imported into Brazil (CONAB, 2020; CERESINI et al., 2018). In 1986, the wheat blast was first reported in northern Parana state, Brazil (IGARASHI, 1986). After that, the pathogen quickly spread to south-central Brazil and other countries such as Argentina, Bolivia, and Paraguay (GLADIEUX et al., 2018; CERESINI et al., 2018). In 2016, the wheat blast was reported outside South America, firstly, in Bangladesh, southeast Asia, and secondly, in 2017, in Zambia, eastern Africa, causing outbreaks that significantly harmed production in both countries (CALLAWAY et al., 2016; ISLAM et al., 2016; TEMBO et al., 2020). These two outbreaks resulted from contaminated seed lots distributed to two continents (SINGH et al., 2021). In 2022, the blast disease was also reported in Germany in central Europe (BARRAGAN et al., 2022).

In 2015, the resistance to QoI fungicides in Brazilian PoTl populations has been reported (CASTROAGUDIN et al., 2015). QoI-resistant (QoI-R) isolates from those PoTl populations were associated with the G143A point mutation in the cytochrome b (*cyt B*) gene (CASTROAGUDIN et al., 2015). Fungicide resistance may change plant pathogens' fitness (HAWKINS and FRAAIJE et al., 2018). In a recent study, QoI-R PoTl isolates carrying the G143A mutation had a higher fitness and competitive advantage than QoI-sensitive (QoI-S) isolates (DORIGAN et al., 2022), contrasting with the evolutionary theory, whose foundation is that fungicide resistance should come with fitness cost (HAWKINS and FRAAIJE et al., 2018). The higher conidial production and higher levels of disease severity on the QoI-R PoTl isolates reinforce their competitive ability against the QoI-S isolates and their potential for continuously causing crop damages on wheat fields after displacing the sensitive PoTl lineages (DORIGAN et al., 2022). On the other hand, the fitness cost has also been reported in blast pathogens, with reduced infection

efficiency and aggressiveness in field isolates of *P. oryzae* on rice with G143A mutation and reduced virulence in the G143A mutant of *Magnaporthe oryzae* from *Perennial ryegrass* (MA and UDDIN, 2009; D' AVILA, 2022; DORIGAN et al., 2023).

This dissertation is organized into three papers (1 review and two original research papers). The first paper published in Pest Management Science is a review entitled "Target and non-target site mechanisms of fungicide resistance and their implications for the management of crop pathogens," which provides background information about fungicide resistance mechanisms and their implications for developing anti-resistance strategies in plant pathogens. In the second paper, entitled "Temporal dynamics of *Pyricularia oryzae Triticum* resistant to quinone outside inhibitor fungicides," we characterize the temporal progress of wheat blast and fit the best nonlinear model, describing the nature of an epidemic of QoI-R PoTl isolate compared with the sensitive isolate (QoI-S), throughout five successive infection cycles of PoTl on wheat leaves and heads. Finally, in the third paper, entitled "Stability of resistance and competitive advantage of *Pyricularia oryzae Triticum* resistant to QoIs," we assess the fungicide resistance stability, fitness, and competitive ability of the QoI-resistant (R) PoTl isolates group throughout nine and five successive infection cycles *in vitro* and *in vivo*, respectively, in the absence of fungicide.

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PART 2

ARTICLES:

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CHAPTHER 1– TARGET AND NON-TARGET SITE MECHANISMS OF FUNGICIDE RESISTANCE AND THEIR IMPLICATIONS FOR THE MANAGEMENT OF CROP PATHOGENS

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ABSTRACT

BACKGROUND: Fungicides are indispensable for high-quality crops, but the rapid emergence and evolution of fungicide resistance have become the most important issues in modern agriculture. Hence, the sustainability and profitability of agricultural production have been challenged due to the limited number of fungicide chemical classes. Resistance to site-specific fungicides has principally been linked to target and non-target site mechanisms. These mechanisms change the structure or expression level, affecting fungicide efficacy and resulting in different and varying resistance levels.

RESULTS: This review provides background information about fungicide resistance mechanisms and their implications for developing anti-resistance strategies in plant pathogens. Here, our purpose was to review changes at the target and non-target sites of quinone outside inhibitor fungicides (QoIs), methyl-benzimidazole fungicides (MBCs), demethylation inhibitor fungicides (DMIs), and succinate dehydrogenase inhibitor fungicides (SDHIs) and to evaluate if they may also be associated with a fitness cost on crop pathogen populations.

CONCLUSION: The current knowledge suggests that understanding fungicide resistance mechanisms can facilitate resistance monitoring and assist in developing anti-resistance strategies and new fungicide molecules to help solve this issue.

1. INTRODUCTION

Fungicide resistance may occur in fungi, which are etiological agents of important pathosystems, and in some cases, the molecules used fail to effectively control crop diseases due to resistance associated not only with target sites but also with non-target site mechanisms.[1]

Since 1980, the emergence of pathogen populations resistant to various fungicides belonging to different chemical classes has risen continuously globally.[2] Consequently, many studies based on genome sequencing, bioinformatics, and gene manipulation have focused on on the modes of action of fungicides and their corresponding resistance mechanisms. Among them, increased activity of efflux pumps, metabolic circumvention, detoxification, standing genetic variations, regulation of stress response pathways, and single nucleotide polymorphisms (SNPs) have been studied.[1, 3, 4] The purpose of this review was to summarise both known mechanisms linked to target sites, such quinone outside inhibitor fungicides (QoIs), methyl-benzimidazole fungicides (MBCs), demethylation inhibitor fungicides (DMIs), and succinate dehydrogenase inhibitor fungicides (SDHIs) and those not linked to target sites as overexpression of drug efflux pumps, detoxification, and *CYP51* paralogs correlated with fungicide resistance.

Moreover, we hope to inspire further efforts in exploring the molecular underpinnings of fungicide resistance and assessing the fitness costs, resistance stability, competitive abilities, and resistance inheritance based on non-target sites of plant pathogens. This knowledge may provide more insights into their impact on disease management, a better understanding of the pathosystem, and the adoption of anti-resistance strategies.

2. MUTATIONS LINKED TO FUNGICIDE RESISTANCE

2.1. Target site alterations

Mutations are inherited alterations in a specific DNA sequence compared to a reference sequence.[5] Non-synonymous mutations in genes that encode the target site are amino acid substitutions at the fungicide target enzyme, reducing fungicide binding.[6] In

agroecosystems, genetic mutation plays an essential role in the evolution of plant pathogens to fungicide resistance.[7]

In some cases, resistance to site-specific fungicides, such as QoIs, occurs due to a single point mutation in the gene encoding the target enzyme. It can confer a high level of resistance, with qualitative resistance occurring. When the QoI fungicide binds to the target protein translated from the *cyt b* gene, ATP synthesis is inhibited by blocking the electron transfer chain reaction, resulting in the death of QoI-sensitive isolates. Mutations in the *cyt b* gene that result in binding site modification consequently have non-attachment between QoI fungicides and the target protein and thus do not compromise ATP production for QoI-resistant isolates (Fig. 1A). For example, the substitution of the amino acid glycine for alanine at the position of codon 143 (G143A) in the cytochrome *b* (*cyt b*) gene occurs by immediate change, significance, and directional selection for reduced sensitivity.[8] However, quantitative resistance occurs when more than one gene contributes to resistance. Quantitative resistance happens by gradual changes in the direction of resistance over time, either to multisite fungicides or to some single-site compounds.[9] For example, fungal populations have the potential to evolve quantitative resistance in the presence of lanosterol demethylation inhibitors (DMIs) (Fig. 1C). Quantitative resistance to DMIs may be associated with mutations in the *CYP51* gene, overexpression of the target site, altered efflux pump activity, metabolic circumvention, and metabolic detoxification of fungicide molecules.[1, 10]

For the chemical classes of fungicides used in agroecosystems, there are several reports of point mutations at the target site of field isolates associated with the evolution of fungicide resistance. The Fungicide Resistance Action Committee (FRAC) and the European and Mediterranean Plant Protection Organization (EPPO) have listed and published information on the resistance of pathogen species to fungicide classes.[11, 12] We focused on point mutations at the target sites of QoIs, MBCs, DMIs, and SDHIs.

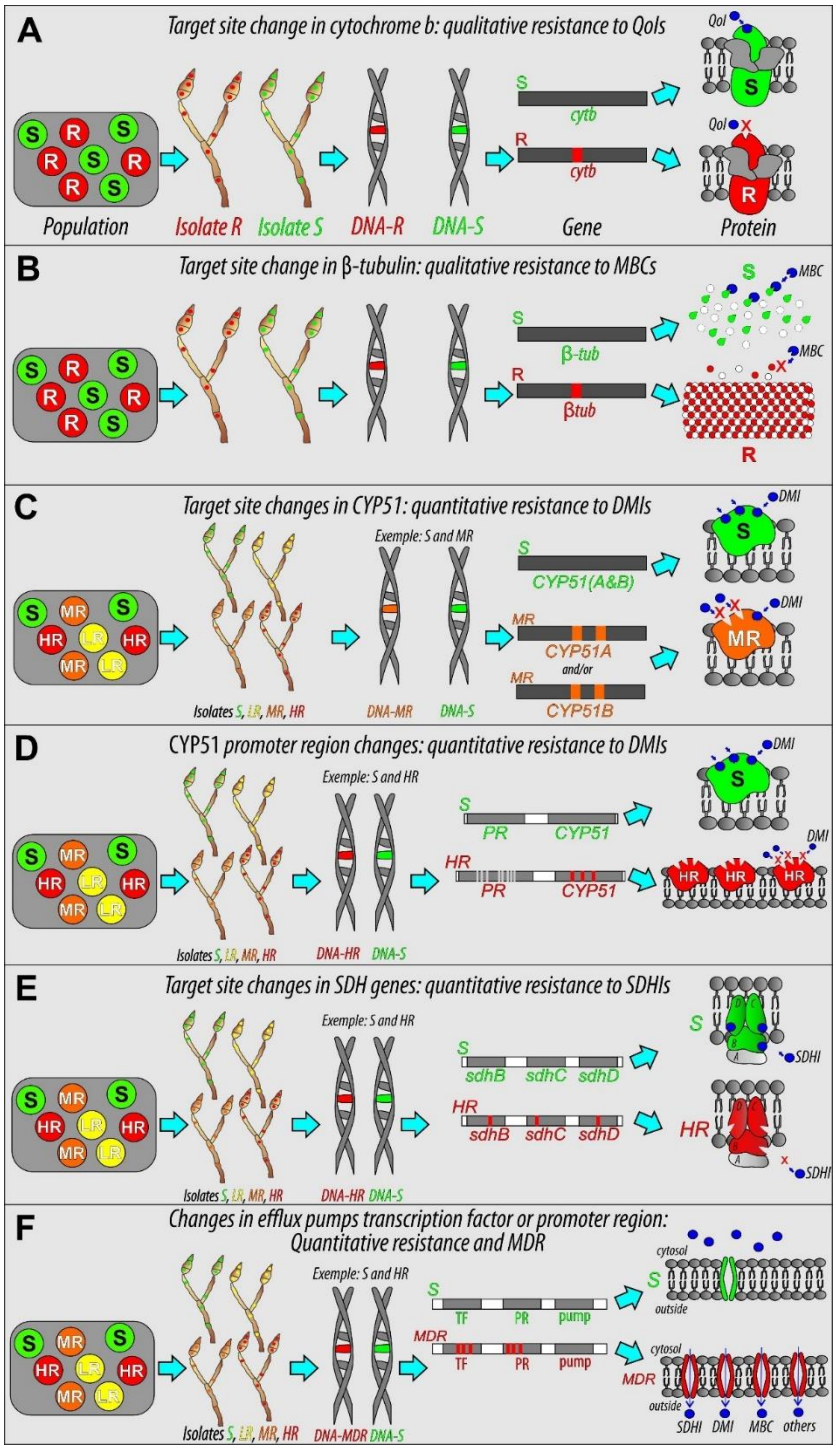

Figure 1. Schematic illustration of different fungicide resistance mechanisms. Within the scheme, for each case, and from left to right shown the populations, individual isolates with different resistance classes, DNA genomic, genes and target proteins produced. Blue balls represent the fungicides molecules in all examples (A to F). A) Target site changes in the bc1 complex (complex III in the electron transport chain) associated with qualitative resistance to quinone outside inhibitor fungicides (QoIs). The isolates (and color codes) are: QoI-sensitive (green) and QoI-resistant (red). B) Target site changes in the *β-tubulin* gene associated with qualitative resistance to methyl-benzimidazole fungicides (MBCs). The isolates (and color codes) are: MBC-sensitive (green) and MBC-resistant (red). In the target protein, the *β-tubulin* is represented by green color for MBC-

sensitive isolates and the red color for MBC-resistant isolates. *Alpha-tubulin*is represented by the white color. C) Target site changes in the *CYP51*gene associated with quantitative resistance to demethylation inhibitor fungicides (DMIs). The isolates (and color codes) are: DMI-sensitive (green), DMI-reduced sensitivity (yellow), DMI-moderately resistant (orange) and DMI-highly resistant (red). Mutations in the *CYP51A* and or *CYP51B* gene of the DMI-moderately resistant isolate are represented by the orange color. D) *CYP51* promoter region changes associated with quantitative resistance to DMIs. The isolates (and color codes) are: DMIs-sensitive (green), DMIs-reduced sensitivity (yellow), DMIs-moderately resistant (orange) and DMIs-highly resistant (red). E) Target site changes in the Succinate dehydrogenase (complex II or succinate-ubiquinone oxidoreductase) associated with quantitative resistance to succinate dehydrogenase inhibitors fungicides (SDHIs). The isolates (and color codes) are: SDHI-sensitive (green), SDHI-reduced sensitivity (yellow), SDHI-moderately resistant (orange) and SDHI-highly resistant (red). F) Changes in efflux pumps transcription factor (TF) or promoter region (PR) associated with quantitative multidrug resistance (MDR). The isolates (and color codes) are: sensitive (green), MDR-reduced sensitivity (yellow), MDR-moderately resistant (orange) and MDR-highly resistant (red).

2.1.1. QoI fungicides

In QoI fungicides, field resistance has been reported in 43 fungal species and is associated with seven mutations in the *cytb* gene (Table 1).[11] The most common mutation identified is G143A, which has been detected in 80% of fungal species.[13] Quantitative polymerase chain reaction (qPCR) assays have shown a predominance of the G143A mutation within and among pathogen species. The frequency of the G143A mutation within some populations is over 90%.[14] The *cytb* G143A substitution has been observed in field isolates of *Botrytis cinerea* from tomato, cucumber, and strawberry,[15] *Plasmopara viticola* from grape,[16] *Zymoseptoria tritici* from wheat,[14] and many other pathosystems (Table 1). However, in some cases, QoI resistance has only been associated with the F129L mutation in the *cytb* gene, for example, populations of *Alternaria solani* from potato,[17] *Phakopsora pachyrhizi* from soybean,[18] *Rhizoctonia solani* from rice,[19] and others (Table 1). In rare cases, the F129L mutation has been reported in *P. viticola*.[20]

Table 1. Description of mutations most frequently associated with fungicide resistance of plant pathogenic fungi.

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Alternaria alternata	F167Y	G143A	-	-	P230A; P230D; P230F; P230I; P230R; N235D; N235E; N235G; N235T; H277L; H277R; H277Y	G79R; H134R; S135R	D123E; H133P; H133R; H133Q; H133T
Alternaria arborescens	-	G143A	-	-	-	-	-
Alternaria solani	-	F129L	-	-	H278R; H277Y	H134Q; H134R	T28A; A47T; D123E; H133R
Alternaria tenuissima	-	G143A	-	-	-	-	-
Botrytis cinerea	E198A; 198K; E198L; 198V; F200Y	G143A	-	-	P225F; P225H; P225L; P225T; N230I; H272L; H272R; H272V; H277Y	P80H; G85A; I93V; M158V; V168I; A187F	H132R; I189L
Botrytis elliptica	-	-	-	-	H272R; H277Y	-	-
Cercospora beticola	F167Y; E198A	F129L; G143A	-	L144F	-	-	-
Cercospora nicotianae	-	F129L; G143A	-	-	-	-	-
Cercospora sojina	-	G143A	-	-	-	-	-
Cladosporium fulvum	E198A; F200Y	-	-	-	-	-	-
Colletotrichum acutatum	-	F129L; G143A	-	-	-	-	-

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Colletotrichum cereale	E198K; E198A; F200Y	-	-	-	-	-	-
Colletotrichum gloesporióides	E198A	G143A	-	-	-	-	-
Colletotrichum graminicola	-	G143A	-	-	-	-	-
Colletotrichum siamense	E198A;	G143A	-	-	-	-	-
Corinespora cassicola	M163I; F167Y; E198A; F200S; F200Y	G143A	-	-	H278R; I280V; H277Y	S73P; N75S	S89P; D95E; H105R; G109V
Didymella bryoniae	-	G143A	-	-	H277R; H277Y	-	-
Didymella rabiei	-	G143A	-	-	-	-	-
Didymella tanaceti	-	-	-	-	H277R; H277Y; I279V	S73P; G79R; H134R; H134Q; S135R	D112E; H122R
Erysiphe graminis f.sp. *hordei*	-	-	-	Y137F; K147Q; K172E; M304I; S509T	-	-	-
Erysiphe graminis f.sp. *tritici*	-	G143A	-	S79T; Y137F; K175N	-	-	-
Erysiphe necator	-	G143A	-	Y136F	H242R; I244V; H277Y	G169D; G169S	-

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Fusarium graminearum	Q73R; F167Y; E198L; 198Q; F200Y	-	-	-	-	A83V; R86C; R86H	-
Helminthosporium solani	E198A	-	-	-	-	-	-
Lasiodiplodia theobromae	E198A; E198K; F200Y	-	-	-	-	-	-
Leveillula taurica	-	L130T; G143A; L210T; L290T; L290F	-	-	-	-	-
Monilinia fructicola	H6Y; E198A; E198K	-	-	G461S	-	-	-
Monilinia laxa	E198A	-	-	-	-	-	-
Microdochium majus	-	G143A	-	-	-	-	-
Microdochium nivale	-	G143A	-	-	H253Q	-	-
Mycovellosiella nattrassii	-	G143A	-	-	-	-	-
Parastagonospora nodorum	-	G143A	-	Y137F; Y144H	-	-	-
Passalora fulva	-	F129L	-	-	-	-	-
Penicillium aurantiogriseum	E198A; E198K; F200Y	-	-	-	-	-	-

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Penicillium digitatum	E198Q; F200Y	-	-	Y137H; Q309H; G459S; F506I	-	-	-
Penicillium expansum	E198A; F167Y; E198K	-	-	-	-	-	-
Penicillium puberulum	E198A; E198K;	-	-	-	-	-	-
Pestalotiopsis longiseta	-	F129L; G143A	-	-	-	-	-
Phakopsora pachyrhizi	-	F129L	-	F120L; Y131F; Y131H; K142R; I145F; I475T	-	I86F	-
Plasmopara viticola	-	G143A	-	-	-	-	-
Podosphaera fusca	E198A	G143A	-	-	-	-	-
Podosphaera leucotricha	-	G143A	-	-	-	-	-
Podosphaera xanthii	-	G143A	-	-	H267Y; H277Y	-	-
Pseudoperonospora cubensis	-	G143A	-	-	-	-	-
Pseudocercospora fijiensis	E198A	G143A	-	Y137F; A313G; A381G; Y461D; G462A; Y463D; Y463H; Y463N; Y461N	-	-	-
Puccinia triticina	-	-	-	Y137F	-	-	-

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Pyrenopeziza brassicae	E198A; 198G; F200Y; L240F	-	-	G460S; S508T	-	-	-
Pyrenophora teres	-	F129L	F489L	-	H277Y	K49E	-
Pyrenophora tritici-repentis	-	F129L; G137R; G143A	-	-	-	-	
Pyricularia grisea	-	F129L; G143A	-	-	-	-	-
Pythium aphanidermatum	-	F129L	-	-	-	-	-
Ramularia collocygni	-	G143A	-	V136A; Y137F; A311S; I381T; I384L; D458G; Y459C; Y459N; G460D; G460V; Y461N; Y461H	N224T; H266R; H266Y; T267I; I268V; H277Y	N87S; G91R; H142R; H146L; H146R; H153R; G171D	-
Rhizoctonia cerealis	-	-	-	-	H277Y	-	-
Rhizoctonia solani	-	F129L	-	-	H246Y	H139Y	H116D; H116Y; G138V; L129Y
Rhynchosporium commune	E198G; F200Y	G143A	-	-	-	-	-
Sclerotinia homoeocarpa	E198K	-	-	-	H277Y; H267Y	G91R; G150R; G159W	-
Sclerotinia sclerotiorum	E198A; F200Y	-	-	-	H273Y	G91R; H146R; G150R	D108K; H132R

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
Sphaerotheca fuliginea	-	G143A	-	-	-	-	-
Stemphylium botryosum	-	-	-	-	H272L; H272R; H272Y; H277Y; P225F; P225L	-	-
Stemphylium vesicarium	-	G143A	-	-	-	-	-
Tapesia acuformis	E198A;E198G; E198Q; F200Y	-	-	-	-	-	-
Tapesia yallundae	E198A; 198G; E198K; L240F; F200Y	-	-	-	-	-	-
Venturia inaequalis	E198A; 198G; E198K; F200Y; L240F	G143A	-	Y137F	T253I	H151R	-
Venturia pirina	E198A;F200Y;	-	-	-	-	-	-
Zymoseptoria tritici	E198A	F129L; G143A	-	Y137F; D107V; D134G; 136A; V136C; V136G; Y137F; M145L; S208T; N284H; H303Y; A311G;	N225I; N225T; R265P; H267L; T268A; T268I; H277Y	T79I; T79N; W80S; S83G; A84F; N86A; N86K; N86S; P127A;	R47W; M114V; D129G

Plant pathogens	***β*-tubulin**[a]	***cytb***[b]	***CYP51A***[c]	***CYP51B***[c]	***sdhB***[d]	***sdhC***[d]	***sdhD***[d]
				G312A; A379G; I381V; A410T; G412A; Y459C; Y459D; Y459H; Y459N; Y459P; Y459S; Δ459; Δ459-460; Δ460; G460D; Y461D; Y461G; Y461H; Y461L; Y461N; Y461S; V490L; G510C; S524T		R151M; R151S; R151T; H152R; V166M; T168R	

[a] Mutations described in the *β-tubulin* gene of plant pathogens associated with resistance to Methyl benzimidazole carbamates (MBCs).

[b] Mutations described in the *cytochrome b (cytb*) gene of plant pathogens associated with resistance to Quinone outside inhibitors (QoIs).

[c] Mutations described in the *cytochrome P450 family 51 subfamily A member 1* and *subfamily B member 1* genes of plant pathogens associated with resistance to Demethylation inhibitors (DMIs).

[d]Mutations described in the *sdhB, sdhC, sdhD genes,* which encode the three subunits of succinate dehydrogenase (SDH; mitochondrial complex II) of plant pathogens associated with resistance to Succinate dehydrogenase inhibitors (SDHIs).

2.1.3. DMI fungicides

For DMIs, field resistance observed in 19 fungal species was associated with 58 mutations in the *CYP51* gene (Table 1).[11] When *CYP51* of DMI fungicides binds to the target protein, ergosterol synthesis is inhibited, resulting in the death of DMI-sensitive isolates. Changes in the *CYP51* gene of DMI-moderately resistant isolates, which provides reduced protein binding affinity, lead to a reduction in the inhibition of ergosterol synthesis. *CYP51* paralogs (*CYP51A* and *CYP51B*) are represented in both DMI-sensitive and DMI-moderately resistant isolates (Fig. 1C). The most common mutation among those identified in the *CYP51A* and *CYP51B* genes and associated with DMI resistance is *CYP51* Y137F, which has been reported in 58% of fungal species.[13] For example, important *CYP51* Y137F mutations related to high DMI resistance have been identified in field isolates of *Erysiphe graminis* f. sp. *tritici* from wheat,[24] *Phakopsora pachyrhizi* from soybean,[25] *Z. tritici* on wheat,[26] and others (Table 1).

2.1.4. SDHIs

For SDHIs, field resistance has been identified in 22 fungal species and associated with 83 mutations in the *sdh* genes (Table 1).[11, 27] Mutations in the *sdhB, sdhC,* and *sdhD* genes of SDHI-highly resistant isolates are represented by red colour (Fig. 1E). When SDHI fungicides bind to the SDH target protein, the activity of succinate dehydrogenase is inhibited, resulting in the death of SDHI-sensitive isolates. Changes in SDHI-highly resistant isolates for certain *sdh* genes provide altered binding affinity, and the SDHI fungicides do not bind to the target protein and do not inhibit fungal respiration (Fig. 1E). The most common mutation among the *sdh*B, *sdh*C, and *sdh*D genes found correlated with SDHI resistance is the *sdhB*-H277Y, which has been observed in 68% of fungal species.[13] This mutation has been identified in field isolates of *B. cinerea* from strawberry,[28] *Rhizoctonia cerealis* from wheat,[29] *Z. tritici* from wheat,[30, 31] and many other pathosystems (Table 1).

2.2. Overexpression of the target site

Although target site alterations are the main fungicide resistance mechanism known, the resistance of plant pathogens to DMIs and MBCs has also been associated with overexpression of the target site.[10] Insertions in the *CYP51* gene promoter region are associated with increased gene expression in *Zymoseptoria tritici* DMI-highly resistant isolates. This resistance mechanism may be associated with the target site change (Fig. 1D). In several fungal species, resistance to DMIs results from overexpression of the sterol 14 *alpha-demethylase* gene *CYP51*.[9, 32] Generally, overexpression of the target site occurs by either rearrangement or mutation in its promoter region.[3, 4]

Some studies with *Mycosphaerella fijiensis* on bananas have reported overexpression of the *CYP51* gene associated with DMI resistance, in which its DMI-resistant-progeny inherits the changes in the *PfCYP51* gene promoter region linked to increased expression.[33, 34] For field isolates of *Cercospora beticola* on beet, DMI resistance occurs by overexpression of the *CYP51* gene.[35] Field isolates of *Monilinia fructicola* on peaches also show DMI resistance mediated by overexpression of the *CYP51* gene.[4] In *Pyrenophora teres* on oat, DMI resistance results from the F489L change in *CYP51A*, which causes inducible overexpression of the *CYP51* gene.[36] Similarly, overexpression of the *CYP51* gene was a DMI resistance mechanism in *Aspergillus flavus, A. niger, A. parasiticus,* and *Pyricularia oryzae* from rice.[37-39] Additionally, in *Colletotrichum gloeosporioides*, DMI resistance in field isolates results from mutations and overexpression of *CYP51*.[40] However, overexpression of the *PdCYP51B* gene confers resistance to DMIs in *Penicillium digitatum*, the causal agent of citrus green mould.[41] Concerning MBCs, benomyl resistance of *Colletotrichum acutatum* from grapes results in overexpression of the *β-tubulin* gene CaTUB1, controlled by CaBEN1.[42]

3. NON-TARGET SITE MECHANISMS OF FUNGICIDE RESISTANCE

Non-target site mechanisms of fungicide resistance have been identified as an important factor related to the emergence of fungicide resistance in many fungal pathogens. This mechanism includes reducing the fungicide's effectiveness without affecting its interaction with the target site. Here, we discuss the types of non-target site mechanisms of fungicide resistance (NTSR) and their impact on fungicide resistance. At least nine non-target site mechanisms of fungicide resistance can provide resistance to the main fungicide classes, such as (i) drug efflux transporters, (ii) *CYP51* paralogs, (iii) mitochondrial heteroplasmy, (iv) alternative respiration, (v) altered sterol metabolism,

(vi) detoxification, (vii) regulation of stress response pathways, (viii) quantitative effects, and (ix) transcription factors.[1] Among the main classes of chemical fungicides, resistance to DMIs appears to be more diverse and correlated with six non-target site mechanisms, likely due to its frequent and prolonged use in both the agricultural and medical fields.[1, 2] This section focuses on the main non-target site mechanisms: overexpression of drug efflux pumps, detoxification, and *CYP51* paralogs correlated with fungicide resistance. An important question to consider is whether one of the most critical implications of non-target site resistance is its potential to confer cross-resistance to multiple fungicides, including those with different modes of action. This is because the resistance mechanism targets the fungicide's detoxification or transport pathways rather than the fungicide's target protein.[1] Thus, fungicide resistance resulting from non-target site mechanisms can be challenging for the management and control of plant pathogens.[1] Furthermore, resistance in the non-target site may have a low impact on fungal fitness, allowing the populations to remain and spread in the absence of fungicides.[1] This persistence may lead to the establishment of resistant populations that are hard to manage. In this sense, non-target site mechanisms of fungicide resistance are a significant concern in agriculture and require careful consideration in fungicide development and management strategies. Understanding the molecular and biochemical mechanisms underlying non-target site resistance and developing effective management strategies to mitigate its impact are crucial for the long-term sustainability of fungicide use in agriculture.

3.1 Overexpression of drug efflux pumps

Drug efflux transporters are integral membrane-bound proteins that facilitate the movement of various substrates across biological membranes, including protein macromolecules, ions, and small molecules.[43] In phytopathogenic fungi and other eukaryotes, two primary groups of drug efflux transporters exist: ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters. Despite the importance of these transporter families in virulence, there appears to be no direct correlation between the abundance of these transporters in the fungal genomes of saprobic and pathogenic isolates.[44]

Initially, ABC transporters have been described as P-glycoproteins (P-gp), but they are also known as PDR proteins (pleiotropic drug resistance) or MDR proteins (multidrug resistance). The ABC superfamily, which appears to be the dominant drug

transporters, is composed of a transmembrane domain (TMD, conferring specificity) and a structurally conserved nucleotide-binding domain (NBD) where ATP is hydrolysed.[1, 43] The tight coupling of ATP cleavage allows ABC transporters to export toxic compounds.[45] An essential characteristic of ABC drug transporters is their low substrate specificity, enabling them to transport a wide variety of structurally different compounds.[46] There are seven subfamilies (A–G) of the ABC transporter classified based on the architecture of the protein.[1, 43]

The MFS transporter carries compounds through the fungal plasma membrane by either chemiosmotic ion gradients or proton motive force.[47] The MFS transporter contains 74 families, and each family is accountable for a specific substrate type.[48] In general, MFS transporters function in drug efflux systems, the secretion of endogenously produced toxins, and as metabolites of the Krebs cycle, organophosphate/phosphate exchangers, and bacterial aromatic permeases.[43]

Overexpression of efflux pumps in MDR isolates may occur by either rearrangement or mutation in their promoter region (PR) or transcription factor (TF). MDR isolates exhibit overexpression of the ATP-binding cassette (ABC) transporters and or the Major Facilitator Superfamily (MFS) transporters, resulting in different molecule types carryied across a biological membrane, including SDHIs, DMIs, and MBCs. Different levels of efflux pump overexpression in MDR isolates may lead to different drug sensitivity levels. No major changes have been observed in the genes of sensitive isolates (Fig. 1F).

In ascomycetes, such as in *P. oryzae*, *B. cinerea*, and *Fusarium* spp., 40 to 50 ABC transporters have been reported, and more than 200 MFS with approximately 100 MFS transporters belong to MDR proteins.[44, 49-52] Studies have reported the involvement of ABC transporters in the development of MDR in phytopathogens (e.g., ABC-G transporter), such as BcATRB and BcATRD from *B. cinerea* on fruits and vegetables, and association with resistance to DMIs, MBCs, and SDHIs. The ABC-G transporter also confers resistance to DMIs, such as MoABC2 in *P. grisea* from rice [53] and ShATRD in *Sclerotinia homoeocarpa* from turfgrass.[54] For *P. digitatum* on citrus, ABC-G transporters, such as PMR1 and PMR5, have been associated with resistance to MBCs.[55] Regarding MFS transporters, the fungal phenotype DMI resistant has been associated with MDR by overexpression of MFS genes, including MgMFS1 in *Z. tritici* from wheat,[52] PdMFS1 in *P. digitatum* from citrus,[56] and BcMFSM2 in *B. cinerea* from fruits and vegetables (Fig. 1F).[57] The fungus *Z. tritici* uses the MgMFS1 protein to protect

against natural toxins and fungicides.[58] However, gene disruption has been associated with increased sensitivity to QoIs in *Z. tritici*, confirming its role in MDR.[43] Furthermore, MFS's host-specific toxins (HSTs) in plant pathogens can affect plant pathogenesis.[44]

3.2 Expression of drug efflux pumps

Several ABC transporter genes have been described, and their expression is upregulated in various natural and synthetic toxic compounds, such as phytoalexins and drugs.[59] Although the expression of efflux transporters occurs between 10 and 15 minutes after exposure to the drugs, this "lag period" seems to be enough for several toxins to diffuse into the pathogen's cells, inhibiting growth. If the appropriate transporters are constitutively expressed, drug absorption is blocked, thus avoiding the "lag period" and conferring drug resistance.[46]

In *B. cinerea*, a pathogen that causes grey mould on fruit and vegetables, MDR phenotypes exhibit overexpression of the ABC transporter BcATRB and the MFS transporter BcMFSM2 (Fig. 1F).[57] BcATRB, which is defined as MDR1, provides tolerance for phytoalexin resveratrol. Its overexpression confers resistance to fludioxonil, carbendazim, cyprodinil, and tolnaftate, whereas BcMFSM2, defined as MDR2, has been correlated with resistance to iprodione, boscalid, tolnaftate, cyprodinil, fludioxonil, fenhexamid, tebuconazole, bitertanol, and cycloheximide.[57-60] Natural hybridisation between MDR1 and MDR2 phenotypes resulted in MDR3.[57] MDR1h, a more potent MDR1 isolate that carries a more significant expression level of BcATRB, has been discovered. Its resistance to fludioxonil and cyprodinil is higher than that of the MDR1 isolate.[61] For *P. tritici-repentis*, the causal agent of wheat tan spot, adaptation to DMI fungicides and the ability to infect plants is dependent on efflux pump gene expression and localisation of their products in the plasma membrane.[60] Another wheat pathogen, *Z. tritici* causes Septoria leaf blotch; overexpression of the MgMFS1 gene occurs with high resistance to DMIs and low resistance to QoIs and SDHIs.[63, 64] However, disruption of the *Mgmfs1* gene has been associated with increased sensitivity to tolnaftate, epoxiconazole, boscalid, QoIs, and a cercosporin compound in *Z. tritici*.[63, 64]

In turn, *P. digitatum* from citrus has presented complete or partial resistance to triflumizole, imazalil, and prochloraz with overexpression of PMR1, PdMFS1, and PdMFS2.[65-67] For field isolates of *S. homoeocarpa*, resistance to propiconazole results from overexpression of the PDR transporter ShATRD. Furthermore, practical field

resistance to propiconazole, iprodione, and boscalid occurs with ShPDR1 overexpression.[68]

3.3 Events underlying the overexpression of drug efflux pumps

3.3.1. Transcription factors

Some changes in the expression levels of efflux transporters play an essential role in the emergence of resistance to many drugs. It regulates the transcription of efflux transporters by different mechanisms, alterations in their promoters, and gain-of-function mutations in the transcription factors controlling their expression (Fig. 1F).[1] TFs are proteins that interact with specific DNA sequences to regulate gene expression and influence biological processes.[69] They have a meaningful role in signal transduction pathways and are the last bridge between signal flow and target gene expression. Amino acid alteration is the most common type of alteration in certain TFs, and it increases the expression levels of efflux pumps. For *B. cinerea,* point mutations in MDR1 occur in all isolates with the MDR1 phenotype. These mutations provide BcATRB overexpression by transforming TFs from a drug-inducible state to a permanently active one.[57]

Furthermore, point mutations in MDR1 *mrr1* transformants are responsible for both permanent MRR1 activation and BcATRB overexpression.[57] In *A. fumigatus,* the transcription factor SltA regulates itraconazole resistance by overexpressing ABC transporter MDR1, and the DMIs target Erg11A.[70] Field isolates of *S. homoeocarpa* from turfgrass contain a gain-of-function mutation (M853T) in ShXDR1. This mutation causes the overexpression of ABC transporters (ShPDR1 and ShATRD) and other genes (*CYP561*, *CYP65*, *CYP68*), leading to multidrug resistance (MDR).[71] Additionally, overexpression of ATRB associated with resistance to fludioxonil is not regulated by a single transcription factor MRR1 in *Botrytis fragariae* from strawberries.[72]

Notably, the regulators of MDR1 (Mrr1) and ShXDR1 contain a Zn_2Cys_6 domain. The transcription factor Zn_2Cys_6 and C6 zinc proteins are a subcategory of zinc finger proteins exclusive to fungi. Zn_2Cys_6 has a well-conserved Cys-X2-Cys-X6-Cys-X5-12-Cys-X2-Cys-X6-9-Cys (C6 domain) with two zinc atoms, a binding domain, and a regulatory domain.[73] Zn_2Cys_6 is involved with drug resistance.[74] For example, in *Rhynchosporium commune* from barley, either TFs or activators containing a fungal-specific Zn_2Cys_6 domain have contributed to DMI resistance.[75] Either overexpression of the ABC transporter or MFS encoding genes increasing fungicide efflux may be a

resistance mechanism.[76] In *P. oryzae* from rice, isoprothiolane resistance occurs with the Zn_2Cys_6 transcription factor encoding MoIRR.[74] For three *P. oryzae* lab mutants, the mutations R343W, R345C, and a 16-bp insertion in MoIRR have been identified and linked to isoprothiolane resistance. In addition, cross-resistance to isoprothiolane and iprobenfos has been observed, suggesting that MoIRR is strongly associated with resistance to choline biosynthesis inhibitors.[74] However, in *Z. tritici* from wheat, SDHI cross-resistance to fluopyram and isofetamid has not been associated with alterations in *sdhB*, *C*, and *D*.[77] Despite the 519-bp MgMFS1 promoter insert in SDHI-resistant isolates, MgMFS1 overexpression appears not to be associated with fluopyram and isofetamid resistance.[77] Furthermore, in transcription factor Zn_2Cys_6 in *C. acutatum* from fruit, the leucine zipper transcription factor CaBEN1 has been associated with resistance to benomyl.[42] For *A. alternata* on tangelo, the stress-responsive transcription factor Yap1 results from clotrimazole, fludioxonil, vinclozolin, and iprodione resistance.[78] For *P. digitatum* from citrus, the zinc finger transcription factor Crz1 confers resistance to DMIs.[79] Therefore, some TFs significantly mediate fungicide resistance by regulating drug efflux pumps in many cases.

3.3.2. Promoter rearrangement

Promoter rearrangement may be caused by complex insertion–deletion events involving transposable element sequences. Detection of promoter rearrangements can provide valuable information for monitoring fungicide resistance in plant pathogens. However, the detection of these events can be challenging, as they may involve large genomic regions and can be difficult to distinguish from other types of mutations.[59, 80] Mernke et al.[80] showed two types of rearrangement (A and B) in the *BcmfsM2* promoter related to MDR for *B. cinerea* in vineyards. In promoter type A rearrangement, there is an insertion of 1326-bp in conjunction with a 678-bp deletion, whereas in promoter type B, there is an insertion of 1011-bp and a 76-bp deletion. MDR2 isolates, which harbour either type A or type B rearrangement, exhibit identical resistance phenotypes and similar levels of *BcmfsM2* overexpression.[80] Previously, the use of the reporter gene confirmed the constitutive activation of the *BcmfsM2* promoter per rearrangement.[57]

Omrane et al. (2017) also showed insertion in the promoter region of an efflux transporter associated with MDR in *Z. tritici*. Thus, three types of insertions occur in the MgMFS1 promoter region, including types I, II, or III, determined by the insertion length

in the MFS1 promoter. Type I insertion, a 519-bp long terminal repeat (LTR)-retrotransposon containing upstream activation sequences (UASs), can lead to its overexpression and thus to an MDR phenotype. The type II insertion also has UASs, and the type II insert appears devoid of regulatory elements. These three insertions in the same promoter have led to multidrug resistance and induced MgMFS1 overexpression.[64]

3.4 Inhibition of multidrug efflux pumps

Efflux pump inhibitors (EPIs) can affect the biological functions of the efflux pump and delay the development of fungicide resistance. EPIs inhibit efflux pump activities; thus, fungicide can restore its efficacy and reach the action site. At least five different modes of action have been described for EPIs, such as the following: (i) interfering with the assembly of the efflux pump,[81] (ii) inhibition of the energy source of the efflux pump,[82] (iii) reduction in the NP expression of the efflux pump genes,[83] (iv) hindrance of the substrate from passing through the efflux pump channel,[84] and (v) competitive/non-competitive inhibition of the efflux pumps.[85] The molecules of efflux EPIs are derived from medicinal plants holding antimicrobial activities, as well as synthetic sources.[86] Verapamil, a calcium blocker that inhibits different types of drug efflux, acts by binding the target site of the multidrug similar to the pump substrates.[87] In a recent study, verapamil reverted the resistance of *C. albicans* to fluconazole (FLC), showed synergism with FLC against *C. albicans*, and reduced fungal recovery.[88] For *Phytophthora capsici* on pepper, verapamil had no inhibitory effect in resistant isolates.[89] However, the MDR isolates of *Phytophthora capsici* could be inhibited by the efflux pump inhibitor amitriptyline,[89] which is one substrate of P-gp belonging to the ABC transporter superfamily.[90] In addition, amitriptyline can recover the activity of fluazinam, chlorothalonil, and oxathiapiprolin in *P. capsici* isolates.[89] For *A. alternata* on tomato, zinc nanoparticles (ZnO-NPSs) are effective against boscalid-resistant and -sensitive isolates and showed enhanced antifungal activity when applied in combination with boscalid or fluazinam (an ATP-synthetase inhibitor).[91] These synergistic/additive effects between fluazinam and ZnO-NPs may be an indication that the toxicity of ZnO-NPs is involved with an efflux mechanism regulating zinc ion homeostasis.[92, 93] Although there are many advantages and potential for EPIs in managing fungicide resistance, they still need more research and interest from industries and more capital. Therefore, this could be key to helping solve resistant fungicides in pathogens.[86]

3.5 Detoxification

Metabolic detoxification is a common resistance mechanism in antibiotics, insecticides, and herbicides. It can occur by oxidation, reduction, and hydrolysis to give modified functional groups conjugated for excretion or deposition.[94] This mechanism has been reported for QoI, MBC, and DMI fungicides.[10,95,96] For MBCs, isolate SD-4 of *Mycobacterium* sp. degrades carbendazim (MBCs) by hydrolase that encodes the Mhel gene. A site directional mutation experiment demonstrated that the critical amino acid sites Cys16 and Cys222 have an important role in the hydrolysis of MBC Mhel.[96] For DMIs, cytochrome P450 monooxygenase CYP684 is responsible for the natural resistance of *Botrytis pseudocinerea* to fenhexamid by oxidation.[97] For multi-site fungicides, the non-essential thiol compound available for detoxifying Captan has been shown as a possible mechanism for *B. cinerea* against this fungicide.[98]

3.6 Paralogs of the *CYP51* gene

In several fungal species with multiple *CYP51* paralogs, resistance to DMIs is often associated with mutations in or overexpression of the sterol 14 *alpha-demethylase* gene *CYP51A*, as described in sections 2.1.3 and 2.2.[39, 99] Several species of ascomycete fungi have multiple *CYP51* paralogs, including *CYP51A* and *CYP51B* in *Aspergillus* spp., *CYP51C* in *Fusarium* spp., and a pseudo-generalised duplication of *CYP51A* called *CYP51A*-p in *R. commune*.[100, 101] *CYP51* paralogs may mediate differential sensitivity to DMIs (Fig. 1C). For example, sensitivity to DMI fungicides increases more in *CYP51A* deletion mutants than in *CYP51C* of *Fusarium graminearum*.[102] The disruption of *CYP51A* associated with increased DMI sensitivity has also been observed in *A. fumigatus* [103] and *F. graminearum*.[39] In both *Colletotrichum fioriniae* and *C. nymphaeae,* disruption of *CYP51A* and *CYP51B* increases sensitivity in eight and five DMI fungicides, respectively.[104] Therefore, the less conserved nature of *CYP51A* may significantly impact DMI sensitivity.[105]

4. FITNESS PENALTY AND FITNESS ADVANTAGE

Studies of virulence evolution in plant pathogens have been limited. Typically, the evolution of plant pathogens is studied by focusing on the host–pathogen interaction, target enzyme, or metabolic pathway.[106] Fungicide resistance can provide fitness advantages to plant pathogens resistant in the field, including higher virulence than wild-type isolates. Still, few reports of plant pathogens have shown both fungicide resistance and higher fitness. Nevertheless, it is essential to investigate whether fitness costs offset these benefits in the absence of fungicides. Resistance mutations may be associated with a fitness cost in plant pathogens, resulting in evolutionary trade-offs.[10] Conceptually, fitness can be defined as the survival and reproductive success of an individual or group of individuals that carry a particular characteristic of fungicide resistance expressed as progeny contribution to the next generation.[107] For a single isolate, the fitness components, such as growth rate and virulence, may represent the isolate's capacity to be pathogenic. Other components, such as conidial production and conidial germination, may demonstrate the isolate's potential to develop infections, reproduce, and spread in field crops. Isolates with higher fitness may be associated with an evolutionary compensatory process.[10] In this sense, it may reduce the fitness costs from a point mutation in the target gene that confers fungicide resistance.[108] In some cases, resistant isolates exhibiting fitness advantages, such as isolates of *Z. tritici* resistant to cyproconazole with no *CYP51* mutations, have higher virulence than wild-type isolates, likely due to efflux pumps against fungicides [109] (Table 4). Similar to what was observed for *Z. tritici*, *P. oryzae* pathotype *Triticum* isolates carrying the G143A mutation in the *cytb* gene also show greater virulence and competitive advantage than sensitive wild-type isolates (Table 2).[110] These results contrast with the evolutionary idea that fungicide resistance can come with fitness costs.[10] Conversely, many plant pathogens with fungicide resistance are associated with a fitness cost and/or absence of fitness cost.

For QoIs, there are some examples of QoI-resistant plant pathogens in which a fitness cost has been associated with G143A/S, F129L mutations in the *cytb* gene, such as *F. fujikuroi*,[111] *M. oryzae*,[112, 113] *M. fructicola*,[114] *P. viticola*,[20, 115] *P. oryzae*,[116] *U. maydis*,[117] and *Z. tritici*[118] (Table 3). There were also reports of no fitness costs in QoI-resistant field isolates, such as *A. alternata*,[119] *A. alternata* pathotype *tangerine*,[120] *B. cinerea*,[121, 122] *C. acutatum*,[123, 124] *E. necator*,[125] *M. grisea*,[126] *P. pachyrhizi*,[18] *P. vitícola*,[16, 127, 128] and *Z. tritici*[129] (Table 2).

Table 2. Description of mutations in the cytochrome b (*cyt b*) gene or non-target-site of plant pathogens associated with resistance, fitness cost and resistance stability to Quinone outside inhibitors (QoIs).

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Alternaria alternata	Pistachio	*Cyt b* alteration	G143A	-	✓	-	NI	119
A. alternata pathotype *tangerine*	Citrus	*Cyt b* alteration	G143A	-	✓	-	Stable	120
A. alternata	Potato	*Cyt b* alteration	G143A	NI	NI	NI	Stable	182
Botrytis cinerea	Strawberry	*Cyt b* alteration	G143A	-	✓	-	NI	121
B. cinerea	Apple	*Cyt b* alteration	G143A	NI	NI	NI	Stable	104
B. cinerea	Vegetable fruits	*Cyt b* alteration	G143A	-	✓	-	NI	121
Brumeria graminis	Wheat	*Cyt b* alteration	G143A	-	-	-	Stable	193
B. graminis f. sp.*tritici*	Wheat	Non-target-site	-	✓	-	-	NI	194
Cercospora beticola	Sugar beet	*Cyt b* alterations	G143S or F129V	-	-	-	Stable	195
C. beticola	Sugar beet	*Cyt b* alterations	G143S or F129V	NI	NI	NI	NI	196
Colletotrichum acutatum	Strawberry	*Cyt b* alteration	G143A	-	✓	-	Stable	123, 124
Colletotrichum lentis	Lentis	*Cyt b* alteration	G143A	NI	NI	NI	NI	197

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Colletotrichum truncatum	Soybean	*Cyt b* alterations	G143A or F129L	NI	NI	NI	NI	198, 199
Corynespora cassiicola	Soybean	*Cyt b* alteration	G143A	NI	NI	NI	NI	200
Erysiphe necator	Grape	*Cyt b* alteration	G143A	-	✓	-	Stable	125
Fusarium fujikuroi	Rice	*Cyt b* alteration	G143A	✓	-	-	NI	111
Glomerella cingulata	Apple	*Cyt b* alteration	G143A	NI	NI	NI	NI	201
Lasiadiplodia theobromae	Mango	Non-target-site	-	-	✓	-	Stable	183
Magnaporthe grisea	Barley	*Cyt b* alteration	G143A	-	✓	-	Stable	126
M. oryzae	Perennial ryegrass	*Cyt b* alteration	G143A	✓	-	-	NI	112
M. oryzae	Rice	*Cyt b* alteration	G143A/S	✓	-	-	Stable	113
Monilinia fructicola	Peach	Non-target-site	-	✓	-	-	Not Stable	114
Mycosphaerella fijiensis, M. musicola and M. thailandica	Bananas	*Cyt b* alteration	G143A	NI	NI	NI	NI	202
Parastagonospora nodorum	Wheat	*Cyt b* alteration	G143A	NI	NI	NI	NI	203

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Phakopsora pachyrhizi	Soybean	*Cyt b* alteration	F129L	-	✓	-	NI	18
Phytophthora cactorum	Strawberry	*Cyt b* alteration	G143A	NI	NI	NI	NI	204
Plasmopara viticola	Grape	*Cyt b* alteration	G143A, F129L	✓	-	-	NI	115, 20
P. viticola	Grape	*Cyt b* alteration	G143A	-	✓	-	Stable	127
P. viticola	Grapevine	*Cyt b* alteration	G143A	-	✓	-	NI	128
Pyricularia oryzae	Rice	*Cyt b* alteration	G143A	✓	-	-	Stable *in vitro* and Not stable *in vivo*	116
P. oryzae Triticum	Wheat	*Cyt b* alteration	G143A	-	-	✓	NI	110
Septoria glycines	Soybean	*Cyt b* alteration	G143A	NI	NI	NI	NI	205
Ustilago maydis	Maize	*Cyt b* alteration	G143A	✓	-	-	NI	117
Venturia inaequalis	Laboratory mutants	*Cyt b* alteration	G143A	-	-	-	NI	206
V. inaequalis	Apple	Non-target-site	-	NI	NI	NI	NI	95
Zymoseptoria tritici	Wheat	*Cyt b* alteration	G143A	✓	-	-	NI	118
Z. tritici	Wheat	*Cyt b* alterations	G143A, G37V	-	✓	-	Stable	129

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Z. tritici	Wheat	*Cyt b* alteration	G143A	NI	NI	NI	NI	207

Mutations in the cytochrome b (*cyt b*) gene or non-target-site of plant pathogens associated with resistance stability to quinone outside inhibitors (QoIs). NI, not investigated

Table 3. Description of mutations in the *β-tubulin* gene of plant pathogens associated with resistance, fitness cost and resistance stability to Methyl benzimidazole carbamates (MBCs).

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Botryosphaeria dothidea	Apple	*β-tubulin* alteration	E198A	-	✓	-	Stable	135
Botrytis cinerea	Ginseng	*β-tubulin* alterations	E198A/V/K	NI	NI	NI	NI	208
B. cinerea	Fruits and vegetables	*β-tubulin* alterations	E198A/V/K	✓	-	-	NI	131
B. cinerea	Fruits and vegetables	*β-tubulin* alterations	F200Y; E198A; M233I	✓	-	-	Stable	130
B. cinerea	Fruits and vegetables	*β-tubulin* alteration	E198G	-	✓	-	NI	21
Cercospora beticola	Sugar beet	*β-tubulin* alteration	E198A	-	-	-	NI	209
Colletotrichum gloeosporioides	Apple	*β-tubulin* alteration	E198A	NI	NI	NI	NI	210
Colletotrichum musae	Banana	*β-tubulin* alteration	F200Y	-	✓	-	NI	136,137

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Colletotrichum truncatum	Soybean	*β-tubulin* alterations	F200Y; E198A	NI	NI	NI	NI	124,199
Colletotrichum spp.	Strawberry	*β-tubulin* alterations	F200Y; E198A	NI	NI	NI	NI	211
Corynespora cassiicola	Soybean	*β-tubulin* alterations	F200Y; E198A	NI	NI	NI	NI	200
Fusarium fujikuroi	Rice	*β-tubulin* alterations	E198K; F200Y	NI	NI	NI	NI	212
Fusarium graminearum	Wheat	-	-	-	✓	-	Stable	184
Fusarium moniliforme	-	*β-tubulin* alteration	T50A	-	-	-	NI	213
Monilinia fructicola	Peach	*β-tubulin* alteration	E198A	-	✓	-	Stable	114
M. fructicola	Stone fruit	*β-tubulin* alteration	E198A	✓			NI	132
M. fructicola	Stone fruit	*β-tubulin* alteration	H6Y	-	✓	-	NI	138
Monilinia laxa	Stone fruit	*β-tubulin* alteration	L240F	✓	-	-	NI	133
Oculimacula sp.	Barley and wheat	*β-tubulin* alteration	F200Y	-	✓	-	NI	139
Penicillium expamsum	Pears	*β-tubulin* alterations	F167Y or E198A/V/K	-	-	✓	NI	214
Pestalotiopsis-like species	Tea	Variations in *β-tubulin*	-	NI	NI	NI	NI	215

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Phomopsis obscurans	Strawberry	*β-tubulin* alterations	E198V; F200T	-	✓	-	NI	140
*Pseudocercosporella fijiense*s	Banana	*β-tubulin* alterations	F167Y or E198A/V/K	-	✓	-	Stable	141
Ustilaginoidea virens	Rice	Variations in *β-tubulin*	-	✓	-	-	Stable	134
Venturia carpophila	Peach	Variations in *β-tubulin*	E198K/G	NI	NI	NI	NI	216

[a]Mutations in the *β-tubulin* gene of plant pathogens associated with resistance stability to Methyl benzimidazole carbamates (MBCs). NI: Not Investigated.

Table 4. Description of mutations in the *CYP51* gene or non-target-site of plant pathogens associated with resistance, fitness cost and resistance stability to Demethylation inhibitors (DMIs).

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Alternaria alternata	Tomato	-	-	-	✓	-	NI	217
A. alternata	*Paris polyphylla* var.*chinensis*	*CYP51* alterations	K715R + Y781C, K715R + D1140G + T1628A	-	✓	-	NI	155

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Aspergillus parasiticus	Lab mutants	*CYP51*alterations	CYP51A-G54W	✓	-	-	NI	38
A. parasiticus	Lab mutants	Overexpression of *CYP51A* gene	-	-	✓	-	NI	38
Blumeria graminis f. sp. *tritici*	Wheat	-	-	✓	-	-	NI	218
B. graminis f. sp. *tritici*	Wheat	Multiple mechanisms	-	-	✓	-	NI	219
Botrytis cinerea	Tomato	*CYP51* alterations and induced expression	G476S, K104E, M231T	✓	-	-	Stable	142
B. cinerea	Tomato	Inducible expression of *CYP51*	-	✓	-	-	Stable	143
B. cinerea	Grape vineyards	*CYP51* alterations	P347S	-	✓	-	NI	156
Cercospora beticola	Sugar beet	*CYP51* alterations	I330T, P384S and E297K	-	-	-	Stable	35
C. beticola	Sugar beet	Overexpression of *CYP51*	-	-	✓	-	Stable	35
Cladosporium cucumerinum	Apple	-	-	✓	-	-	NI	220
Colletotrichum acutatum	Chili	*CYP51* alterations and *CYP51*s overexpression	CYP51A:Y128H or T207M	-	✓	-	NI	157

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Colletotrichum gloeosporioides	Chili pepper	*CYP51* alterations and overexpression	CYP51A: V18F, L58V, S175P, P341A, A340S, T379A, N476T, CYP51B: D121N, T132A, F391Y, T262A	✓	-	-	NI	40
C. gloeosporioides	Grape	*CYP51* alterations	single deletion mutants of CYP51A or -B	✓	-	-	NI	144
Colletotrichum siamense	Strawberry	*CYP51* alterations	deletion mutants of CYP51A or -B	NI	NI	NI	NI	221
C. siamense	Chili	*CYP51* alterations	CYP51A: D115V, R306K and E397D. CYP51B:R266H	-	-	-	NI	222
Colletotrichum spp.	Lab mutants	*CYP51* alterations	CYP51A or –B deletion	-	-	-	NI	105
Colletotrichum truncatum	Chili pepper	Inducible expression of *CYP51* and *CYP51* alterations	M376L/H373N	✓	-	-	Stable	96
C. truncatum	Soybean	*CYP51* alterations	-	NI	NI	NI	NI	198
Erysiphe necator	Grapevine	Altered sterol composition	-	-	✓	-	NI	223
Fusarium culmorum	Small-grain cereals	Major facilitator and overexpression of FcABC1	-	-	-	-	Stable	224
Fusarium fujikuroi	Rice	Metabolization	-	-	-	-	NI	225

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
F. fujikuroi	Rice	*CYP51* alterations	CYP51B: F511S or S312T/F511S	NI	NI	NI	NI	226
Fusarium graminearum	Lab mutants	-	-	-	✓	-	Stable	227
F. graminearum	Wheat	*CYP51* alterations	G443S	NI	NI	NI	NI	227
F. graminearum	Lab mutants	*CYP51* alterations and overexpression *CYP51A, -B* and *-C*	D243N and 103Q&V157L	✓	-	-	Stable	145
Fusarium solani	-	*CYP51* alterations	L218	NI	NI	NI	NI	228
Lasiodiplodia theobromae	Papaya	Overexpression of *CYP51*	-	✓	-	-	NI	229
L. theobromae	Mango	Overexpression of *CYP51*	-	✓	-	-	NI	230
Magnaporthe oryzae	Rice	Induced overexpression of *CYP51A* and *CYP51* alterations	Y126F, I125L	✓	-	-	Stable	146
Monilinia fructicola	Peach	-	-	-	✓	-	Not stable	185
M. fructicola	Stone fruits	Constitutive*CYP51* overexpression, promoter insert (Mona) and *CYP51* alterations	Y136F	✓	-	-	Stable	147

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
M. fructicola	Stone fruits	Overexpression of *CYP51*	-	-	✓	-	NI	231
M. fructicola	Stone fruits	Non-target-site	-	-	✓	-	NI	175
M. fructicola	Peach	*CYP51* alterations	G461S	✓	-	-	Stable	148
M. fructicola	Stone fruits	*CYP51* alterations	G461S	✓	-	-	Not stable	149
Oculimacula acuformis	Wheat	Mechanism unknown	-	✓	-	-	NI	232
O. acuformis and O. yallundae	Wheat	Mechanism unknown	-	-	✓	-	NI	232
Penicillium expansum	Apple	-	-	✓			NI	233
Phakopsora pachyrhizi	Soybean	*CYP51* alterations	F120L + Y131H, Y131F + K142R, Y131F + I475T orF120L +Y131F + I475T	✓	-	-	NI	150,151
Phomopsis obscurans	Strawberry	Mechanism unknown	-	-	✓	-	NI	140
Phytophthora nicotianae	Tobacco	-	-	✓	-	-	Stable	152
Pyrenophora teres	Barley	-	-	-	✓	-	NI	234
P. teres	Barley	*CYP51* alterations and overexpression of*CYP51A* and *CYP51*	F489L	-	-	-	NI	37

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Pyricularia oryzae Triticum	Wheat	Non-target-site	-	NI	NI	NI	NI	235
Ramularia collocygni	Barley	*CYP51* alterations	I325T, I328L	NI	NI	NI	NI	236
Rhizocotonia solani	Rice	*CYP51* alterations	S94A, N406S, H793R, L750P	✓	-	-	NI	153
Sclerotinia homoeocarpa	Turfgrass	-	-	✓	-	-	NI	237
Stagonosporopsis citrulli	Watermelon and muskmelon	*CYP51* alterations	G463S, I444M, Y446H, A464G	NI	NI	NI	NI	238
Ustilago maydis	Lab mutants	Altered sterol composition	-	-	✓	-	NI	239
Ustilaginoidea virens	Rice	Induced overexpression of *CYP51*	-	-	✓	-	NI	240
Venturia effusa	Pecans	*CYP51* alterations	CYP51A: G444D; CYP51B:G357H, I77T/I77L	NI	NI	NI	NI	241
Venturia inaequalis	Apple	Constitutive *CYP51*	-	-	✓	-	NI	242
Villosiclava virens	Lab mutants	*CYP51* alterations	Y136H	-	✓	-	NI	158
Zymoseptoria tritici	Wheat	*CYP51* alterations	I381V, V136A	-	-	-	NI	243
Z. tritici	Wheat	*CYP51* alterations	Y137F, V136A and I381V	✓	-	-	NI	154

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Z. tritici	Wheat	*CYP51* alterations	50S, S188N, A379G,459/460, G460D, Y461H, or N513K,	-	✓	-	NI	154
Z. tritici	Wheat	*CYP51* alterations	V136C, I381V, Y461H, S524T	NI	NI	NI	NI	207
Z. tritici	Wheat	Non-target-site	-	-	-	✓	NI	108
Z. tritici	Wheat	Non-target-site	-	-	-	-	NI	178
Z. tritici	Wheat	-	-	✓	-	-	NI	178

[a]Mutations in the *CYP51* gene or non-target-site of plant pathogens associated with resistance stability to Demethylation inhibitors (DMIs). NI: Not Investigated.

Table 5. Description of mutations in the *sdh* gene or non-target-site of plant pathogens associated with resistance, fitness cost and resistance stability to Succinate dehydrogenase inhibitors (SDHIs).

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Alternaria alternata	Lab mutants	*Sdh* alterations	sdhB-S221P, sdhB -H267N, sdhB-H267Y, or sdhD-D129E	-	✓	-	Stable	159
A. alternata	Lab mutants	*Sdh* alterations	sdhC-H134R, sdhD-H133R, and sdhD-D123E	✓	-	-	NI	159
A. alternata	Peach	*Sdh* alterations	sdhB-H277Y/R, sdhC-H134R, and sdhD-H133R	-	✓	-	Stable	8
A. alternata	Almond	*Sdh* alterations	sdhB- H277L, sdhC-H134R, G79R	NI	NI	NI	NI	244
A. alternata and *A. solani*	Potato	*Sdh* alterations	sdhD-D123E, sdhB-H277/8 Y/R, sdhC-H134R, or sdhD-H133R	-	✓	-	NI	167
Botrytis cinerea	Lab mutants	-	-	✓	-	-	NI	245
B. cinerea	Apple	-	-	✓	-	-	Stable	161

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
B. cinerea	Apple	*Sdh* alterations	sdhB-H272R/Y/L, sdhB-N230I, and sdhB-P225F	✓	-	-	NI	121
B. cinerea	Lab mutants	*Sdh* alterations	sdhB-H267L/R P225L/H272R	✓	-	-	Stable	161
B. cinerea	Strawberry	*Sdh* alterations	sdhB-H272R, sdhB-H272Y, sdhB-H272L, sdhB-P225F, and sdhB-N230I	-	✓	-	NI	160
B. cinerea	Apple, cherry,blueberry, pear and strawberry	*Sdh* alterations	shB-G85A, sdhB-I93V, sdhB-M158V and sdhB-V168I	✓	-	-	NI	246
B. cinerea	Vegetable fruits	*Sdh* alterations	sdhB-P225F/H and sdhB-I274V	✓	-	-	NI	121
B. cinerea	Vegetable fruits	*Sdh* alterations	sdhB-P225L, sdhC-G85A and I93V	-	✓	-	Stable	162
Colletotrichum truncatum	Soybean	*Sdh* alterations	sdhB-S208Y	NI	NI	NI	NI	199

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Colletotrichum gloeosporioides and C. acutatum	Various plants	Non-target-site	-	NI	NI	NI	NI	247
Corynespora cassiicola or D. bryoniae	Lab mutants	*Sdh* alterations	-	-	✓	-	NI	159
C. cassiicola	Fruit and vegetable	*Sdh* alterations	sdhB-H278Y, sdhB-I280V and sdhD-H105R	✓	-	-	NI	163
C. cassiicola	Fruit and vegetable	*Sdh* alterations	sdhB-H278R, sdhD-D95E and sdhD-G109V	-	✓	-	NI	163
C. cassiicola	Fruit and vegetable	*Sdh* alterations	Double mutations (B-I280V+D-D95E/D-G109V/D-H105R, B-H278R+D-D95E/D-G109V, B-H278Y+D-D95E/D-G109V)	-	✓	-	NI	168

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
C. cassiicola	Cucumber	*Sdh* alterations	sdhB-H278Y/L, I280V, sdhC-S73P, N75S, H134R, sdhD-D95E, G109G	NI	NI	NI	NI	248
Erysiphe necator	Vineyards	*Sdh* alterations	sdhC-I244V, G25R, H242R	NI	NI	NI	NI	249
Fusarium graminearum	Wheat	*Sdh* alterations	sdhA-Y182F, sdhB-H53Q, C90S, A94V and sdhC-S31F	-	✓	-	NI	170
F. graminearum	Wheat	*Sdh* alterations	sdhC-A78V	-	✓	-	NI	169
Fusarium pseudograminearum	Wheat	*Sdh* alterations	sdhA-R18L, V160M, sdhB-D69V, D147G, C257R and sdhC-W78R	-	✓	-	NI	170
Penicillium expamsum	Lab mutants	*Sdh* alterations	sdhB-H272Y	-	✓	-	NI	171
Phakopsora pachyrhizi	Soybean	*Sdh* alterations	sdhC-I86F	✓	-	-	Stable	164
Pyricularia oryzae Triticum	Wheat	Non-target-site	-	NI	NI	NI	NI	250

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
Ramularia collocygni	Lab mutants	*Sdh* alterations	sdhB-N224I and sdhC-H142R	-	✓	-	Stable	172
R. collocygni	Lab mutants	*Sdh* alterations	sdhC-H142Q	-	✓	-	Not stable	172
R. collocygni	Barley	*Sdh* alterations	sdhC-H146R, H153R	NI	NI	NI	NI	236
Rhizoctonia cerealis	Lab mutants	*Sdh* alterations	sdhB-N246Y, sdhC-H139Y and sdhD-H116Y	✓	-	-	Stable	29
Rhizoctonia solani	Rice	-	-	✓	-	-	NI	251
Sclerotinia sclerotiorum	Soybean and dry bean	*Sdh* alterations	sdhB-A11V,sdhC-I22V, A31V	NI	NI	NI	NI	252
S. sclerotiorum	Soybean	*Sdh* alterations	sdhB-A11V, R5G, L269S, V113A, W229R, sdhD-K35E, A55E, S59R, K78N	✓	-	-	NI	165
Stemphylium solani	Lab mutants	-	-	✓	-	-	Stable	253
Verticillium dahliae	Lab mutants	-	-	-	✓	-	Stable	254
Ustilago nuda	Barley	-	-	-	✓	-	NI	255
Zymoseptoria tritici	Wheat	*Sdh* alterations	sdhB-S221P/T, sdhB-	-	✓	-	NI	30

Plant pathogens	Host	Resistance Mechanism	Mutation	Fitness penalty	No Fitness penalty	Fitness advantage	Mutation stability[a]	Reference
			H267F/L/N/Y, sdhB-I269V andsdhD-D129E/G/T					
Z. tritici	Lab mutants	*Sdh* alterations	sdhC-H152R	✓	-	-	NI	166

[a]Mutations in the sdh gene or non-target-site of plant pathogens associated with resistance stability to Succinate dehydrogenase inhibitors (SDHIs).
NI: Not Investigated.

For MBCs, plant pathogens, such as *B. cinerea*,[130, 131] *M. fructicola*,[132] *M. laxa*,[133] and *Ustilaginoidea virens*,[134] exhibit fitness costs linked to E198A/V/K, F200Y, L240F, and M233I mutations in the *β-tubulin* gene (Table 3). No fitness costs associated with MBC resistance have been found in *Botryosphaeria dothidea*,[135] *B. cinerea*,[21] *C. musae*,[136, 137] *M. fructicola*,[114, 138] *Oculimacula* sp.,[139] *Phomopsis obscurans*,[140] and *P. fijiense*[141] (Table 3).

For DMIs, many isolates that are DMI resistant, such as *A. parasiticus*,[38] *B. cinerea*,[142,143] *C. gloeosporioides*,[40,144] *C. truncatum*,[96] *F. graminearum*,[145] *M. oryzae*,[146] *M. fructicola*,[147-149] *P. pachyrhizi*,[150,151] *P. nicotianae*,[152] *R. solani*,[153] and *Z. tritici*,[154] carry *CYP51* gene mutations and show fitness costs (Table 4). However, isolates with *CYP51* gene mutations, such as *A. alternata*,[155] *A. parasiticus*,[38] *B. cinerea*,[156] *C. acutatum*,[157] *Villosiclava virens*,[158] and *Z. tritici*,[154] show no fitness costs associated with DMI resistance (Table 4).

For SDHIs, many field and lab mutant isolates resistant to SDHIs and linked to the *sdh* gene, such as *A. alternata*,[159] *B. cinerea*,[121, 122, 160-162] *C. cassiicola*,[163] *P. pachyrhizi*,[164] *R. cerealis*,[29] *S. sclerotiorum*,[165] and *Z. tritici*,[166] have associated fitness costs. However, mutations found in the *sdh* gene of *A. alternata*,[8, 159] *A. solani*,[167] *B. cinerea*,[160, 162] *C. cassiicola*,[163, 168] *F. graminearum*,[169, 170] *Fusarium pseudograminearum*,[170] *P. expamsum*,[171] *R. collo-cygni*,[172] and *Z. tritici*,[30, 166] have no fitness costs (Table 5).

In addition to target site alterations, mutations at the non-target site may also be associated with fitness costs. For overexpressed drug efflux pumps, energetic costs may occur due to active transport. These energetic costs are due to ATP-binding cassette transporters hydrolysing ATP and MFS transporters using transmembrane proton-motive force.[60] Isolates of *B. cinerea* suffer a fitness cost associated with MDR1 (*atr*B overexpression) and MDR2 (*mfs*M2 overexpression), resulting in a reduced resistance frequency over the winter months.[173] In another study, MDR1h and MDR1 isolates (*atr*B overexpression) with multiple target-site resistance mutations did not show fitness costs in mycelial growth and conidial production *in vitro*.[174] Further, field isolates of *M. fructicola* with non-target-site resistance to propiconazole have no fitness cost.[175] In contrast, flusilazole-resistant mutants of *A. parasiticus* with *mdr* overexpression have fitness costs showing a reduction in growth, sporulation, conidial germination and loss of aflatoxin production.[176] Similarly, MDR mutants of *F. graminearum* have reduced growth and virulence.[177] Interestingly, even without *CYP51* mutations, isolates of *Z.*

tritici with cyproconazole resistance have higher virulence than wild-type isolates, probably due to either efflux transporters or other resistance mechanisms.[109] However, in isolates of *Z. tritici*, quantitative trait locus *PKS1* has been associated with fitness costs resulting in slower growth.[178]

Many mechanisms conferring fungicide resistance to a wide range of plant pathogens have been reported. Therefore, it is also necessary to assess the molecular basis of fungicide resistance, fitness costs, and inheritance of non-target site-based resistance to contribute insights into crop pathogen management.[1]

5. FUNGICIDE RESISTANCE STABILITY

Resistance stability, fitness, and competitive abilities of field-resistant isolates are important risk factors in developing fungicide resistance in crop fields.[179, 180] Fungicide resistance stability in plant pathogens may be characterised by the ability to retain the same level of fungicide insensitivity after successive generations of exposure or no exposure to the target fungicide.[181]

For QoIs, *A. alternata* pathotype *tangerine*,[120] *A. alternata*,[182] *B. cinerea*,[104] *C. acutatum*,[124] *E. necator*,[125] *L. theobromae*,[183] *M. grisea*,[126] *P. vitícola*,[127] and *Z. tritici*[129] exhibit stable QoI resistance and no fitness costs (Table 2). Nevertheless, *M. oryzae* [113] and *P. oryzae*[116] have stable QoI resistance, which is associated with fitness costs (Table 2). Interestingly, QoI resistance in *M. fructicola* not related to mutations in the *cyt b* gene is not stable, and it has been associated with fitness costs (Table 3).[114]

For MBCs, some plant pathogens, such as *Botryosphaeria dothidea*,[135] *F. graminearum*,[184] *M. fructicola*,[114] and *P. fijienses*,[141] exhibit stable resistance with no fitness costs (Table 3). In contrast, *B. cinerea* has stable resistance to MBCs and a fitness cost for mycelial growth, conidial production, virulence, and sclerotium production.[130]

For DMIs, the stable resistance by either *CYP51* mutations or induced expression, such as in *B. cinerea*,[142, 143] *C. beticola*,[35] *C. truncatum*,[96] *F. graminearum*,[145] *M. oryzae*,[146] and *M.fructicola*,[147, 148] is associated with a fitness cost (Table 4). In contrast, DMI resistance stability with no fitness cost has also been found in some species, including *A. alternata*,[155] *C. beticola*,[35] *Fusarium graminearum*,[177] and *P. nicotianae*[152] (Table 4). In contrast, DMI resistance may not be stable in crop fields, and reversion to fungicide sensitivity can occur without selection pressure, e.g., in *M. fructicola*[149, 185] (Table 4). This phenomenon may have relevant implications for disease management

programmes in crop fields. After using discontinuous DMIs for three years, there was a decrease in the frequency of DMI-resistant isolates and an increase in the sensitive populations of *M. fructicola*. This re-establishment of the sensitive population may be associated with low adaptability and low competitiveness of DMI-resistant isolates compared to sensitive isolates.[149] There could be high fitness costs in resistant populations compared to wild-type populations,[10] and resistant isolates can be effectively controlled using anti-resistance management strategies.

For SDHIs, lab mutants and field isolates from *A. alternata* with *sdh* mutations associated with stable resistance do not show fitness cost[8, 159] (Table 5). *Botrytis cinerea*,[161, 179] *P. pachyrhizi*,[164] and *R. cerealis*[29] with stable resistance to SDHIs show a fitness cost, while *Ramularia collocygni* and *B. cinerea* show both unstable resistance to SDHIs and no fitness cost[162, 172] (Table 5).

6. MANAGING THE EVOLUTION OF FUNGICIDE RESISTANCE

Adopting resistance management strategies to optimise the activity of available fungicides in the future is necessary. These include the introduction of more host species, cultivating disease-resistant varieties and planting different crop cultivars possessing different resistance genes, avoiding applying overused fungicides, rotation between fungicides of different classes, and crop rotation.[186-190] Integrated disease management is the most sustainable solution to avoiding the selection of pathogens with resistant genes that can increase over time, spread to neighbouring areas, and cause epidemics.[191] Moreover, another strategy that can be adopted is inhibitor efflux pump activity (EPI). Blocking efflux pump activity may be an effective method for restoring the efficacy of drugs that are ineffective for combating microorganism resistance.[86, 192]

7. FINAL CONSIDERATIONS

Fungicides are essential tools for crop protection against fungal pathogens, but the overuse of these chemicals has led to the emergence of resistant isolates. The molecular mechanisms underlying fungicide resistance are complex and multifaceted. In recent years, the molecular basis of fungicide resistance has been extensively studied, and many mutations associated with resistance have been identified. Understanding the molecular

mechanisms related to fungicide resistance is essential for the development of effective management strategies, but it remains challenging.

Either mutations at the target site or those at non-target site mechanisms involved with the evolution of fungicide resistance can also confer fitness advantages, such as higher levels of virulence for plant pathogens. Nevertheless, there are many other cases in which these mutations linked to fungicide resistance have been associated with fitness costs, indicating the need for further investigations of each pathosystem to gain more insights into crop field management. In addition, in some cases, among non-target site mechanisms in plant pathogens, the expression of drug efflux and mutations in transcription factors have also been associated with either fitness costs or no fitness costs. In such a scenario, future studies exploring the potential of molecules that inhibit multidrug efflux pumps can also be promising to help solve this issue.

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9. CONFLICT OF INTEREST DECLARATION

No conflict of interest.

10. DATA AVAILABILITY STATEMENT

Data that supports the findings of this study are available upon request.

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CHAPTHER 2 - TEMPORAL DYNAMICS OF *Pyricularia oryzae* Triticum RESISTANT TO QUINONE OUTSIDE INHIBITOR FUNGICIDES

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ABSTRACT

Wheat blast, caused by *Pyricularia oryzae* Triticum lineage (PoTl), can infect wheat leaves and heads. The pathogen biology and disease epidemiology of the isolates PoTl with higher fitness and adaptative advantages due to resistance to Quinone outside inhibitor fungicides (QoI-R) are still unknown. This study aimed to characterize the temporal progress of wheat blast and to fit the best nonlinear model, describing the nature of an epidemic of QoI-R PoTl isolate compared with the sensitive isolate (QoI-S), over the course of five successive infection cycles of PoTl on wheat leaves and heads. Wheat blast occurred in all infection cycles. The secondary inocula produced by QoI-R and QoI-S PoTl isolates, on symptomatic wheat leaves, caused blast symptoms when inoculated on wheat heads. The area under the disease progress curve (AUDPC) was calculated based on disease severity. In all infection cycles, the AUDPC of QoI-the R isolate was significantly higher than that of the QoI-S isolate. Between the 1st and 5th infection cycle, a significant reduction was observed in the AUDPC of the PoTl isolates. The nonlinear logistic model had the best fit to describe the intensity of the disease progress curves (DPCs) of PoTl isolates on wheat leaves and heads, fitting classic sigmoid-shaped curves. Our findings indicate that the PoTl isolates did not keep the same temporal dynamics after five successive infection cycles. These findings may imply the integrated management of the disease wheat blast pathogen in Brazilian fields.

Keywords Wheat blast · Ecology and epidemiology · Progress curve · Nonlinear model · Disease control · Pest management

1. INTRODUCTION

Wheat (*Triticum aestivum*) is one of the most important cereal staple crop produced worldwide contributing to global food security (ACEVEDO et al., 2018). In 2019, Brazil produced 6.6 million tons of wheat, representing 0.9% of the 735 million tons produced globally (COLUSSI et al., 2022). Wheat production can be threatened by several pests and diseases, though (FAOSTAT, 2020). Wheat blast, caused by *Pyricularia oryzae* Triticum lineage (PoTl), is one of the major diseases threatening the crop yields. Wheat blast disease was first reported in 1986 in Parana state, Brazil. Since then, the pathogen has rapidly spread to wheat fields in south-central Brazil and neighboring countries, including Argentina, Bolivia, and Paraguay (CERESINI et al., 2018a, GLADIEUX et al., 2018). In 2016 the disease was reported outside South America, firstly, in Bangladesh, southeast Asia, and secondly, in 2017, in Zambia, eastern Africa, causing outbreaks that significantly harmed production in both countries (CALLAWAY, 2016, ISLAM et al., 2016, TEMBO et al., 2020). These two outbreaks have been linked to introductions via contaminated seed lots (SINGH et al., 2021). The fungal pathogen is able to infect both wheat leaves and heads, but head blast is the most destructive symptom (CASTROAGUDIN et al., 2015). Head blast associated yield losses ranging from 10 to 100% have been reported in wheat crops from South America countries, Bangladesh and Zambia (BONJEAN et al., 2016, ISLAM et al., 2016, TEMBO et al., 2020). More recently, the blast disease was found in Germany, in 2022, in central Europe (BARRAGAN et al., 2022).

Managing wheat blast disease is still challenging. The deployment of wheat resistant cultivars and calendar-based preventive fungicide spraying are the main management strategies in Brazil (GOULART et al., 2007, PAGANI et al., 2014). However, resistance is not considered durable across geographical regions due to the pathogen´s high diverse in virulence, and to the limited efficacy of fungicides (CERESINI et al., 2018a, CERESINI et al., 2018b). For many years, the limited efficacy of systemic site-specific fungicides has been associated with application technologies difficulties in systematically reaching the pathogen infection sites on wheat heads where infection occurs; with the highly favorable environmental conditions for the disease; and with the high susceptibility of wheat cultivars. It was only recently, though, that the inefficacy of the systemic site-specific fungicides was associated with the widespread distribution of fungicide resistance in the country (CERESINI et al., 2018b). In fact, resistance was

reported for the major fungicide classes labeled for managing wheat disease: Quinone outside inhibitors (QoIs) and triazoles or demethylation inhibitors (DMIs) (CERESINI et al., 2018b; CASTROAGUDIN et al., 2015; DORIGAN et al., 2019; POLONI et al., 2021; DORIGAN et al., 2022).

Despite the fact that these two molecules with distinct mode of action were reportedly high-risk fungicides for resistance, no anti-resistance strategies have been adopted in the country, worsening the scenario of fungicide inefficacy (LUCAS, 2015; VICENTINI et al., 2022). For instance, the systemic single-site high-risk QoI fungicides, in particular, have been sprayed as single molecule formulations or in mixtures with DMIs, for which resistance has also been reported (STEVENSON, 2018). Not surprisingly, the efficacy of this spraying approach in managing wheat blast has rarely been higher than 60% (CERESINI et al., 2018b). To make the situation even worse, PoTl has a wide host range, including other poaceous hosts, which grow near wheat fields and may be an important reservoir or source of QoI- and/or DMI-resistant (R) inoculum in the early phases of a wheat blast epidemic, hindering the management of the disease (CASTROAGUDIN et al., 2015, CASTROAGUDIN et al., 2016, DORIGAN et al., 2019).

Resistance to QoI fungicide in PoTl has been reported in Brazil since 2015, in several geographical populations across the major wheat cropping areas (CASTROAGUDIN et al., 2015). QoI-resistant (QoI-R) isolates from those PoTl populations were associated with the G143A point mutation in the cytochrome b (*cyt B*) gene (CASTROAGUDIN et al., 2015). Fungicide resistance provides changes in fitness for plant pathogens. For example, QoI-R PoTl isolates carrying the G143A mutation had a higher fitness and competitive advantage than QoI-sensitive (QoI-S) isolates, contrasting with the evolutionary theory, whose foundation is that fungicide resistance should come with fitness cost (HAWKINS and FRAAIJE, 2018). The higher conidial production and higher levels of disease severity on the QoI-R PoTl isolates reinforce their competitive ability against the QoI-S isolates and their potential for continuously causing crop damages on wheat fields after displacing the sensitive PoTl lineages (DORIGAN et al., 2022). On the other hand, the fitness cost has been also reported in some cases, with reduced infection efficiency and aggressiveness in *P. oryzae*, reduced virulence in *Magnaporthe oryzae* and *Zymoseptoria tritici* field isolates with G143A and reduced pathogenicity in some *Fusarium fujikuroi* lab mutants with G143A (MA B. and UDDIN, W. 2009; HAGERTY, 2016; D' AVILA, 2022; SONG et al., 2022).

Although wheat blast has been reported in Brazil for more than 30 years, there are still unanswered questions about the pathogen´s ecology and disease epidemiology, especially related to the timing and temporal dynamics of wheat heads infection. Few studies have clarified the epidemiological importance of PoTl secondary inocula produced from symptomatic wheat leaves to the temporal progression of blast on heads, and much more research is needed to better characterized the incubation period and latent period on wheat leaves and heads (RIOS et al., 2016). Furthermore, it is unknown whether the fitness advantage associated with QoI-R in populations of the wheat blast pathogen keeps the same temporal dynamics after successive cycles of infection. Knowledge about variation in the long-range temporal progress curves of QoI-R PoTl isolates is essential to guide the adoption of anti-resistance strategies that include timing for fungicide spraying. For instance, as a smarter alternative for the calendar based preventive and successive fungicide sprays, epidemiology-based advise on the right timing for fungicide spraying could be applied instead, delaying the directional selection of the QoI-R PoTl populations with the G143A mutation.

Several statistical models have been applied to describe the temporal dynamics of epidemics and to compare epidemic-based management tactics (VANDERPLANK, 1963, CAMPBELL and MADDEN, 1990). Applying such models could help determining: i) if the wheat blast disease progress curves (DPCs) maintain the same shape over many infection cycles; ii) whether the parameters rate (r) and initial inoculum (y0), which are fit in the regression models, varied along successive infection cycles; and iii) if the disease severity in the fungal progeny arisen from each infection cycle remains at the same levels. On barley, G143A mutants of *P. oryzae* tested under both saprophytic and infections conditions did not show differences in fitness parameters compared to the QoI-S isolate throughout four infection cycles (AVILA-ADAME and KOLLER, 2003). Therefore, the aim of this study was (i) to verify whether the QoI-R and QoI-S PoTl secondary inoculum produced in symptomatic wheat leaves during five successive infection cycles could develop blast on wheat heads; (ii) to characterize the temporal dynamics of wheat blast, the area under the disease progress curve (AUDPC), incubation period and latent period for both wheat leaves and heads over the course of five successive infection cycles of QoI-R and QoI-S PoTl isolates; (iii) to verify whether the fitness advantage of the QoI-R PoTl isolate was maintained (in terms of blast severity) after five successive infection cycles on wheat leaves and heads; and (iv) to fit empirical models to

describe the wheat blast dynamics in each infection cycle of QoI-R and QoI-S PoTl isolates in the absence of fungicide selection pressure.

2. MATERIALS AND METHODS

2.1. Fungal material, inoculum preparation, and plant material

The *Pyricularia oryzae* Triticum lineage (PoTl) QoI-resistant (R) 121146 isolate and the QoI-sensitive (S) Py6038 isolate were used for testing the relevant hypotheses in this study. The QoI-R isolate had the G143A mutation in its *cyt B* gene (CASTROAGUDIN et al., 2015). These isolates were deposited in the Molecular Plant Pathology fungal collection from São Paulo State University, Ilha Solteira, SP. The QoI-R isolate was obtained in 2012 from symptomatic heads from wheat fields in Mato Grosso do Sul (MS) state, Brazil. The QoI-S isolate was collected in Goiás (GO) state, Brazil, in 2006, using the same procedure. The sampling methods adopted in wheat fields were performed as described by (CASTROAGUDIN et al., 2015).

For inoculum preparation, mycelial discs (7-mm diameter) of the QoI-R and QoI-S PoTl isolates were transferred from a colony with 7 days of growth to 15 plates containing PDA medium (42 g L^{-1} potato-dextrose-agar, KASVI, India) and 15 plates containing oatmeal agar medium (OA, 60 g of oatmeal flour, 12 g of agar). For each plate containing PDA or OA, 50 μg mL^{-1} streptomycin sulphate and chloramphenicol were added. These plates were incubated for 15 days at 25 °C under constant light using 1,060-lumen Osram® fluorescent lamps. To facilitate the release of conidia, 4 mL of distilled water with the surfactant Tween 80 (10 μL L^{-1}) was added to each plate containing the culture medium. The conidial suspensions were obtained by scraping the mycelia with a sterile spatula. The conidial concentration was measured using a Neubauer chamber.

Concomitantly with inoculum preparation, three 'Anahuac 75' wheat plants were grown in a 770-mL plastic pot containing plant substrate Tropstrato HT potting mix (Vida Verde, Campinas, São Paulo, Brazil). The plants were kept in a greenhouse and irrigated daily, and every 15 days, a dose of 0.84 grams of N-P_2O_5-K_2O (10-10-10) was applied per pot. The QoI-R and QoI-S PoTl isolates were inoculated on the leaves of 1-month-old wheat plants, at growth stage 14 (ZADOKS et al., 1974). On heads, the inoculations were carried out at the beginning of anthesis, at growth stage 60, in 2-month-old immature heads (ZADOKS et al., 1974). Using a manual sprayer, conidial suspensions with 10^5 conidia mL^{-1} of QoI-R and QoI-S PoTl isolates were uniformly inoculated either onto the

adaxial leaf surfaces or onto the heads until draining. For inoculation of each isolate, 25 mL of the conidial suspension were spread in twelve wheat heads distributed on four pots, each containing three plants. For the first 24 hours after inoculation, the plants were incubated in a growth chamber in the dark at 25 °C and >90% relative humidity. Then, the plants were kept in a growth chamber under the same conditions but with a 12-hour photoperiod supplied by 33,354-lumen Osram® sodium vapour lamps (400W, model HQI-T NDL E40 5200K) for 21 days. An Even® digital thermo-hygrometer was used to monitor the temperature and relative humidity.

In the growth chamber, the wheat plants inoculated with each isolate were incubated separately and completely isolated from one another using transparent plastic bags, avoiding lateral contamination. Between one infection cycle and another, every 21 days, the conidia produced by PoTl isolates on wheat leaves were inoculated on healthy plants of the subsequent cycle. For the secondary inoculum production on infected wheat leaves with typical blast lesions in each infection cycle, the plants were kept in a growth chamber for 21 days. Twelve wheat leaves with sporulating lesions from each isolate were collected from four pots containing three wheat plants. The wheat leaves were placed into plastic tubes containing 10 mL of distilled water. The conidia were dislodged from the blast lesions by vortexing the plastic tube for 1 minute. The number of conidia recovered from sporulated wheat leaves was quantified using a Neubauer chamber. The secondary inoculum obtained from wheat leaves with typical blast lesions was inoculated simultaneously on healthy wheat leaves and heads from the subsequent cycle. These consecutive inoculations were carried out until five successive infection cycles were obtained. The 1st, 3rd, and 5th infection cycles were assessed.

2.2. Experimental design

The experimental design was completely randomized, with four replicates per treatment. The experiments were repeated once. The temporal dynamics of QoI-R PoTl isolate was assessed among infection cycles, and the factorial arrangement was 5 × 2, combining five infection cycles and two treatments, the isolate (QoI-R) 121146 and isolate (QoI-S) Py6038, which were inoculated on wheat leaves and heads of different plants. For each experiment, four pots containing three plants and/or three wheat heads each were prepared for inoculation of each isolate, and disease severity, incubation period and latent period were assessed in twelve wheat leaves and heads.

2.3. Assessment of disease severity and the ability of PoTl secondary inoculum to develop wheat blast over time

For QoI-R and QoI-S PoTl isolates, in each infection cycle, the ability of the secondary inoculum produced on symptomatic wheat leaves to develop blast on wheat heads was assessed based on disease severity values. For each infection cycle, the disease severity on wheat leaves and heads was determined at 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 d.a.i (days after inoculation). The non destructive method was used to measure the disease severity on wheat leaves and heads over the course of each infection cycle. A Canon® digital camera (EOS Rebel T1i model) was used to photograph wheat leaves and heads with typical blast symptoms. The digital camera was attached to a 1.55-m-high monopod at a distance of 20 cm from the wheat leaves and heads. Assess 2.0 (APS, St. Paul, Minnesota), a software program for analyzing digital photographs, was used to determine the percentage of each wheat leaf or head area affected in relation to the total leaf or head area (i.e., the disease severity caused by QoI-R and QoI-S PoTl isolates on wheat leaves and heads). Two sides of each head were photographed, and the disease severity was calculated as the arithmetic mean between the two images obtained. For each infection cycle, disease severity values of QoI-R and QoI-S PoTl isolates on wheat leaves and heads were plotted, obtaining the disease progress curves (DPCs). The infection rate of QoI-R and QoI-S PoTl isolates was determined on wheat leaves and heads.

2.4. Area under the disease progress curve

The AUDPC was calculated to compare the disease severity of QoI-R and QoI-S PoTl isolates within each cycle and between infection cycles. The disease severity values from the 12 blast assessments were integrated as AUDPC for wheat leaves and heads, according to SHANER and FINNEY (1977):

$$AUDPC = \sum_{i=1}^{n-1} \frac{(Y_i + Y_{i+1})}{2} * (T_{i-1} - T_i)$$

where AUDPC = area under the disease progress curve, Yi = proportion of disease at the i^{th} observation, Ti = time in days at the i^{th} observation, and n = total number of observations.

2.5. Assessment of incubation period and latent period

For each infection cycle, the incubation period and latent period were estimated by monitoring wheat leaves inoculated until the first symptoms and signals of the fungal

reproductive structures. The length of the incubation period in days was estimated between inoculation and appearance of the first symptoms of the disease. The length of the latent period in days was assessed between inoculation and the first conidia emergency (reproductive structures) on wheat leaves inoculated with QoI-R and QoI-S PoTl isolates.

2.6. Model fitting to disease progress curves

For each infection cycle, nonlinear models were fitted, and the data from the 12 assessments of disease severity of QoI-R and QoI-S PoTl isolates on wheat leaves and heads were used for the fitting. Different models were fitted, including exponential $y = (y0) \times \exp(rt)$ (1), Gompertz's $y = \exp(-(-\ln(y0)) \times \exp(-rt))$ (2), logistic $y = 1/[1 + ((1/y0) -1) \times \exp(-rt)]$(3), monomolecular $y = 1 - (1 - y0) \times \exp(-rt)$ (4), and linear ($y = y0 + r \times t$) (5). For these equations, y represents the disease severity percentage at time t, y0 is the disease severity at time t0 or the initial inoculum and r is the disease progress rate of each model, with time in days (CAMPBELL and MADDEN, 1990). **The best model was chosen according to the highest coefficient of determination in the regression analysis (R^2), the smallest mean-square of the residuals (MSR), the significance of the parameters of the fitted regression models, and the lowest dispersion of points in the residuals plot.**

2.7. Data analysis

For the analysis of data, results from two replicates of each experiment were combined. The value of the quotient between the largest and smallest square mean residual from two replicates of each experiment was less than seven, admitting the homogeneity of residual variances, which allowed the joint analysis of the data (GOMES, 1990). The Shapiro–Wilk tests was applied to assess the assumptions of the ANOVA for blast severity, AUDPC, incubation period and latent period ($p = 0.05$). The severity, incubation period and latent period of QoI-R and QoI-S PoTl isolates on wheat plants were compared by ANOVA's F test using a factorial arrangement between isolate and cycle. The QoI-R and QoI-S PoTl isolates were compared within each cycle and between infection cycles. Within each cycle, the isolates were compared by ANOVA's F test and between them with the Scott-Knott test ($p \leq 0.05$). All analyses were performed using the ExpDes.pt package of the statistical software RStudio version 1.2.5033 (FERREIRA and CAVALCANTI, 2009).

3. RESULTS

3.1. Disease progress curve and disease progress rate

The wheat blast occurred in all infection cycles, and symptoms were observed on both wheat leaves and heads. For each infection cycle, the secondary PoTl inoculum produced on symptomatic 'Anahuac 75' wheat leaves at 21 d.a.i. was able to develop blast symptoms on wheat heads. There was continuous progress of blast symptoms on leaves and heads, from 0.0 to 60.0-100.0% severity throughout 15 days. The isolate QoI-R had maximum disease intensity at 10 d.a.i, (i.e., earlier than the 15 d.a.i of QoI-S) (Fig. 1).

The sigmoid-shaped disease progress curve of the isolate QoI-R was maintained from the first to the last infection cycle on wheat leaves and heads (Fig. 1). There was a significant interaction between infection cycle and isolate on 'Anahuac 75' wheat leaves and heads ($p \leq 0.001$). In all infection cycles, at 7 d.a.i, the isolate QoI-R caused the highest disease severity on wheat leaves and heads ($p \leq 0.001$) (Fig. 1 and Fig. 2).

On the heads, the highest levels of disease severity for the isolate QoI-R, at 7 d.a.i, was at 1st infection cycle (Fig. 1). At the end of the 1st and 5th cycles, at 15 d.a.i, there was 100.0% disease severity on wheat leaves and heads inoculated with the isolates of PoTl (Fig. 1 and Fig. 2). In contrast, at the end of the 3rd infection cycle, for wheat plants inoculated with isolate QoI-R, there was 98.7% and 82.7% disease severity on wheat leaves and heads, respectively, while for plants inoculated with isolate QoI-S, the disease severity was 79.8% and 63.9%, respectively (Fig. 1).

Within each infection cycle, the disease severity was significantly different between isolates ($p \leq 0.001$). For the 5th infection cycle on wheat leaves, at 7 d.a.i, the disease severity of isolate QoI-R was 1.96 times higher than that of isolate QoI-S ($p \leq 0.001$) (Fig. 1 and Fig. 2). On the heads, at 7 d.a.i, the disease severity of isolate QoI-R was 3.96 times higher than that of isolate QoI-S ($p \leq 0.001$) (Fig. 1 and Fig. 2).

Within each infection cycle, there were significant differences between infection rate of isolate QoI-R and isolate QoI-S inoculated on wheat leaves ($p \leq 0.01$) and wheat heads ($p \leq 0.1$), according to ANOVA's F test. In all infection cycles, the infection rate of QoI-R and QoI-S PoTl isolates increased with the continuous progress of blast symptoms on leaves and heads, which occurred from 5 to 10 d.a.i. (Fig. 1). For the 1st and 5th infection cycle on wheat leaves and heads, at 7 d.a.i, the infection rate of QoI-R isolate was higher than that of isolate QoI-S ($p \leq 0.001$) (Fig. 1 and Fig. 2). Between the 10 and 15 d.a.i, the infection rate of QoI-R and QoI-S PoTl isolates decreased in the 1st

and 5th infection cycle, since 100% blast symptoms on leaves and heads were reached (Fig. 1).

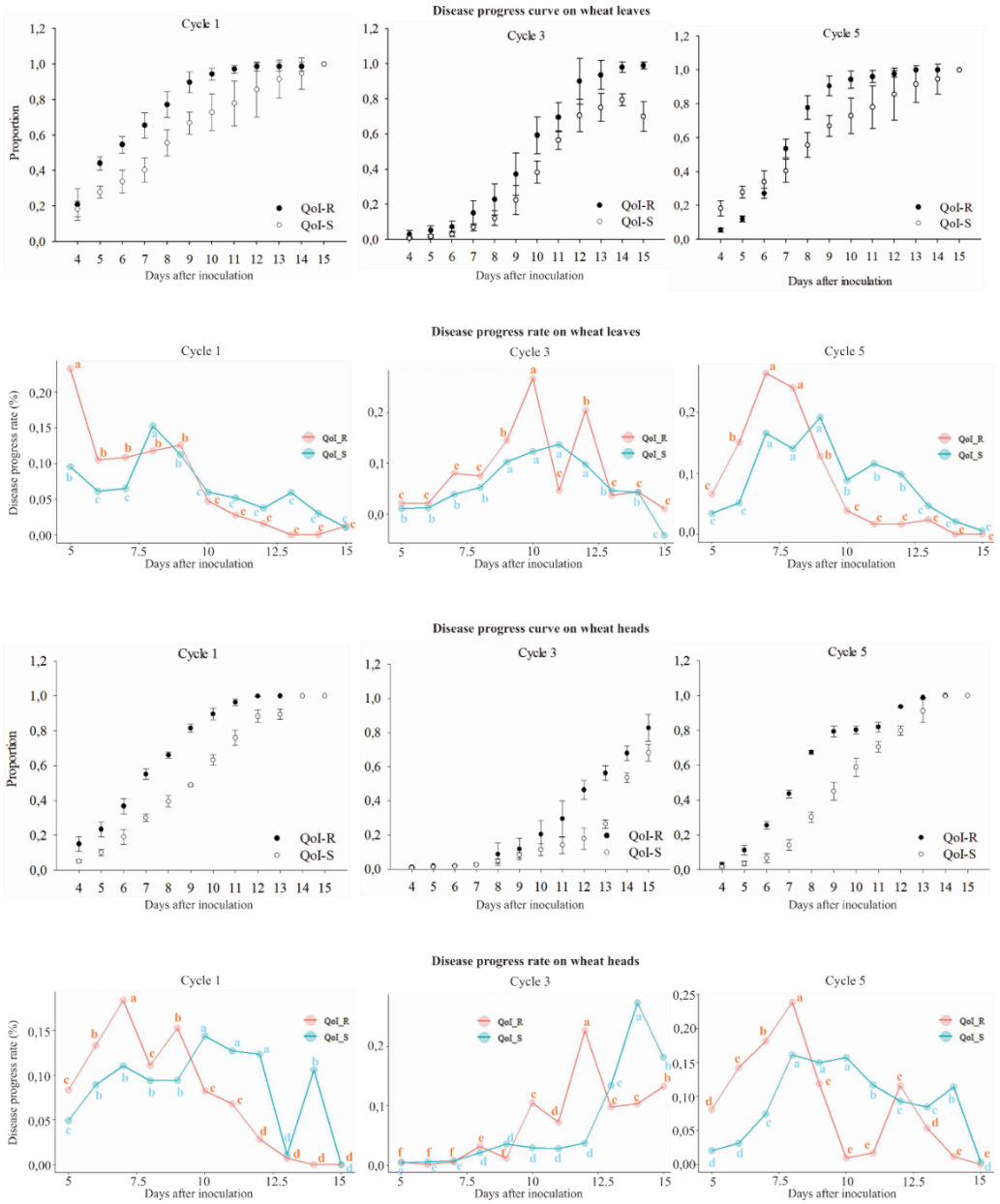

Figure 1. Disease progress curves (DPCs)[a] and disease progress rate[b] of wheat blast on wheat leaves and heads in the 1st, 3rd and 5th cycles of the *Pyricularia oryzae* Triticum lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide. [a]DPCs included severity values of the PoTl isolates in percentage/100 at indicated days after inoculation (DAI). [a]The error bars in each DPC point represent the average standard error of the severity values. [b]*Pyricularia oryzae* Triticum lineage isolates resistant (R) (red line) and sensitive (S) (blue line) to Quinone outside inhibitor (QoI) fungicide. [b]Means followed by the same letter are not significantly different from the Scott-Knott test ($p \leq 0.001$).

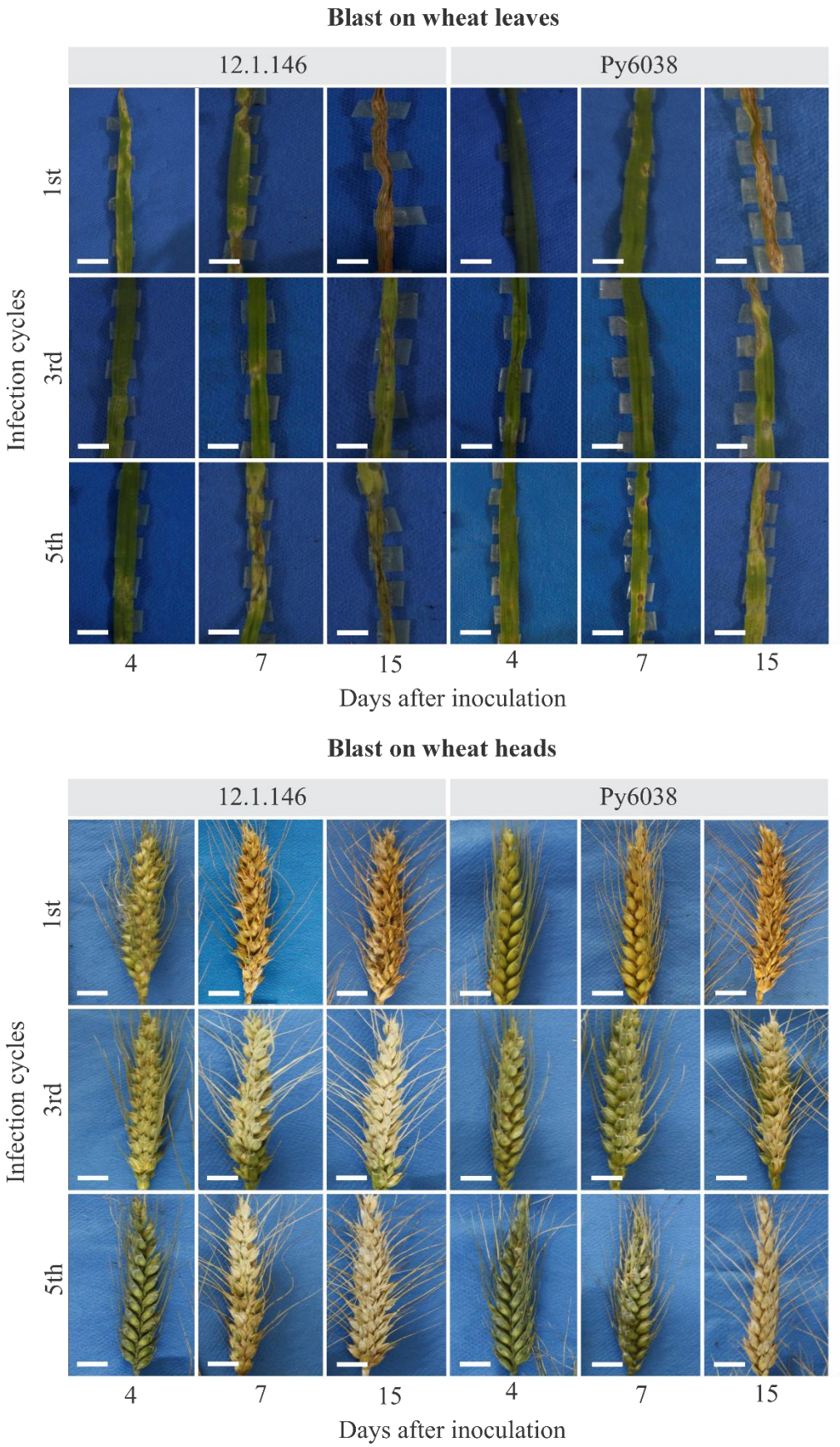

Figure 2: Images of blast symptoms on the leaves and heads of wheat cv. Anahuac 75 at 4, 7 and 15 days after inoculation (d.a.i) with *Pyricularia oryzae* Triticum lineage isolate 12.1.146 and Py6038 after successive infection cycles[a]. Bars: 10mm.

3.2. Area under the disease progress curve

For AUDPC within each infection cycle, there were significant differences between isolate QoI-R and isolate QoI-S ($p \leq 0.001$), according to ANOVA's F test. Among the infection cycles, the interaction between cycle and isolate on wheat leaves and heads were statistically significant ($p \leq 0.001$). In all infection cycles, higher AUDPC values were observed on the wheat leaves and heads inoculated with the isolate QoI-R, and the AUDPC was lower for the isolate QoI-S ($p \leq 0.001$) (Table 1). The highest AUDPC values were observed for PoTl isolates on wheat leaves and heads at 1st infection cycle (Table 1). Between the 1st and 5th infection cycles, the AUDPC on wheat leaves had a significantly reduction of 12.42 and 5.07 % for PoTl isolates 12.1.146 and Py6038, respectively (Table 1). Similarly, between the 1st and 5th infection cycle, the AUDPC on wheat heads had a significantly reduction of 4.78 and 12.26 % for PoTl isolates 12.1.146 and Py6038, respectively (Table 1).

Table 1. Area under the disease progress curve (AUDPC) of *Pyricularia oryzae Triticum* lineage isolates and effects of successive infection cycles.

Isolates[a]	**AUDPC**					
	Leaves			**Heads**		
	1st [b]	**3rd** [b]	**5th** [b]	**1st** [b]	**3rd** [b]	**5th** [b]
QoI-R	915.11Aa	685.56Cc	801.37Bb	803.64Aa	287.76Cb	765.20Ba
QoI-S	704.10Aa	522.57Cb	668.39Ba	627.88Aa	168.93Cc	550.87Bb

[a]The area under the disease progress curve (AUDPC) based on the severity values of blast on wheat leaves and heads in the 1st, 3rd and 5th infection cycles of the *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide were obtained by the joint analysis of two experiments.

[b] Means followed by the same letter are not significantly different from the *Scott-Knott* test ($p \leq 0.001$). The capital letter compares AUDPC between infection cycles, while lowercase letters compare AUDPC of the *Pyricularia oryzae Triticum* lineage isolates.

3.3. Incubation period and latent period

Within each infection cycle, significant differences between QoI-R and QoI-S PoTl isolates for incubation period and latent period ($p \leq 0.001$) were identified by ANOVA's *F* test. Among the infection cycles, there were a significant interaction between cycles and PoTl isolates for incubation period on wheat leaves ($p \leq 0.001$) and heads ($p \leq 0.1$) and latent period ($p \leq 0.001$). In all infection cycles, a shorter incubation period

and latent period were observed on the wheat leaves and heads inoculated with the isolate QoI-R, and the incubation period and latent period were longer for the isolate QoI-S ($p \leq 0.001$) (Table 2). At the end of the 5th infection cycle, the incubation time of the QoI-R and QoI-S PoTl isolates on wheat leaves increased significantly from 2.6 to 3.2 and from 3.2 to 3.7 days, respectively ($p \leq 0.001$) (Table 2). Similarly, between the 1st and 5th infection cycle, the incubation period on wheat heads increased significantly from 2.7 to 2.9 days for QoI-R PoTl isolate and from 3.7 to 3.9 days for QoI-S PoTl isolate ($p \leq 0.001$) (Table 2). For the latent period of the QoI-R PoTl isolate on wheat leaves, there was no effect of the infection cycles in the time necessary for conidial production. Conversely, the latent period of the QoI-S PoTl isolate on wheat leaves increased significantly from 6.6 to 7.2 days between the 1st and 5th infection cycle ($p \leq 0.001$) (Table 2).

3.4. Model fitting to disease progress curves

In all infection cycles, for both wheat leaves and heads, the logistic model was the best fit for the wheat blast progress curve (Fig. 3). In the infection cycles, on wheat plants inoculated with the isolates QoI-R and QoI-S, overall, the logistic model resulted in the highest R^2 and the lowest MSR on wheat leaves and heads (Table 3 and Table 4). Additionally, in all infection cycles, for both wheat leaves and heads, the disease progress rate (r) and the initial inoculum (y0) under the logistic model were significant parameters for isolates of PoTl (Table 3 and Table 4). When comparing the disease progress rate (r) of PoTl on leaves between the 1st infection cycle (rc1) and the 5th infection cycle (rc5) under the logistic model, the rc5 of QoI-R was 1.71 times higher than rc1, while in heads the rc5/rc1 ratios was 1.07.

Table 2. Incubation period and latent period on wheat plants of *Pyricularia oryzae Triticum* lineage isolates and effects of successive infection cycles.

Isolates[a]	**Incubation period (days)**						**Latent period (days)**		
	Leaves			**Heads**			**Leaves**		
	1st[b]	**3rd**[b]	**5th**[b]	**1st**[b]	**3rd**[b]	**5th**[b]	**1st**[b]	**3rd**[b]	**5th**[b]
QoI-R	2.67 Cb	3.04 Aa	3.21 Ba	2.73 Bb	3.65 Aa	2.94 Bb	6.29 Cc	6.21 Aa	6.46 Ba
QoI-S	6.67 Cc	4.97 Aa	3.77 Bb	3.71 Ba	4.13 Aa	3.96 Ba	6.67 Cc	8.21 Aa	7.21 Bb

[a] The number of days until the appearance of blast symptoms (incubation period) on wheat leaves and heads and emergence of conidia (latent period) on wheat leaves in the 1st, 3rd and 5th cycles of the *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide were obtained by the joint analysis of two experiments.

[b] Means followed by the same letter are not significantly different from the *Scott-Knott* test ($p \leq 0.001$). The standard errors resulted from four repetitions. The capital letter compares incubation period and latent period between infection cycles, while lowercase letters compare incubation period and latent period of the *Pyricularia oryzae Triticum* lineage isolates.

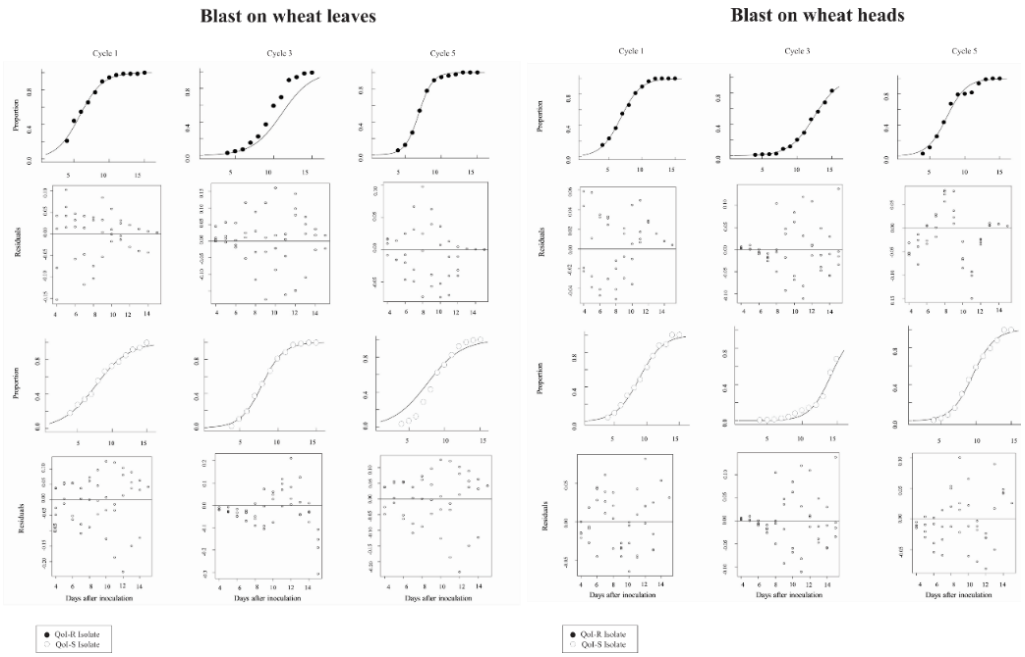

Figure 3. Scatter plot of residuals and fitted disease severity for wheat leaves and heads by the logistic model in the 1st, 3rd and 5th cycles of the *Pyricularia oryzae* Triticum lineage isolates resistant R (dark circle) and sensitive S (clear circle) to quinone outside inhibitor fungicide (QoI)[a]. [a]The severity values of the *Pyricularia oryzae* Triticum isolates are in percentage/100, at indicated days after inoculation (DAI). The plots represent the fitted disease severity and dispersion of points in the residuals of PoTl (QoI-R) (black filled circles) and (QoI-S) (white filled circles) in each infection cycle.

Table 3. Parameters used in the regression analysis to fit the nonlinear models for blast disease progress curve on wheat leaves, inoculated with two isolates of *Pyricularia oryzae Triticum*.

Cycle[b]	Isolate[a]	Models	Rate (*r*)[f]	y_0[e]	R^2[c]	MSR[d]
1st	QoI-R	Linear	0.066***	0.151**	0.80	0.01355
		Monomolecular	0.349***	-2.285***	0.95	0.00317
		Exponential	0.074***	0.373***	0.71	0.01987
		Logistic	0.616***	0.027***	0.96	0.00251
		Gompertz	4.7e-01*	3.8e-05	0.96	0.00255
	QoI-S	Linear	0.077***	-0.093*	0.90	0.00760
		Monomolecular	0.201***	-1.007***	0.88	0.00963
		Exponential	0.111***	0.207***	0.84	0.01284
		Logistic	0.419***	0.039***	0.92	0.00642
		Gompertz	0.308***	0.001***	0.91	0.00709
3rd	QoI-R	Linear	0.106***	-0.5081***	0.92	0.01133
		Monomolecular	0.180***	-1.4040***	0.82	0.02719
		Exponential	0.186***	0.0720***	0.84	0.02321
		Logistic	0.743***	0.0007*	0.96	0.00522
		Gompertz	Na	Na	Na	Na
	QoI-S	Linear	0.085***	-0.4445***	0.89	0.01086
		Monomolecular	0.123***	-0.8959***	0.83	0.01686
		Exponential	0.199***	0.0459***	0.80	0.01968
		Logistic	0.524***	0.0030*	0.92	0.00742
		Gompertz	3.5e-01***	0.0000	0.94	0.00559
5th	QoI-R	Linear	0.091***	-0.1588*	0.80	0.02558
		Monomolecular	0.336***	-3.1513***	0.92	0.00975
		Exponential	0.105***	0.24747***	0.66	0.04324
		Logistic	1.052***	0.00071**	0.99	0.00126
		Gompertz	Na	Na	Na	Na
	QoI-S	Linear	0.077***	-0.0938*	0.90	0.00760
		Monomolecular	0.201***	-1.0074***	0.88	0.00963
		Exponential	0.111***	0.2072***	0.84	0.01284
		Logistic	0.419***	0.0399***	0.92	0.00642
		Gompertz	0.308***	0.0013	0.91	0.00709

[a]121146 (G143A mutant QoI-R isolate resistant to azoxystrobin) and Py6038 (QoI-S isolate sensitive to azoxystrobin).

[b]On wheat leaves, five successive infection cycles of the *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide were performed.

[c]Coefficient of determination fitted in the regression analysis (R^2). The significance codes used were: *** $p< 0.001$; ** $p< 0.01$; * $p< 0.05$. Na: not fitted.

[d]Mean square of the residual of the linear regression analysis (MSR).

[e]$y_{0:}$ initial severity of wheat blast.

[f] $r_{:}$ progression rate of wheat blast.

Table 4. Parameters used in the regression analysis to fit the nonlinear models for blast disease progress curve on wheat heads inoculated with two isolates of *Pyricularia oryzae Triticum*.

Cycle[b]	Isolate[a]	Models	Rate (*r*)[f]	y_0[e]	R^2[c]	MSR[d]
1st	QoI-R	Linear	0.0835***	-0.0741	0.87	0.0119
		Monomolecular	0.2985***	-2.1418***	0.94	0.0055
		Exponential	0.1001***	0.2646***	0.76	0.0232
		Logistic	0.6664***	0.0107***	0.99	0.0008
		Gompertz	4.8e-01***	5.9e-08***	0.98	0.0017
	QoI-S	Linear	0.0964***	-0.3581***	0.97	0.0024
		Monomolecular	0.1960***	-1.3709***	0.90	0.0106
		Exponential	0.1547***	0.1141***	0.89	0.0125
		Logistic	0.5540***	0.0074***	0.98	0.0013
		Gompertz	3.8e-01***	0.0000	0.97	0.0026
3rd	QoI-R	Linear	0.0755***	-0.4412***	0.86	0.0110
		Monomolecular	0.0918***	-0.6603***	0.76	0.0191
		Exponential	0.2848***	0.0123***	0.94	0.0048
		Logistic	0.5625***	0.0009***	0.96	0.0027
		Gompertz	Na	Na	Na	Na
	QoI-S	Linear	0.0521***	-0.3202***	0.72	0.0126
		Monomolecular	0.0571***	-0.3942***	0.65	0.0159
		Exponential	0.3669***	0.0024	0.63	0.0189
		Logistic	6.6e-01***	8.9e-05*	0.95	0.0020
		Gompertz	Na	Na	Na	Na
5th	QoI-R	Linear	0.0934***	-0.2335***	0.89	0.0124
		Monomolecular	0.2743***	-2.2367***	0.95	0.0057
		Exponential	0.1207***	0.1940***	0.76	0.0280
		Logistic	0.7153***	0.0049***	0.97	0.0026
		Gompertz	5.0e-01***	0.0000	0.98	0.0015
	QoI-S	Linear	0.1042***	-0.4913***	0.97	0.0038
		Monomolecular	0.1818***	-1.4175***	0.87	0.0167
		Exponential	0.1841***	0.0739***	0.88	0.0157
		Logistic	0.6473***	0.0021***	0.98	0.0014
		Gompertz	Na	Na	Na	Na

[a]121146 (G143A mutant QoI-R isolate resistant to azoxystrobin) and Py6038 (QoI-S isolate sensitive to azoxystrobin).

[b]On wheat heads, five successive infection cycles of the *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide were performed.

[c]Coefficient of determination fitted in the regression analysis (R^2). The significance codes used were: *** $p< 0.001$; ** $p< 0.01$; * $p< 0.05$. Na: not fitted.

[d]Mean square of the residual of the linear regression analysis (MSR).

[e]y_0: initial severity of wheat blast.

[f] r: progression rate of wheat blast.

4. DISCUSSION

After the recent reports of fitness advantage for the QoI-R PoTl isolates carrying the G143A mutation compared with the sensitive isolates, in this study, we assessed the effect of successive infection cycles of the QoI-R and QoI-S PoTl isolates on the temporal development of blast on wheat leaves and heads. Knowledge about the biology and epidemiology of the pathogen may lead us to better understand and to apply the anti-resistance strategies against PoTl populations that cause blast in wheat fields in Brazil. This information may also provide a safer basis for employing models capable of supporting decision making in the application of QoI fungicides in controlling the wheat blast disease.

Firstly, our study reports that the QoI-R and QoI-S secondary inoculum produced on wheat leaves was pathogenic and efficient in causing wheat blast on wheat leaves and heads throughout five successive infection cycles. Moreover, the secondary inoculum of the isolates PoTl harvested from wheat leaves with typical blast symptoms and sporulated lesions caused blast symptoms when inoculated on wheat heads. This result reveals an important role for the PoTl inoculum produced on symptomatic wheat leaves in the development of blast on wheat heads. The PoTl inoculum is capable of infecting wheat plants from vegetative stages of the host up to the head stage (CRUZ et al., 2015, CRUZ and VALENT, 2017, MARTÍNEZ et al., 2019). The development of wheat blast symptoms in the middle canopy of plants in combination with the natural senescence of basal leaves contributes to the formation of an important source of secondary inoculum of PoTl (CRUZ et al., 2015). Similar to what was observed for *P. oryzae Triticum* on wheat, the PoTl inoculum and blast symptoms also spread from leaves to heads, following a vertical movement (GONGORA-CANUL et al., 2020). In our study, the time between inoculation and the first conidia emergency on wheat leaves (latent period) was shorter for QoI-R PoTl isolate than that of the isolate QoI-S in all infection cycles (Table 2), which was consistent with previous report in QoI-R PoTl populations (DORIGAN et al.,

2022). In contrast, QoI-R isolates of *P. oryzae* on rice had a longer latent period compared to the sensitive isolates (D' AVILA, 2022).

Secondly, in all infection cycles, the time period between inoculation and the first symptom on wheat leaves and heads (incubation period) was shorter for QoI-R isolate than that of the QoI-S isolate (Table 2), which was also consistent with previous report in QoI-R PoTl populations (DORIGAN et al., 2022). As part of the latent period, the incubation period is the time between host infection and the expression of disease symptoms. After that period, the inoculum formation in the lesions begins, marking the end of the latent period and the beginning of the infectious period. The number of pathogen generations is determined by the duration of the latent period (in hours, days, or weeks). Thus, the latent period affects the upper limit of the explosiveness of an epidemic. The more generations a pathogen has in the growth period of hosts, the more inoculum will be produced by the pathogens; hence, the disease can build up at high levels (Jürgen Kranz, 2002). Knowledge regarding the temporal dynamics, incubation period, and latent period of plant disease could be used as a decision support tool for more targeted timing of fungicides (MOLITOR et al., 2012) and to develop better pathogen management strategies. Thus, our results about incubation and latent period may provide detailed information for disease prediction models to help predict the appearance of the first visible symptoms, the fungicide application timing and effectiveness against wheat blast.

The wheat blast severity from both QoI-R and QoI-S isolates increased over time on wheat leaves and heads. At 7 d.a.i, the disease intensity of QoI-R PoTl isolate on leaves and heads at the initial infection cycle was 1.54 and 1.82 times higher than that of the QoI-S PoTl isolate, respectively. Similarly, to what was observed for the initial infection cycle, in successive infection cycles, at 7 d.a.i, QoI-R PoTl isolate caused higher disease severity than the QoI-S PoTl isolate. These results indicated that the fitness advantage of the QoI-R PoTl isolate (in terms of blast severity on wheat leaves and heads) was maintained over the course of five successive infection cycles. Although the disease severity was lower at the 3rd infection cycle, the infection levels went back up in later cycles. These differences in the 3rd infection cycle on wheat leaves and heads can be explained by several factors, including longer incubation of PoTl isolates (Table 2), as well as lower infection rate and expansion of lesions on infected wheat leaves and heads (Fig. 1 and Fig. 2).

For the 1stand 5th infection cycles, the levels of disease severity of QoI-R PoTl isolate were higher (more than 60% at 9 d.a.i), and the rate of disease progress was higher at the 5th cycle than at the initial infection cycle on leaves and heads. Similarly, to what was observed for QoI-R PoTl isolate, *P. oryzae* isolate have caused a high level of infection, more than 60% of the disease incidence at 10 d.a.i, in combination with the increase in the rate of disease progression (RIOS et al., 2016). Furthermore, our results demonstrate a continuously decreasing infection rate of PoTl isolates ranging from near 0.0 between 10 and 15 d.a.i (Fig. 1). Therefore, decreasing the probability of occurrence of secondary infections on wheat leaves 15 d.a.i (Fig. 1).

Thirdly, regarding the AUDPC, based on the disease severity values on wheat leaves and heads, the isolate QoI-R had higher values than the isolate QoI-S in all infection cycles (Table 1). When inoculated on wheat leaves and heads for each infection cycle, there were differences in the behaviour of the temporal progress of blast and AUDPC between the isolate QoI-R and the isolate QoI-S. The AUDPC is a better indicator of disease expression over time for temporal analyses of plant disease. Based on our results, the differences in AUDPC values of PoTl isolates in each infection cycle may provide a better understanding of the wheat blast epidemic behavior and develop effective disease management strategies (DUARTE et al., 2012). Furthermore, between the 1st and 5th infection cycles, a significant reduction in the AUDPC of the PoTl isolates was observed (Table 1). These results indicate that the PoTl isolates did not keep the same temporal dynamics after five infection cycles.

Fourthly, the nonlinear model that best fit the sigmoid progress curves over the course of time, for both the isolate QoI-R and the isolate QoI-S, was the logistic model (Fig. 3). This model was also selected by other authors to describe the wheat blast epidemic, although on crop fields (GOMES et al., 2019, GONGORA-CANUL et al., 2020). The Gompertz model has also been cited for its temporal fit of wheat blast (GONGORA-CANUL et al., 2020). Both logistic and Gompertz models have similar curve shapes and are often used to describe and model polycyclic-like epidemics (Mouen BEDIMO et al., 2007, VAN DEN BOSCH et al., 2014, GOMES et al., 2019, FERNANDEZ-CAMPOS et al., 2020).

Therefore, we can highlight some important findings of this study: i) for each infection cycle, the secondary inoculum of QoI-R PoTl isolate and QoI-S PoTl isolate produced on symptomatic wheat leaves infected and colonized wheat heads; ii) disease severity in both QoI-R and QoI-S isolates increased over the course of time in each

infection cycle on wheat leaves and heads; iii) the PoTl isolates did not keep the same temporal dynamics after five infection cycles. As a final highlight, iv) the logistic model was the best model for wheat blast epidemics caused by PoTl isolates under controlled conditions.

Regarding the effect of successive cycles of infection of QoI-R PoTl isolates with higher fitness and fitness advantages under the severity of wheat blast, we obtained relevant information about this evolutionary advantage of QoI-R PoTl populations. QoI-R PoTl populations with higher fitness are likely to have higher advantages throughout the infection process and blast progression on wheat leaves and heads. This high variability between QoI-R PoTl isolate and QoI-S PoTl isolate may be attributed to the extensive use of QoI fungicides to control wheat blast in recent years. This probably has resulted in a selection of QoI-R PoTl isolates with fitness advantages (CASTROAGUDIN et al., 2015; DORIGAN et al., 2022). Moreover, there is no fitness cost associated with QoIs resistance in PoTl populations (DORIGAN et al., 2022). Hence, resistant genotypes of the PoTl populations with fitness advantages may prevail on wheat fields, even after successive infection cycles in the absence of selection pressure, and QoIs may lose their efficacy permanently. In this sense, the most effective action to manage the resistance to QoIs in PoTl populations would be to remove the selection pressure to QoIs. Similarly, to what was reported by DORIGAN et al. (2022), no fitness cost associated with QoIs resistance was also reported by AVILA-ADAME and KOLLER (2003) in *P. oryzae* on barley, over three consecutive infection cycles, in the absence of azoxystrobin (DORIGAN et al., 2018; AVILA-ADAME and KOLLER, 2003). Conversely, few studies also reported a fitness cost associated with the G143A mutation for QoIs resistance, including *P. oryzae* on rice, *Ustilago maydis* on corn, *P. oryzae* on perennial ryegrass, *Z. tritici* on wheat, and some lab mutants of *F. fujikuroi* (MA and UDDIN, 2009; HAGERTY, 2016; D' AVILA, 2022; SONG et al., 2022, ZIOGAS, 2002).

When analyzing the likely contributions of our results to the management of the QoI-R PoTl, the questions and answers about successive wheat crop cycles in Brazil and the prevalence of fitness advantage of isolates QoI-R PoTl (in terms of blast severity) have important implications for the integrated management of wheat blast in wheat fields. Certain cultural practices should be implemented, for example, the management of secondary inoculum of QoI-R PoTl in wheat fields, in basal leaves, which is a potentially important inoculum source for new epidemics, as well as the crop planting date, which according to GOULART et al. (2007) should be adjusted as a strategy to escape infection

by the QoI-R PoTl pathogen (GOULART et al., 2007, CRUZ et al., 2015). Other anti-resistance strategies can be implemented and contribute to the reduction of QoI-R PoTl populations in wheat fields, for example, applications of multisite fungicides with a low risk of resistance in mixtures with effective active ingredients of single-site inhibitors (e.g., DMIs). Multisite fungicides can be employed as mixing partners with other fungicides at an early stage of the disease epidemic (COOK et al., 1999, HOBBELEN et al., 2011, VAN DEN BOSCH et al., 2014). Among site-specific fungicides, tebuconazole and benzovindiflupyr can still be used for the management of wheat blast, as well as co formulation with multisite fungicides (CAZÓN et al., 2023). These important anti-resistance strategies would prevent the selection of the PoTl populations with higher fitness, adaptative advantages and resistance to fungicides in Brazilian wheat fields.

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6. CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

7. DATA AVAILABILITY STATEMENT

The epidemiological data that supports the findings of this study are available upon request.

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CHAPTHER 3 – STABILITY OF RESISTANCE AND COMPETITIVE ADVANTAGE OF *Pyricularia oryzae Triticum* RESISTANT TO QoIs

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ABSTRACT

Wheat blast, caused by *Pyricularia oryzae Triticum* lineage (PoTl), is one of the most important and devastating fungal diseases damaging wheat crops. The stability of the Quinone outside inhibitor fungicides (QoIs) resistance of PoTl isolates with higher fitness, and adaptative advantages are still unknown. In this study, the fungicide resistance stability, fitness, and competitive ability of the QoI-resistant ® PoTl isolates group were determined throughout nine and five successive infection cycles *in vitro* and *in vivo*, respectively, in the absence of fungicide. No changes in the sensitivity of the QoI-R and sensitive (S) PoTl isolate groups were observed for azoxystrobin after successive infection cycles *in vitro* and *in vivo*. The QoI-R PoTl isolates group increased mycelial growth and conidial germination ability over time. For the QoI-R isolate group, the leaf and head disease, conidial production, and latent period on wheat leaves remain unchanged between the 1st and 5th infection cycle. In all infection cycles, the highest leaf and head disease and the highest amount of conidia harvested from wheat leaves were found in isolate mixtures (0S:80R or 0S:100R) with the predominance of resistant isolates. At discriminatory doses of the fungicide, *in vitro* and *in vivo*, the conidia of the 20S:80R and 0S:100R mixtures harvested remained resistant after successive infection cycles. The G143A mutation conferring QoIs resistance remains stable after five infection cycles of the 0S:100R mixtures on wheat leaves. Our results provide evidence that the QoIs resistance and adaptative advantages of the QoI-R PoTl isolate group remain unchanged even after successive infection cycles. We discuss the ecological implications of the wheat blast population's adapted and QoIs resistance remained stable.

Keywords: Wheat blast, fungicide sensitivity, azoxystrobin, EC_{50}, fitness

1. INTRODUCTION

Wheat blast, caused by *Pyricularia oryzae Triticum* lineage (PoTl), syn. *P. graminis-tritici* (CERESINI et al., 2018; GLADIEUX et al., 2018), affects important cereal crops, including wheat, rice, barley, millet, and oats (CERESINI et al., 2018). In countries or regions where the wheat blast is endemic, PoTl may cause 10-100% grain losses (DUVEILLER, 2016; CERESINI et al., 2018, BONJEAN, et al., 2016). In 2020, Brazilian wheat production was 6.6 million tons, representing 0.9% of the 735 million tons produced globally (COLUSSI, 2022). Due to the many diseases, there is a lack of quality Brazilian wheat and low internal production and supply. Consequently, Brazil still imports 4 million tons of wheat grain annually (CERESINI et al., 2018). The wheat blast was first reported in 1986 Parana state, Brazil (IGARASHI, 1986). After that, this devastating pathogen was quickly disseminated to south-central Brazil and other countries such as Argentina, Bolivia, and Paraguay (GLADIEUX et al., 2018; CERESINI et al., 2018). Until April 2016, the wheat blast was restricted to South America (CERESINI et al., 2018). Then, there was the first report of this disease in Bangladesh, southeast Asia, and Zambia, southern Africa, in 2016 and 2017, respectively (ISLAM et al., 2016; TEMBO et al., 2020). More recently, in 2022, the blast disease was detected in Germany, in central Europe (BARRAGAN et al., 2022). In Brazil, the fungicide application has been widely employed to manage wheat blast, even with efficacy rarely over 60% of disease control (CERESINI et al., 2018). The reduced efficiency of molecules with a specific target action and lack of varietal resistance make wheat blast management difficult in wheat crops.

In 2015, there was the first report of selection for resistance to Quinone outside inhibitor fungicides (QoI) in Brazilian populations of PoTl. The mutation G143A in the cytochrome b (*cytb*) gene was associated with QoI-resistance in PoTl populations (CASTROAGUDIN et al., 2015). In addition to the target-site-based mechanisms of fungicide resistance, non-target site mechanisms, such as overexpression of drug efflux pumps, lack of conversion to the active components, and detoxification may also be associated with QoI-resistance in phytopathogens (HAWKINS and FRAAIJE, 2018, HU, 2021; DORIGAN et al., 2023). Recently, PoTl populations have also been associated with resistance to two other chemical groups, both triazoles or demethylation inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs) (VICENTINI et al., 2022, POLONI et al., 2021).

The fungicide resistance can lead to fitness advantages on phytopathogens. Probably, the fitness advantages may be associated with an evolutionary compensatory process (HAWKINS and FRAAIJE, 2018). However, mutations linked to fungicide resistance can also be associated with a fitness cost, resulting in evolutionary trade-offs (HAWKINS and FRAAIJE, 2018). Fitness is defined as the survival and the reproductive success of an allele, individual, or group of individuals carrying a particular character of fungicide resistance, expressed as progeny contribution to the next generation (PRINGLE and TAYLOR, 2002). Fitness can also be employed to demonstrate all estimated parameters for a single isolate, such as mycelial growth, conidial production, germination, and virulence (KARAOGLANIDIS et al., 2001). Many fitness parameters were assessed for a QoIs resistant (QoI-R) PoTl isolate group carrying G143A mutation (DORIGAN et al., 2022). These isolates had greater (mycelial growth, virulence, conidial production, and competitive ability) than sensitive (QoI-S) ones (DORIGAN et al., 2022). This result contrasted with Evolutionary Theory, which affirms that fungicide resistance can come with fitness costs (HAWKINS and FRAAIJE, 2018).

Although the QoIs resistance has become pervasive and persisted in PoTl populations in south-central Brazil for seven years from 2012 to 2019 (VICENTINI et al., 2022), very little is known about the stability of the G143A mutation and fitness advantage of QoI-R PoTl populations over time. Resistance stability is the pathogen's capacity to retain the same insensibility level to fungicide after successive generations of either exposition or without exposition to the target fungicide (VEGA and DEWDNEY, 2014). For the wheat blast pathosystem, few studies clarify if the fitness advantage of QoI-R PoTl populations carrying G143A mutation is stable even after successive infection cycles. Therefore, investigating both fungicide resistance stability, fitness advantage stability, and competitive abilities of PoTl populations resistant to QoIs may likely improve the anti-resistance strategies that reduce the decrease of sensibility of PoTl populations to QoIs in wheat fields.

For other phytopathogenic fungi species carrying the G143A mutation, such as *Colletotrichum acutatum* from strawberries, the resistance to QoI persisted even after four cycles *in vitro* in the absence of fungicide (FORCELINI et al., 2018). In *Erysiphe necator*, the fungal causing grape powdery mildew, isolates show stability of QoI resistance throughout four years after the last exposure to fungicides, while the isolates of *Plasmopara viticola* from grapes were resistants to QoI for four consecutive cycles (RALLOS et al., 2014; GENET, 2006). Further, G143A mutants of *Pyricularia oryzae*

on barley did not show reversion to sensitivity throughout three successive cycles (AVILA-ADAME and KOLLER, 2003). Similar to what was observed for *P. oryzae* on barley, *Botrytis cinerea* on apple was also stable even after four consecutive cycles (CHEN et al., 2016).

In such a scenario, the objective of this study was (i) to determine if the resistance of QoI-R PoTl field isolates remained stable after nine and five successive infection cycles *in vitro* and *in vivo*, respectively, without fungicide (ii) to verify if the fitness parameters and competitive abilities of the QoI-R PoTl field isolates are stable (i.e., no decrease) after successive cycles of infection *in vitro* and *in vivo* in the absence of fungicide selection pressure and (iii) to confirm if the mutation G143A in the *cytb* gene of QoI-R PoTl field isolates remain stable after successive infection cycles *in vitro* and *in vivo* in the absence of fungicide selection pressure.

2. MATERIALS AND METHODS

2.1 Fungal material and plant material

Eight QoIs resistant (QoI-R) or sensitive (QoI-S) *Pyricularia oryzae Triticum* lineage isolates characterized in previous studies were used in this present study (CASTROAGUDIN et al., 2015, VICENTINI et al., 2022, DORIGAN et al., 2022). The PoTl isolates were collected from wheat fields in Amambaí, Mato Grosso do Sul (MS), in 2012, Goiânia, Goiás (GO), in 2006, Londrina, Paraná (PR), in 2018 and Itapetininga, in the São Paulo (SP) state, Brazil, in 2018, from leaves and heads with typical blast symptoms (Table 1). Sampling was carried out following the procedure described by (CASTROAGUDIN et al., 2015). These isolates were deposited in the Molecular Plant Pathology fungal collection from São Paulo State University, Ilha Solteira, SP, Brazil.

Table 1. Description of *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) obtained from wheat plants that were used in the fungicide resistance stability and fitness study.

Isolates	Origin, year	Sensitivity to fungicides[a]	Reference
Py6038	Goiânia, GO, 2005	QoI-S	Castroagudin, 2015
Py22.2	Goiânia, GO, 2005	QoI-S	Castroagudin, 2015
12.1.129	Amambai, MS, 2012	QoI-S	Castroagudin, 2015
12.1.132	Amambai, MS, 2012	QoI-S	Castroagudin, 2015
12.1.146	Amambai, MS, 2012	QoI-R	Castroagudin, 2015
18SPM6	Itapetininga, SP, 2018	QoI-R	Vicentini 2022
12.1.312	Londrina, PR, 2012	QoI-R	Castroagudin, 2015
18PRH9	Londrina, PR, 2018	QoI-R	Vicentini 2022

Legend: [a] R = Resistant; S = Sensitive.

For conidial production, mycelial discs (7-mm diameter) of QoI-R and QoI-S PoTl isolate groups were transferred from 7-day-old cultures of growth to 15 plates containing PDA medium (42 g L^{-1} potato-dextrose-agar, KASVI, India) and 15 plates with oatmeal agar medium (OA, 60 g of oatmeal flour, 12 g of agar). In each plate containing PDA or OA medium, 50 μg mL^{-1} streptomycin sulfate and chloramphenicol were added. These plates were incubated at 25 °C under constant light for fifteen days using 1,060-lumen Osram® fluorescent lamps. Four mLs of distilled water with the surfactant Tween 80 (10 μL L^{-1}) were added to the culture medium to facilitate the release of conidia. The conidial suspensions were obtained by scraping the mycelia produced in the plates using a sterilized spatula. For QoI-R and QoI-S PoTl isolate groups, the adjustment of the conidia concentration, $2{,}2 \times 10^4$ conídia mL^{-1}, was performed using a Neubauer chamber.

Simultaneously conidial production, three wheat 'Anahuac 75' plants were grown in each plastic pot containing 770 mL of the plant substrate Tropstrato HT potting mix (Vida Verde). The wheat growth was conducted in a greenhouse, and a 0.84-gram dose of N-P_2O_5-K_2O (10-10-10) was applied per pot every 15 days. The wheat plants were watered daily. The inoculations of PoTl isolates on wheat leaves were performed when the plants were a 1-month-old plants at growth stage 14 (ZADOCKS et al., 1974). On

heads, the inoculations were carried out at the beginning of anthesis, at growth stage 60, on 2-month-old immature spikelets (ZADOCKS et al., 1974).

2.2 Experimental design

The experimental design was completely randomized. Four replicates were prepared for each treatment, and all experiments were repeated once. Four plates from each concentration were prepared for azoxystrobin sensitivity assessment. The effect of successive infection cycles under the stability of fitness parameters was assessed using the factorial arrangements 9 × 2 and 5 × 2, *in vitro* and *in vivo*, respectively. The infection cycle factor was combined with two treatments: QoI-R and QoI-S PoTl isolate groups. For the competitive ability assays, QoI-R and QoI-S PoTl isolates were evaluated using the factorial arrangements 9 × 5 and 5 × 5, *in vitro* and *in vivo*, respectively. The infection cycle factor was combined with five treatments: 0S:100R, 20S:80R, 50S:50R, 80S:20R, and 100S:0R obtained from PoTl isolate mixtures of the 121146 QoI-resistant (R) and Py6038 QoI-sensitive (S) isolates. An experimental repetition consisted of a single plate and/or a pot containing three wheat plants and/or three wheat heads per treatment. For the *in vitro* assays, treatments were inoculated on four plates of PDA and/or OA medium, and when tested *in vivo*, the treatments were inoculated in four pots containing three wheat plants and/or three wheat heads.

2.3 Resistance stability of QoIs resistant PoTl isolate group

The effect of successive infection cycles *in vitro* and *in vivo* of the QoI-R PoTl isolate group under resistance stability to QoIs was studied. Nine successive infection cycles *in vitro* of the QoI-R and QoI-S PoTl isolate groups were carried out without fungicide pressure. The azoxystrobin sensitivity of PoTl isolates was determined at the 1st, 5th, and 9th infection cycles. Between one infection cycle and another, mycelial discs (7-mm diameter) of isolates were transferred from 7-day-old colony growth to Petri dishes (90-mm diameter) containing PDA medium. The plates were incubated at 25 °C under a 12-h photoperiod using 1,060-lumen Osram® fluorescent lamps.

Five successive infection cycles of the QoI-R and QoI-S PoTl isolate groups on wheat leaves and heads were performed in the absence of fungicide pressure. The inoculated plants were kept in a growth chamber at 25 °C under a 12-h photoperiod using

33.354-lumen Osram® sodium vapor lamps (400W, model HQI-T NDL E40 5200K). The infection cycles of the PoTl isolates on leaves and heads lasted 21 days. An Even® digital thermo-hygrometer monitored the temperature and relative humidity in the growth chamber. Before completing another infection cycle, the wheat plants were removed from the growth chamber to avoid cross infection. The sensitivity of the PoTl isolates to azoxystrobin was determined at the 1st, 3rd, and 5th infection cycles. Between one infection cycle and another, every 21 days, the conidia produced by QoIs resistant and sensitive PoTl isolate groups on wheat leaves were inoculated on healthy plants of the subsequent cycle. Both wheat plants and the inoculum of PoTl isolates were prepared following the same procedures described in the 'Fungal material and Plant material' section.

2.4 Effect of successive infection cycles on the sensibility of the QoIs resistant and sensitive PoTl **isolate groups to azoxystrobin**

For both *in vitro* and *in vivo* assays, the effective concentration that inhibits 50% of mycelial growth (EC_{50}) was used to assess the resistance stability of PoTl isolates to QoIs. A QoI fungicide azoxystrobin stock solution (250 g L^{-1} a.i., Syngenta, Basel, Switzerland) was prepared at 1,000 μg mL^{-1} after dilution in deionized water. Aliquots of the stock solution were added to autoclaved PDA medium, cooled, at 45 °C, to obtain the final concentrations of 0, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5.0, 7.0, and 10.0 μg mL^{-1}. There was added 0.5 mM salicyl hydroxamic acid (SHAM) in all concentrations of QoI to inhibit the alternative oxidase pathway of fungal respiration (AOX) (MA et al., 2003). For each isolate group, mycelial discs (7-mm diameter) from a 7-day-old colony were transferred to plates containing PDA medium supplemented with different concentrations of azoxystrobin. The mycelial growth of PoTl isolates was determined based on the diameter of the fungal colony. It was measured by aiding a caliper seven days after incubation at 25 °C and a 12-hour photoperiod. EC_{50} values of PoTl isolates were determined using the fungi's relative growth (RG), for each dose, in relation to the control without fungicide. EC_{50} values were obtained using *RGi* fungi, and final concentrations of azoxystrobin were converted in log_{10} using the ED50plus v1.0 program (VARGAS, 2000).

2.5 Effect of successive infection cycles on fitness parameters of the QoIs resistant and sensitive PoTl isolate groups

For fitness parameters, the QoI-R and QoI-S PoTl isolate groups were evaluated in both *in vitro* and *in vivo* experiments. The variables studied *in vitro* were mycelial growth, conidial production, and conidial germination ability. For the *in vivo* assays, incubation period, latent period, disease severity on wheat leaves and heads, conidial production, and conidial germination ability were also determined. The evaluations of infection cycles *in vitro* and *in vivo* were carried out to conform to what was described in the 'Resistance stability of QoIs resistant PoTl isolate group.'

Mycelial growth on PDA. The PoTl isolates were grown on a PDA medium at 25 °C under constant light for seven days. For evaluating the mycelial growth, colony diameter (mm) was determined using a caliper.

In vitro conidial production. The conidial production of either QoI-R or QoI-S isolate groups of PoTl isolates was carried out following the same procedures described in the 'fungal material and plant material' section. A repetition was composed of 10 mL conidial suspension for each isolate group.

In vitro germination ability. Conidial suspension of QoI-R and QoI-S isolate groups PoTl were obtained, as described in the '*In vitro* conidial production' section. Both 200 µL of PD medium (potato dextrose, Sigma-Aldrich, St. Louis, Missouri, USA) and 200 µL of conidial suspension supplemented with 0.5 mM of SHAM were mixed and transferred to a 24-well culture plate. The plates were incubated in the shaker at 110 rpm and 25 °C for 12 h in the dark. The germination percentage of each isolate group was calculated by counting 50 conidia for each repetition. The conidia were considered to have germinated when the germ tube length had twice or more of the conidia length (OLAYA et al., 1998).

Incubation period and latent period. The incubation and latent periods were determined by monitoring wheat leaves inoculated until the first symptoms and signals of the fungal reproductive structures. The incubation period is the time from inoculation to the first symptoms appearing. The latent period is characterized by the time from inoculation to the first conidia emergency (reproductive structures) on wheat leaves inoculated with PoTl isolates.

Disease severity. After preparation of both wheat plants and conidial suspension from QoI-R and QoI-S isolate groups PoTl as described in the 'Fungal material and Plant material' section, the wheat plants were inoculated with the fungal isolates. For each isolate group, the conidial suspension was adjusted to 2.2×10^4 conidia mL^{-1}. The inoculations of isolates were carried out using a manual sprayer until draining. For each isolate, 25 mL of the conidial suspension were inoculated in twelve wheat plants distributed on four pots, each containing three plants. Afterward, the wheat plants were kept in a growth chamber under nebulization at 25°C, with relative humidity at 90%, and in the dark for the first 24 h. After this period, the wheat plants were incubated under a 12-hour photoperiod as described in the 'Resistance stability of QoIs resistant PoTl isolate group' section. An Even® digital thermo-hygrometer was used to monitor the temperature and relative humidity. A Canon® digital camera (EOS Rebel T1i model) was used to photograph wheat leaves and heads with typical blast symptoms. The software program to analyze digital photographs, Asses 2.0 (APS, St. Paul, Minnesota), was used to determine the percentage of foliar area lesioned concerning the total size of the leaf (i.e., the blast severity on leaves or heads at 7 days after inoculation (d.a.i)). On heads, two faces of each head were photographed, and the severity value was determined by the arithmetic to mean among two images obtained.

In vivo conidial production. The conidial production of QoI-R and QoI-S isolate groups PoTl was determined on wheat leaves, at 21 d.a.i, on typical blast lesions. Three sporulated wheat leaves were placed into plastic tubes containing 10 mL of distilled water. After that, the plastic tubes were agitated in a vortex for 20s to dislodge conidia from leaf fragments containing blast lesions. The number of recovered conidia from lesions was quantified in a Neubauer chamber. For each isolate group, the conidial suspensions were obtained from plastic tubes containing three wheat leaves.

In vivo germination ability. The germination percentage *in vivo* of QoI-R and QoI-S isolate groups PoTl were obtained as described in the '*In vitro* germination ability' section.

2.6 Effect of successive infection cycles on competitive abilities of the QoIs resistant and sensitive PoTl **isolates**

Two experiments of competitive ability were performed, one *in vitro* and another *in vivo*. The conidial mixtures of the QoI-resistant PoTl isolate 121146 (R) and QoI-sensitive PoTl isolate Py6038 (S) were used without fungicide pressure. The conidial suspensions of the R and S isolates adjusted to $2{,}2 \times 10^4$ conidia mL^{-1} were mixed to obtain the proportions of (100S:0R, 80S:20R, 50S:50R, 20S:80R and 0S:100R).

In vitro competitive ability. Aliquots of each mixture (20 µL) were transferred to 15 plates of either PDA or oatmeal agar medium, and the plates were incubated at 25° C under constant light for 15 days. Nine successive infection cycles were performed following the procedures described in the 'Fungal material and plant material' section. An aliquot of 20 µL conidial suspensions of each mixture obtained from each cycle was used in the subsequent cycle. For each mixture, the conidial production was quantified using a Neubauer chamber.

In vivo competitive ability. The wheat plants were inoculated following the procedures described in the 'Disease severity' section. For each mixture, 12 wheat plants distributed in four pots were inoculated. For each mixture, wheat blast severity and conidial production on wheat leaves were evaluated at 7 and 21 d.a.i, respectively. Five successive infection cycles were carried out as described in the 'Resistance stability of QoIs resistant PoTl isolate group' section.

2.7 Effect of successive infection cycles on frequency of QoIs resistant conidia of mixtures of the PoTl isolates

The frequency of QoI-R conidia *in vitro* obtained from PDA or oatmeal agar medium at 15 d.a.i. and *in vivo* from wheat blast lesions at 21 d.a.i. were determined for each mixture of QoI-R and QoI-S isolates (DORIGAN et al., 2022).

2.8 Effect of successive infection cycles of QoIs resistant PoTl isolate group on frequency of G143A mutation in *cytochrome b*

The frequency of G143A mutation in the cytochrome *b* (*cytb*) gene was assessed for QoI-R and QoI-S PoTl mixtures tested in the '*In vivo* competitive ability' section. The inoculation of PoTl mixtures on wheat leaves followed the procedure described in the

'Disease severity' section. The PoTl 121146 alone (QoI-R) (0S:100R) and PoTl Py6038 alone (QoI-S) (100S:0R) isolates of the 1st and 5th infection cycles were obtained from wheat leaves with typical blast symptoms at 7 d.a.i. For each mixture, twenty single cultures were isolated from typical blast lesions of twelve wheat plants distributed on four pots containing three plants. Genomic DNA of every single culture was extracted from mycelial growth on PDA at 25 °C and 12-h photoperiod. Genomic DNA was extracted using the kit Wizard® Genomic DNA Purification (Promega, Madison, WI). DNA was quantified using a NanoDrop® Lite spectrophotometer (Thermo Fisher Scientific, USA) and diluted to a final concentration of 25 ng mL^{-1}. For the QoI-R isolates, the PCR primers of the partial amplification of *cytb* gene PoTl (5'-ATGAGAGATGTTAATAACGGGTGAT-3') and PoTlR (5'-TTAGTAATAACTGTAGCAG-3') were used to amplify a fragment of 245-bp. The primers used to amplify a product similar to QoI-S isolates were PoTl and PoTlS (5'-TTAGTAATAACTGTAGCAGC-3'). Polymerase chain reactions (PCR) were performed to amplify the cytochrome *b* (*cytb*) gene (CASTROAGUDIN et al., 2015). The PCR products were visualized under ultraviolet light after electrophoresis on a 0.8% agarose gel with 1× Tris-acetate-EDTA and stained with GelRed. The PCR products were sequenced to determine the frequency of the G143A mutation and whether it was stable after five infection cycles. All sequences generated in this work were deposited in GenBank (Table 5).

2.9 Germination, penetration and colonization of QoI-R and QoI-S PoTl isolates by scanning electron microscopy (SEM)

The germination, penetration and colonization of the PoTl isolates 121146 (0S:100R) and Py6038 (100S:0R) were analyzed using scanning electron microscopy (SEM). The detached wheat leaves and/or head rachis were treated with 10 µg mL azoxystrobin. The wheat leaves and/or head rachis untreated were sprayed with distilled water (0 µg mL). Forty-eight hours after fungicide application, some micro-droplets of 20 µL containing conidial suspension from each isolate were inoculated on the abaxial face of wheat leaves and or head rachis. Eight detached wheat leaves and/or head rachis were inoculated for each isolate, with an experimental unit represented by a single leaf and/or head rachis. Each wheat leaves and or head rachis was fixed with transparent scotch tape at the bottoms of plastic trays (39 × 24 × 7 cm in length × width × height,

respectively). After that, the plastic trays containing inoculated leaves and or head rachis were kept under the wet chamber at 25 °C in the dark during the whole experiment. The relative humidity was maintained at approximately 90% using cotton plugs moistened with distilled water placed on the sides of the trays. Conidial germination and penetration were evaluated at 6, 12 and 24 hours after inoculation (hpi) in the presence and absence of fungicide selection pressure. Eight samples (5-mm-diameter) were collected using a manual cutter at each sampling time. The samples of each treatment were preserved in a Karnovsky fixative solution [2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.05M sodium cacodylate buffer, $CaCl_2$ 0.001M, pH 7.2 and stored at 4°C for 24 h. After fixation, each sample was washed in 0.05M cacodylate buffer three times and the time between one wash and another was 10 min. The samples were transferred to 1% osmium tetroxide in water for 1 h. After that, the samples were washed in distilled water three times and dehydrated in an acetone gradient (25%, 50%, 75%, 90% and 100%), staying for 10 minutes in each concentration and three times for 10 minutes in the concentration of 100%. The samples were treated in a Balzers CPD 030 critical point drier, in which CO_2 replaced the acetone. The specimens were metalized with a gold layer in the metallizer Balzers SCD 050 sputtering. Finally, images were obtained using a scanning electron microscope LEO EVO 40 and edited using Corel Draw X9® software.

2.10 Data analysis

The data for the experiments *in vitro* and *in vivo*, repeated in time, were submitted to a joint analysis among two experimental replicates to identify differences among the results of the two experiments. The Shapiro–Wilk and Bartlett tests were used to assess the assumptions of analysis of variance (ANOVA) for fitness parameters and competitive ability of the QoI-R and QoI-S PoTl isolates ($p = 0.05$). For the *in vitro* and *in vivo* assays, the statistical differences between fitness parameters and competitive ability of PoTl isolates on wheat leaves and heads were compared by ANOVA's *F* test. The factorial arrangement between PoTl isolate groups and infection cycles was used, comparing the PoTl isolate groups within each cycle and between nine and five infection cycles *in vitro* and *in vivo*, respectively. Within each cycle, the QoI-R and QoI-S PoTl isolate groups were compared by ANOVA's *F* test and between them with the Scott-Knott test ($p \leq 0.05$). For the *in vitro* and *in vivo* assays, Person's correlation was carried out using data

from competitive ability to explore the relationship between infection cycles and mixtures, amount of conidia produced, disease severity and frequency of germination of QoI-R conidia harvested. The test *t* was used to verify the correlation significance. All analyses were performed using the ExpDes.pt package of the statistical software RStudio version 1.2.5033 (FERREIRA and CAVALCANTI, 2009).

3. RESULTS

3.1 Effect of successive infection cycles on the sensibility of the QoIs resistant and sensitive PoTl isolate groups to azoxystrobin

The sensitivity of QoI-R and QoI-S PoTl isolate groups for azoxystrobin was tested throughout nine and five successive infection cycles *in vitro* and *in vivo*, respectively. For both experiments, the EC_{50} value of the QoI-R PoTl isolate group was higher than 10 µg mL^{-1}, indicating that the resistance of PoTl isolates to azoxystrobin remains stable (Figure 1). For the QoI-S PoTl isolate group, no changes in sensitivity to azoxystrobin were observed at the end of the 9th and 5th successive infection cycle *in vitro* and *in vivo*, respectively (Figure 1).

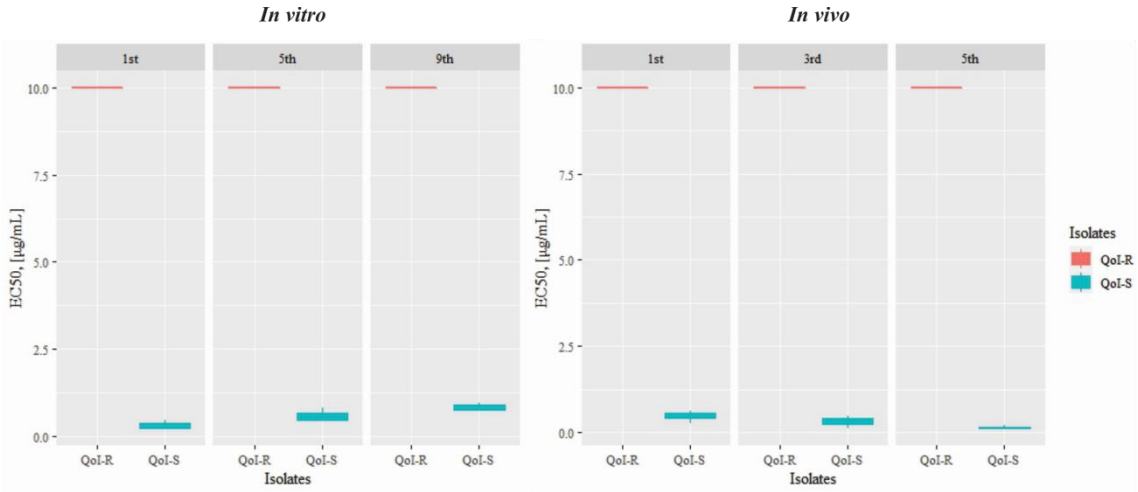


Figure 1. Boxplot showing the distribution of EC_{50} of *Pyricularia oryzae Triticum* lineage isolate group resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) fungicide after successive infection cycles in the absence of azoxystrobin[a].

3.2 Effect of successive infection cycles on fitness parameters of the QoIs resistant and sensitive PoTl isolate groups

In vitro fitness parameters. Within each infection cycle, ANOVA's *F* test detected significant differences between QoI-R and QoI-S PoTl isolates for mycelial growth ($p \leq 0.001$), conidial production ($p \leq 0.001$), and germination ability ($p \leq 0.001$). Among the infection cycles, a significant interaction between cycles and PoTl isolate groups was observed for mycelial growth ($p \leq 0.001$), conidial production ($p \leq 0.001$), and germination ability ($p \leq 0.05$). Between the 1st and the 9th infection cycles, the mycelial growth had significantly increased from 52.2 to 53.2 mm for the QoI-R PoTl isolates group and from 40.4 to 51.2 mm for the QoI-S PoTl isolates group ($p \leq 0.001$) (Table 2). Similarly, there was a significant increase in germination ability from 50.7 to 63.6% for the QoI-R PoTl isolates group and from 38.1 to 51.8 % for the QoI-S PoTl isolates group ($p \leq 0.001$) (Table 2). In contrast, the conidial production reduced significantly for QoI-R and QoI-S PoTl isolate groups between the 5th and the 9th infection cycles ($p \leq 0.001$) (Table 2).

In vivo fitness parameters. Within each infection cycle, significant differences between QoI-R and QoI-S PoTl isolates for incubation and latent period ($p \leq 0.001$), disease severity ($p \leq 0.001$), conidial production ($p \leq 0.001$), and germination ability ($p \leq 0.001$), were identified by ANOVA's *F* test. Among the infection cycles, there was a significant interaction between cycles and PoTl isolate groups for incubation period on wheat heads ($p \leq 0.01$), latent period ($p \leq 0.05$), conidial production ($p \leq 0.001$) and disease severity on wheat leaves ($p \leq 0.001$) and heads ($p \leq 0.05$). Non-significant interaction was found between infection cycles and germination ability ($p = 0.1074$) and incubation period on leaves ($p = 0.1163$). At the end of the 5th infection cycle, the incubation time of the QoI-R and QoI-S PoTl isolate groups on wheat leaves increased significantly from 2.1 to 2.5 and from 2.9 to 3.1 days, respectively ($p \leq 0.001$) (Table 3). In contrast, there was no effect of the infection cycles in the incubation time on wheat heads of the QoI-R and QoI-S PoTl isolate groups ($p \leq 0.001$) (Table 3). On wheat leaves, there was no effect of the infection cycles in the time necessary for conidial production of the QoI-R and QoI-S PoTl isolate groups ($p \leq 0.001$) (Table 4). Similarly, the disease severity on wheat leaves and heads did not suffer the effect of infection cycles, at 7 d.a.i, indicating that QoI-R and QoI-S PoTl isolate groups did not change their disease severity between the 1st and the 5th infection cycles ($p \leq 0.001$) (Table 3; Figure 2).

Table 2. Fitness parameters (mycelial growth, conidial production and conidial germination) of *Pyricularia oryzae Triticum* lineage isolate group resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide, and effects of successive infection cycles *in vitro*.

Isolates[a]	**Mycelial growth (mm)**			**Conidial production (spores mL^{-1})**			**Conidial germination (%)**[b]		
	1st[c]	**5th**[c]	**9th**[c]	**1st**[c]	**5th**[c]	**9th**[c]	**1st**[c]	**5th**[c]	**9th**[c]
QoI-R	52.20 **C***a*	48.19 **B***b*	53.29 **A***a*	1.31×10^5 **A***a*	7.20×10^4 **B***b*	6.35×10^4 **B***b*	50.77 **C***b*	52.75 **B***b*	63.68 **A***a*
QoI-S	40.46 **C***b*	50.56 **B***a*	51.21 **A***a*	1.24×10^5 **A***a*	3.00×10^4 **B***b*	1.52×10^4 **B***b*	38.12 **C***c*	44.87 **B***b*	51.87 **A***a*

[a] Fitness parameters of the *Pyricularia oryzae Triticum* lineage (PoTl) isolate group resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) fungicides were obtained by the joint analysis of two experiments.

[b] The mean values of spores germinated in PD (potato dextrose) medium without quinone outside inhibitor (QoI).

[c] Three evaluations were performed at the end of the 1st, 5th and 9th infection cycles. The mean values of the fitness parameters of the PoTl were obtained by the joint analysis of two experiments. The significance tests were conducted at a level of nominal significance ($p = 0.05$). Means followed by the same letter are not significantly different by Scott-Knott test at $p \leq 0.001$. The capital bold letter compares the fitness parameters of the PoTl isolate groups among the 1st, 5th, and 9th infection cycles. The lower italic letter compares the fitness parameters among isolates in each infection cycle.

Table 3. Fitness parameters (incubation period and disease severity on wheat leaves and heads) of *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide, and effects of successive infection cycles *in vivo*.

Isolates[a]	**Incubation period (days)**						**Disease severity (%)**					
	Leaves			**Heads**			**Leaves**			**Heads**		
	1st [c]	**3rd** [c]	**5th** [c]	**1st** [c]	**3rd** [c]	**5th** [c]	**1st** [c]	**3rd** [c]	**5th** [c]	**1st** [c]	**3rd** [c]	**5th** [c]
QoI-R	2.13**B***b*	2.47**A***a*	2.50**A***a*	3.04**A***b*	3.36**A***a*	3.02**A***b*	46.21**A***a*	39.24**A***b*	36.15**A***b*	72.43**A***a*	54.70**B***b*	68.31**A***a*
QoI-S	2.97**B***b*	3.23**A***a*	3.06**A***b*	3.83**A***a*	3.49**A***b*	3.59**A***b*	15.59**A***b*	17.32**A***b*	24.85**A***a*	42.13**A***b*	36.19**B***b*	60.95**A***a*

[a] Fitness parameters of the *Pyricularia oryzae Triticum* lineage (PoTl) isolate group resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) fungicides were obtained by the joint analysis of two experiments.

[b] Three evaluations were performed at the end of the 1st, 3rd and 9th infection cycles. The mean values of the fitness parameters of the PoTl were obtained by the joint analysis of two experiments. The significance tests were conducted at a level of nominal significance ($p = 0.05$). Means followed by the same letter are not significantly different by Scott-Knott test at $p \leq 0.001$. The capital bold letter compares the fitness parameters of the PoTl isolate groups among the 1st, 3rd and 5th infection cycles. The lower italic letter compares the fitness parameters among isolates in each infection cycle.

Table 4. Fitness parameters (latent period, conidial production and conidial germination) of *Pyricularia oryzae Triticum* lineage isolates resistant (R) and sensitive (S) to Quinone outside inhibitor (QoI) fungicide, and effects of successive infection cycles *in vivo*.

Isolates[a]	**Latent period (days)**			**Conidial production (spores mL^{-1})**			**Conidial germination (%)**[b]		
	1st[c]	**3rd**[c]	**5th**[c]	**1st**[c]	**3rd**[c]	**5th**[c]	**1st**[c]	**3rd**[c]	**5th**[c]
QoI-R	6.63**A***a*	6.60**A***a*	6.68**A***a*	1.52 × 10^5**B***b*	6.19 × 10^5**A***a*	6.55 × 10^5**A***a*	78.31**A***a*	63.56**B**b	78.33**A**a
QoI-S	6.99**A***b*	7.29**A***a*	7.09**A***b*	7.7 × 10^4**B***b*	2.39 × 10^5**A***a*	2.40 × 10^5**A***a*	57.00**A***a*	34.75**C**c	49.31**B**b

[a] Fitness parameters of the *Pyricularia oryzae Triticum* lineage (PoTl) isolate group resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) fungicides were obtained by the joint analysis of two experiments.

[b] The mean values of spores germinated in PD (potato dextrose) medium without quinone outside inhibitor (QoI).

[c] Three evaluations were performed at the end of the 1st, 3rd and 5th infection cycles. The mean values of the fitness parameters of the PoTl were obtained by the joint analysis of two experiments. The significance tests were conducted at a level of nominal significance (p = 0.05). Means followed by the same letter are not significantly different by Scott-Knott test at $p \leq 0.001$. The capital bold letter compares the fitness parameters of the PoTl isolate groups among the 1st, 3rd and 5th infection cycles. The lower italic letter compares the fitness parameters among isolates in each infection cycle.

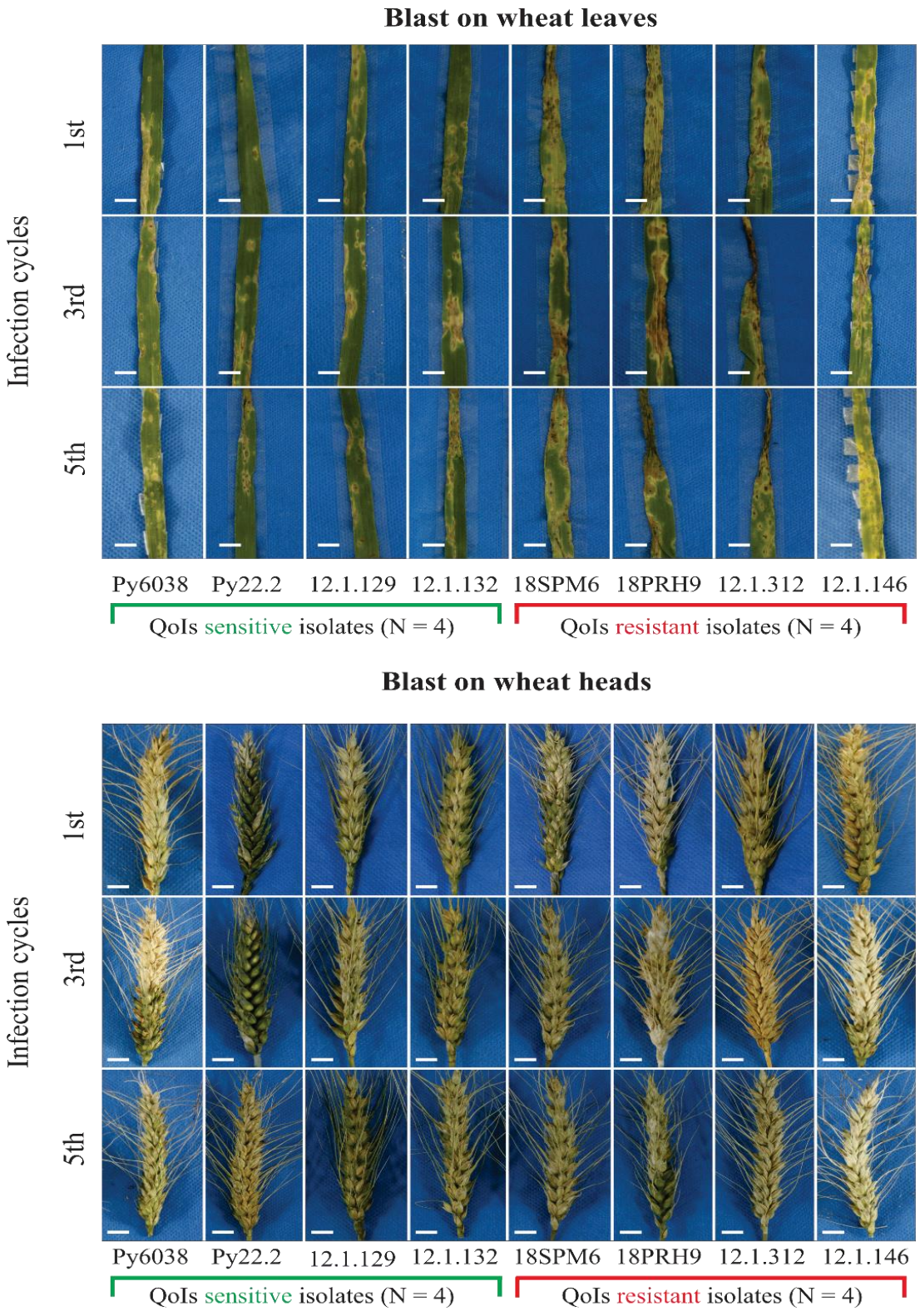


Figure 2. Photographs of blast symptoms on the leaves and heads of wheat cv. Anahuac 75 at 7 days after inoculation (d.a.i) with *Pyricularia oryzae Triticum* lineage isolate group resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) fungicide after successive infection cycles in the absence of azoxystrobin[a]. Bars: 10mm.

On wheat leaves, 21 d.a.i, the conidial production increased significantly from 1.5 × 10^5 to 6.5 × 10^5 conidia mL^{-1} for QoI-R PoTl isolates group and from 7.7 × 10^4 to 2.4 × 10^5 conidia mL^{-1} for QoI-S PoTl isolates group between the 1st and the 5th infection cycle ($p \leq 0.001$) (Table 4). No changes occurred in the germination ability of the QoI-R isolate group between the 1st and the 5th infection cycle. At the same time, a significant reduction from 57.0 to 49.3 was identified for the QoI-S isolate group ($p \leq 0.001$) (Table 4).

3.3 Effect of successive infection cycles on competitive abilities of the QoIs resistant and sensitive PoTl isolates

In vitro competitive ability. Within each infection cycle, significant differences between mixtures of PoTl 121146 (QoI-R) and Py6038 (QoI-S) isolates and conidial production obtained from PDA or oatmeal agar medium at 15 d.a.i. were detected by ANOVA's *F* test ($p \leq 0.001$). Among the infection cycles, there was a significant interaction between the infection cycles and conidial production of mixtures of the QoI-R and QoI-S isolates ($p \leq 0.001$). After nine successive infection cycles, there was an increase in the conidial production capacity for both PoTl 121146 alone (QoI-R) (0S:100R) and PoTl Py6038 alone (QoI-S) (100S:0R) isolates, ($p \leq 0.001$) (Figure 4). Consequently, a positive significant correlation was detected among infection cycles and conidial production of mixtures ($r = 0.35$) ($p \leq 0.01$) (Figure 5). On the other hand, a negative correlation was found between conidial production and mixtures of the QoI-R and QoI-S isolates ($r = -0.26$), ($p \leq 0.10$) (Figure 5).

In vivo competitive ability. Within each infection cycle, ANOVA's *F* test found significant differences between mixtures of QoI-R and QoI-S isolates for disease severity at 7 d.a.i and conidial production obtained from wheat blast lesions at 21 d.a.i. ($p \leq 0.001$). Among the infection cycles, there were significant interactions between infection cycles and the disease severity on wheat leaves and heads inoculated with mixtures of the QoI-R and QoI-S isolates ($p \leq 0.001$). At the end of the 5th infection cycle, a decrease in the conidial production capacity and the severity of disease on wheat leaves and heads were observed for both PoTl 121146 alone (QoI-R) (0S:100R) and PoTl Py6038 alone (QoI-S) (100S:0R) isolates, ($p \leq 0.001$) (Figure 3 and Figure 4). Thus, a negative significant correlation was observed among infection cycles and conidial production of mixtures ($r = -0.88$), ($p \leq 0.001$), besides between infection cycles and disease severity ($r = -0.36$), (*p*

≤ 0.01) (Figure 5). On the other hand, a positive significant correlation was found between conidial production and disease severity of mixtures (r = 0.57) ($p \leq 0.001$) (Figure 5).

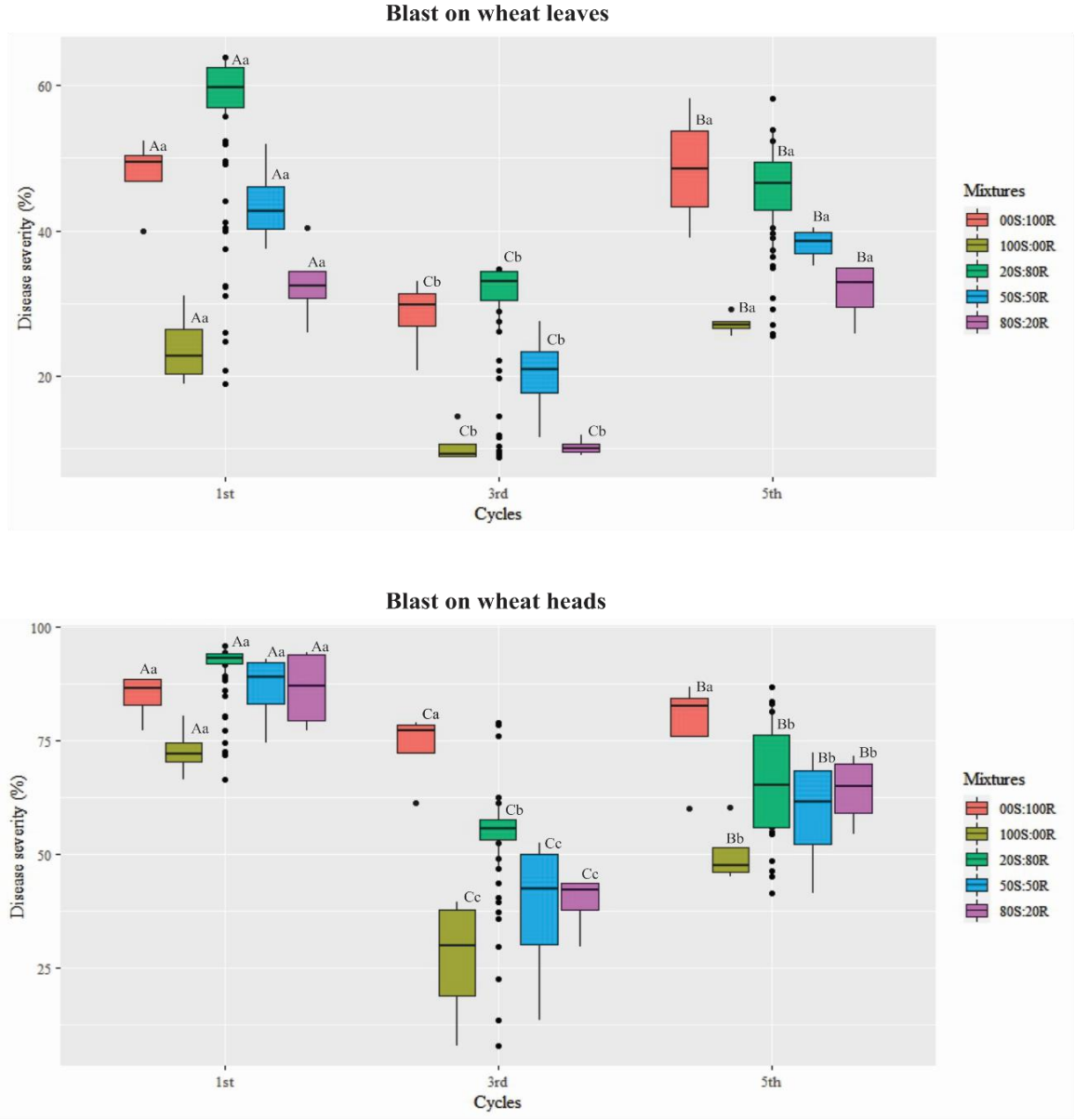


Figure 3. Boxplot distribution of wheat blast severity on the leaves and heads at 7 days after inoculation (d.a.i.) with *Pyricularia oryzae* Triticum lineage conidia mixtures of conidial resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) over the course of five successive infection cycles[a]. [a] Five mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were used in the following proportions: 0S:100R, 20S:80R, 50S:50R, 80S:20R, 100S:0R and 0S:0R, in which R corresponds to the QoI-resistant isolate 121146 and S corresponds to the QoI-sensitive isolate Py6038. Means followed by the same letter are not significantly different by the *Scott-Knott* test ($p \leq 0.001$). The capital letter compares the severity values of the mixtures among the infection cycles. The lower letter compares the severity values among mixtures in each infection cycle. The error bars in each box represent the average standard error of the severity values.

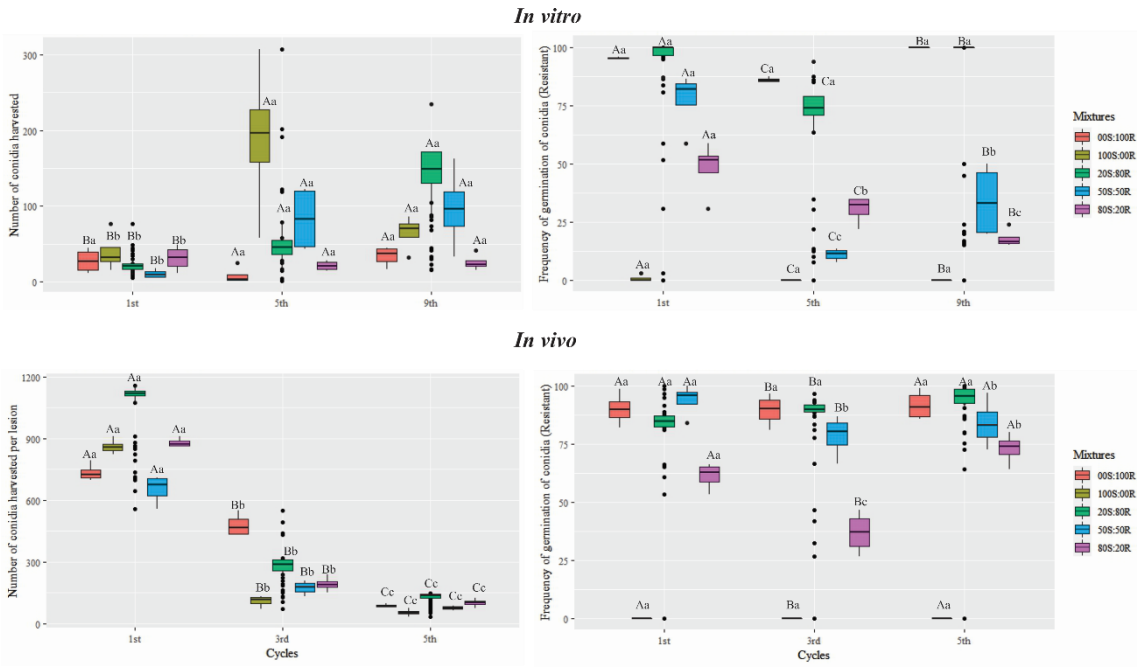
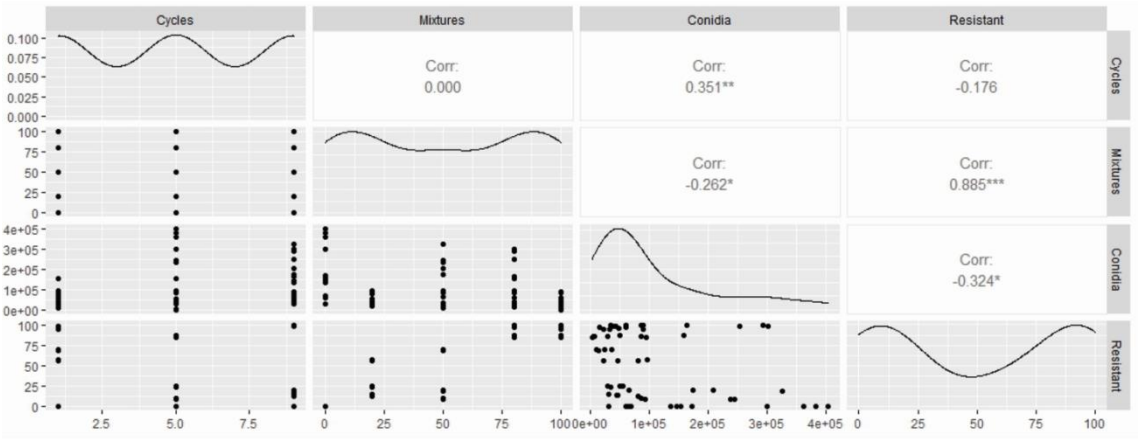


Figure 4. Boxplot showing the number of conidia and frequency of germination of conidia (resistant) of the *Pyricularia oryzae* Triticum lineage obtained from PDA or oatmeal agar medium at 15 d.a.i. and from wheat blast lesions at 21 d.a.i. for mixtures of conidial resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) throughout nine successive infection cycles *in vitro* and five successive infection cycles *in vivo*[a]. [a] Five mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were used in the following proportions: 0S:100R, 20S:80R, 50S:50R, 80S:20R, 100S:0R and 0S:0R, in which R corresponds to the QoI-resistant isolate 121146 and S corresponds to the QoI-sensitive isolate Py6038. Means followed by the same letter are not significantly different by the *Scott-Knott* test ($p \leq 0.001$). The capital letter compares the number of conidia harvested and the frequency of germination of conidia (resistant) of the mixtures among the infection cycles *in vitro* and *in vivo*. The lower letter compares the number of conidia harvested and the frequency of germination of conidia (resistant) among mixtures in each infection cycle *in vitro* and *in vivo*. The error bars in each box represent the average standard error from the number of conidia harvested and the frequency of germination of conidia (resistant) *in vitro* and *in vivo*.

In vitro



In vivo

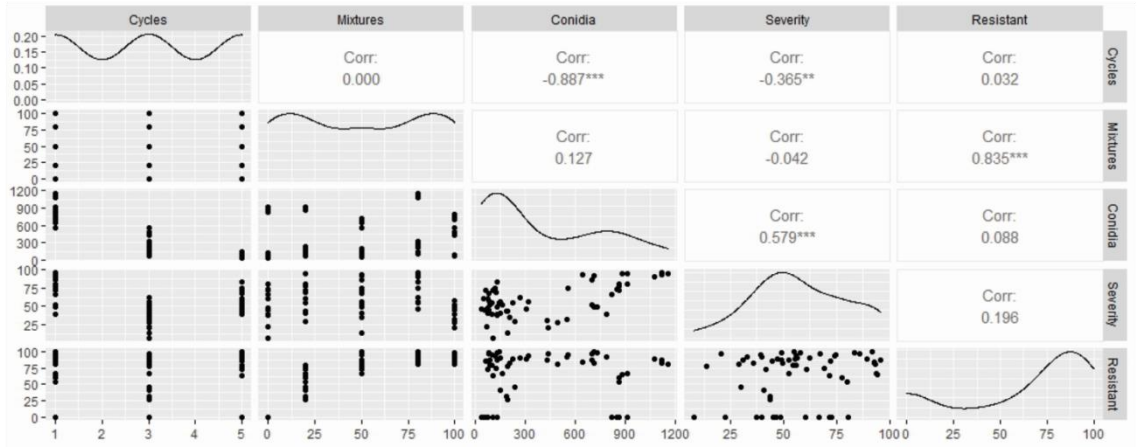


Figure 5. Pearson correlation analysis among the infection cycles and disease severity values, conidial production, and frequency of germination of conidia (resistant) obtained from PDA or oatmeal agar medium at 15 d.a.i. and from wheat blast lesions at 21 d.a.i. for *Pyricularia oryzae* Triticum lineage (PoTl) conidia mixtures of conidial resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI). Diagonal frames represent the distribution of the infection cycles, mixtures of isolates at different ratios, disease severity values, conidial production and frequency of germination of conidia (resistant) throughout nine successive infection cycles *in vitro* and five successive infection cycles *in vivo*; frames below, the bivariate dispersion; frames above, the correlations values and the significance levels. Significance of the correlation was determined by *t* tests. *p* values were represented by: *** = 0.001; ** = 0.01; * = 0.1.

3.4 Effect of successive infection cycles on frequency of QoIs resistant conidia of mixtures of the PoTl isolates

Within each infection cycle, the ANOVA's *F* test detected significant differences between mixtures of QoI-R and QoI-S isolates for frequency of QoI-R germinated conidia obtained from PDA or oatmeal agar medium at 15 d.a.i. and from wheat blast lesions at 21 d.a.i. ($p \leq 0.001$). Among the infection cycles, there were significant interactions between infection cycles and the frequency of germinated conidia QoI-R *in vitro* and *in vivo* ($p \leq 0.001$). At 10 μg mL^{-1} of azoxystrobin, no changes in the frequency of germinated conidia QoI-R for 20S:80R and 0S:100R (100.0%) mixtures were observed after nine successive infection cycles *in vitro* ($p \leq 0.001$) (Figure 4). However, when grown in 10 μg mL^{-1} of azoxystrobin, a decreasing frequency of germinated conidia QoI-R for mixtures was observed between the 1st and 5th infection cycle *in vivo* ($p \leq 0.001$) (Figure 4). For all infection cycles, the frequency of QoI-S conidia with the 100S:0R mixture remained 100.0% *in vitro* and *in vivo* (Figure 4). At 10 μg mL^{-1} of azoxystrobin, a high degree of correlation was observed between mixtures of the QoI-R and QoI-S isolates and conidia resistants *in vitro* ($r = 0.88$) and *in vivo* ($r = 0.83$), ($p \leq 0.001$) (Figure 5).

3.5 Effect of successive infection cycles of QoIs resistant PoTl isolate group on frequency of G143A mutation in *cytochrome b*

A comparison of the sequences generated from the QoI-resistant isolate (100S:0R) and QoI-sensitive isolate (0S:100R) allowed us to confirm the presence of the G143A mutation. For the 1st and the 5th infection cycles, the G143A mutation was found in 100% of the QoI-resistant isolates, indicating that the G143A mutation of the PoTl QoI-resistant isolates remains stable (Table 5, Figure 6A and Figure 6C). The mutation G143A was not found in the sequences of the QoI-sensitive isolates (Table 5, Figure 6B and Figure 6D).

Table 5. Detection of the G143A mutation in the cytochrome *b* (*cytb*) gene of *Pyricularia oryzae Triticum* lineage (PoTl), after successive infection cycles *in vivo*[u].

Isolates[v]	Infection cycle	QoI sensitivity phenotype[x]	Mutation[y]	Number of Isolates	Mutation G143A frequency	NCBI Genbank acession number
100S:0R	1st	S	Gly (GGT)	20	0	OR463358
0S:100R	1st	R	Ala (GCT)	20	100	OR463357
100S:0R	5th	S	Gly (GGT)	20	0	OR463360
0S:100R	5th	R	Ala (GCT)	20	100	OR463359

[u] DNA fragment of the *cytb* gene of 245 bp was successfully amplified and sequenced from 4 isolates of the *Pyricularia oryzae Triticum* lineage (PoTl).

[v] PoTl 121146 alone (QoI-R) (0S:100R) and PoTl Py6038 alone (QoI-S) (100S:0R) isolates of the 1st and 5th infection cycles were obtained from wheat leaves with typical blast symptoms at 7 days after inoculation.

[x] QoI sensitivity phenotypes: S, sensitive; R, resistant.

[y] *cyt b* gene that carries the transition from GGT (G) to GCT (C) at codon 143 confers resistance in *Pyricularia oryzae Triticum* to Quinone oxidase inhibitor fungicide (QoI).

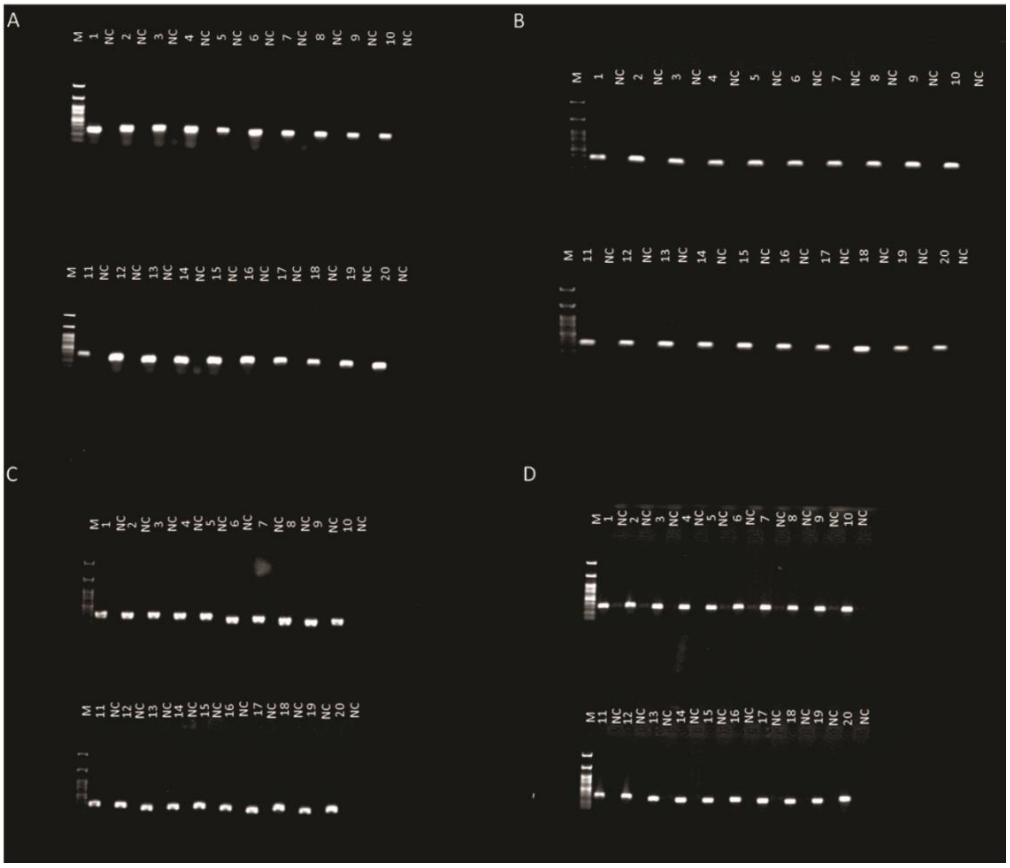


Figure 6. Detection of azoxystrobin-sensitive and resistant isolates of *Pyricularia oryzae* Triticum lineage after successive infection cycles in the absence of azoxystrobin by polymerase chain reaction with the primers used to amplify a product similar to QoI-R (0S:100R) isolates, PoTl/PoTlR and QoI-S (100S:0R) isolates, PoTl/PoTlS, respectively, amplifying a 245-bp product[a]. [a]Twenty single QoI-R (0S:100R) isolates were obtained from typical blast lesions at the 1st infection cycle (A) and 5th infection cycle (C). Twenty single QoI-S (100S:0R) isolates were obtained from typical blast lesions at the 1st infection cycle (B) and 5th infection cycle (D). The negative control (NC) primers were PoTl/PoTlS and PoTl/PoTlR for QoI-R and QoI-S isolates, respectively.

3.6 Germination, penetration and colonization of QoI-R and QoI-S PoTl isolates by scanning electron microscopy (SEM)

There was variation in conidial germination and colonization of the PoTl 121146 (QoI-R) (0S:100R) and PoTl Py6038 (QoI-S) (100S:0R) isolates on the abaxial surface of the wheat leaves and/or head rachis treated with 10 µg mL azoxystrobin and untreated. For both isolates, the germination tubes were observed 6 hpi on the abaxial surface of the wheat leaves and/or head rachis untreated (Figure 7A, 7C and Figure 8A, 8C).

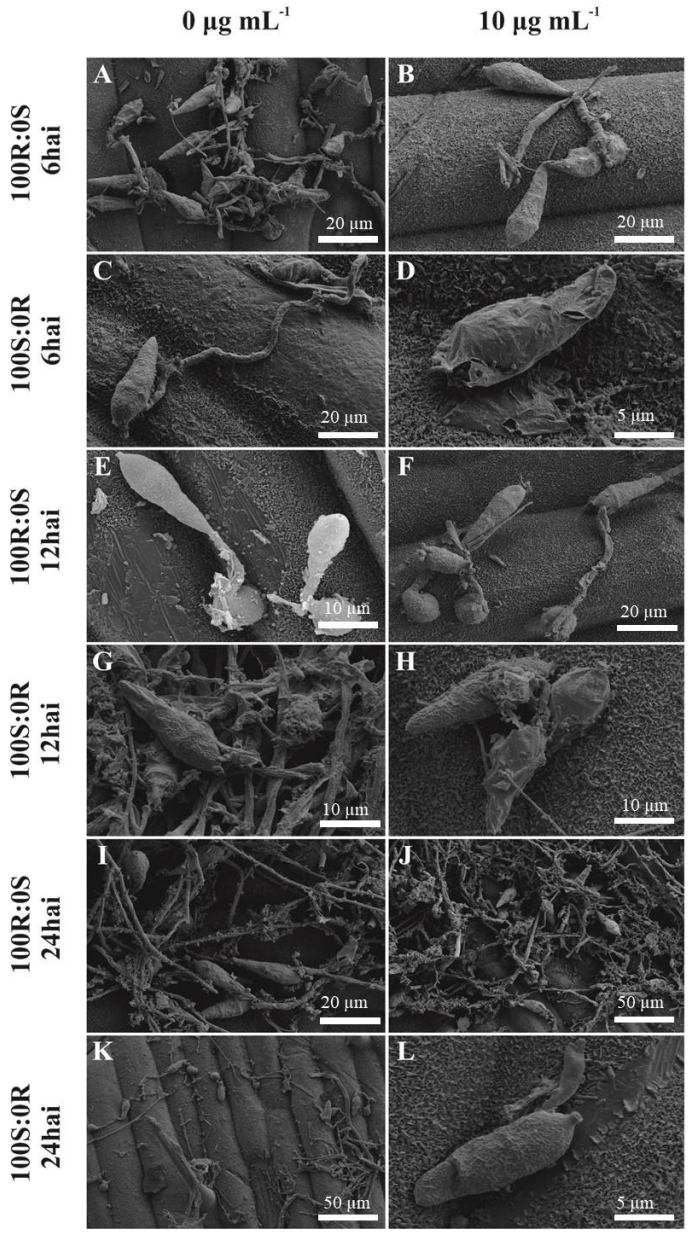


Figure 7. Scanning electron micrographs of germination and penetration of *Pyricularia oryzae Triticum* lineage (PoTl) in 'Anahuac 75' wheat leaves untreated (0 µg mL^{-1}) and treated with 10.0 µg mL^{-1} of azoxystrobin. Conidia germinating at 6, 12, and 24 hours after inoculation[a]. [a]Two mixtures of isolates resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) fungicide were inoculated on wheat leaves following proportions: 0S:100R and 100S:0R, in which R corresponds to the QoI-resistant isolate 121146 and S corresponds to the QoI-sensitive isolate Py6038. Conidia germinating at 6 (A), 12 (E), and 24 (I) hours after inoculation of the 0S:100R isolate on wheat leaves untreated (0 µg mL^{-1}). Conidia germinating at 6 (B), 12 (F), and 24 (J) hours after inoculation of the 0S:100R isolate on wheat leaves treated with 10.0 µg mL^{-1} of azoxystrobin. Conidia germinating at 6 (C), 12 (G), and 24 (K) hours after inoculation of the 100S:0R isolate on wheat leaves untreated (0 µg mL^{-1}). Conidia germinating at 6 (D), 12 (H), and 24 (L) hours after inoculation of the 100S:0R isolate on wheat leaves treated with 10.0 µg mL^{-1} of azoxystrobin.

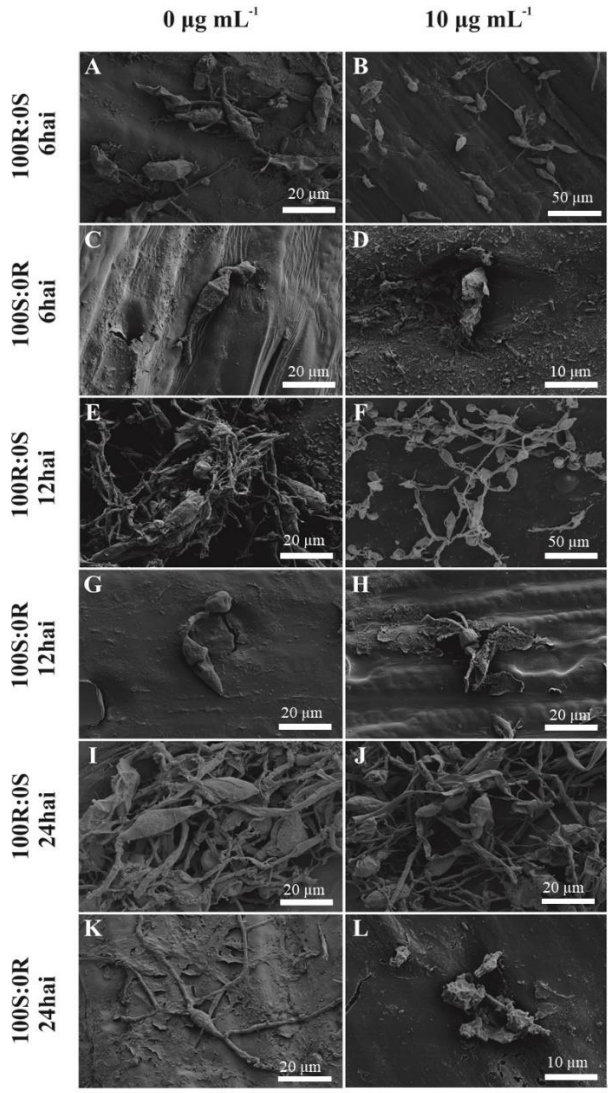


Figure 8. Scanning electron micrographs of germination and penetration of *Pyricularia oryzae Triticum* lineage (PoTl) in 'Anahuac 75' wheat head rachis untreated (0 µg mL^{-1}) and treated with 10.0 µg mL^{-1} of azoxystrobin. Conidia germinating at 6, 12, and 24 hours after inoculation[a]. [a]Two mixtures of isolates resistant (R) and sensitive (S) to quinone oxidase inhibitor (QoI) fungicide were inoculated on wheat head rachis following proportions: 0S:100R and 100S:0R, in which R corresponds to the QoI-resistant isolate 121146 and S corresponds to the QoI-sensitive isolate Py6038. Conidia germinating at 6 (A), 12 (E), and 24 (I) hours after inoculation of the 0S:100R isolate on wheat head rachis untreated (0 µg mL^{-1}). Conidia germinating at 6 (B), 12 (F), and 24 (J) hours after inoculation of the 0S:100R isolate on wheat head rachis treated with 10.0 µg mL^{-1} of azoxystrobin. Conidia germinating at 6 (C), 12 (G), and 24 (K) hours after inoculation of the 100S:0R isolate on wheat head rachis untreated (0 µg mL^{-1}). Conidia germinating at 6 (D), 12 (H), and 24 (L) hours after inoculation of the 100S:0R isolate on wheat head rachis treated with 10.0 µg mL^{-1} of azoxystrobin.

At a dose of 10 µg mL azoxystrobin, the germinal tubes only occurred for the 0S:100R isolate inoculated on the abaxial surface of the wheat leaves and/or head rachis (Figure 7J and Figure 8J). There was no conidial germination for the mixture containing only the QoI-sensitive isolate (100S:0R) inoculated on the abaxial surface of the wheat leaves and/or head rachis treated with 10 µg mL azoxystrobin (Figure 7D, 7H, 7L and Figure 8D, 8H, 8L).

4. DISCUSSION

For over 30 years, the wheat blast in Brazil was considered one of the main obstacles to expanding Brazilian wheat crops. The wheat blast control depends on the fungicides' presence, but their efficacy is rarely over 60% (CERESINI et al., 2018). So far, PoTl populations have also been associated with resistance to three different chemical groups, including QoI, DMI, and SDHI fungicides (VICENTINI et al., 2022). When contrasting populations of the PoTl from Brazil, no change in the sensibility to QoIs was found between older (2012) and newer (2018) populations (VICENTINI et al., 2022). We hypothesize that the persistence of QoI-R PoTl populations with higher fitness and adaptive advantages occurs due to the intensive use of fungicides on wheat crops, exerting strong selection pressure in these populations (DORIGAN, 2022, DORIGAN et al., 2023). In the present study, we assessed the stability of fungicide resistance, fitness advantages, and competitive ability of the QoI-R PoTl isolates over successive infection cycles. Knowledge about the biological and ecological characteristics of the QoI-R PoTl isolate may help to develop anti-resistance strategies. Suppose there is no fitness cost associated with QoIs resistance in PoTl populations. In that case, resistant genotypes may persist on wheat fields, even after successive infection cycles without selection pressure, and QoIs may lose their efficacy permanently. Our results evidenced that the sensitivity, higher fitness, and competitive advantages of the QoI-R PoTl remain stable over time under controlled conditions. This scenario reinforces an urgent need to develop and implement effective anti-resistance strategies against QoI-R PoTl populations in Brazil.

No changes in the sensibility of PoTl were observed for the azoxystrobin over the nine and five successive infection cycles *in vitro* and *in vivo*, respectively. This result is consistent with the previous reports in some fungi species where QoIs resistance was stable, including *B. cinerea* on apple (CHEN et al., 2016), *Cercospora beticola* on sugar beet (MALANDRAKIS et al., 2006), *C. acutatum* on strawberries (FORCELINI et al.,

2018), *E. necator* on grapes (RALLOS et al., 2014), *Lasiodiplodia theobromae* on mango (HE, 2021), *P. viticola* on grapes (GENET, 2006), *P. oryzae* on barley (AVILA-ADAME and KOLLER, 2003). Contrary to the above, QoI-R isolates of *Pyricularia oryzae* on rice (D' ÁVILA, 2022) and *Monilinia fructicola* on peach (CHEN et al., 2014) had no stable resistance to QoIs.

Initially, we determined the fitness parameters of QoI-R and QoI-S PoTl isolate groups *in vitro* after nine infection cycles in the absence of azoxystrobin. Surprisingly, between the 1st and the 9th infection cycles, the QoI-R and QoI-S PoTl isolate groups showed a significant increase in mycelial growth and germination ability, except for conidial production. For the fitness variable *in vivo*, no changes were found for disease severity on wheat leaves and heads, incubation period, latent period, conidial production, and germination ability of the QoI-R PoTl isolates group. Our results for most of the fitness components indicated that the fitness advantage of the QoI-R PoTl isolate group remained stable and with higher fitness than the QoI-S PoTl isolate group throughout nine and five infection cycles *in vitro* and *in vivo*, respectively. These results are consistent with previous studies of PoTl fitness, where QoI-R isolates also showed higher fitness than sensitive ones (DORIGAN et al., 2022).

Posteriorly, we compared the competitive ability of the QoI-R and QoI-S PoTl isolates on wheat leaves and heads. From the 1st to 5th infection cycle, there was a decrease in the disease severity and conidial production on wheat leaves for both QoI-R 121146 alone (0S:100R) and QoI-S Py6038 alone (100S:0R) isolates. Nevertheless, the values of disease severity and conidial production on wheat leaves of the 0S:100R mixture were higher than those from the 100S:0R mixture during all infection cycles (Figure 3 and Figure 4). At discriminatory doses of the fungicide, *in vitro* and *in vivo*, the frequency of germination of conidia (resistant) of the 20S:80R and 0S:100R mixtures harvested remained resistant after successive infection cycles, indicating that no change occurred in the QoIs resistance and competitive advantage of the QoI-R PoTl isolate (Figure 4). These results are consistent with *Erysiphe graminis* f. sp. *tritici*, where some QoI-R isolates competed better than sensitive ones (CHIN et al., 2001). In our present study, it could be explained that the fitness advantage and higher competitive ability of the QoI-R PoTl isolates than its wild-type isolates might be associated with an evolutionary compensatory process (HAWKINS and FRAAIJE, 2018; DORIGAN et al., 2023).

For the competitive abilities assay, the frequency of the 0S:100R isolates with G143A was 100%, even after five infection cycles (Table 5). This result confirms that

PoTl resistance to QoI fungicides associated with G143A mutation is stable without selection pressure. A similar result was observed in some fungi species carrying G143A mutation where QoIs resistance was stable, including *Alternaria alternata* pathotype *tangerine* on citrus (VEGA and DEWDNEY, 2014), *Alternaria alternate* on potato (DING et al., 2009), *Botrytis cinerea* on apple (CHEN et al., 2016), *Colletotrichum acutatum* on strawberries (FORCELINI et al., 2018), *Erysiphe necator* on grapes (RALLOS et al., 2014), *Magnaporthe grisea* on barley (AVILA-ADAME, 2003), *Phakopsora vitícola* on grapes (GENET, 2006), and *Z. tritici* (FOUCHÉ et al., 2022) on wheat exhibit stable QoI resistance and no fitness costs. On the other hand, *Magnaporthe oryzae* on perennial ryegrass (MA and UDDIN, 2009) and *Pyricularia oryzae* on rice (D'ÁVILA, 2022) have no stable QoI resistance, which is associated with fitness costs.

Taken together, our results confirm and extend earlier findings that resistance to QoIs in PoTl populations is stable, suggesting that the spread of resistant mutants may persist after successive infection cycles under field conditions. Moreover, there was no change in fitness advantage and competitive ability of the QoI-R PoTl isolates, even after planta infection cycles, suggesting its persistence in wheat fields over time. It is consistent with the absence of fitness cost associated with QoI resistance in *Colletotrichum acutatum* on strawberries and *Phakopsora pachyrhizi* on soybeans (FORCELINI et al., 2018, KLOSOWSKI et al., 2016). Our results, per si, contrast with the Evolutionary Theory, whose foundation is that fungicide resistance is associated with a fitness cost (HAWKINS and FRAAIJE, 2018).

Both stability of resistance to QoIs and persistence of higher fitness of QoI-R PoTl populations reported in our present study may lead to relevant consequences in the integrated management of wheat blast. Therefore, the use of fungicides belonging to different chemical classes or application of QoIs combined with multi-site fungicides with a low risk for resistance has been some adopted strategies to reduce the spread of QoI-R PoTl populations (VAN DEN BOSCH et al., 2014). Although both strategies have been used in practice, fungicide mixtures have become routine resistance management strategies when the fitness cost is not associated with resistance (BARDAS et al., 2008). Among site-specific fungicides, tebuconazole and benzovindiflupyr can still be used for the management of wheat blast, as well as co-formulation with multisite fungicides (CAZÓN et al., 2023). Adopting these anti-resistance strategies would prevent the selection of the PoTl populations with higher fitness, adaptative advantages, and resistance to fungicides in Brazilian wheat fields. In the future, more research should

investigate whether the higher fitness and competitive advantage of the QoI-R PoT populations may increase or be reduced under fungicide selection pressure.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

7. DATA AVAILABILITY STATEMENT

The fitness data that supports the findings of this study are available upon request.

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