UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE ENGENHARIA
CAMPUS DE ILHA SOLTEIRA

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UNDERSTANDING THE EVOLUTION OF FUNGICIDE RESISTANCE IN FIELD POPULATIONS OF THE WHEAT BLAST PATHOGEN FROM BRAZIL

Ilha Solteira

2022

PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

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Tese apresentada à Faculdade de Engenharia de Ilha Solteira – UNESP como parte dos requisitos para obtenção do título de Doutora em Agronomia. Especialidade: Sistemas de Produção.

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2022

FICHA CATALOGRÁFICA
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V633u

Vicentini, Samara Nunes Campos.
Understanding the evolution of fungicide resistance in field populations of the wheat blast pathogen from brazil / Samara Nunes Campos Vicentini. -- Ilha Solteira: [s.n.], 2021
116 f. : il.

Tese (doutorado) - Universidade Estadual Paulista. Faculdade de Engenharia de Ilha Solteira. Especialidade: Sistemas de Produção, 2021

Orientador: Paulo Cezar Ceresini
Coorientador: Silvino Intra Moreira
Inclui bibliografia

1. Integrated disease management. 2. Epidemic monitoring. 3. *Pyricularia oryzae Triticum lineage*. 4. Strobilurin. 5. Triazoles. 6. SDH inhibitors.

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CRB/8 - 9999

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Ilha Solteira

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Understanding the evolution of fungicide resistance in field populations of the wheat blast pathogen from Brazil

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AGRADECIMENTOS

Agradeço primeiramente a Deus por toda luz que tenho recebido para trilhar os meus caminhos e toda a força que tem me concedido, para que eu possa continuar a alcançar meus sonhos e objetivos.

Aos meus pais, José do Carmo Silva Campos e Maria Luzinete Nunes Campos, por serem meus exemplos de vida, que me inspiram.

Ao meu marido Alan Henrique Vicentini, pelo amor incondicional, pela confiança, cumplicidade, suporte emocional e todo apoio durante todos esses anos de relacionamento.

Aos meus irmãos, sogros, cunhados e sobrinhos pelo apoio, amizade, troca de ensinamentos e experiências ao longo dessa caminhada.

À Universidade Estadual Paulista “Júlio de Mesquita Filho”, em especial à Faculdade de Engenharia, Campus de Ilha Solteira e ao Programa de Pós-graduação em Agronomia pelo ensino, estrutura e funcionários.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de doutorado concedida e pela possibilidade de realização do doutorado sanduíche (Processo número: 88887.570719/2020-00).

Ao NIAB - National Institute of Agricultural Botany, Cambridge, Reino Unido, por ter me recebido no meu tão sonhado intercâmbio na Inglaterra, mesmo que tenha sido em tempos tão difíceis de pandemia.

Ao meu orientador, Prof. Dr. Paulo Cezar Ceresini, por toda a confiança que depositou em mim e ao meu trabalho, pela compreensão, paciência e valiosos ensinamentos que me transmitiu ao longo de todos esses anos.

Aos amigos do Programa de Pós graduação em Agronomia da UNESP por compartilharem todos os momentos durante o doutorado.

Aos amigos de trabalho do Laboratório de Fitopatologia Molecular da UNESP, em especial Abimael, Loane, Tatiane, Diego, Silvino, Ana Carolina, Suzany, Maikon, Fabio, Tamires, Katherin, Gabriela, Bruna, Lucas e Félix, pela troca de experiências, amizade e companheirismo ao longo desses anos.

Aos pesquisadores do NIAB e amigos do Disease Control Group, em especial ao Dr. Bart Fraaije e a Dra. Nichola Hawkins pelo acolhimento e ensinamentos durante meu doutorado sanduíche.

Aos profissionais do IDR – Paraná, Londrina e à empresa Geoclean que nos deram suporte nas viagens e nas coletas realizadas neste trabalho.

Aos membros da minha banca examinadora por terem aceitado meu convite, pelas contribuições e por dedicarem um tempo valioso para a leitura do meu trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

“…There’s nothing you can know that isn't known
Nothing you can see that isn't shown
Nowhere you can be that isn't where you're meant to be…

… nothing you can do that can’t be done...

All you need is love

The Beatles

RESUMO

Algumas doenças de plantas estão se tornando difíceis de serem controladas devido à falta de cultivares resistentes dos hospedeiros e à disponibilidade limitada de fungicidas para proteção das culturas. A fim de aumentar a vida útil dos novos princípios ativos fungicidas atualmente disponíveis, estratégias de manejo integrado de doenças baseadas em "evolução inteligente" (i.e., guiada) dos fitopatógenos são necessárias. Neste contexto, o trabalho visou estudar os cenários históricos e atuais de resistência a fungicidas do fitopatógeno *Pyricularia oryzae* linhagem *Triticum* (*PoT*l), agente causal da brusone do trigo, doença fúngica importante à cultura no Brasil, e de manejo difícil, porque vários grupos de fungicidas (por exemplo, inibidores da esterol desmetilase, da quinona e da succinato desidrogenase) tornaram-se (ou estão se tornando) ineficazes. No primeiro capítulo, populações de campo brasileiras do patógeno da brusone do trigo, amostradas de diferentes regiões geográficas em 2012 e 2018 mostraram ser resistentes aos fungicidas QoI (estrobilurinas) e DMI (azoles), também, níveis moderados de resistência a SDHI foram detectados em cinco das seis populações de campo amostradas em 2012 e na maioria das cepas isoladas em 2018. No segundo capítulo, objetivamos testar se a resistência aos fungicidas DMI e SDHI detectada em *PoT*l era devida ao(s) mecanismo(s) MDR mediados por bomba de efluxo, caracterizando a sensibilidade de isolados de *PoT*l a quatro substratos antifúngicos. Os 16 isolados avaliados foram agrupados em quatro fenótipos distintos de resistência a múltiplas drogas (MDRPs), com base na resistência a combinações de fungicidas e substratos antifúngicos da bomba de efluxo. No terceiro capítulo foi realizado o monitoramento de populações do patógeno da brusone do trigo em Londrina, Paraná, com o uso de um dispositivo automatizado de amostragem de ar, acoplado a um ensaio quantitativo de PCR em tempo real (qPCR). O inóculo de *PoT*l foi detectado em amostras coletadas do ar durante as safras de trigo em 2019 a 2021 e a prevalência de alelos do citocromo b G143A resistente a QoI (QoI-R) em populações de *PoT*l de amostras coletadas do ar também foi determinada para um subconjunto de 10 amostras de DNA positivas para *PoT*l. Para o controle eficaz da brusone é necessário uma melhor compreensão da epidemiologia da doença, conhecimento do cenário atual da sensibilidade/resistência aos principais fungicidas e de novas estratégias para manejo da doença. Este trabalho utilizou ferramentas que certamente podem ser aplicadas para estudar a evolução e

seleção de mecanismos para resistência a fungicidas em outros fitopatógenos que são importantes para o Brasil.

Palavras-chave: manejo integrado de doenças; monitoramento de epidemias; *Pyricularia oryzae* linhagem *Triticum;* Estrobilurinas; Triazóis; inibidores da SDH.

ABSTRACT

Some plant diseases are becoming very difficult to control due to lack of host resistance cultivars and limited availability of fungicides for crop protection. In order to increase the shelf life of new and currently available fungicide actives, “evolution-smart” (i.e., guided) integrated pest management strategies are needed. In this context, this work aimed to study the historical and current scenarios of the plant pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l), the causal agent of wheat blast, an important fungal disease in Brazil, which has difficult control, because several groups of fungicides (e.g. sterol demethylase, quinone outside and succinate dehydrogenase inhibitors) became (or having become) ineffective. In the first chapter, Brazilian field populations of the wheat blast pathogen sampled from different geographical regions in 2012 and 2018 were shown to be resistant to both QoI (strobilurin) and DMI (azole) fungicides, also moderate levels of SDHI resistance were detected in five out of the six field populations sampled in 2012 and in most of the strains isolated in 2018. In the second chapter we tested whether resistance to DMI and SDHI fungicides detected in *PoT*l was due to efflux pump mediated MDR mechanism(s) by characterising the isolates sensitivity to four antifungal substrates. The 16 tested isolates were grouped into four distinct multidrug resistance phenotypes (MDRPs) based on resistance to combinations of fungicides and antifungal efflux pump substrates. In the third chapter, we use to monitoring wheat blast pathogen population at Londrina in Paraná State, an automated air sampling device, coupled with a quantitative real-time PCR (qPCR). *PoT*l inoculum was consistently detected in aerosols during the wheat cropping seasons in 2019 to 2021 and the prevalence of QoI resistant (QoI-R) cytochrome *b* G143A alleles in aerosol populations was also determined for a subset of 10 *PoT*l positive DNA samples. To improve wheat blast control, a better understanding of the disease epidemiology, the fungicide sensitivity/resistance scenario and new disease management strategies are needed. This work focused on generic tools that can be applied to study evolution and selection of fungicide resistance mechanisms in other pathogens that are important to Brazil.

Keywords: integrated disease management; epidemic monitoring; *Pyricularia oryzae Triticum* lineage; Strobilurin; Triazoles; SDH inhibitors.

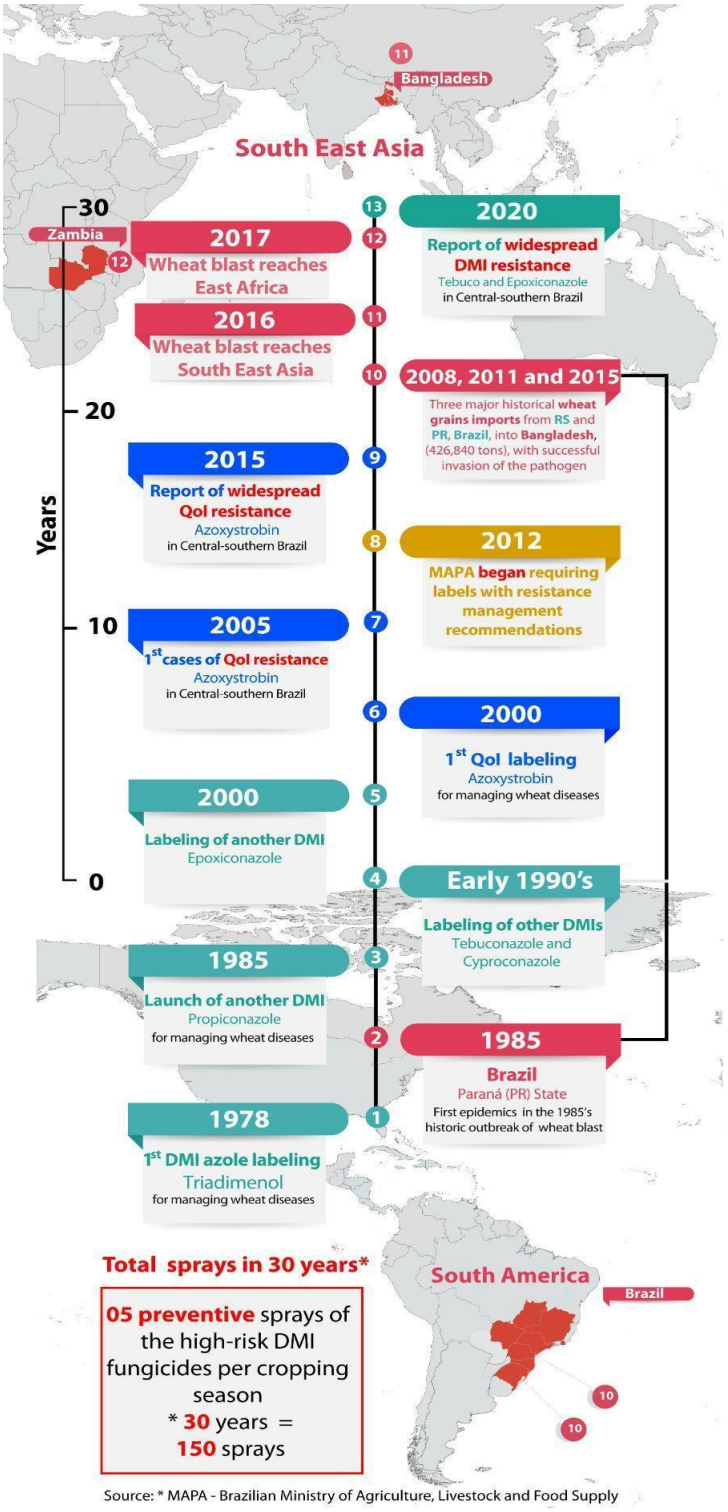
SUMMARY

INTRODUCTION

Wheat production is threatened by recent events of intercontinental expansion of the devastating blast disease, caused by the ascomycetous fungus *Pyricularia oryzae Triticum* lineage (*PoT*l). Since its first emergence in Paraná (PR) State, Brazil in 1985 (Igarashi *et al.*, 1986; Ceresini *et al.*, 2018), wheat blast became well established across all wheat cropping regions from Central-Southern Brazil, as well as from Argentina, Bolivia, and Paraguay, in South America (Ceresini *et al.*, 2019). The pathogen range expansion in South America has been associated with its efficient dispersal by contaminated seeds (CERESINI *et al*., 2018; CERESINI *et al*., 2019). After a series of wheat grains imports from Brazil by the Bangladesh milling industry, between 2008 and 2015 (Figure 1) supposedly followed by the inadvertently utilization as seeds, wheat blast emerged in several wheat cropping regions from Bangladesh, Southeast Asia, in 2016 (ISLAM *et al.*, 2016). One year later, the disease was then reported in Zambia, Eastern Africa (Figure 1), with no clear tracking of the origin of the inoculum that founded this newly established population (TEMBO *et al.*, 2020). Now that the pathogen has reached three continents, the spread to other wheat cropping areas that are still disease free has become a food security concern for countries where wheat is a major staple crop (SINGH *et al.*, 2021).

The impact of the disease in infected wheat fields can be devastating because yield losses in susceptible cultivars can reach up to 100% (KOHLI *et al.*, 2011; CRUZ *et al.*, 2019). Populations of the pathogen can survive and persist in the agroecosystem across cropping seasons mainly because of its wide host range spanning many cultivated grass species including barley, oats, perennial ryegrass (*Lolium perenne*), and signal grass (*Urochloa brizantha*), besides native and invasive grass species (e.g. *Chloris distichophylla*, *Cynodon* spp, *Digitaria insularis*, *Equinochloa crusgalli*, *Panicum maximum*, *Rhynchelytrum repens*, and *Sorghum sudanense* (CASTROAGUDÍN *et al.*, 2017). Therefore, besides the invasive grass species, important forage crop species such as signal grass (MACIEL *et al.*, 2022) and perennial ryegrass are susceptible *PoT*l hosts that can keep a yearlong bridge of fungal inoculum in between wheat cropping seasons. The pathogen can also survive in the off-season in infected seeds (CERESINI *et al*., 2018; CERESINI *et al*., 2019), or as saprophytic mycelium in crop residues, under favorable environmental conditions (PIZOLOTTO *et al.*, 2019).

Figure 1. Timeline of events spanning the emergence and spread of the wheat blast pathogen in South America, the evolution for fungicide resistance in Brazil, and the recent invasion into new areas in Southeast Asia and Africa.



Source: Information derived from Castroagudín *et al.* (2015), Ceresini *et al.* (2018), Ministério da Agricultura, Pecuária e Abastecimento - MAPA (2022), Islam *et al.* (2016), Poloni *et al.* (2021), and Tembo *et al.* (2020).

Knowledge about the duration and importance of each survival strategy is still unknown. No isolated management strategy deployed singly achieves a satisfactory level of control, rendering wheat blast as a very challenging disease for management. Despite this challenge, the two most common strategies for wheat blast within an integrated disease management approach include the deployment of resistant cultivars and fungicides sprays (GOULART *et al.*, 1996; URASHIMA *et al.*, 2004; CRUZ *et al.*, 2016; ASCARI *et al.*, 2021). However, due to the virulence diversity of *PoT*l populations, durable resistance in wheat cultivars is lacking. Besides, the common strategy of deploying wheat varieties with single major gene resistance on a large spatial scale favoured selection for virulence (CRUZ *et al.*, 2010; MCDONALD; LINDE 2014; CRUZ *et al*., 2016; CERESINI *et al*., 2018). Similarly, fungicide spray programs have not been fully effective on controlling the wheat blast disease (PAGANI; DIANESE; CAFÉ-FILHO *et al.*, 2014; SHARMA, 2017; ASCARI *et al*., 2021). This was due to the common strategy of deploying single site-specific fungicide actives on a large favoured, which selects for the prevalence of fungicide resistant lineages of *PoT*l (CASTROAGUDÍN *et al.*, 2015; CERESINI *et al*., 2018; POLONI *et al.*, 2021).

Although the field efficacy of fungicides in wheat fields is considered low, resulting in only a small decrease in blast severity on symptomatic spikes, DMI and QoI fungicides are sprayed regularly and intensively still today for the management of rusts and other wheat foliar and ear associated diseases in Brazil as they have been for the last three decades (REIS *et al.*, 1977; MACIEL, 2011; NAVARINI; BALARDIN, 2012; TORMEN *et al.*, 2013; PAGANI; DIANESE; CAFÉ-FILHO, 2014; DEBONA *et al.*, 2009). A total of 49 fungicides are labelled for managing wheat blast in Brazil, including 17 triazoles and seven mixtures of triazoles (sterol demethylation inhibitors - DMIs) and strobilurin (quinone outside inhibitors - QoIs) (MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO - MAPA, 2022).

Many factors are likely associated with the ineffectiveness of fungicides for managing wheat blast, including: i) the limitations of the current spraying technology to efficiently reach and cover the fungal infection sites in the wheat ears; ii) the lack of an accurate widely adopted disease forecasting and risk monitoring of infection periods, based on weather conditions or inoculum prevalence; iii) the extremely favorable weather conditions for the disease; iv) the pathogen´s high evolutionary potential for virulence and fungicide resistance derived from genetically diverse populations; v) the ability for short and long distance dispersal of fungicide resistant

strains of the pathogen; and vi) the intrinsic inefficacy of some active ingredients (MACIEL *et al.*, 2014; CASTROAGUDÍN *et al.*, 2015; CASTROAGUDÍN *et al.*, 2016; CERESINI *et al.*, 2018). In addition, the wide variety of invasive grass species as alternative hosts to *PoT*l provides a survival niche for the pathogen and a continuous external source of new inoculum (URASHIMA *et al.*, 1993; CASTROAGUDÍN *et al.*, 2015). These plants are located near wheat fields, on which fungicides are not sprayed, playing an important role in the survival and generation of genetic variation in *PoT*l populations, which have a predominantly mixed reproductive system that combines both sexual reproduction and asexual clonal dispersal (URASHIMA; IGARASHI; KATO, 1993; MACIEL *et al*., 2014; CASTROAGUDÍN *et al*., 2015; CERESINI *et al*., 2018; DORIGAN *et al.*, 2019). These factors should be considered when deciding for spraying fungicides to control wheat blast in Africa and Asia.

The assertion that the low efficacy of fungicides against wheat blast in Brazil might have resulted from fungicide resistance is a simple plausible explanation. In fact, *PoT*l populations in Brazil have shown prevalence of high levels of double resistance to DMI and QoI fungicides currently used to wheat blast management (CASTROAGUDÍN *et al*., 2015; OLIVEIRA *et al.*, 2015; DORIGAN *et al*., 2019; POLONI *et al*., 2021; VICENTINI *et al.*, 2022). Therefore, there is an urgent need to identify new, and more effective fungicide molecules and formulations that control *PoT*l on wheat, but as the emergence of fungicide resistance is basically a non-stop process, the fate of new site-specific, narrow spectrum, high risk systemic fungicide molecules towards losing efficacy are hardly avoidable (CERESINI *et al*., 2019).

A timeline of events spanning the historical evolution of resistance to the two major fungicide classes in Brazil is summarized in Figure 1. This timeline virtually began in 1978 with the labelling and wide usage, in Brazil, of the first DMI fungicide for the management of wheat diseases (Figure1). A few years later, the first epidemics of wheat blast was reported with the historic outbreak in Londrina, Paraná State, in 1985, and DMI's were the fungicide choice due to their systemic prophylactic properties. In the same and in subsequent years, few other DMI fungicides were labelled for controlling wheat diseases, which included propiconazole in 1985, tebuconazole and cyproconazole in the early 1990s, and epoxiconazole in the early 2000s (MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO - MAPA, 2022). Despite the reports that fungicides for wheat blast, including the DMIs, were not fully effective on controlling the disease, resistance to DMI fungicides was not explored as a plausible

explanation, until 2020. Even though DMI´s are recognized as a medium to high-risk fungicide group for the emergence of resistance according to FRAC (FRAC, 2022), they were sprayed as single active formulations, with no precautions of adopting anti-resistance strategies (at least not on-label until 2012). In fact, DMI´s were intensively used in calendar-based sprays programs (up to five sprays, on-label, per cropping season) targeting several wheat diseases, including rusts, necrotic leaf spots, Fusarium head blight, and wheat blast - (MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO - MAPA, 2022). With such non-stop selection pressure for up to 30 years of DMI fungicides sprays, the evolutionary outcome was prejudicial since it resulted in the emergence of a highly resistant *PoT*l population across all wheat producing areas from Central-southern Brazil (POLONI *et al*. 2021; Figure 1).

The evolutionary fate of resistance to the high risk QoI fungicides in *PoT*l populations was somewhat distinct, as it happened more quickly. Azoxystrobin was the first QoI fungicide labelled in Brazil in early 2000s (Figure 1). Although the first report of QoI resistance was from 2015, that study included *PoT*l isolates sampled in 2005, only five years after its labelling for managing wheat diseases (CASTROAGUDÍN *et al*., 2015). QoI resistance is now widespread in all wheat cropping regions from Brazil (CASTROAGUDIN *et al*., 2015). Similarly, to the path that resulted in resistance to DMIs, the high risk QoI fungicides were sprayed intensively, on calendar-based schedules, as a single active formulation, exerting a high selection pressure on populations of *PoT*l. Unfortunately, it was only in 2012 that pesticide labelling policies were introduced in Brazil, requiring risk assessment of resistance for pesticides and commercial labels containing resistance management recommendations (Figure 1). The adoption of such anti-resistance strategies is challenging for growers and wheat health extensionists alike without an alternative system to the prevalent calendar-based spraying (POLONI *et al.*, 2021).

In recent years, new fungicide formulations have been labelled and introduced for managing wheat diseases in Brazil. These fungicides include the second-generation carboxamide fluxapyroxad, a succinate dehydrogenase inhibitor (SDHI) that are considered at supposed medium risk for fungicide resistance emergence. Unexpectedly from the point of view of anti-resistance strategies, these fungicides were co-formulated with high risk QoI or DMI molecules (pyraclostrobin or epoxiconazole), for which resistance has been reported for *PoT*l populations

(MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2022; Poloni *et al.*, 2021).

The onset of double fungicide resistance in populations of *PoT*l in Brazil indicated that the anti-resistance strategies (if any at all) adopted in the country in the last two decades have failed, requiring fast actions and additional approaches, blended with the integrated management (IDM) of wheat blast (VICENTINI *et al*. 2022). The joint adoption of anti-resistance and IDM strategies should be coordinated locally, taking into account the particular circumstances of fungicide resistance in each country or region (METHA 2014; CERESINI *et al*., 2018). These strategies should be based on knowledge of the pathogen's population biology, and factors related to the epidemiology of the disease, which unfortunately are still missing.

In order to rationalize fungicide inputs (e.g. choice, timing, dose-rate, spray number and mixing/alternation), to test anti-resistance strategies aimed to delay evolution and spread of resistance of existing fungicides (azoles and QoIs) and new actives (SDHIs) and to future new actives entering the market, we need high performance monitoring tools, enabling quantitative measurement of pathogen´s inoculum levels and detection of fungicide resistant alleles, in combination with disease forecasting. Airborne inoculum dissemination is one of the most important ways of transportation of the pathogen to neighbouring fields (Urashima *et al.*, 2009). Monitoring data of *PoT*l conidia in the air can be important to develop forecasting models for blast disease (DANELLI *et al.*, 2019), although airborne *PoT*l monitoring studies are still scarce.

This research has three chapters to elucidate the historical and current scenarios of *PoT*l fungicide resistance in Brazil. In the first chapter of this Thesis presents a paper published on *Plant Pathology*, we determined the SDHI baseline sensitivity of *PoT*l populations from 2012 and monitored the prevalence of resistance to QoI, DMI and SDHI fungicides in current Brazilian field populations of the wheat blast pathogen sampled in 2018 - 2019.

Because *PoT*l isolates showing reduced sensitivity to SDHI reveleaded no target-site mutations at the sdh-B, C or D genes [besides showing multiple resistance to azoles and QoI] we suspect that the resistance to SDHI may be due to the mechanism described as overexpression of efflux pumps encoded by ATP-binding cassette (ABC) transporters and/or major facilitator superfamily (MFS) transporters (OMRANE *et al.*, 2015; HAWKINS; FRAAIJE, 2018). Therefore, the second chapter of

this Thesis presents a paper already accepted for publication by Agronomy, in which we tested the hypothesis that QoI-, DMI- and/or SDHI-resistant isolates of *PoT*l are also resistant to efflux pump substrates.

The third chapter in this Thesis brings a unique contribution on the application of inoculum surveillance tools based on real-time quantitative detection of the pathogen spores in air samples, using newly developed, compact, high-volume cyclone samplers able to process 10 L of air min^{-1}, from Burkard Agri, United Kingdom (https://www.youtube.com/watch?v=1qehH5zlq6w). Levels of airborne spores determined based on a real time quantitative PCR (RT qPCR) assay were linked to weather data and these data sets could be used to improve the prediction of wheat blast epidemics. Effect of wheat free cropping period between seasons on airborne inoculum qPCR detection was measured and QoI resistant *cytB*-associated allele detected and quantified using pyrosequencing of PCR amplicons from positive samples.

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CHAPTER 1: MONITORING OF BRAZILIAN WHEAT BLAST FIELD POPULATIONS REVEALS RESISTANCE TO QoI, DMI AND SDHI FUNGICIDES

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https://doi.org/10.1111/ppa.13470

Received: 18 February 2021 | **Accepted:** 18 August 2021

ABSTRACT

Wheat blast is one of the most important and devastating fungal diseases of wheat in South America, South-east Asia, and now in southern Africa. The disease can reduce grain yield by up to 70% and is best controlled using integrated disease management strategies. The difficulty in disease management is compounded by the lack of durable host resistance and the ineffectiveness of fungicide sprays. New succinate dehydrogenase inhibitor (SDHI) fungicides were recently introduced for the management of wheat diseases. Brazilian field populations of the wheat blast pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l) sampled from different geographical regions in 2012 and 2018 were shown to be resistant to both QoI (strobilurin) and DMI (azole) fungicides. The main objective of the current study was to determine the SDHI baseline sensitivity in these populations. Moderate levels of SDHI resistance were detected in five out of the six field populations sampled in 2012 and in most of the strains isolated in 2018. No association was found between target site mutations in the *sdhB*, *sdhC*, and *sdhD* genes and the levels of SDHI resistance, indicating that a pre-existing resistance mechanism not associated with target site mutations is probably present in Brazilian wheat blast populations.

Keywords: fluxapyroxad; fungicide resistance; nontarget site resistance; *Pyricularia oryzae Triticum* lineage; second-generation carboxamides; succinate dehydrogenase inhibitors.

1 INTRODUCTION

Wheat blast caused by the hemibiotrophic ascomycete fungal pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l, also known *Pyricularia graminis-tritici*) (CASTROAGUDÍN *et al*., 2016) has been a major disease across central and southern Brazil since it was first described there in Paraná (PR) State in 1985 (CERESINI *et al*., 2018). Following the emergence of wheat blast in Bangladesh in 2016 (Islam *et al*., 2016) and Zambia in 2017 (TEMBO *et al*., 2020), *PoT*l came to the attention of Asian and African governments and the broader international community of plant pathologists, bringing to light an urgent need to develop an action plan to contain the spread of this destructive pathogen in Asia and Africa (ISLAM *et al*., 2016; TEMBO *et al*., 2020).

The two most common disease management strategies (i.e., deployment of resistant cultivars and fungicides) are prone to failure if deployed in isolation. Major gene resistance is likely to be broken rapidly due to emergence of virulent races from the extremely variable *PoT*l population (CERESINI *et al*., 2018). No sources of durable host resistance to *PoT*l have been identified to date (CRUZ *et al*., 2010), and fungicides were shown to be only partially effective (CRUZ *et al*., 2018; PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014). Therefore, integrated disease management (IDM) strategies are needed to minimize crop losses without impacting environmental sustainability. IDM includes cultural and sanitary practices (CERESINI *et al*., 2018; MEHTA, 2014), dynamic deployment of resistant cultivars, sensitive and accurate detection of infected seeds and seed treatment (CERESINI *et al*., 2018), regional disease forecasting systems to improve timing of fungicide applications (FERNANDES *et al*., 2017), improved disease surveillance through training of extension agents and growers (MEHTA, 2014), development of more effective fungicides and spray technologies for treating leaves and ears (CASTROAGUDÍN *et al*., 2015; CRUZ *et al*., 2018; PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014; RIOS *et al*., 2016; ROCHA *et al*., 2014), biological control, and alternative strategies such as wheat biofortification with silicon and phosphite (CERESINI *et al*., 2018; PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014). The adoption of IDM strategies should be coordinated across a local landscape, taking into account the specific climatic and agronomic factors associated with each affected country or region. Effective strategies for managing wheat blast should be developed and deployed based on knowledge of *PoT*l biology and wheat

blast epidemiology, including the pathogen's disease cycle, survival strategy, means of spread, host range, reproductive mode(s), and the most conducive weather conditions for disease development (CERESINI *et al*., 2018).

Fungicides are regularly used to manage wheat blast and other ear-associated diseases in Brazil. Azole (sterol demethylation inhibitor [DMIs]) fungicides have been used since 1989 and quinone outside inhibitors (QoIs) since 2001. However, the field efficacy of fungicides is considered low, resulting in only small decreases in blast severity on symptomatic spikes (CRUZ *et al*., 2018; PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014). Disease control and reduced crop losses were found only when mixtures of azoles and QoIs were applied early in the season on moderately resistant wheat varieties under low or moderate disease pressure (PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014; RIOS *et al*., 2016; ROCHA *et al*., 2014). In such cases, the effectiveness of applications at early heading and early grain-filling stages seemed to be associated with a reduction in *PoT*l inoculum produced on the lower leaves, leading to a reduction in ear infections (CRUZ *et al*., 2015). Recent recommendations to manage the disease in Asia and Africa included spraying of DMIs combined with QoIs (i.e., tebuconazole with trifloxystrobin). In Brazil, these fungicides provided only partial control under high disease pressure, reducing disease incidence by only 50% in comparison to nontreated plots (CERESINI *et al*., 2018; CRUZ *et al*., 2018).

The limited efficacy of fungicide treatments on wheat blast in Brazil is probably due to many factors, including the difficulty of reaching the infection sites on spikelets, the high genotypic and virulence diversity of *PoT*l strains, highly favorable weather conditions for disease dev environmental sustainability development coupled with high levels of varietal susceptibility (CRUZ *et al*., 2010; FERNANDES *et al*., 2017), and the inherent inefficacy of some active ingredients (CRUZ *et al*., 2018; PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014). Another facet of wheat blast that compromises the efficacy of chemical management is that *PoT*l has a broad host range, including several invasive grass species growing in or near wheat fields, on which fungicides are not sprayed (CASTROAGUDÍN *et al*., 2015; DORIGAN *et al*., 2019), but which then provide a continuous external source of new inoculum (CASTROAGUDÍN *et al*., 2016). A total of 49 fungicides have been labelled for wheat blast in Brazil, including 17 azoles and seven mixtures of azoles and QoIs. These two fungicide groups have been used intensively for management of rusts and other wheat foliar diseases for one to three decades (MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO -

MAPA, 2021). Guidelines from MAPA recommend that sprays of fungicides should be carried out preventively, every 14 days, from the onset of the wheat flowering stage to the appearance of the first blast symptoms, up to a maximum of four applications per crop cycle. Although these fungicides were used intensively over many years to manage wheat diseases, it was never suggested that their low efficacy against wheat blast was a result of fungicide resistance.

Resistance to both azole (tebuconazole and epoxiconazole) and QoI fungicides (azoxystrobin and pyraclostrobin) was recently found to be pervasive in *PoT*l populations across the major wheat-growing areas of central and southern Brazil (CASTROAGUDÍN *et al*., 2015; CERESINI *et al*., 2018; DORIGAN *et al*., 2019; POLONI *et al*., 2021). All six Brazilian populations of *PoT*l tested exhibited high levels of resistance to azoxystrobin, pyraclostrobin, and to both tebuconazole and epoxiconazole in in vitro fungicide sensitivity assays. QoI resistance was linked with a mutation resulting in the substitution of glycine by alanine in codon 143 (G143A) of cytochrome *b*, a well-known mutation conferring high levels of resistance in many other pathogens (CASTROAGUDÍN *et al*., 2015). The resistance mechanism for azoles was not clear, with a few strains carrying an alteration, R158K, in the azole target protein sterol 14α-demethylase (CYP51A) (DORIGAN *et al*., 2019; POLONI *et al*., 2021).

New fungicide formulations introduced in 2017 for managing wheat diseases in Brazil are based on mixtures with the second-generation carboxamide fluxapyroxad, a succinate dehydrogenase inhibitor (SDHI), combined with the QoI pyraclostrobin or the azole epoxiconazole, two active ingredients to which *PoT*l populations were already found to be resistant (CASTROAGUDÍN *et al*., 2015; DORIGAN *et al*., 2019; POLONI *et al*., 2021). Until 2021, MAPA has labelled a total of nine similar fungicide coformulations with fluxapyroxad as active ingredient and a single one with the new SDHI bixafen. Because none of these formulations was labelled for wheat blast, their effectiveness against *PoT*l has yet to be determined. The average number of sprays of SDHI fungicide recommended per cropping season varies from three to four, depending on the commercial product labelled, with a concentration ranging from 42.8 to 58.7 g a.i./ha per spray (MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO – MAPA, 2021).

The SDHI fungicides are grouped into two categories: (a) first generation SDHIs, such as carboxin and oxycarboxin, which have been used since the 1960s against rust fungi, but have limited activity against ascomycetes; and (b) second-generation SDHIs,

which include several molecules independently developed by different agrochemical companies (e.g., bixafen [Bayer], boscalid [BASF], fluopyram [Bayer CropScience], fluxapyroxad [Mitsui Chemicals], isopyrazam [Syngenta], penthiopyrad [Mitsui Chemicals], and sedaxane [Syngenta]). Second-generation SDHI fungicides have broad-spectrum activity on cereal fungal pathogens, including ascomycetes (AVENOT; MICHAILIDES, 2010; SIEROTZKI; SCALLIET, 2013).

The target site of SDHI fungicides is succinate dehydrogenase (Sdh), also referred to as complex II or succinate-ubiquinone oxidoreductase (SQR), an important component of the mitochondrial respiratory chain (AVENOT; MICHAILIDES, 2010). The SDHI molecules bind specifically to the ubiquinone site (Q-site) of complex II, thereby inhibiting fungal respiration and preventing energy production (MIYAMOTO *et al*., 2010). Complex II is composed of two peripheral hydrophilic subunits, SdhA and SdhB, and two subunits integrated into the hydrophobic mitochondrial inner membrane, SdhC and SdhD. These two latter subunits are part of the biggest and the smallest subunit of SDH, respectively (OMURA; SHIOMI, 2007). The binding pocket of SDHI fungicides is formed by Sdh subunits B, C, and D.

The second-generation SDHIs are assessed as medium to high resistance risk fungicides (SIEROTZKI; SCALLIET, 2013). Resistance to SDHI fungicides has been largely associated with single-site mutations (single nucleotide polymorphisms, SNPs) in genes encoding SdhB, SdhC, and SdhD in a range of plant pathogens, including *Botrytis cinerea* on apples, strawberries, and several other hosts (AMIRI *et al*., 2020), and *Corynespora cassicola* causing leaf spot on cucumber (MIYAMOTO *et al*., 2010). Reduced sensitivity (above baseline) to SDHIs was reported in Europe for populations of *Pyrenophora teres* and *Ramularia collo-cyani* on barley (REHFUS *et al*., 2016) and *Zymoseptoria tritici* causing septoria leaf blotch on wheat (DOOLEY *et al*., 2016).

So far, in Brazil, there has been no report of the occurrence of resistance to SDHI fungicides in populations of cereal fungal pathogens, including wheat blast. However, as a medium to high risk fungicide class, we should consider the risk for the emergence of resistance to SDHIs if anti-resistance strategies are not adopted. Such strategies aiming to decrease the fungicide selection pressure on the pathogen populations include limiting the number of SDHI sprays during the cropping season and using fungicide coformulations in mixtures with different modes of action (CERESINI *et al*., 2018).

Because new SDHI formulations were recently labelled for the management of

wheat diseases in Brazil, the main objective of this study was to determine the SDHI baseline sensitivity of older Brazilian populations of the wheat blast pathogen for which resistance to both QoI and DMI fungicides has been reported in previous studies (CASTROAGUDÍN *et al*., 2015; CERESINI *et al*., 2018; DORIGAN *et al*., 2019; POLONI *et al*., 2021). In addition, we investigated the fungicide sensitivity to azoles, QoIs, and SDHIs in newer *PoT*l populations sampled from different geographical areas in Brazil. In cases where isolates of SDHI-resistant wheat blast were found, we also investigated whether target-site mutations were the underlying mechanism of resistance.

2 MATERIALS AND METHODS

2.1 OLDER WHEAT BLAST FIELD POPULATIONS

Six populations of *P. oryzae Triticum* lineage (*PoT*I) from wheat and one of *P. oryzae* (or *P. oryzae Oryza* lineage, *PoO*I) from rice were screened to measure their baseline sensitivity to the SDHI fungicide fluxapyroxad. We then determined the frequency distribution of the resistant and sensitive phenotype categories according to the observed range of EC_{50} values. These isolates of *PoT*I were obtained from infected wheat heads sampled during the 2012 growing season. The population of *P*. *oryzae* was supplied by EMBRAPA Rice and Beans and was sampled in 2007 (Table 1). A total of 157 *PoT*I and nine *PoO*I isolates were screened. All these isolates represented distinct multilocus microsatellite genotypes and were grouped into seven distinct geographical or host populations (CASTROAGUDÍN *et al*., 2015).

2.2 NEWER WHEAT BLAST FIELD POPULATIONS

During the 2018 growing season, 183 *PoT*I strains grouped into three geographical populations were isolated from wheat fields in Minas Gerais (MG), Paraná (PR), and São Paulo (SP) States (Table 1). Isolations were made from ears excised from symptomatic bleached wheat heads. Briefly, each rachis sample was cut into 0.2 - cm fragments, which were surface sterilized in 70% ethanol, transferred to 1% sodium hypochlorite solution for 1 min, and washed in distilled water. The next steps of the isolation process were to determine the frequency of QoI and/or DMI resistant strains within each pathogen population.

Table 1. Description of populations of the *Pyricularia oryzae Triticum* lineage from wheat and *P. oryzae Oryza* lineage from rice chosen to determine baseline sensitivity to the SDHI fungicide fluxapyroxad

	Sampling year	Population	Number of isolates	Wheat/rice varieties	Localities
Pyricularia oryzae Triticum lineage	2012	DF + GO_2012	22	BRS 254, BR 18	Brasília, Distrito Federal; Rio Verde, Goiás
		MG_2012	25	BRS 264, BR 18	Patrocínio and Perdizes, Minas Gerais
		MS_2021	38	BRS Guaramirin	Amambal and Aral Moreira, Mato Grosso do Sul
		PR_2012	33	CD 104	Londrina, Paraná
		RS_2012	17	Unknown	Passo Fundo, São Luiz Gonzaga, São Borja and Três de Mato, Rio Grande do Sul
		SP_2012	22	CD 116	Itaí, São Paulo
Pyricularia oryzae Oryzae lineage	2007	TO_2007	9	Epagri 109, Epagri 114, Piracema, Best 2000	Formoso, Tocantins
Total for older populations [a]			166		
P. oryzae Triticum lineage	2018	MG_2018	9	Unknown	Uberaba, Minas Gerais
		PR_2018	40	Unknown	Londrina, Paraná
		SP_2018	134	Unknown	Itapetininga, São Paulo
Total newer populations			183		

[a]These isolates represent distinct multilocus microsatellite genotypes.

Twelve rachis fragments were transferred to three Petri plates containing potato dextrose agar (PDA; 20.7 g L^{-1} potato dextrose, 15 g L^{-1} agar) medium either amended or not amended with fungicides, including (a) a plate containing only PDA for isolation of sensitive strains; (b) a plate containing PDA amended with azoxystrobin at the discriminatory concentration of 10 µg ml^{-1} to isolate and identify QoI-resistant strains (CASTROAGUDÍN *et al*., 2015); and (c) a plate containing tebuconazole at the discriminatory concentration of 0.3 µg ml^{-1} to isolate and identify DMI-resistant strains (DORIGAN *et al*., 2015); and a plate containing tebuconazole at the discriminatory concentration of 0.3 µg ml^{-1} to isolate and identify DMI-resistant strains (DORIGAN *et*

al., 2019; POLONI *et al*., 2021). Plates were sealed and incubated at 24°C for 3 days after which *PoT*l strains growing in each medium were recovered. For long-term storage of the isolates, sterile filter paper disks were placed on top of pure cultures and left for 15 days until they were completely colonized. Colonized paper disks were then transferred to cryotubes containing silica gel and stored at −20℃ until further use.

2.3 SDHI BASELINE SENSITIVITY TESTING OF OLDER BRAZILIAN WHEAT BLAST POPULATIONS AND SELECTED ISOLATES FROM 2018

The *PoT*l and *PoO*l isolates were reactivated in PDA. After 7 days of growth, mycelium discs from the edges of the new colonies were transferred to oatmeal agar (OA; 60 g L^{-1} oatmeal flour, 15 g L^{-1} agar) and incubated for 15 days at 24 °C with a 12-h photoperiod for sporulation. To harvest spores, the superficial mycelium was scraped with a sterilized spatula, and distilled sterilized water amended with Tween 80 (two drops per litre) was added. The number of spores in suspension was determined using a Neubauer chamber and adjusted to 2×10^4 spores ml^{-1}. The spore suspension was kept for 18 h in a growth chamber at 24 °C with a 12-h photoperiod to induce germination of spores. The experiment with the older populations consisted of 157 *PoT*l and nine *PoO*l isolates, hierarchically grouped by population, as described in Table 1.

To determine the EC_{50} for fluxapyroxad, six fungicide doses were used: 0.0, 0.01, 1, 3, 5, and 50 µg ml^{-1}. A modification of the microtitre plate method was adopted to measure indirectly the fungal growth in each fungicide dose, as described by Fraaije *et al*. (2012). A total volume of 130 µl was dispensed into each microtitre plate well and, for each test and control isolate, the following treatments were included: positive control (120 µl of potato dextrose [PD] broth with- out fungicide + 10 µl of spore suspension, only), negative controls (120 µl of PD broth amended with each of the fungicide doses + 10 µl of sterile distilled water only), and the six doses of fluxapyroxad (120 µl of PD broth amended with different doses of the fungicide + 10 µl of spore suspension). Salicylhydroxamic acid (SHAM) at 0.5 mM was added to the PD broth to suppress alternative oxidase (AOX) activity, following the procedures described by Ma *et al*. (2006). Adding SHAM to the fungicide sensitivity assessment test is warranted to preserve the accuracy of the method by avoiding the detection of false positives for resistance to SDHIs (Figure S1). After incubation for 120 h at 24 °C, the growth at each

fungicide dose was determined indirectly based on light absorbance values measured at 620 nm using the Multiskan FC microplate photometer (Thermo Fisher Scientific). EC_{50} values (concentration sufficient to inhibit 50% of the fungal mycelial growth compared to the positive control) for fluxapyroxad were determined using the software ED50plus v. 1.0 (Instituto Nacional de Enfermedades Respiratorias, Mexico) and converting fungicide concentrations (*x*) into log *x*. Based on EC_{50} values, isolates were grouped into four different sensitivity phenotype classes for fluxapyroxad, similar to those proposed by Amiri *et al*. (2014) for another pathosystem: sensitive (S, EC_{50} < 3 μg ml^{-1}), reduced sensitivity (RS, EC_{50} > 3 and < 20 μg ml^{-1}), moderately resistant (MR, EC_{50} > 20 and < 50 μg ml^{-1}), and highly resistant (HR EC_{50} > 50 and < 100 μg ml^{-1}).

The experimental design consisted of complete randomized blocks, with five replicates per treatment. The entire experiment was repeated once. Analysis of variance (ANOVA) and the Scott– Knott test (at 5% probability) for means comparison were performed in the R environment using the statistical package Agricolae (available at the URL: https://CRAN.R-project.org/package=agricolae).

In another experiment with the 2018 populations, the EC_{50} was determined as described above for 22 *PoT*l isolates (six from MG_2018, nine from PR_2018, and seven from SP_2018), and the following control isolates: nine from *PoO*l, four from *Pyricularia grisea*, one from *Pyricularia* sp., and four *PoT*l isolates from 2012 (12.1.087, 12.1.146, 12.1.183, and 12.1.312). In addition to SDHI sensitivity, we also investigated the sensitivity of these isolates to the QoI fungicide azoxystrobin and to the azole fungicide tebuconazole, following methodology from Castroagudín *et al*. (2015) and Poloni *et al*. (2021). The following fungicide sensitivity phenotype classes were used: S = sensitive, RS = reduced sensitivity, MR = moderately resistant, R = resistant; HR = highly resistant. For the QoI fungicide, considering the qualitative nature of the resistance, isolates were grouped only into two categories: sensitive (S, EC_{50} < 10 μg ml^{-1}), and resistant (R, EC_{50} >10 μg ml^{-1}); for the DMI fungicide tebuconazole, isolates were grouped into five different sensitivity phenotype classes: sensitive (S, EC_{50} < 0.05 μg ml^{-1}), reduced sensitivity (RS, EC_{50} > 0.05 – 0.50 μg ml^{-1}), moderately resistant (MR, EC_{50} > 0.50 – 1.0 μg ml^{-1}), resistant (R, EC_{50} >1.0 – 1.5 μg ml^{-1}), and highly resistant (HR EC_{50} > 1.5 – 2 μg ml^{-1}), as described by Poloni *et al*. (2021); and for the SDHI fluxapyroxad, isolates were grouped into the four different sensitivity phenotype classes as described above.

2.4 SDHI SENSITIVITY STATUS OF NEWER BRAZILIAN POPULATIONS OF THE WHEAT BLAST PATHOGEN SAMPLED IN 2018

Reactivation of *PoT*l fungal cultures, growth conditions for spore production, harvesting, and preparation of spore suspensions were conducted as described above. About 60 % (*n* = 103) of the total number of *PoT*l isolates obtained in our survey (*N* = 183) were randomly selected to determine their sensitivity to the SDHI fungicide fluxapyroxad. We determined the relative growth of each isolate at the discriminatory concentration of 3 µg ml $^{-1}$ and at 0 µg ml $^{-1}$ fluxapyroxad in the presence of 0.5 mM SHAM using the same method as described for testing the SDHI baseline sensitivity. Fungal growth was based on the absorbance measured at 620 nm using the same microplate reader after incubation for 120 h at 24 °C. From the absorbance values obtained at 3 and 0 µg fluxapyroxad ml $^{-1}$, we calculated the relative growth ratio for each isolate. The experimental design consisted of complete randomized blocks, with four replicates per treatment. The entire experiment was repeated once. ANOVA and the Scott–Knott test (at 5% probability) for means com- parison were performed in the R environment using the statistical package Agricolae.

2.5 PHENOTYPING OF IN PLANTA SDHI SENSITIVITY OF TWO GROUPS OF CONTRASTING *PoTL* ISOLATES

For this study, three fluxapyroxad-sensitive (12.1.165, 18MGH19, and 18PRH9) and three-resistant (12.1.312, 12.1.146, and 18SPM6) *PoT*l isolates were chosen. The experimental design adopted was completely randomized with three replicates. Each replicate was represented by one 770 ml pot containing three wheat plants of the susceptible wheat cv. Anauhac 75 with a minimum of three ears in total. The experiment was repeated once. Pots contained Topstrato HT substrate (Vida Verde) and the plants were grown at 25 – 30 °C in a greenhouse, where the pots were fertilized (0.82 g/pot) with the N:P_2O_5:K_2O formula (10:10:10) every 15 days and irrigated daily. For fungal inoculum preparation, 7 mm mycelial discs of each isolate were transferred to 15 Petri dishes containing OA and 15 Petri dishes containing PDA, both amended with 50 µg ml $^{-1}$ chloramphenicol and 50 µg/ml streptomycin. The plates were incubated for 15 days at 25 °C in continuous light for sporulation. After this period, spore suspensions were prepared as described above and adjusted to 2 × 10^4 spores ml $^{-1}$. Ears with immature spikelets from 2-month-old wheat plants were sprayed with one of

two fluxapyroxad concentrations (0 and 5 µg ml $^{-1}$), at the beginning of anthesis. Approximately 48 h after the fungicide treatment, for each *PoT*l isolate, 25 ml of spore suspension was sprayed directly onto the plant ears using a hand sprayer. Negative controls were represented by noninoculated wheat ears sprayed with the two fungicide concentrations. After inoculation, the plants were incubated for 24 h in the dark, at 25 °C and 90% relative humidity and subsequently transferred to a growth chamber with the same conditions and a 12-h photoperiod provided by Osram 33,354 lumen sodium vapour lamps (400 W, Model HQI-T NDL E40 5200K) for 7 days. A digital thermohygrometer (Even) was used to monitor the temperature and relative humidity in the chamber. The levels of wheat blast severity on ears were determined according to the percentage of diseased area assessed in the two opposite sides of the wheat ears, photographed using a Canon digital camera. The assessment was conducted using the Assess image analysis software v. 2.0 (American Phytopathological Society). ANOVA and the Scott–Knott test (α = 5%) for means comparison were performed in the R environment using the statistical package Agricolae. We analysed the effects of groups of isolates by SDHI sensitivity phenotype (N = 2, df = 1), concentration of fluxapyroxad (N = 2, df = 1), and the interaction between SDHI sensitivity phenotype × concentration of fluxapyroxad (df = 1).

2.6 SEQUENCING OF GENES ENCODING FUNGICIDES TARGETS IN A SUBSET OF FUNGICIDE RESISTANT WHEAT BLAST ISOLATES

We examined 17 isolates from the 2012 populations and 22 isolates from the 2018 populations, representing different levels of sensitivity to fluxapyroxad, for variation in the *sdhB*, *sdhC*, and *sdhD* genes encoding the SDH subunits that form the SDHI binding site. To allow sequence comparisons with closely related species, we also sequenced isolates from *P*. *grisea* from *Digitaria sanguinalis* (12.0.082, 12.0.264, 12.0.713, and 12.0.733), *PoO*l (284, 421, 364, 611, 674, 656, 658, 678, and 704), and other *Pyricularia* sp. from *D*. *sanguinalis* (363) available in our fungal collection. For *PoT*l isolates from the 2018 populations, we also assessed variation in the *cytB*, and *CYP51A* and *CYP51B* genes, encoding the target proteins for QoI and DMI fungicides, respectively. The *cytB*, *CYP51A*, and *CYP51B* sequences were deposited in GenBank (accessions MW355014 to MW355026). Genomic DNA was extracted from freeze-dried fungal mycelium using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-

Aldrich), according to the manufacturer's instructions. DNA was quantified on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and diluted to 25 ng µl $^{-1}$ final concentration. PCR amplifications of the *cytB*, *CYP51A*, and *sdh* genes were carried out using the primers listed in Table 2. PCR was conducted in 30-µl volumes containing ultrapure distilled water, 25 ng template DNA, 0.3 µM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 3 µl of 10× PCR reaction buffer, and 0.05 U *Taq* DNA polymerase (Thermo Fisher Scientific). Amplifications were performed in a ProFlex PCR thermal cycler (Applied Biosystems), with cycling conditions as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 1 min, 1 min at the specific annealing temperature for each primer pair (Table 2), and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products were purified and sequenced by the Center for Biological Resources and Genomic Biology (Crebio, UNESP Jaboticabal Campus, Brazil), using an ABI 3730xl DNA Analyzer (Applied Biosystems). DNA sequences were analysed and aligned using the Geneious R v. 9.0.5 (Biomatters) program to identify alleles, haplotypes, and nonsynonymous mutations leading to amino acid changes in inferred proteins sequences. For annotation and inference of protein sequences from the *sdh* sequences, we included the following reference sequences obtained from the NCBI/GenBank: KX231838 (*PoO*l MCR) and NC_017852.1 (*PoO*l 70-15), both from the rice blast pathogen, for *sdhB*; CP034206 (*PoO*l MZ5-1-6, from the blast pathogen on *Eleusine coracana*) and NC_017849 (*PoO*l 70-15) for *sdhC*; and NC_017852 (*PoO*l 70-15) for sdhD.

Table 2. Specific primers for PCR amplifications and for assessment of variation in the cytB, CYP51A, CYP51B, and sdh genes of *Pyricularia oryzae Triticum* lineage from wheat

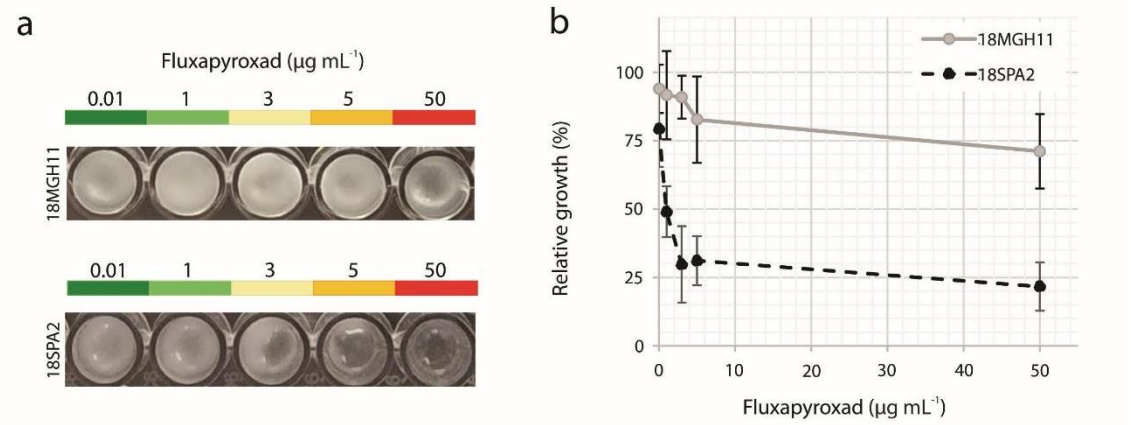
Gene	Primer	Sequences (5' – 3')	T_a (°C)	Reference
cytB	PgCytb-F1	AGTCCTAGT GTAATGGAAGC	55.0	Castroagudín *et al*. (2015)
	PgCytb-R1	ATCTTCAACGTG TTTAGCACC		
CYP51A	*Cyp51A*_278F	CTTTTGTCACTTGTTCTCTGCC	55.0	Dorigan *et al*. (2019)
	*Cyp51A*_662F	GCCCCCATCAACTTCCTAG		
	*Cyp51A*_757R	TGAGGTCCATGTAAACATCG		
	*Cyp51A*_1345R	CAAAGGGCAGGTAAGGACTC		
	*Cyp51A*_1749R	AGAGATATGCCTCATTGCTAAA		
CYP51B	*Cyp51B*_867F	CCTGAGATATAAATGACGGCAGAGA	55.0	Poloni *et al*. (2021)
	*Cyp51B*_2772 R	CTTGTTCTGATCTTCATCTTGGCTG GCTCCCTAGA TTCAATTTTCCT CAG		
	*Cyp51B*_912F	ACCACCCACCAAGATATTGTTTTTG		
	*Cyp51B*_2704 R	AGAAGTTTATGAAGATCGCCCTCAC		
	*Cyp51B*_1556F			
sdh	SDH-B F	GCTTATGTGAGTGTGACCCAAA	59.6	This study
	SDH-B R	TTGATCGACCTGTACTGCTTG		
	SDH-C F	CCAATCATCTAGCTGCTCTTC	56.2	This study
	SDH-C R	CTCTCACCCCTCAAAACACT		
	SDH-D F	AGCAAGTAAACTCGGCAAAGA	59.6	
	SDH-D R	CATCGTTGGTCTCAAACTCGT		

Abbreviation: T_a, annealing temperature.

3 RESULTS

Increasing doses of the SDHI fungicide fluxapyroxad reduced fungal growth (Figure 1). The mean EC_{50} values for the different wheat blast field populations sampled in 2012 and for *PoT*I isolates within these populations differed significantly (Figure 2, Table 3), indicating significant phenotypic variation for fluxapyroxad sensitivity exists across and within field populations. The older *PoT*I populations from DF + GO, MG, MS, and PR_2012 were less sensitive to fluxapyroxad (with mean EC_{50} ranging from 10.96 to 13.15 µg ml $^{-1}$) than the SP and RS_2012 populations (mean EC_{50} ranging from 6.52 to 7.55 µg ml $^{-1}$; Figure 2).

Figure 1. In vitro sensitivity of two phenotypically distinct isolates of *Pyricularia oryzae Triticum* lineage (*PoT*I) to increasing doses of fluxapyroxad in a microtitre assay. (a) Comparison of a highly resistant (18MGH11) with a sensitive (18SPA2) isolate of *PoT*I. (b) Relative growth of the two isolates exposed to different concentrations of fluxapyroxad. Error bars indicate the standard deviation of the sample mean calculated based on eight replicates of each treatment



Analyses of the frequency distribution of fluxapyroxad sensitivity indicated that most of the *PoT*I and *PoO*I isolates screened (*n* = 152, 91.6%) showed reduced sensitivity levels (EC_{50} values between 3 and 20 µg ml $^{-1}$) for fluxapyroxad (Figure 2). Moderate fluxapyroxad resistance (EC_{50} between 20 and 50 µg ml $^{-1}$) was detected in 6.0% of the isolates (*n* = 10), in five out of the six tested field populations of *PoT*I and in the one *PoO*I population. Fluxapyroxad sensitivity (EC_{50} <3 µg ml $^{-1}$) was detected in only 2.4% of the isolates tested (*n* = 4; Figure 2).

Our more recent geographical population sampling from three infected wheat fields in south-eastern Brazil during the 2018 growing season resulted in a total of 183 *PoT*l isolates (Tables 1 and 4). Among these, 20 isolates (10.9%) were resistant either to the QoI fungicides azoxystrobin at the discriminatory concentration 10 µg ml $^{-1}$ or to the DMI fungicide tebuconazole at 0.3 µg ml $^{-1}$, while 163 isolates (89.1%) were resistant to both fungicides (Table 4). Supporting these observations of QoI and DMI resis- tance, the 510 bp fragment of the *cytB* gene for a set of 22 isolates from the 2018 populations (Table 5) was identical to the *PoT*l H1 haplotype carrying the nucleotide change G:C at position 428 in the *cytB* gene (the amino acid change G143A) associated with resistance to QoI fungicides (CASTROAGUDÍN *et al*., 2015). Similarly, the 1551 bp *CYP51A* sequences from all 22 isolates examined were identical to the H1 haplotype found in DMI-resistant *PoT*l isolates carrying the nucleotide change G : A at position 473 (unique amino acid substitution R158K) described by Dorigan *et al*. (2019) and Poloni *et al*. (2021).

The screening of a subset of 103 isolates (56.3% of the total) from the newer populations of *PoT*l to determine their sensitivity to the SDHI fungicide fluxapyroxad indicated that 47.6% (*n* = 49) of the isolates sampled from Minas Gerais, São Paulo, and Paraná wheat fields in 2018 had reduced sensitivity at the discriminatory concentration of 3 µg ml $^{-1}$. The group of resistant isolates showed significantly higher relative growth on fluxapyroxad than the group of sensitive isolates from all three populations (Figure 3). The fluxapyroxad EC_{50} values for 22 randomly selected isolates from these three 2018 populations showed that moderate resistance was detected in 15 isolates (EC_{50} varying from 4.97 to 45.33 µg ml $^{-1}$) and highly resistant phenotypes were detected in five isolates (EC_{50} values ≥ 50 µg ml $^{-1}$). Only two isolates were sensitive to fluxapyroxad (Table 5). Three out of five isolates of *P*. *grisea*, included as additional species in this experiment, also showed high levels of resistance to fluxapyroxad (Table 5).

Figure 2. Frequency distribution of sensitivity to the succinate dehydrogenase inhibitor fungicide fluxapyroxad based on EC_{50} values in populations of *Pyricularia oryzae Triticum* lineage (*PoT*I) sampled from wheat fields in Distrito Federal and Goiás (DF – GO), Minas Gerais (MG), Mato Grosso do Sul (MS), Paraná (PR), Rio Grande do Sul (RS), and São Paulo (SP) in 2012. The population of the sister species *P. oryzae Oryza* lineage (*PoO*l) from rice blast sampled from Tocantins (TO) was included in the assay for comparison. (a) Map showing geographic locations of wheat fields; (b) range of EC_{50} values within different sensitivity phenotypes for fluxapyroxad; (c) frequency distribution of sensitivity phenotypes among populations; (d) distribution of EC_{50} values among isolates in different populations; dashed vertical arrows indicate the boundaries between sensitive, reduced sensitivity, and resistant categories; notched boxplots indicate the median and the distribution of EC_{50} for each population. Shared upper case letters on top of the boxplots indicate that the mean EC_{50} values are not significantly different by the Scott–Knott test at α = 0.05

a. Geographical populations of *Pyricularia oryzae Triticum* lineage sampled from wheat fields

b. Sensitivity phenotypes to the SDHI fungicide fluxapyroxad

EC_{50} µg.mL^{-1}	Categories of sensitivity phenotype
< 3	Sensitive (S)
> 3 to 20	Reduced sensitivity (RS)
> 20 to 50	Moderately resistant (MR)
> 50	Highly resistant (HR)*
	*[detected in *Pg* and in *PoT*I from 2018]

c. Frequency distribution of sensitivity and resistance to the SDHI fungicide fluxapyroxad

Species	Population	Resistance HR	Resistance MR	Sensitivity RS	Sensitivity S
*PoT*I	DF+GO_2012	-	1	20	1
	MG_2012	-	1	23	1
	SP_2012	-	1	21	-
	MS_2012	-	1	37	-
	PR_2012	-	3	30	-
	RS_2012	-	-	17	-
*PoO*l	TO 2007	-	3	4	2
	Total [a]	-	10	152	4
	Percentage	-	**6.0**	**91.6**	**2.4**

d. Resistance to the new SDHI fungicide fluxapyroxad in Brazilian populations of the wheat blast pathogen sampled in 2012

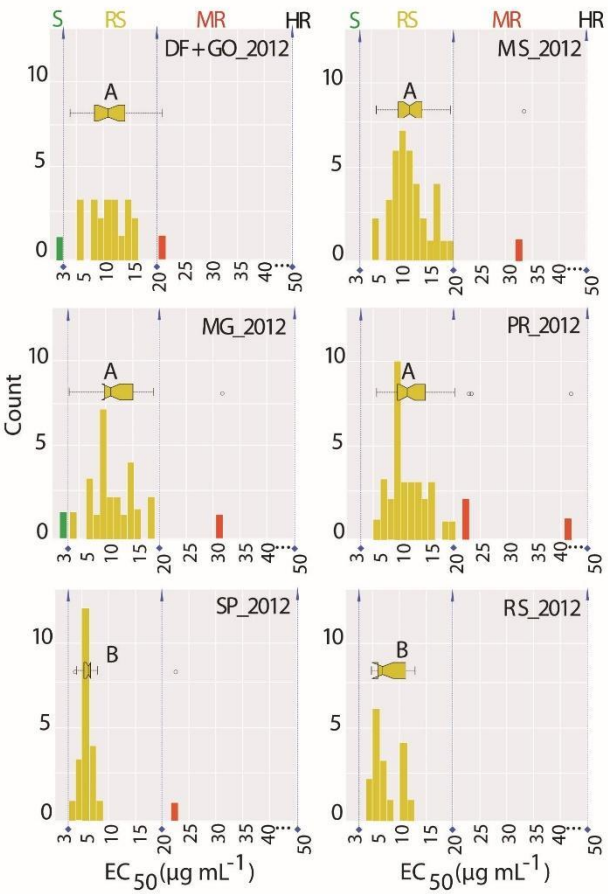


Table 3. Analysis of variance of the EC_{50} values for the succinate dehydrogenase inhibitor fungicide fluxapyroxad seven geographical and host populations of *Pyricularia oryzae Triticum* lineage from wheat and *P. oryzae Oryza* lineage from rice

Source of Variation	*df*	Mean square	*F*	*p*
Populations	5	334.5	36.894	$<2 \times 10^{-16***}$
Isolates (population)	161	113.2	3.128	$<2 \times 10^{-16***}$
Blocks	9	185.4	5.126	$<3 \times 10^{-6***}$
Residual	1474	36.2		

Note: Because no significant interaction was detected between replicates of the experiments and other sources of variation, the two replicates were combined.
***Significant by *F* test at $p \leq 0.001$.

Table 4. Sensitivity to quinone outside inhibitor (QoI) and sterol demethylation inhibitor (DMI) fungicides of field isolates of *Pyricularia oryzae Triticum* lineage from newer populations sampled in 2018

		Resistant isolates			
Location	**No. of isolates**	**QoI**	**DMI**	**Either**	**Both**
Uberaba, MG	9	9	9	0	9
Londrina, PR	40	35	34	11	29
Itapetininga, SP	134	134	125	9	125
Total	183	178	168	20	163

Note: Resistance isolates calculated by growth on potato dextrose agar amended with fungicides at discriminatory concentrations (CASTROAGUDÍN *et al*., 2015; DORIGAN *et al*., 2019; POLONI *et al*., 2021). QoI: azoxystrobin 10 µg ml $^{-1}$; DMI, tebuconazole 0.3 µg ml $^{-1}$.

Table 5. Field isolates of *Pyricularia* spp. from older (2007/2012) and newer (2018) populations according to their category of sensitivity phenotype to three major classes of fungicide, selected for assessing variation of the *sdh* genes

Species and isolate [a]	Population	Sensitivity phenotype[b] QoI azoxystrobin	DMI tebuconazole	Mean EC_{50} (µg ml^{-1})	SDHI fluxapyroxad	Mean EC_{50} (µg ml^{-1})
*PoT*I older populations sampling year: 2012 (*n* = 17)						
12.1.165	DF + GO_2012	S	R	1.26 C	S	1.15 D
12.1.005	MG_2012	R	R	1.20 C	S	2.88 D
12.1.045i	SP_2012	R	R	1.09 D	RS	3.30 D
12.1.087	MG_2012	R	R	1.50 B	RS	3.34 D[c]
12.1.030i	SP_2012	R	R	1.39 B	RS	3.96 D
12.1.037	DF + GO_2012	R	R	1.23 C	RS	3.98 D
12.1.015	MG_2012	S	R	1.33 B	RS	11.63 D
12.1.146	MS_2012	R	R	1.27 C	RS	17.06 C[c]
12.1.176	MS_2012	R	R	1.45 B	RS	17.49 C
12.1.097	MG_2012	R	R	1.44 B	RS	18.48 C
12.1.130	MS_2012	S	HR	1.70 A	RS	19.31 C
12.1.069	DF + GO_2012	R	R	1.35 B	RS	14.01 D
12.1.299	PR_2012	R	R	1.24 C	MR	21.83 C
12.1.314	PR_2012	R	R	1.46 B	MR	21.97 C
12.1.017i	PR_2012	S	MR	0.98 D	MR	22.27 C
12.1.183	MS_2012	R	R	1.03 D	MR	27.02 C[c]
12.1.312	PR_2012	R	R	1.12 D	MR	42.28 B [c]
*PoT*I current population sampling year: 2018 (*n* = 22)						
18PRH9	PR_2018	R	R	1.44 B	S	2.73 D[c]
18SPA2	SP_2018	R	MR	0.79 E	S	2.94 D[d]
18MGH19	MG_2018	R	R	1.31 B	RS	4.97 D
18MGH25	MG_2018	R	MR	0.62 E	RS	4.91 D
18PRD3	PR_2018	R	R	1.41 B	RS	6.41 D
18PRA7	PR_2018	R	R	1.26 C	RS	6.46 D
18SPF5	SP_2018	R	R	1.36 B	RS	6.49 D[d]
18PRA2	PR_2018	R	HR	1.62 A	RS	11.40 D
18PRA6	PR_2018	R	R	1.38 B	RS	18.46 C
18SPK6	SP_2018	R	R	1.19 C	RS	17.86 C[c]
18PRC5	PR_2018	R	R	1.42 B	RS	18.34 C[d]
18SPQ9	SP_2018	R	R	1.17 C	RS	24.71 C
18SPT7	SP_2018	R	R	1.05 D	MR	24.71 C
18PRB5	PR_2018	R	R	1.34 B	MR	29.65 C
18MGF3	MG_2018	R	R	1.18 C	MR	31.51 C
18PRA1	PR_2018	R	R	1.46 B	MR	35.82 B
18MGH21	MG_2018	R	R	1.36 B	MR	45.33 B[d]
18MGF23	MG_2018	R	R	1.16 C	HR	≥50 A
18PRC9	PR_2018	R	R	1.11 D	HR	≥50 A
18MGH11	MG_2018	R	R	1.31 B	HR	≥50 A[c]

*PoT*I current population sampling year: 2018 (*n* = 22)						
18SPC10	SP_2018	R	R	1.33 B	HR	≥50 A
18SPM6	SP_2018	R	R	1.41 B	HR	≥50 A
*PoO*I older population sampling year: 2007 (*n* = 9)						
611		S	S	0.009 G	RS	9.76 D
674		S	S	0.019 G	RS	11.42 D
364		S	S	0.006 G[c]	RS	12.32 D[d]
678		S	S	0.014 G	RS	15.35 D
658		S	S	0.027 G	MR	23.79 C
284		S	S	0.039 F	MR	26.89 C
704		S	S	0.007 G[c]	MR	43.67 B
Pyricularia sp. and Pg older population sampling year: 2012 (*n* = 5)						
363		R	S	0.07 F[c]	S	3.62 D
12.0.082		S	MR	0.79 E[c]	MR	23.49 C[c]
12.0.713		R	MR	0.87 E	HR	≥50 A[d]
12.0.733		R	R	1.22 C	HR	≥50 A[d]
12.0.264		R	R	1.01 D	HR	≥50 A[d]

Note: Means followed by the same upper case letters are not significantly different according to the Scott–Knott test at $p \leq 0.05$; bold or regular upper case letters delimit comparisons within each of two distinct experiments.

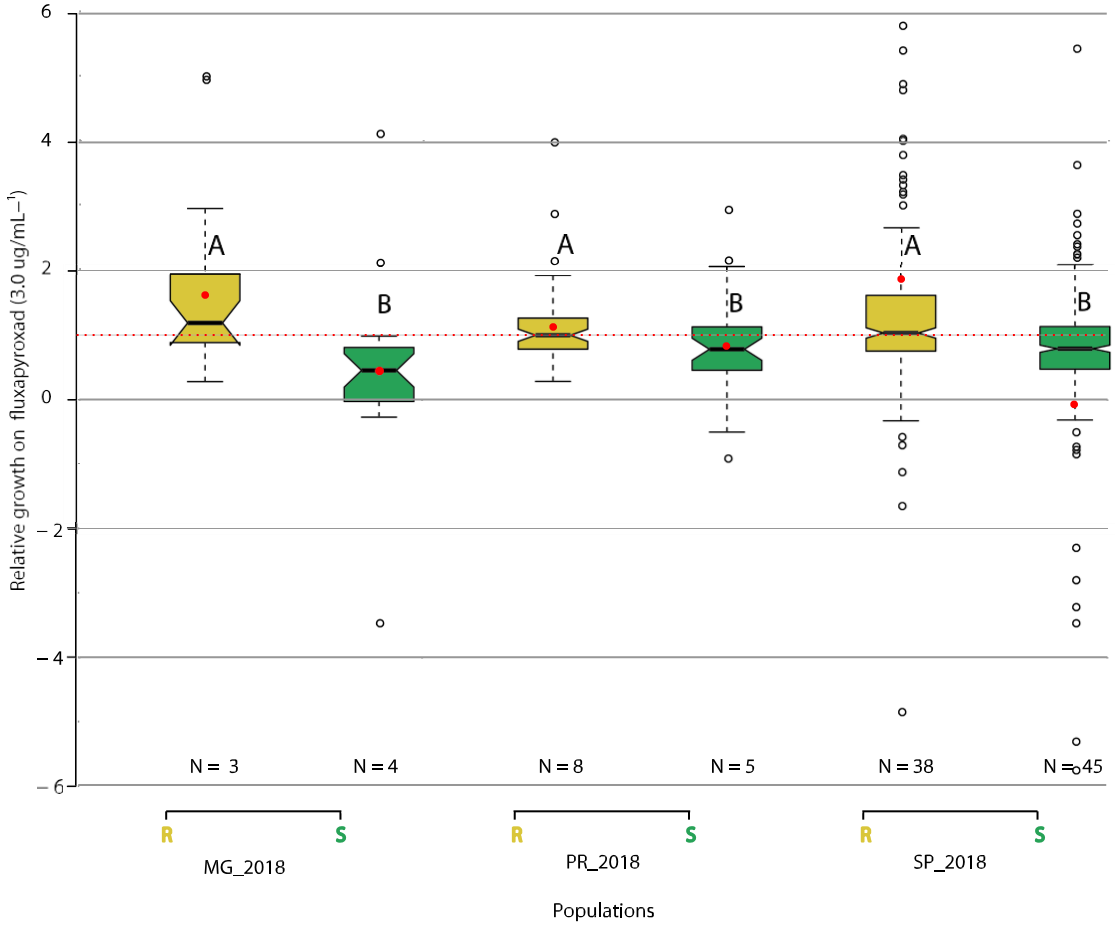
[a] *PoT*I, *Pyricularia oryzae Triticum* lineage; *PoO*I, *Pyricularia oryzae Oryza* lineage; Pg, *Pyricularia grisea*.

[b] Fungicide sensitivity phenotype classes: S, sensitive; RS, reduced sensitivity; MR, moderately resistant; R, resistant; HR, highly resistant. For the DMI fungicide tebuconazole, isolates were grouped into five different sensitivity phenotype classes: S, EC_{50} < 0.05 µg ml $^{-1}$; RS, EC_{50} between 0.05 and 0.5 µg ml $^{-1}$; MR, EC50 between 0.5 and 1 µg ml $^{-1}$; R, EC50 between 1 and 1.5 µg ml $^{-1}$; HR EC50 between 1.5 and 2 µg ml $^{-1}$, as described by Poloni *et al*. (2021). For the SDHI fluxapyroxad, isolates were grouped into four different sensitivity phenotype classes: S, EC_{50} < 3 µg ml $^{-1}$, RS, EC_{50} between 3 and 20 µg ml $^{-1}$; MR, EC_{50} between 20 and 50 µg ml $^{-1}$; HR, EC_{50} between 50 and 100 µg ml $^{-1}$, similar to the sensitivity phenotypes proposed by Amiri *et al*. (2014) for another pathosystem. Isolates were classed as QoI resistant if they grew at the discriminatory concentration of 10 µg ml^{-1}; they all carried the amino acid change G143A mutation (nucleotide change G to C at position 428 in the *cytB* gene; Castroagudín *et al*. (2015) and this study).

[c] Isolates used as controls in distinct experiments.

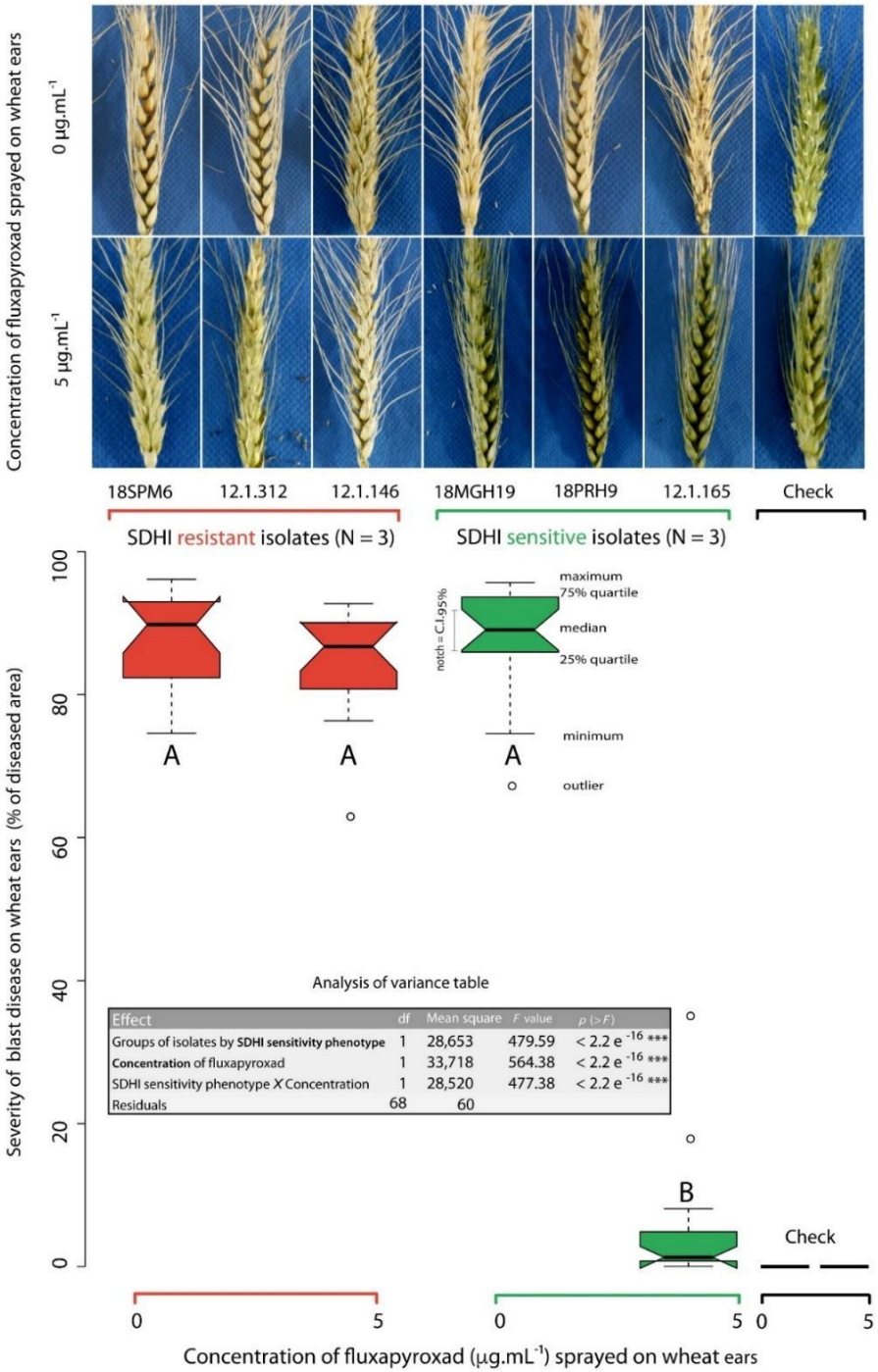
[d] EC_{50} determination using mycelial fragment growth for strains that did not sporulate.

Figure 3. Screening of sensitivity to the succinate dehydrogenase inhibitor fungicide fluxapyroxad in three groups of *Pyricularia oryzae Triticum* lineage (*PoT*l) isolates sampled from Minas Gerais (MG), Paraná (PR), and São Paulo (SP) wheat fields in 2018. Relative growth is the ratio of growth of the isolates on potato dextrose broth supplemented with the discriminatory concentration of 3 µg ml $^{-1}$ fluxapyroxad and 0 µg ml $^{-1}$. Green boxplots indicate sensitive isolates while yellow boxes indicate reduced sensitivity. Notched boxplots indicate the median and the range of relative growth for each group of isolates within population. Shared upper case letters on top of a boxplot indicate that the mean relative growth values (red dots) are not significantly different by the Scott–Knott test at α = 0.05



The phenotyping of in planta SDHI sensitivity fully confirmed the in vitro assessment for both groups of sensitive and resistant isolates of *PoT*l; the group of resistant isolates caused similar blast disease severity levels on fluxapyroxad sprayed (5 µg fluxapyroxad ml $^{-1}$) and on nonsprayed ears from the wheat susceptible cultivar Anahuac 75, and the group of SDHI sensitive isolates caused disease only on the nonsprayed wheat ears (Figure 4).

Figure 4. In planta confirmation of decreased succinate dehydrogenase inhibitor (SDHI) fungicide efficacy by phenotyping the disease severity of two contrasting groups of fluxapyroxad-sensitive and fluxapyroxad-resistant isolates of *Pyricularia oryzae Triticum* lineage (*PoT*I) on fungicide sprayed and nonsprayed ears from the wheat susceptible cultivar Anauhac 75. Boxplots contain the distribution of the blast disease severity values on wheat ears around a median for the distinct groups of sensitive or resistant *PoT*I isolates on plants sprayed with 0 or 5 µg/ml fluxapyroxad. Treatment means followed by the same upper case letters are not significantly different by the Scott–Knott test at α = 0.05



Analyses of the *sdhB*, *C*, and *D* genes in 17 isolates from the 2012 *PoT*l population and 22 isolates from the 2018 populations showed an absence of nonsynonymous mutations, regardless of the fluxapyroxad sensitivity of the isolates (Table 6). All the isolates surveyed grouped into a single *sdhB* haplotype (*sdhB* H1), a single *sdhC* haplotype (*sdhC* H1), and two *sdhD* haplotypes. The *sdhD* H1 haplotype was detected in most of the isolates, while *sdhD* H2, carrying three synonymous sub- stitutions, was identified in isolates 12.1.183, 18PRA7, and 18PRH9.

Inclusion and comparison of reference sequences from *PoO*l and of experimentally derived sequences from available isolates of *P*. *grisea* and *Pyricularia* sp. resulted in detection of additional mutations, both synonymous and nonsynonymous, in all three genes. For the *sdhB* gene, five other haplotypes distinct from the predominant *PoT*l *sdhB* H1 were identified. For instance, *sdhB* H3 (from the reference sequence *PoO*l MCR KX231838) had a single nonsynonymous mutation leading to the amino acid change H245L (Table 6). Additional *sdhB* sequencing of nine *PoO*l isolates from our collection identified haplotypes *sdhB* H5 (*PoO*l 284, 364, 611, 656, and 704) and *sdhB* H6 (*PoO*l 421, 658, 674, and 678), which differed from *sdhB* H3 by two or one synonymous mutations, respectively. However, the *sdhB* H4 from four *P. grisea* isolates (12.0.082, 12.0.264, 12.0.713, and 12.0.733) had 40 mutations, five of which were nonsynonymous leading to amino acid substitutions A123V, S125A, A165S, L233Q, and V274A (Table 6) and these isolates showed varying levels of SDHI resistance (Table 5). The *sdhC* gene from the SDHI-sensitive reference isolate *PoO*l 70-15 NC_017849 and the *Pyricularia* sp. isolate 363 from *Digitaria* both carried the mutation SdhC A27D. And finally, the *sdhD* gene from the SDHI-sensitive *Pyricularia* sp. isolate 363 carried the mutation SdhD P30S.

Table 6. Summary of variation at *sdhB*, *sdhC* and *sdhD* genes from field isolates of *Pyricularia oryzae Triticum* lineage (*PoT*l), *P*. *oryzae Oryza* lineage (*PoO*l), *P*. *grisea* (*Pg*) and *Pyricularia* sp. (*P*. sp.) from Brazil with distinct resistance phenotypes to the succinate dehydrogenase inhibitor fungicide fluxapyroxad

Gene	Length of amplicon sequence (bp)	Coding region (bp)	Protein length (amino acids)	Reference sequence deposited at GenBank NCBI	Synonymous mutations, insertions deletions[a]	Haplotypes detected	Species -sampling year	*n*	SDHI resistance phenotypic class[b]	Nonsynonymous mutations detected/ position	Nonsynonymous a.a. change/ position
sdhB	1051 (from 287 to 1338)	825	274	MT268226	0	*sdhB* H1	PoTl - 2018	5	HR	0	0
					0	*sdhB* H1	PoTl - 2018	5	MR	0	0
				MT268225	0	*sdhB* H1	PoTl - 2012	5	MR	0	0
					0	*sdhB* H1	PoTl - 2012	10	RS	0	0
				MT268226	0	*sdhB* H1	PoTl - 2018	10	RS	0	0
				MT268225	0	*sdhB* H1	PoTl - 2012	2	S	0	0
				MT268226	0	*sdhB* H1	PoTl - 2018	2	S	0	0
				MT268221	3	*sdhB* H2	*P*. sp. 363	1	S	0	0
				KX231838	6	*sdhB* H3	PoOl MCR	1	n.d.	CAC:CTC/1220	H:L/245
				MT268222	35	*sdhB* H4	Pg 12.0.082	1	MR	GCT:GTT/776	A:V/123
							Pg 12.0.713	3	HR	TCC:GCC/782	S:A/125
							Pg 12.0.733		HR	GCG:TCA/902	A:S/165
							Pg 12.0.264		HR	CTG:CAG/1185 and GTA:GCA/1308	L:Q/233 and V:A/274
				MT268223	6	*sdhB* H5	PoOl 656	1	S	0	0
							364, 611	2	RS	0	0
							284, 704 - 2007	2	MR	0	0
				MT268224	5	*sdhB* H6	PoOl 421	2	S	0	0
							674, 678	1	RS	0	0
							658 - 2007	1	MR	0	0
				NC_017852.1 (1560 bp)			PoOl 70-15	1	n.d.	0	0

Gene	Length of amplicon sequence (bp)	Coding region (bp)	Protein length (amino acids)	Reference sequence deposited at GenBank NCBI	Synonymous mutations, insertions deletions[a]	Haplotypes detected	Species -sampling year	*n*	SDHI resistance phenotypic class[b]	Nonsynonymous mutations detected/ position	Nonsynonymous a.a. change/ position
sdhC	997 (from 86 to 1082, 7 gaps)	609	202	MT268229	0	*sdhC* H1	PoTl - 2018	5	HR	0	0
					0	*sdhC* H1	PoTl - 2018	5	MR	0	0
				MT268228	0	*sdhC* H1	PoTl - 2012	5	MR	0	0
					0	*sdhC* H1	PoTl - 2012	10	RS	0	0
				MT268229	0	*sdhC* H1	PoTl - 2018	10	RS	0	0
				MT268228	0	*sdhC* H1	PoTl - 2012	2	S	0	0
				MT268229	0	*sdhC* H1	PoTl - 2018	2	S	0	0
				MT268227	1; insertion of (G)5 at position 1001[c]	*sdhC* H2	*P.* sp 363	1	S	GCC:GAC/453	A:D/27
				NC_017849 (1369 bp)	1; insertion of (G)7 at position 1001[c]	*sdhC* H3	PoOl 70-15	1	n.d.	GCC:GAC/453	A:D/27
				CP034206	0; insertion of (G)1 at position 1001 [c]	*sdhC* H4	PoOl MZ5-1-6	1	n.d.	0	0

Gene	Length of amplicon sequence (bp)	Coding region (bp)	Protein length (amino acids)	Reference sequence deposited at GenBank NCBI	Synonymous mutations, insertions deletions[a]	Haplotypes detected	Species -sampling year	*n*	SDHI resistance phenotypic class[b]	Nonsynonymous mutations detected/ position	Nonsynonymous a.a. change/ position
sdhD	890 (46–935)	516	171	MT268234	0	*sdhD* H1	PoTl - 2018	5	HR	0	0
					0	*sdhD* H1	PoTl - 2018	5	MR	0	0
				MT268233	0	*sdhD* H1	PoTl - 2012	4	MR	0	0
					0	*sdhD* H1	PoTl - 2012	10	RS	0	0
				MT268234	0	*sdhD* H1	PoTl - 2018	9	RS	0	0
				MT268233	0	*sdhD* H1	PoTl - 2012	2	S	0	0
				MT268234	0	*sdhD* H1	PoTl - 2018	1	S	0	0
				MT268235	3	*sdhD* H2	PoTl 12.1.183	1	MR	0	0
				MT268236			PoTl 18PRH9	1	S	0	0
							18PRA7 - 2018	1	RS	0	0
				MT268230	5	*sdhD* H3	*P.* sp 363	1	S	CCC:TCC/250	P:S/30
				MT268231	5	*sdhD* H4	PoOl 421, 656	2	S	0	0
				MT268232			364, 611, 674, 678	4	RS	0	0
				MT268231			284, 658, 704	3	MR	0	0
				NC_017852 (1197 bp)			PoOl 70-15	1	n.d.	0	0

[a] Synonymous mutations in the coding region. The haplotype could still carry other mutations in noncoding regions of the sequence.

[b] Fungicide sensitivity phenotype classes: S, sensitive; RS, reduced sensitivity; MR, moderately resistant; HR, highly resistant.

[c] The insertions of (G)n in the *sdhC* were detected in the noncoding region of the gene.

4 DISCUSSION

The main objective of our study was to determine the SDHI baseline sensitivity of Brazilian wheat blast populations sampled across different geographical areas. In addition to older populations sampled in 2012, we included three newer populations from 2018 and determined the frequency of resistance to QoI and DMI fungicides. The newer *PoT*l populations were isolated from similar agroecosystems to the older populations. In the older populations of *PoT*l, 89% of the isolates were resistant to QoIs while 100% were resistant to DMIs (CASTROAGUDÍN *et al*., 2015; CERESINI *et al*., 2018; DORIGAN *et al*., 2019; POLONI *et al*., 2021).

The outcome of the screening of *PoT*l strains for sensitivity to both QoI and DMI fungicides at discriminatory concentrations revealed that double resistance to both fungicide classes is pervasive (≥ 84 % of the strains tested from the different locations) and persisted in the Brazilian agroecosystem for the 6 years included in our survey. We hypothesize that the persistence of double resistance to QoIs and DMIs in field populations of *PoT*l is because these fungicides have been in continuous use in spray programmes to manage wheat diseases, maintaining intensive selection pressure on wheat blast populations (CERESINI *et al*., 2018; POLONI *et al*., 2021). This strong selection pressure is reinforced by the intensive double-cropping systems practised in tropical and subtropical Brazilian agroecosystems, which include rotations among wheat, maize and/or soybean. In these cropping systems, wheat is directly sprayed with QoI, DMI, and SDHI fungicides to target aerial pathogens such as *PoT*l during the typical autumn-winter cropping season, while other Poaceae hosts that typically invade maize or soybean fields are indirectly sprayed with the same fungicides that are applied to the alternating crop in the subsequent spring-summer cropping season (OSAKI *et al*., 2019). An alternative hypothesis is that no fitness costs are associated with QoI and azole resistance, resulting in the persistence of resistant genotypes even in the absence of selection pressure. *PoT*l can survive between growing seasons on other invasive grass host species growing adjacent to wheat fields (CASTROAGUDÍN *et al*., 2015) or on crop residues (PIZOLOTTO *et al*., 2019), which are not subjected to fungicide treatments. QoI- or azole-resistant strains of *P*. *grisea, Pyricularia pennisetigena, and Pyricularia urashimae* have also recovered from several poaceous hosts growing near wheat fields, indicating that these pathogens have multiple hosts, including wheat, and suggesting that significant fitness costs are not associated with fungicide resistance (CASTROAGUDÍN *et al*., 2015; DORIGAN *et al*.,

2019).

For SDHI sensitivity, fluxapyroxad-resistant isolates were detected in five out of the six older field populations of *PoT*l. Reduced sensitivity was detected in 91.6% of the isolates (*n* = 152). This finding is consistent with intrinsic or standing variation in SDHI resistance, given that these 2012 populations had not yet been exposed to selection by second-generation SDHI fungicides. For the three 2018 populations, isolated after introduction of SDHIs in Brazil, about half of *PoT*l isolates showed a further reduction in sensitivity to fluxapyroxad, and five highly resistant isolates (with EC_{50} values > 50 µg ml $^{-1}$) were detected among the 27 isolates tested. Our in planta experiment corroborated the in vitro detection of SDHI resistance.

In the current and previous studies, *PoT*l isolates from geographically distinct older and newer populations of the wheat blast pathogen were characterized for their sensitivity to QoI, DMI, and SDHI fungicides (CASTROAGUDÍN *et al*., 2015; Ceresini *et al*., 2018; Dorigan *et al*., 2019; Poloni *et al*., 2021; and this study). Mutations in the target genes *cytB* (for QoI resistance) and *CYP51A* (DMI resistance) have been described. High levels of QoI resistance were linked to alteration of the G143A cytochrome *b* in both the 2012 (CASTROAGUDÍN *et al*., 2015) and 2018 populations. In contrast, although six distinct haplotypes and four nonsynonymous substitutions were detected in *CYP51A* (DORIGAN *et al*., 2019), none of these variants correlated with EC_{50} values, suggesting that other mechanisms could be responsible for the observed DMI resistance. The lack of correlation between SDHI (log *y*, where *y* = EC_{50} SDHI) and DMI sensitivity (log *x*, where *x* = EC_{50} DMI) (non- significant *F* statistic, with 1 and 42 *df* for the linear model = 0.315, *p* = 0.578, multiple R^2 = 0.007), indicated that there was no multidrug resistance for both SDHI and DMI fungicides achieved by efflux pumps such as described for the MgMFS1 transporter system involved in multidrug resistance in *Z. tritici* field isolates (OMRANE *et al*., 2015).

In this study, we tested the hypothesis that the levels of resistance to SDHIs in *PoT*l were associated with point mutations in genes encoding succinate dehydrogenase (*sdh*) subunits B, C or D. In 39 representative *PoT*l isolates coming from five distinct SDHI sensitivity classes in both older and newer populations, no nonsynonymous variants were found in the *sdhB*, *sdhC*, or *sdhD* genes, even in isolates with high levels of resistance (EC_{50} between 20 and 50 µg/ml). We conclude that resistance to the SDHI fungicide fluxapyroxad is not correlated with target-site mutations in the *sdh* genes in the wheat blast pathogen. This stands in contrast to

previous reports of SDHI resistance in other plant pathogens (AMIRI *et al*., 2020; AVENOT; MICHAILIDES, 2010; REHFUS *et al*., 2016). The detection of fluxapyroxad-resistant *PoT*l strains in multiple locations in 2012, before the introduction of second generation SDHIs into the cereal fungicide market in Brazil, indicates that the wheat blast pathogen may be intrinsically resistant to SDHI due to a pre-existing nontarget site resistance mechanism.

One possible mechanism to explain resistance to SDHIs, in particular, in *PoT*l is increased efflux pump activity (HAWKINS; FRAAIJE, 2018). This occurs through the overexpression of ATP-binding cassette (ABC) transporters or by efflux pumps belonging to the major facilitator superfamily (MFS). Overexpression of efflux pumps can confer resistance to many unrelated structural and functional toxic components (HAWKINS; FRAAIJE, 2018; OMRANE *et al*., 2015). Field isolates of *Z*. *tritici* classified as multidrug resistant (MDR) have promoter changes in *MgMFS1* that lead to the overexpression of this transporter (OMRANE *et al*., 2015). It is also possible that multiple transporters with different substrate specificities contribute to fungicide resistance, as shown for *B. cinerea* (LEROUX; WALKER, 2013).

Target-site SDHI resistance remains a future risk for populations of *PoT*l as nonsynonymous mutations with amino acid changes have already been detected in sister species. For instance, SdhB H245L was detected in the rice blast pathogen *P*. *oryzae* isolate *PoO*l MCR included as a reference sequence in our study. This SdhB alteration is equivalent to H277Y in the *P*. *teres* f. sp. *teres* archetype protein sequence. Orthologous mutations conferring SDHI resistance have been described in laboratory mutants and field isolates of a range of plant pathogens, including *Z*. *tritici* (FRAAIJE *et al*., 2012). The five *SdhB* mutations A123V, S125A, A165S, L233Q, and V274A, found in field isolates of *P*. *grisea* (and equivalent to T155V, K157A, A197S, D265Q, and T306A in *P*. *teres* f. sp. *teres*), could also be associated with resistance to SDHI fungicides, because these isolates were characterized from moderate to extremely resistant (EC_{50} between 20 and 50 µg/ml). However, a range of nonsynonymous *Sdh* mutations not affecting SDHI sensitivity was reported in other pathogens. Studies of laboratory mutants using protein modelling (e.g., FRAAIJE *et al*., 2012; SCALLIET *et al*., 2012) indicate that the SdhB residues listed here for *P. grisea* are unlikely to form part of the binding pocket and/or have an impact on SDHI binding. Interestingly, another target site-related mechanism for SDHI resistance was recently reported for *Z*. *tritici* involving the presence of a second dispensable SdhC

paralogue in some isolates (STEINHAUER *et al*., 2019) and this was responsible for the observed standing genetic variation in field populations (Yamashita; Fraaije, 2018).

In conclusion, double resistance to QoI and DMI fungicides is pervasive and persistent in field populations of *PoT*l from the Brazilian agroecosystem. SDHI-resistant isolates were detected in both older pre-SDHI (2012) and newer post-SDHI (2018) populations of the wheat blast pathogen. No mutations were detected in the *sdhB*, *sdhC*, and *sdhD* genes from fluxapyroxad-resistant *PoT*l isolates, indicating a pre-existing nontarget site resistance mechanism is probably operating.

The onset of multiple fungicide resistance in populations of the wheat blast pathogen in Brazil indicates that the anti-resistance strategies adopted in the country before 2012 have failed, requiring immediate action and additional measures.

Remarkably, it was only after 2012 that pesticide resistance was officially recognized as a liability for the country's food production and security. This recognition led policymakers and pesticide regulators to introduce a very comprehensive policy on pesticide labelling requiring risk assessment data on resistance to support the registration process of new pesticides or for the renewal of already labelled pesticides. Such policies, if enacted in full (i.e., mandated by the federal agency regulating the fungicide labelling; Ministério da Agricultura Pecuária e Abastecimento – MAPA, 2021, abided by the fungicide sector advised by FRAC Brazil, and eventually adopted by farmers), could prevent the negative social, economic, and environmental impacts resulting from the spread of pesticide resistance in agriculture. However, with the new labels policy, the pesticide sector is legally exempt from any liability arising from the loss of efficacy of a particular fungicide. Consequently, managing fungicide resistance has become the sole responsibility of local farmers. A question remains whether the new label recommendations to prevent fungicide resistance are feasible and practical for adoption by all farmers, including those who are untrained and of poor education.

Instead of recommending to farmers such anti-resistance strategies that are difficult to implement, strategies to delay the emergence of fungicide resistance may be more useful. These include the regulation of mandatory industrial coformulations of new fungicides containing active ingredients with different modes of action, implemented prior to the labelling process. Thus, new fungicides with active components that have only a single-site mode of action, and so have a high risk of subsequent development of fungicide resistance, should only be labelled and

launched to the agricultural sector if coformulated with low-risk, protectant, multisite fungicides with broad-spectrum activity. These coformulated mixtures provide adequate disease control while minimizing the risk for development of resistance without any further requirement for management strategies (Mikaberidze *et. al*., 2014). Among the currently labelled SDHI fungicides available in Brazil, there is only a single commercial product that follows this principle, which is a coformulation of the single-site fluxapyroxad with the multisite fungicide copper oxychloride, from Oxiquímica Agrociência Ltd., Brazil (Ministério da Agricultura Pecuária e Abastecimento - MAPA, 2021). However, it is still only labelled for controlling the Asian soybean rust (*Phakopsora pachyrhizi*).

Acknowledgments: This study was funded by the São Paulo Research Foundation, Brazil (FAPESP) through regular research grants to Paulo Cezar Ceresini (2013/10655-4, 2015/10453-8; 2020/07611-9); Fundação Araucária to Adriano Augusto de Paiva Custódio. (FPS2020011000057); a Newton Fund grant from the Biotechnology and Biological Sciences Research Council (BBSRC) and UK Research and Innovation (UKRI), with FAPESP as partner institution in Brazil to P.C.C. (2017/50456-1; 2018/21197-0) and to S.I.M. (postdoctoral scholarship 2019/12509-1); by an Embrapa –Monsanto –Macro Program II (02.11.04.006.00.00) research grant to J.L.N.M. P.C.C. is supported by research productivity scholarships from Brazilian National Council for Scientific and Technological Development -CNPq (Pq-2 307361/2012-8; Pq-2 307295/2015-0 and Pq-1D 313825/2018-1). We also thank CAPES for sponsoring the establishment of the “Centro de Diversidade Genética no Agroecossistema” (Pro-equipamentos 775202/2012) and our International Research Network “Towards a sustainable bioeconomy for the Cerrado’s agriculture” (CAPES PrInt/UNESP Program 2019 -2023). We also thank Dr Márcio Elias Ferreira, National Center for Genetic Resources -Embrapa Cenargen for providing *Pyricularia oryzae* isolates from rice. B.A.F. received funding from the Newton Fund through grant BB/S018867/2 awarded by BBSRC under the BBSRC-FAPESP AMR and Insecticide Pest Resistance in Livestock and Agriculture Programme.

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CHAPTER 2: EFFLUX PUMPS AND MULTIDRUG-RESISTANCE IN *Pyricularia Oryzae Triticum* LINEAGE

Accepted for publication by Agronomy

Received: 29 June 2022 | **Accepted:** 5 August 2022

ABSTRACT

Widespread resistance to QoIs, DMI and SDHIs fungicides has been reported for Brazilian populations of the wheat blast pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l). A pre-existing resistance mechanism not associated with target site mutations has been indicated for resistance to DMIs and SDHIs, with strong indication that *PoT*l has multidrugresistance (MDR). Therefore, the main objective of this study was to test the hypothesis that resistance to DMI and SDHI fungicides detected in *PoT*l was due to efflux pump mediated MDR mechanism(s) by characterizing the sensitivity to antifungal efflux pump substrates. Four antifungal substrates were tested: tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL). TPCL and RH6G were considered the most relevant indicators for enhanced MDR activity. Among the 16 *PoT*l isolates tested, 9 were insensitive to TPCL, 1 to TOL, 16 to RH6G and 1 to CHX. The *PoT*l isolates were grouped into four distinct multidrug resistance phenotypes (MDRPs) based on resistance to combinations of fungicides and antifungal efflux pump substrates. Insensitivity to TPCL, RH6G and or TOL correlated well with DMI insensitivity, but MDR was not associated with SDHI resistance. The identification of multiple MDRP phenotypes associated with DMI resistance in our study warrants further research aimed at revealing the exact mechanisms of multidrug resistance in the wheat blast pathogen, including efflux pumps overexpression via transcriptomic analyses of differentially expressed genes; identification and discovery of mutations associated with changes in promoter regions or transcription factors of efflux transporters associated with multidrug resistance.

Keywords: wheat blast; MDR; QoI; DMI; SDHI.

1 INTRODUCTION

Wheat blast is caused by the ascomycete fungus *Pyricularia oryzae Triticum* lineage (*PoT*l) (CASTROAGUDÍN *et al.*, 2016; CERESINI *et al.*, 2018). This is a very aggressive disease, and its management is hampered by the absence of durable host plant resistance sources due to the extremely variable pathogen populations, both genetically and phenotypically (CRUZ *et al.*, 2010; CERESINI *et al.*, 2019). In addition, the frequent and extensive use of site-specific fungicides without adopting anti-resistance strategies, including other integrated disease management strategies, has resulted in a general loss of fungicide efficacy for management of the disease (PAGANI; DIANESE; CAFÉ-FILHO *et al*., 2014; CERESINI, *et al*., 2018).

Resistance to the site-specific fungicides QoI (quinone-outside inhibitors) and DMI (sterol 14α-demethylation inhibitors) has become pervasive and persistent in *PoT*l populations from all wheat-cropping regions throughout Brazil over a time span of seven years, from 2012 to 2019 (CASTROAGUDÍN *et al.*, 2015; OLIVEIRA *et al.*, 2015; DORIGAN *et al.*, 2019; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022). Resistance to QoI was mainly associated with cyt*B* target site alteration G143A (substitution of glycine (G) by alanine (A) at codon 143), which has been reported for many plant pathogens (CASTROAGUDN *et al*., 2015; MAIR *et al.*, 2016). Due to the absence of *CYP51* mutations and promoter inserts linked with azole resistance, the mechanism(s) conferring azole resistance remains unclear for *PoT*l populations in Brazil.

Moreover, resistance to the new generation carboxamide fluxapyroxad (a succinate dehydrogenase (Sdh) inhibitor (SDHI) fungicide) was detected in *PoT*l populations sampled in 2012 and 2019 (VICENTINI *et al.*, 2022). Since SDHI resistance was detected in Brazil before the introduction of this fungicide MOA in the cereals market and no changes in Sdh subunits B, C and D, forming the SDHI binding site, were detected, it was suggested that resistance is mediated by a pre-existing non-target site resistance mechanism.

Reduced sensitivity phenotype to multiple chemically unrelated compounds is defined as multidrug resistance (MDR), which is often caused by overexpression of efflux transporters with broad substrate specificity, as has been reported for a range of plant pathogens (KRETSCHMER *et al.*, 2009; OMRANE *et al.*, 2015; FISHER *et al.*,

2018; WANG *et al.*, 2018). In fungi, the major drug efflux pumps are members of the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter proteins (PERLIN *et al.*, 2014). Together, these two superfamilies of integral membrane transporters represent roughly half of all the genes coding for transporters in fungal genomes (PAULSEN *et al.*, 1998). As an example, *Zymoseptoria tritici* genome analysis indicated the presence of 47 ABC transporters and 288 MFS transporters, of which at least 87 of the latter are putative multidrug transporters (ROOHPARVAR, 2007).

ABC transporters consists of transmembrane domains (TMD) and structurally conserved nucleotide-binding domains (NBDs) and function as molecular machines by coupling ATP binding, hydrolysis, and phosphate release to the translocation of diverse substrates across membranes (THOMAS; TAMPÉ, 2020). In comparison, MFS transporters use proton motive forces to drive substances across membranes. These proteins play fundamental roles, such as the transport of small molecules, metabolites, and ions, as well as fungicides, toxins, and heavy metals removal as protection against biotic and abiotic stresses (DRIESSEN *et al.*, 2000; YAN, 2015; THOMAS; TAMPÉ, 2020). ABC or MFS transporters are located in different types of membranes, from the external plasma membrane to intracellular compartments membranes such as vacuole, endoplasmic reticulum, peroxisomes and mitochondria (PERLIN; ANDREWS, 2014; TOMAS; TAMPÉ, 2020; ESQUIVEL, 2017; SUN *et al*., 2006).

Overexpression of efflux pumps can be caused by modifications in their promoter regions or due to gain-of-function mutations in transcription factors that control expression. Omrane *et al*. (OMRANE *et al.*, 2017) identified for the Septoria leaf blotch pathogen *Z. tritici* a 519-bp long terminal repeat (LTR) insert (relic of transposon activity) in the *MFS1* promoter co-segregating with the MDR phenotype using a bulk progeny sequencing approach. Through gene replacement, they showed that the promoter insert was responsible for *MFS1* overexpression and the MDR phenotype. In addition, different types of shorter promoter inserts were found in more recent MDR strains. In MDR strains of human pathogen *Aspergillus fumigatus*, the C_2H_2 zinc finger transcription factor *SltA* was pointed as relevant for azole resistance by coregulating the expression of the fungicide target gene *CYP51A* and the efflux pump *Mdr1* (DU *et al.*, 2021). In MDR isolates of the rice blast pathogen *PoO*l*,* single point mutations in the *MoIRR* gene encoding a Zn_2Cys_6 zinc finger transcription factor-like protein confers resistance to isoprothiolane and to iprobenfos plus tricyclazole

(WANG *et al*., 2018). This Zn_2Cys_6 domain is also present in the *Mrr1* transcription factor found in the opportunistic human fungal pathogen *Candida albicans* and in grape's gray mold pathogen *Botrytis cinerea* (Kretschmer, *et al*. 2009; Schubert *et al.*, 2011). Specifically, mutations in the *Mrr1* regulates gene expression of *CDR1* (ABC) transporter in *C. albicans* and the *BcAtrK* (ABC) transporter in *B. cinerea* with MDR phenotype MDR1. Another mechanism reported for MDR2 in *B. cinerea* is the *BcmfsM2* (MFS) transporter gene promoter region rearrangement (Nakajima *et al.*, 2001; Kretschmer, *et al*., 2009). MDR3 in *B. cinerea*, generated through sexual crossing, combines both the MDR1 and MDR2 mechanisms, resulting in increased substrate range, and resistance to fludioxonil, cyprodinil and tolnaftate.

In general, multidrug transporters have a wide range of substrates with differences in specificity roles according to transporter types, their abundance and recognition of a domain's structure. For example, the *MgMFS1* transporter system in *Z. tritici* has redundancy roles in the transport of rhodamine 6G, once its gene deletion is compensated by expression changes in other transporters with an affinity for this drug (Zwiers *et al.*, 2003). However, the knockout of *MgMFS1* caused an increase in sensitivity to QoI fungicides, with no redundancy in this case. In fact, there is no obvious correlation between homology in the transporter amino acid sequence and specificity to substrates. For instance, the *PoO*l ABC2 transporter has high amino acid homology with BMR1 (BcatrK) in *B. cinerea*, although they have different ranges of substrates (LEE *et al.*, 2005). The *PoO*l ABC2 transports the DMI tebuconazole, cycloheximide, bitertanol, myclobutanil and camptothecin, while BMR1 extrudes ibrobenfos and polyoxin.

Chemically unrelated antifungal compounds have been used as efflux pump substrates in order to test hypotheses about the role of MDR mechanisms and to distinguish different MDR phenotypes caused by enhanced activity of MFS and/or ABC transporters. Triphenyltin chloride, tolnaftate, rhodamine 6G and cycloheximide are amongst the most common efflux pump substrates used as indicators of MDR phenotypes (LEROUX; WALKER, 2010; OMRANE *et al*., 2015; CHANG *et al.*, 2018; YAMASHITA; FRAAIJE, 2018). In our study, we tested the hypothesis that resistance to DMI and SDHI fungicides detected in *PoT*l was due to efflux pump mediated MDR mechanism(s) by characterizing the sensitivity to these four antifungal efflux pump substrates. Based on the sensitivity levels to the efflux pump substrates, we can also

identify if there are different MDR phenotypes amongst the *PoT*l strains previously sampled in 2012 and 2018.

2 MATERIALS AND METHODS

2.1 FUNGAL STRAINS

To carry out this study, twenty-one isolates of *Pyricularia* spp. previously classified according to their insensitivity levels to QoI, DMI and SDHI fungicides were randomly selected from our collections spanning 2007, 2017, 2018 and 2019 sampling years (CASTROAGUDÍN *et al*., 2015; POLONI *et al*. 2021; VICENTINI, *et al*., 2022) The strains selected harbor distinct combinations of resistance phenotypes from fully sensitive isolates to fully resistant isolates to DMI and SDHI fungicides.

2.2 SENSITIVITY TO EFFLUX PUMP SUBSTRATES

The antifungal efflux pump substrates tested were tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL) (all from Sigma-Aldrich, USA). These drugs were dissolved to 1 mg mL^{-1} in acetone followed by dilution in deionized water to obtain a 100 µg mL^{-1} stock solution.

Sensitivity tests with efflux pump substrates were conducted with flat-bottomed 96-well microtiter plates (Kasvi, India) using mycelial fragments. Mycelium fragments were obtained from fungal colonies that were grown for 10 days in PDA at 25 °C under 12 h photoperiod using a protocol adapted from Brito et al. (BRITO *et al.*, 2020) and Arango Isaza *et al.* (ARANGO ISAZA *et al.*, 2006). Pathogen mycelial samples from 10-day old colony from a single agar plate were transferred to a mortar and macerated using a pestle in 3 mL sterilized water and Tween 20 (one drop L^{-1}). Each 1 mL of the macerate was transferred to tubes containing a volume of 1 mL of sterile 2 mm glass beads and vortexed for two minutes. The mycelial fragments suspension recovered from tubes were mixed and pre-diluted to a final volume of 15 mL, counted under a microscope using a Neubauer chamber and adjusted to 10^5 fragments mL^{-1}. Each microplate well was filled with 50 µL of inoculum suspension and 100 µL of PD broth (20.7 gL^{-1} of potato dextrose (Kasvi, India), 1 L of distilled water)) amended with different concentrations of the efflux pump substrates tested and in the presence of chloramphenicol and streptomycin (50 µL mL^{-1} each).

Stock solutions from each of the substrates were added to the wells to obtain the following final concentrations: tolnaftate: 0.0, 0.032, 0.16, 0.8, 4 and 20 µg mL^{-1}; cycloheximide, rhodamine 6G and triphenyltin chloride: 0.0, 0.016, 0.08, 0.4, 2 and 10 µg mL^{-1}. For strains extremely sensitive to triphenyltin chloride, the following dilution series was used: 0.0, 0.000128, 0.00064, 0.0032, 0.016 and 0.08 µg mL^{-1}. The microplates were incubated at 25° C for 5 days under a 12 h photoperiod. After incubation, mycelial growth (*mg*) was measured based on absorbance values at 620 nm ($mg = t_5 - t_0$; where t_0 = reading (absorbance at 620 nm) immediately after plate assembly; t_5 = reading after five days incubation) using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific TM, USA).

Sensitivity to efflux-pump substrates was determined as 50% effective concentration to inhibit fungal growth (EC_{50} in µg mL^{-1}), estimated using the software *ED50 plus* v1.0 (Vargas, 2000). Resistance factors (RF) were determined to estimate the relative fold-inhibition of all the strains tested based on their EC_{50} values and normalized by the lowest EC_{50} for each substrate. For EC_{50} means comparison within each substrate (TPCL, TOL, RH6G or CHX), the experimental design consisted of complete randomized blocks, with four replicates per treatment and experiments in duplicate.

2.3 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) by the *F* test and means comparison by the Scott-Knott test (at 5% probability) were performed using the R environment with the statistical libraries *agricolae* and *laercio* (Team, 2019).

Pearson's correlation analysis was performed using EC_{50} data from all fungal strains to explore the relationships among sensitivity to efflux pump substrates and the fungicides tebuconazole (DMI) and fluxapyroxad (SDHI). The DMI and SDHI EC_{50} data were obtained from previous studies (Poloni, *et al*., 2021; Vicentini, *et al*., 2022). To verify the correlation significance, *t* tests were applied using the R environment with the statistical library *performance analytics* (Team, 2019).

3 RESULTS

3.1 SENSITIVITY TO EFFLUX PUMPS SUBSTRATES

The statistical analysis indicated no significant difference between replicates (R) of the experiments, as well no significant interaction between replicates and the strain (S) factor (R*S). This indicated the reproducibility of the observations allowing for the joint analysis of the replicates. The strain effect was highly significant ($p < 0.01$) for all four efflux pump substrates checked (Table 1).

Table 1. Analysis of variance of the effect of strains on the EC_{50} values for efflux pump substrates

Source of variation	*df*	Triphenyltin Chloride		Tolnaftate		Rhodamine 6G		Cycloheximide	
		Mean square	*F* values	Mean square	*F* values	Mean square	*F* values	Mean square	*F* values
Strain (S)	20	0.0802	53.923 **	5,043	43.241 **	2.946	6.325 **	0.5575	20.851 **
Block	3	0.0021	1.431 ^{NS}	0.0965	0.828 ^{NS}	0.1237	0.265 ^{NS}	0.0881	3.295 ^{NS}
Residuals	122	0.0015		0,1166		0.4659		0.0267	

** Significance by the *F* test at $p < 0.01$; ^{NS} non-significant. The experiment was repeated once and a joint analysis of the data was performed, since no interaction strain vs. experiment was detected in the individual analyses.

Nine out of the 16 *PoT*l strains tested were insensitive to the efflux pumps substrate triphenyltin chloride (TPCL), as shown by a greater than 5-fold elevated EC_{50} level in comparison with the average EC_{50} value of 0.0085 µg mL^{-1}, recorded for the two fungicide sensitive *PoO*l strains 421 and 656 (Table 2). The strains *PoT*l 18SPK6 (Figure 1A), and 18SPC10 showed the highest significant EC_{50} values for TPCL, 0.31 µg mL^{-1}, resulting in a resistance factor (RF) of ~36-fold in comparison with the average EC_{50} value of the two fungicides sensitive *PoO*l strains (Table 2). The RFs for the other seven TPCL insensitive *PoT*l strains ranged from 7.1 to 31-fold, while the two other *PoT*l strains were sensitive, showing RFs < 5.

Only one *PoT*l strain, 12.1.130 (Figure 1D), was insensitive to tolnaftate (TOL), with an EC_{50} of 4.40 µg mL^{-1}, showing a RF of 13 in comparison with the average EC_{50} value of 0.34 µg mL^{-1} measured for the two fungicide sensitive *PoO*l strains 421 and

656 (Figure 1B and Table 2). All other 15 *PoT*I strains and 2 *PoO*I strains tested were sensitive, showing RFs between 0.7 and 3.9.

All 16 *PoT*I strains and 1 *PoO*I strain (674) showed raised EC_{50} values to rhodamine 6G (RH6G) (Table 2). The RFs ranged from 13- to 61-fold for the *PoT*I strains with the highest EC_{50} value, 2.13 µg mL^{-1}, measured for strain 12.1.299 (Figure 1B). A lower RF value of 7.1 was measured for the RH6G sensitive *PoO*I strain 674.

The cycloheximide (CHX) EC_{50} values for the 20 *Po* strains tested ranged from 0.09 to 1.24 µg mL^{-1} (Table 2). The fungicide sensitive *PoT*I reference strain 421 was insensitive and showed a RF of 6.7 in comparison with *PoT*I strain 656. Only one *PoT*I strain, 18MGF23, was also insensitive with an EC_{50} value of 1.24 µg mL^{-1} and a RF of 10 (Figure 1C).

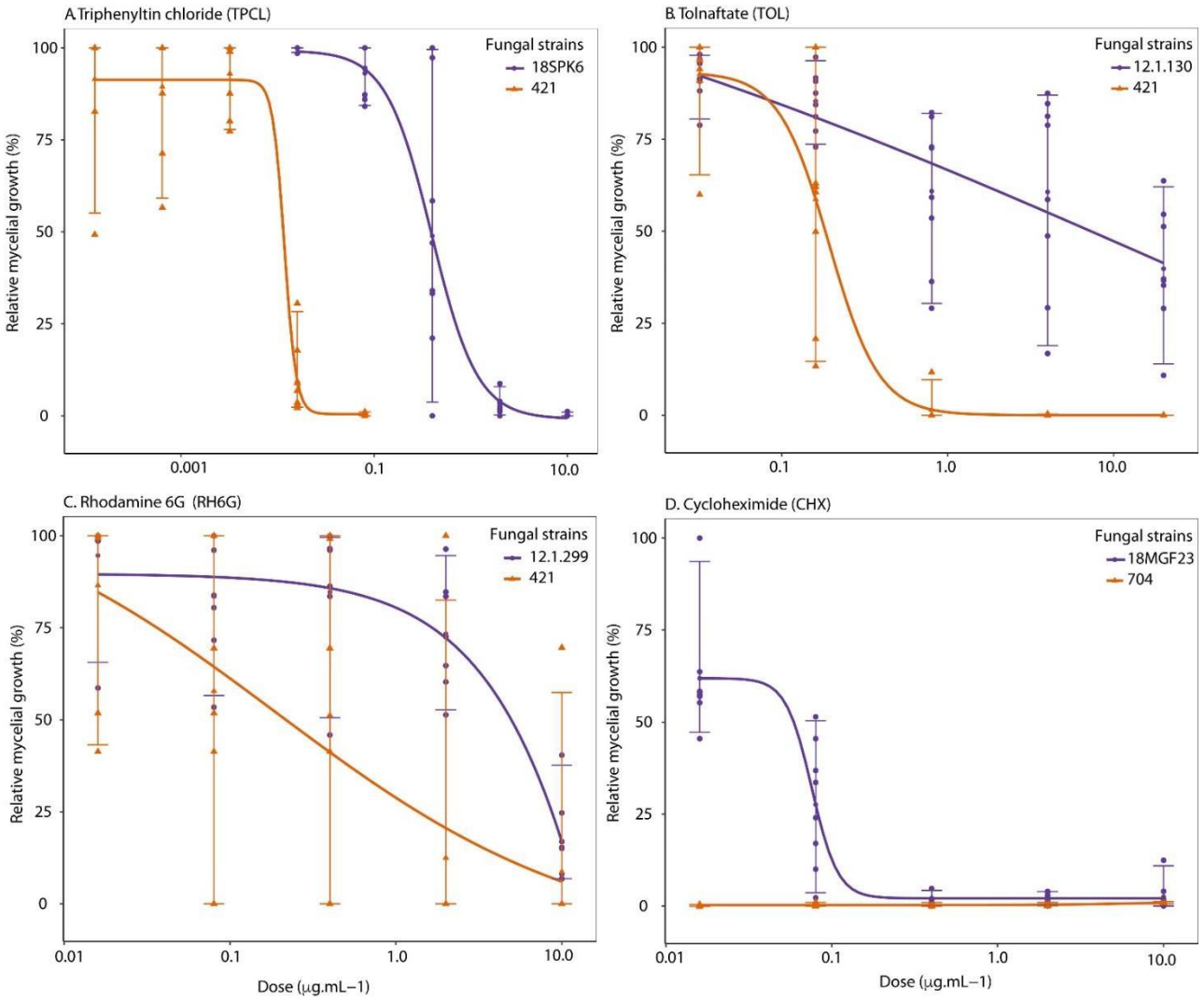
Table 2: Field isolates of *Pyricularia* spp. sampled in Brazil in 2012 and 2018 and respective resistance phenotype classes to three major fungicide classes and sensitivity to efflux pump substrates and respective resistance factors

[a] Species and isolates	[b] Previously determined fungicide resistance phenotype			[d, e, f, g] Mean EC_{50} (µg.mL^{-1}), standard deviation and RF												[h] MDR phenotype
	[c] QoI azoxystrobin	[d] DMI tebuconazole	[e] SDHI fluxapyroxad	Triphenyltin chloride (TPCL)		RF TPCL	Tolnaftate (TOL)		RF TOL	Rhodamine 6G (RH6G)		RF RH6G	Cycloheximide (CHX)		RF CHX	
Pyricularia oryzae Triticum lineage – *PoT*I																
12.1.015	S	R	RS	0.030 (0.01)	d	3.5	0.25 (0.1)	d	0.73	1.01 (0.9)	b	29	0.10 (0.08)	d	0.83	MDRP1
12.1.045i	R	R	RS	0.036 (0.01)	d	4.2	0.36 (0.1)	d	1.1	1.26 (0.41)	b	37	0.13 (0.09)	d	1.1	MDRP1
12.1.037	R	R	RS	0.04 (0.002)	d	4.7	0.31 (0.3)	d	0.91	1.22 (0.8)	b	35	0.09 (0.07)	d	0.75	MDRP1
18MGH25	R	MR	RS	0.06 (0.03)	d	7.1	0.62 (0.33)	d	1.8	0.80 (0.54)	c	23	0.18 (0.11)	d	1.5	MDRP2
18MGH11	R	R	HR	0.04 (0.005)	d	4.7	0.25 (0.07)	d	0.74	0.47 (0.27)	c	13	0.17 (0.14)	d	1.4	MDRP1
12.1.005	R	R	S	0.04 (0.03)	d	4.7	0.55 (0.29)	d	1.6	1.76 (1.1)	a	50	0.18 (01.5)	d	1.5	MDRP1
12.1.183	R	R	MR	0.16 (0.06)	b	19	0.87 (0.49)	c	2.6	1.07 (0.83)	b	31	0.42 (0.15)	c	3.5	MDRP2
18MGH19	R	R	RS	0.12 (0.02)	c	14	1.02 (0.30)	c	3.0	0.73 (1.08)	c	21	0.27 (0.45)	c	2.3	MDRP2
18SPK6	R	R	RS	0.31 (0.09)	a	36	1.33 (0.68)	b	3.9	1.77 (0.80)	a	51	0.15 (0.05)	d	1.3	MDRP2
12.1.299	R	R	MR	0.17 (0.04)	b	20	0.79 (0.57)	c	2.3	2.13 (0.65)	a	61	0.11 (0.09)	d	1.1	MDRP2
12.1.312	R	R	MR	0.19 (0.08)	b	22	0.89 (0.64)	c	2.6	1.03 (0.34)	b	29	0.11 (0.04)	d	0.92	MDRP 2
18MGF23	R	R	MR	0.15 (0.03)	b	18	0.31 (0.12)	d	0.91	1.35 (0.29)	b	39	1.24 (0.53)	a	10	MDRP4
18SPC10 b	R	R	HR	0.31 (0.04)	a	36	0.50 (0.31)	d	1.5	1.25 (1.28)	b	36	0.13 (0.03)	d	1.1	MDRP2
12.1.165	S	R	S	0.27 (0.05)	a	32	0.36 (0.09)	d	1.1	1.47 (1.00)	b	42	0.16 (0.06)	d	1.3	MDRP2

[a] Species and isolates	[b] Previously determined fungicide resistance phenotype			[d, e, f, g] Mean EC_{50} (µg.mL^{-1}), standard deviation and RF													[h] MDR phenotype
	[c] QoI azoxystrobin	[d] DMI tebuconazole	[e] SDHI fluxapyroxad	Triphenyltin chloride (TPCL)		RF TPCL	Tolnaftate (TOL)		RF TOL	Rhodamine 6G (RH6G)		RF RH6G	Cycloheximide (CHX)		RF CHX	
12.1.130	S	HR	RS	0.04 (0.002)	d	4.7	4.40 (2.2)	a	13	1.87 (0.6)	a	53	0.11 (0.03)	d	0.92	MDRP3
18PRH9	R	R	S	0.03 (0.004)	d	3.5	0.87 (0.45)	c	2.6	0.50 (0.6)	c	14	0.12 (0.03)	d	1.0	MDRP1
*Pyricularia oryzae Oryza lineage – PoO*l																	
656	S	S	S	0.013 (0.007)	d	1.53	0.45 (0.28)	d	1.32	0.06 (0.05)	d	1.71	0.12 (0.1)	d	1.0	-
421	S	S	S	0.004 (0.001)	d	0.47	0.23 (0.08)	d	0.68	0.01 (0.01)	d	0.29	0.80 (0.34)	b	6.7	-
674	S	S	RS	0.033 (0.03)	d	3.8	0.42 (0.33)	d	1.24	0.25 (0.17)	c	7.14	0.09 (0.09)	d	0.75	-
704	S	S	MR	0.004 (0.001)	d	0.47	0.28 (0.09)	d	0.82	0.05 (0.03)	d	1.43	0.09 (0.06)	d	0.75	-
Pyricularia grisea - Pg																	
363	R	S	S	0.04 (0.004)	d	10	0.26 (0.08)	d	0.76	1.67 (1.4)	a	48	0.11 (0.05)	d	0.92	-

[a] The *PoT*l strain coding indicates the respective sampling year: '12' for 2012 populations and '18' for 2018 populations; *PoO*l and *Pg* isolates were from 2012 populations.
[b] Sensitivity phenotype classes: **S** = sensitive, **RS** = reduced sensitivity, **MR** = moderately resistant, **R** = resistant, **HR** = highly resistant.
[c] According to Castroagudín *et al*. (2015): **R** isolates grew at the discriminatory dose of 10 µg.mL^{-1}.
[d] According to Poloni *et al*. (2020): **S**, EC_{50} = 0.007 – 0.07 µg mL^{-1}; **MR** , EC_{50} = 0.62 – 0.98 µg.mL^{-1}; **R**, EC_{50} = 1,03 – 1,44 µg.mL^{-1}; **HR** = > 1,62 – 1,70 µg.mL^{-1}.
[e] According to Vicentini *et al.* (2021): **S**, EC_{50} = 1.15 – 2.94 µg.mL^{-1}; **RS**, EC_{50} = 3.30 – 19.31 µg.mL^{-1}; **MR**, EC_{50} = 21.83 – 43.67 µg.mL^{-1}; **HR** = > 50 – 100 µg.mL^{-1}.
[d] Effective concentration to inhibit fungal growth in 50% (EC_{50}) using mycelial fragment method evaluation by absorbance at 620 *nm* in microplates.
[e] Means followed by the same letters in the column did not differ by the Scott-Knott test (at $p \leq 0.05$) . Standard deviation in parenthesis.
[f] Resistance factor (RF) indicates the ratio of the strain's EC_{50} tested to the mean average EC_{50} value for strains *PoO*l 421 and 656 for each antifungal- isolate combination, with exception for cycloheximide were only the EC_{50} value of *PoO*l 656 was used due to insensitivity of strain *PoO*l 421.
[g] RF within each substrate: dark purple = very high, orange = high, light purple = medium.
[h] Multidrug-resistance phenotype of *PoT*l strains (MDRP) classification according to the combination of resistance to different efflux pump substrates and resistance to tebuconazole (DMI) and/or fluxapyroxad (SDHI).

Figure 1. Contrasting in vitro relative growth of *Pyricularia oryzae Triticum* and *Oryza* lineages in different efflux pump substrates in PD broth on a microplate assay. (**A**). Triphenyltin chloride (TPCL)-resistant strain 18SPK6 from *P. oryzae Triticum* lineage (*PoT*I), and the sensitive strain 421 from *P. oryzae Oryza* lineage (*PoO*I). (**B**)**.** Tolnaftate (TOL)-resistant *PoTI* 12.1.130 and the sensitive strain *PoO*I 421. (**C**)**.** Rhodamine 6G (RH6G)**-**resistant strain *PoT*I 12.1.299 and the sensitive strain *PoO*I 421. (**D**)**.** Cycloheximide (CHX)-resistant strain *PoT*I 18MGF23 and the sensitive strain *PoO*I 704. Bars contain the sample median and outer quantiles at 95% confidence interval (the 2.5th and the 97.5th percentiles as the lower and the upper confidence limits). Data points are distributed along the bar



The strain *P. grisea* 363 was resistant only to RH6G, while *PoO*I 421 was resistant only to CHX (Table 2). *PoT*I strains were subsequently classified into multidrug-resistance phenotype patterns (MDRP) based on the resistance levels to the four different efflux pump substrates tested (Table 2). Isolates resistant to either one

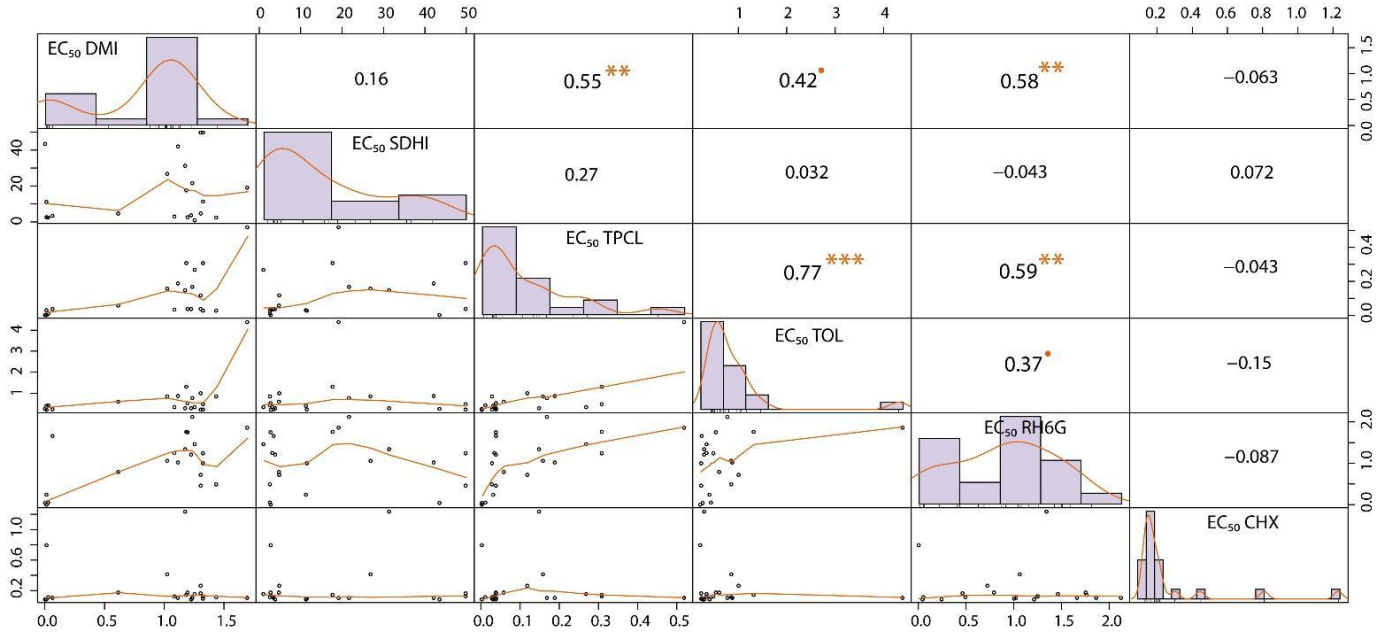
of the target-site fungicides, DMI or SDHI, or both (Table 2), and also to at least one unrelated efflux pump substrate, were considered as expressing MDR (Table 2). The resistance to QoIs was not used as a parameter, because high levels of resistance in these strains was linked to the target-site alteration G143A in cytochrome *b* gene.

Four distinct MDR phenotypes (MDRP1-4) were observed amongst the 16 *PoT*I strains (Table 2): (i) strains with high levels of RH6G and DMI resistance, and with varying levels of SDHI insensitivity (MDRP1, six strains); (ii) strains resistant to RH6G, TPCL and DMI with different levels of SDHI insensitivity (MDRP2, eight strains); (iii) strains resistant to TOL, RH6G, DMI and SDHI (MDRP3, strain 12.1.130) and (iv) strains resistant to TPCL, RH6G, CHX, DMI and SDHI (MDRP4, strain 18MGF23). *PoO*I strains 656 and 704 were sensitive to all four efflux pump substrates, but only *PoO*I 704 was also resistant to the SDHI tested. *PoO*I strain 421 showed insensitivity to CHX but was sensitive to both DMI and SDHI, while *PoO*I strain 674 was insensitive to both RH6G and SDHI but sensitive to DMI.

3.2 CORRELATION ANALYSIS

Positive significant correlation ($p \leq 0.01$) was observed among the EC_{50} values of the fungicide tebuconazole (DMI) and of the substrates TPCL (55%) and RH6G (58%), besides lower significance ($p \leq 0.10$) between DMI and TOL (42%) (Figure 2). However, the EC_{50} values for fluxapyroxad (SDHI) did not correlate with any chemical tested (Figure 2). The sensitivity levels between the substrates TPCL and TOL were significantly correlated ($p \leq 0.001$) with high positive linear relationship (close to 80%) (Figure 2). The TPCL also correlated with RH6G ($p \leq 0.01$), in which the linear relationship was close to 60%. For TOL and TPCL the correlation was lower than 40% (significance at $p \leq 0.1$) (Figure 2). The EC_{50} values for the efflux pump substrate CHX did not correlate with any chemical tested (Figure 2).

Figure 2. Pearson correlation analysis among the EC_{50} values for the efflux pump substrates tolnaftate (TOL), cycloheximide (CHX), rhodamine 6G (RH6G) and triphenyltin chloride (TPCL) and the site-specific fungicides tebuconazole (DMI) and fluxapyroxad (SDHI) determined for *Pyricularia oryzae Triticum* and *Oryza* lineages and *P. grisea*. Diagonal frames represent the distribution of the EC_{50} values for each chemical; frames below, the bivariate dispersion and adjusted lines; 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; *= 0.05; ^{o} = 0.1



4 DISCUSSION

Resistance to site-specific fungicides in fungi can vary for different unrelated chemicals according to the recognition domain's structures and the expression patterns of different transporter's genes, and this variation is population- and strain-associated. Therefore, it is plausible that resistance to the site-specific DMI and SDHI fungicides could occur both due to target-site mechanisms in association with the efflux pump's overexpression in the MDR isolates (Perlin; Andrews; Toh, 2014). A pre-existing mechanism not associated with target site mutations has been associated with the resistance to DMI and SDHI fungicides, with a strong indication that *PoT*l has evolved efflux-based multidrug-resistance (MDR).

We tested this hypothesis by testing a selection of 20 *Po* strains (16 *PoT*l and 4 *PoO*l) with different resistance levels to DMI and SDHI fungicides for sensitivity to four antifungal efflux pump substrates, cycloheximide (CHX), triphenyltin chloride (TPCL), rhodamine 6G (RH6G) and tolnaftate (TOL).

CHX is an antifungal and antibiotic isolated from *Streptomyces griseus* and inhibits protein synthesis. CHX exerts its effect by interfering with the movement of tRNA molecules on the mRNA-ribosomal complex, paralyzing the translocation step of protein synthesis (LAWANA *et al.*, 2014). CHX has been frequently used in efflux pumps studies with transporters such as *PoO*l ABC2 (LEE *et al*., 2005), *AtrD* in *B. cinerea* (KRETSCHMER, *et al*., 2009) and *PdPMR1* in *P. digitatum* (HU; CHEN, 2021). Yeast heterologous gene expression studies with ABC and MFS transporters from both *Z. tritici* (ROOHPARVAR, 2007) and *C. Albicans* (Lamping *et al.*, 2007) show CHX can act as a substrate for both efflux pump families. In comparison with the fungicide sensitive strain *PoO*l 656, only two strains, *PoT*l 18MGF23 and *PoO*l 674, showed raised levels of CHX insensitivity, with RFs of 10 and 6.7, respectively. However, *PoO*l 674 did not have a MDR phenotype, being sensitive to QoI, DMI and SDHI fungicides, and MDR in the remaining *PoT*l strains (*PoTl* strain 18MGF23) is therefore also not likely associated with CHX insensitivity.

Triphenyltin chloride (TPCL) and/or rhodamine 6G (RH6G) can be considered as the most useful indicators of efflux pump mediated MDR activity, since seven strains were insensitive to RH6G alone (MDRP1 and MDRP3) and nine out of 16 fungicides-resistant P*oT*l strains were insensitive to both RH6G and TPCL (MDRP2 and MDRP4), using a RF of 5 as a cut-off, with a high correlation between their EC_{50}s and the EC_{50}s

recorded for the DMI tebuconazole. Fentin compounds such as TPCL inhibit mitochondrial ATPase activity, affecting membrane permeability and calcium influx regulation (HOLLINGWORTH, 2001). *Saccharomyces cerevisiae* and *Z. tritici* are examples of fungi screened for MDR using this substrate (Man *et al.*, 2009; Yamashita; Fraaije, 2018). Rhodamine 6G (RH6G), known to be an inhibitor of mitochondrial oxidative phosphorylation (Gear, 1974), has been used as a transporter substrate to test hypotheses on MDR in many other fungal species, in particular ABC or MFS transporter systems such as *C. albicans* (*Cdr1*) (MAESAKI *et al.*, 1999), *Cryptococcus neoformans* and *C. gattii* (*Afr1*, *Afr2*, *Mdr1*) (Chang *et al*., 2018). RH6G is a fluorescent dye with chemical properties appropriate for accumulation or export assays based on fluorescence detection of its activity, which has been applied, for instance, to determine the activity of the ABC transporters *CgCDR1* and *CgCDR2* in *C. glabrata* (POSTERARO *et al.*, 2006).

In our study, only one *PoT*I strain, 12.1.130, was insensitive to tolnaftate (TOL) (Table 2). This strain, representing MDRP3, was also insensitive to RH6G and showed the highest level of DMI resistance (Table 2). Furthermore, a high level of cross-resistance was found between TOL and RH6G (Figure 2). TOL is a synthetic thiocarbamate used as an antifungal agent, acting as an inhibitor of squalene epoxidase and, consequently, inhibiting the sterol biosynthesis (GEORGOPAPADAKOU; Bertasso, 1992). Tolnaftate has been used for MDR phenotyping in *Z. tritici* (*MgMFS1*)*, B. cinerea* (*AtrB*, *mfsM2*) and *C. albicans* (*Cdr1* and *2*) [13,14,32,45].

In summary, four different MDR phenotypes (MDRP1-4) were observed amongst the 16 *PoT*I strain tested based on insensitivity levels to multiple fungicides and four antifungal efflux pump substrates (TPCL, TOL, RH6G and CHX). Insensitivity to DMI correlated well with insensitivity levels to TPCL and/or RH6G, and the Pearson's correlation between the DMI's EC_{50} with both TPCL and RH6G EC_{50} values corroborated this observation.

Regarding DMI fungicides, the widespread reduced triazole sensitivity of the wheat blast pathogen in Brazil could have evolved to its current high levels of resistance in response to intensive exposure of *PoT*I populations to triazoles over 30 years of selection pressure due to repeated spray applications of DMI fungicides in the local wheat agroecosystem (POLONI *et al*., 2021). Although the authors detected five different *PoT*I *CYP51A* haplotypes (H1 to H5), four of them all carrying non-

synonymous substitutions in *CYP51A* at high frequencies within field populations, none of these substitutions correlated with elevated EC_{50} values. For a selection of strains, including *PoT*I 12.1.299, sequencing of regulatory and coding sequences of both *CYP51A* and *CYP51B* revealed no alterations and the operation of a non-target site resistance mechanism was suggested (Poloni *et al*., 2021). In our present study this strain was categorized as MDRP2, with resistance to the transporter substrates TPCL and RH6G (Table 2).

In the sister species *PoO*I, the rice blast pathogen, an ABC2 transporter system was identified as a relevant mechanism for azole resistance. Disruption of genes associated with the ABC transport resulted in increased sensitivity to tebuconazole in *PoO*I, and also to CHX and other efflux pump drugs (Lee *et al*., 2005). Many other examples of ABC transporters linked to azoles resistance have been reported for different fungi species such as *A. nidulans* (*AtrG*), *A. fumigatus* (*Mdr1, abcE*), *B. cinerea* (*BcatrB*), *C. albicans* (*Cdr1p* and *2p*), *C. neoformans* (*Afr1p*) and *Penicillium digitatum* (*PdPMR1*). Moreover, MFS efflux pumps have been associated with DMI resistance in *A. fumigatus* (*mfsC*), *Z. tritici* (*MgMFS1*), *B. cinerea* (*Bcmfs1*) and *C. albicans* (*CaMdr1p*) (NAKAUNE *et al.*, 2002; DE WAARD *et al.*, 2006; PASRIJA *et al.*, 2007; CANNON *et al.*, 2009; OMRANE *et al*., 2015; DU *et al.,* 2021).

Regarding the mechanisms of the resistance to the SDHI fungicide fluxapyroxad, none of the SDHI-resistant *PoT*I strains harbored any amino acid substitutions in SdhB, C or D (VICENTINI, *et al*., 2022), also pointing to the existence of a non-target site associated resistance mechanism such as enhanced efflux pump activity. For instance, *MgMFS1* promoter insertions conferring resistance to many SDHI molecules, including fluxapyroxad, have been reported for *Z. tritici* (Omrane *et al*. 2017), and *Mrr1* transcription factor changes altering the ABC-*AtrB* and the MFS-*mfsM2* expression levels have been reported to confer resistance to the SDHI fungicide boscalid (KRETSCHMER *et al*., 2009). However, one DMI-sensitive *PoO*I strain without a MDR phenotype, isolate 704, was found moderately resistant to fluxapyroxad (Table 2), whereas three *PoT*I strains, 12.1.005, 12.1.165 and 18PRH9, showing high levels of resistance to the DMI tebuconazole and classified as MDRP1 or MDRP2 (12.1.005, 12.1.165 and 18PRH9), were sensitive to the SDHI fluxapyroxad (Table 2), indicating another resistance mechanism might be responsible. Interestingly, another non-target site resistance mechanism for SDHI resistance was recently suggested to operate in some *Z. tritici* strains (YAMASHITA; FRAAIJE, 2018), but further research

revealed that duplication of a SdhC paralog was responsible for this phenotype (STEINHAUER *et al.*, 2019). The presence of an additional SdhC paralog was found in several other fungi, including *Fusarium graminearum* and *Alternaria alternata*, but multiple copies have not been predicted for *Magnaporthe oryzae*.

The identification of multiple MDRP phenotypes associated with DMI resistance in our study warrants further research aimed at revealing the mechanisms of multidrug resistance in the wheat blast pathogen, including efflux pumps overexpression via transcriptomic analyses of differentially expressed genes; identification and discovery of mutations associated with changes in promoter regions or transcription factors of efflux transporters associated with multidrug resistance.

If allelic variation in the promoter regions, in the transcription factors or even in the coding region of the transporter genes could be unequivocally associated with MDR phenotypes, these alleles could be converted into molecular markers for monitoring, in real time, the spread of different MDR lineages of the pathogen in airborne inoculum (WEST *et al.*, 2008; WEST; KIMBER, 2015; KING *et al.*, 2017). This would foster the adoption of rational and sustainable anti-resistance strategies to manage wheat blast, using real time spatial and temporal disease surveillance tools based on the molecular detection of resistant inoculum of the pathogen in air samples from wheat fields. The adoption of such a smart, real-time anti-resistance strategy would minimize unneeded sprays and guide the choice of fungicides that are still effective when MDR is detected.

Future studies could be conducted by testing the role of specific transporter inhibitors such as *BLT-4* [N-(2-methoxyphenyl)-N′-2-naphthalenyl-urea, ≈ *INF271*, a MFS transporter type inhibitor] and verapamil hydrochloride (for ABC transporter type inhibition) (TEGOS *et al.*, 2008; PRATES *et al.*, 2011; PERLIN; ANDREWS; TOH, 2014; WONG *et al.*, 2014; SHARMA *et al.*, 2019). The main objective would be to assess if these two superfamily-distinct transporter inhibitors could interfere with the ability of *PoT*I strains for MDR resistance. One would look at any phenotypic changes induced by *BLT-4* and verapamil hydrochloride in the fungal strain's resistance responses to DMI and SDHI fungicides (i.e., altering the *PoT*I strain's distinct MDRPs phenotypic patterns for MDR and restoring fungicide sensitivity).

Over the past few years, strategies have been prospected to combat MDR yeasts and bacterial human pathogens, as well as multi-resistant cancer cells, based on the concomitant administration of efflux pump exporters inhibitors using chemicals

in synergism with azoles and other chemo drugs to restore their efficacy (FULTANG *et al.*, 2020; GREEN *et al.*, 2020; VEGA-CHACÓN *et al.*, 2021). Perhaps this strategy could also be applied for managing MDR plant pathogens in the agroecosystem. Further studies could also be carried out in planta to assess whether the use of efflux pump inhibitors in association with DMI or SDHI fungicides would have an additive effect, restoring the efficacy of the fungicides for wheat blast control.

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CHAPTER 3: AEROBIOLOGY OF THE WHEAT BLAST PATHOGEN: INOCULUM MONITORING AND FUNGICIDE RESISTANCE ALLELE DETECTION

ABSTRACT

Wheat blast is considered one of the most important and devastating fungal diseases affecting wheat crops worldwide. The disease is caused by the ascomycetous fungus *Pyricularia oryzae Triticum* lineage (*PoT*l). Disease control is heavily dependent on fungicide use, but resistance to sterol demethylase (DMI), quinone outside (QoI) and succinate dehydrogenase inhibitors (SDHI) have been reported in Brazil. These are the three major groups of fungicides used intensively for managing wheat diseases. In order to rationalize fungicide inputs (e.g. choice, timing, dose-rate, spray number and mixing/alternation) for managing wheat blast, we need monitoring tools, enabling quantitative measurement of pathogen´s inoculum levels and detection of fungicide resistant alleles, in combination with disease forecasting. To monitor wheat blast aerosol populations at Londrina in Paraná State, a major wheat cropping region in Brazil, we used a high-volume cyclone, an automated air sampling device, coupled with a quantitative real-time PCR (qPCR) assay. The objectives of our study were: 1) To monitor the amount of *PoT*l airborne conidia during 2019-2021 based on DNA detection, 2) To reveal the prevalence of QoI resistant (QoI-R) cytochrome *b* alleles in aerosol populations of wheat blast and 3) To determine the impact of weather on the dynamics of wheat blast aerosol populations and spread of QoI resistant alleles. *PoT*l inoculum was consistently detected in aerosols during the wheat cropping seasons in 2019 to 2021, but amounts varied significantly between seasons, with highest amounts detected in 2019. High peaks of PoTl DNA were also continuously detected during off-season in 2020 and 2021. The prevalence of QoI resistant (QoI-R) cytochrome *b* G143A alleles in aerosol populations was also determined for a subset of 10 PoTl positive DNA samples with frequencies varying between 10 and 91 % using a combination of PCR-amplification and SNP detection pyrosequencing. Statistically significant but low correlations were found between the levels of pathogen and the weather variables, indicating that a system based on weather variables has very low predictive value for determining levels of airborne *PoT*l inoculum. This observation

does not invalidate the potential of the continuous direct monitoring of *PoT*I airborne inoculum as prior detection of airborne spore levels of the pathogen can prevent major crop yield losses by ensuring that timely and appropriate disease management practices are adopted.

Keywords: *Pyricularia oryzae Triticum* lineage; airborne spores; epidemic predictors; integrated disease management.

1 INTRODUCTION

Wheat blast is a globally important disease caused by the hemibiotrophic ascomycetous fungal pathogen *Pyricularia oryzae Triticum* lineage (*PoT*l). Since the emergence of the disease in wheat fields from Paraná State, Brazil, in 1985 (Igarashi, 1985) the pathogen has spread to most of the Brazilian wheat cropping regions, and also to other countries in South America, including Argentina, Bolivia, and Paraguay (KOHLI *et al.*, 2011; CERESINI *et al.*, 2018; Cruz *et al.*, 2019). The efficient geographical range expansion of the pathogen in South America has been associated with spread via contaminated seed lots (CERESINI *et al*., 2018). The most recent outbreaks of wheat blast outside South America dates back to 2016 in Bangladesh, Southeast Asia (ISLAM *et al.*, 2016), and to 2017 in Zambia, East Africa (Tembo *et al.*, 2020), raising an urgent need to develop strategies to stop the further spread of this destructive pathogen to other disease-free wheat cropping areas (MALAKER *et al.*, 2016; MCDONALD; STUKENBROCK, 2016; SADAT; CHOI, 2017). The first intercontinental dispersal of the pathogen to Southeast Asia was linked to a South American origin (ISLAM *et al.*, 2016).

Knowledge about the life cycle and population biology of *PoT*l on wheat has increased in the last decade, and some recent studies have shed light on our understanding of the pathogen´s major reproductive mode and genetic structure [2,10,11]. For instance, contemporary populations of *PoT*l carry high genotypic and virulence diversity. This is consistent with a mixed reproductive system in which cycles of sexual reproduction are followed by the dispersal of locally adapted clones (MACIEL *et al.*, 2014; CERESINi *et al*., 2018; PIZOLOTTO *et al.*, 2019). In addition, populations either in close proximity or even separated by more than 2,000 km were very similar, consistent with a high degree of gene flow across both short and large spatial scales. Gene flow was also detected between wheat and non-wheat derived populations of *PoT*l, including from common signal grass (*Urochloa brizantha*) pastures and from 12 other invasive or cultivated grass species. The high gene flow can reflect an efficient wind-dispersal of conidia and/or ascospores from surviving perithecia on crop residues over short to moderate distances (PIZOLOTTO *et al.*, 2019; CASTROAGUDÍN *et al.*, 2017; URASHIMA *et al.*, 2007), as well as a long-distance dispersal via infected seed

of wheat and *Urochloa* spp. (PIZOLOTTO *et al.*, 2019; URASHIMA *et al.*, 2007; GOMES *et al.*, 2017; GOULART *et al.*, 1995).

Currently, the two most common management strategies to reduce the intensity of the disease on wheat fields include the deployment of resistant cultivars and fungicide spray applications (GOULART; PAIVA, 1993; URASHIMA *et al.*, 2004). Notwithstanding, wheat blast has become a very challenging disease to manage due the lack of cultivars with durable resistance (CRUZ *et al.*, 2010), and the low efficacy of fungicides sprays, especially in Brazil due to the evolution of fungicide resistance (CERESINI *et al*., 2018; CRUZ *et al*., 2019; TORRES *et al*., 2022; SHARMA, 2017). In fact, Brazilian populations of *PoT*l have shown moderate to high levels of resistance to all three groups of site-specific systemic high risk fungicides labeled for the management of wheat diseases in the country, which include the azoles (sterol demethylation inhibitor - DMIs) (POLONI *et al.*, 2021), quinone outside inhibitors - QoIs (CASTROAGUDÍN *et al.*, 2015; OLIVEIRA *et al.*, 2015), and the second-generation carboxamide fluxapyroxad, a succinate dehydrogenase inhibitor - SDHI (VICENTINI *et al.*, 2022). Fungicide sprays provide only partial control of the disease, even after multiple applications (PAGANI; DIANESE; CAFÉ-FILHO *et al.*, 2014).

In order to maximize the effective life of new and currently available fungicides for managing wheat blast and other diseases alike, there is a need for designing optimal and effective, evolutionary-smart anti-resistance strategies aimed to delay the evolution and spread of resistance to the existing (azoles and QoIs), the new (SDHIs) and upcoming fungicide actives, that growers and extension wheat pathologists can use as soon as new products are entering the market (CORKLEY *et al.*, 2022). For this purpose, high performance devices for monitoring populations of the pathogen, enabling quantitative measurement of inoculum levels and early detection of fungicide resistant alleles, in combination with disease forecasting, could be applied as smart tools. These tools would then guide the implementation of anti-resistance measures based on the rationalization of fungicide inputs (e.g. choice, timing, dose-rate, spray number and mixing/alternation). For example, an automated air sampler coupled with molecular DNA-based detection can be used to quantify the inoculum levels of air-dispersed pathogens (VAN DER HEYDEN *et al.*, 2021).

Airborne conidia released from infected wheat seedlings or from other grass hosts in neighboring fields are thought to provide an important source of *PoT*l inoculum for infection of wheat spikes (URASHIMA *et al.*, 2009; CRUZ *et al.*, 2015). Airborne

inoculum of *PoT*l is more abundant under conducive weather conditions that include high humidity and warm temperatures. These favorable weather conditions include long and frequent moisture periods (24–40 h), associated with high temperatures (25–30°C) (CARDOSO *et al.*, 2008; FERNANDES *et al.*, 2017). If high levels of *PoT*l airborne inoculum coincides with the wheat heading stage it can result in severe ear infection and high yield losses (GOMES *et al*., 2017; FERNANDES *et al.*, 2021).

Studying the dynamics of airborne populations of plant pathogens such as *PoT*l by quantifying the amount of spores in air samples (i.e., its aerobiology) can help in forecasting disease epidemics. In principle, aerobiology-based disease forecasting models are distinct from the climate-based models such as Sisalert (Plant Disease Epidemic Risk Prediction System) (FERNANDES *et al.*, 2017), which rely upon systematic monitoring of weather parameters across distinct wheat-growing regions to forecast infection periods. While they both should provide valuable tools to predict the occurrence and magnitude of wheat blast epidemics (WEST; KIMBER, 2015; Cruz *et al.*, 2016; NICOLAU *et al.*, 2016; WEST *et al.*, 2017), aerobiology-based models are still lacking behind to allow any comparison on differences in accuracy and reliability and fine tuning used a combined model.

Research on fungal aerobiology has been developed worldwide for more than two decades with the aim of providing real-time on-site quantitative measurements on airborne inoculum of plant pathogens to improve disease management (VAN DER HEYDEN *et al.*, 2021). Innovations have been made in air sampling devices to improve capture efficiency at high air volume collection rates for fungal aerobiology studies (WEST; KIMBER, 2015), since the first fully automated device for detection of airborne spores of *Sclerotinia sclerotiorum* was introduced in 1952 (HIRST, 1952). In recent years, spore-trapping combined with quantitative real-time polymerase chain reaction (qPCR) have been used successfully to detect and quantify airborne spores of wheat pathogens such as *Puccinia striiformis* (DEDEURWAERDER *et al.*, 2011), *Mycosphaerella graminicola* (*Zymoseptoria tritici*) (FRAAIJE *et al.*, 2005; DUVIVIER *et al.*, 2013) and *Blumeria graminis* (CAO *et al.*, 2016). Using DNA based SNP detection the frequency of fungicide resistant alleles within pathogen aerosol populations can also be measured (FRAAIJE *et al.*, 2005).

Automated collection of weather data coupled with continually updated on-site *PoT*l aerobiology data could provide a warning of the likely risk of wheat blast infection periods (WEST; KIMBER, 2015; WEST *et al*., 2017). Data on how much primary and/or

secondary inoculum is produced can provide a direct prediction of impending disease risk (WEST *et al*., 2017; CARISSE *et al.*, 2012) and allows real-time decision making for the most effective fungicide spraying schedule in reducing yield losses while decreasing the environmental impact and the selection pressure for fungicide resistance.

To monitor wheat blast aerosol populations at Londrina in Paraná State, a major wheat cropping region in Brazil, we used a high-volume cyclone, an automated air sampling device, coupled with a quantitative real-time PCR (qPCR) assay. The objectives of our study were: 1) to monitor the amount of *PoT*l airborne conidia during 2019-2021 based on DNA detection, 2) to detect QoI resistant (QoI-R) G143A cytochrome *b* alleles in aerosol populations of wheat blast (CASTROAGUDÍN *et al.*, 2015), and 3) to determine the impact of weather on the dynamics of wheat blast aerosol populations and spread of QoI resistant alleles.

2 MATERIALS AND METHODS

Airborne spores were collected daily in 2 ml tubes using the automated high-volume cyclone, which is available from Agri Samplers Ltd (Cressex Enterprise Centre, Cressex Business Park, Lincoln Rd. High Wycombe, Buckinhamshire, HP12 3RL, United Kingdom) (Figure 1). The air flow was set according to the manufacturer' standard setting of 270 L min^{-1}.

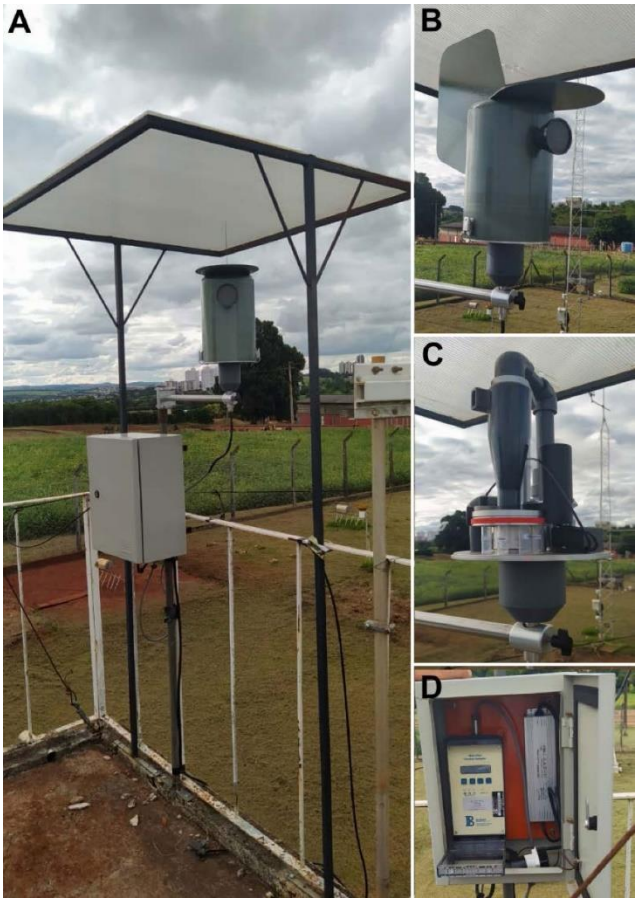
The high volume cyclone air sampler captured aerosols in Paraná state during 2019-2021, for 12 hours per day, from 6:00 to 18:00 h. It operated at the IDR Paraná Experimental Station (former IAPAR) (23° 21' 34.2"S; 51° 09' 52.9"W), in Londrina County, positioned within the limits of the weather station at 5 m high above the ground level and a minimum of 2 km from a wheat crop.

2.1 FUNGAL DNA EXTRACTION FROM AIR SAMPLES AND PURIFICATION

For DNA extraction, 0.5 g of sterile glass beads (400 – 455 µm diameter; Sigma, San Louis, Missouri, USA) were added to each 2 mL tubes together with 440 µL of extraction buffer (400 mM Tris-HCl; 50 mM EDTA pH 8; 500 mM NaCl; 2% polyvinylpyrrolidone; 5 mM 1,10-phenanthroline monohydrate, and just before use, 0.1 % β-mercaptoethanol). Tubes were subsequently transferred to a FastPrep homogenizer (Savant FastPrep BIO101 Homogenizer, Thermo Fisher, Waltham, MA, USA) for three cycles of 6.0 m s^{-1} for 40 s, with 2 min cooling on ice between cycles. After that, a total of 400 µl 2% SDS (sodium dodecyl sulfate) was added to the tubes, which were inverted several times for homogenizing the solution, and then incubated at 65 °C in a water bath for 30 min, while mixing every 10 mins. Subsequently, 800 µL of phenol: chloroform (1:1) was added to each tube, which were vortexed briefly and then centrifuged at 13 k rpm for 10 min at 4 °C. To another set of 1.5 mL eppendorf tubes we added 30 µL 7.5 M ammonium acetate, 480 µl isopropanol and 1 µL glycoblue. The supernatant from the centrifuged solution was pipetted into the new set of tubes and gently mixed and placed in a -20 °C freezer overnight. The tubes were then centrifuged at 13 k rpm for 30 min at 4 °C and the pellet was washed with 200 µL of 70 % ethanol and centrifuged at 13 k rpm for 5 min. The DNA pellet, made visible by glycoblue, was air-dried in a laminar flow cabinet (approx. 30 mins) and resuspended in 100 µl of 10 mM Tris pH 8.0, and then placed in a water bath at 65 °C for 5 mins to fully resuspend DNA, before storage at −20 °C. The DNA was purified

using EchoCLEAN DNA CleanUp column (BioEcho Life Sciences, Cologne, North Rhine-Westphalia, Germany) according to manufacturer's instructions.

Figure 1. (A) High-volume cyclone air sampler from Agri Samplers Ltd. (UK), installed in Londrina, PR, operating at the IDR Paraná (Paraná Agricultural Development Institute) Experimental Station; (B) Front view of the sampler unit; (C) Details of the uncovered spore sampler unit and (D) Control panel by which sampler parameters are defined.



2.2 DETECTION OF *PoT*L IN AIR SAMPLES USING qPCR

For DNA extraction, 0.5 g of sterile glass beads (400 – 455 µm diameter; Sigma, San Louis, Missouri, USA) were added to each 2 mL tubes. PCR reactions were performed in 15-µL reaction (in capped 96 well PCR plates) consisting of 2 µl DNA sample, 13 µl solution containing 7.5 µL of KAPA probe fast qPCR Master Mix (Biosystem, Foster city, California, USA) and 5.425 µl sterile distilled water containing primers MoT3_1F and MoT3_1R, and 5'FAM and 3'BHQ1 labeled probe MoT3_FAM2 for *PoT*l detection targeting a 361 bp amplicon according Pieck *et al*. (2017) and Rox reference dye (Invitrogen; 0.075 µl per reaction). The cycling conditions included 2 mins at 98 °C, followed by 50 cycles of 10 secs at 95 °C and 30 secs at 60 °C. Each

reaction was carried out in duplicate in an AriaMx Real-time PCR System (Agilent, Santa Clara, California, USA). In each qPCR run, standard samples consisting of calibrated amounts of target DNA from *Po**T*I (0.02 pg; 0.2 pg; 2 pg; 20 pg; 200 pg; 2000 pg; 20000 pg; 100 ng; 500 ng were included for the standard curve. Non-templates controls (NTC, *i.e*. sterilized distilled water) were also included in every qPCR run. Data were analyzed using the Agilent AriaMx software.

Table 1. Primers and probe used for real-time PCR detection of *Pyricularia oryzae Triticum* lineage and *cyt b* primers for detection of the A143 allele associated with QoI - resistance using a nested-polymerase chain reaction (nested-PCR) assay and pyrosequencing from fungal DNA extracted from airborne spore samples.

Oligonucleotide name	**Type**	**Oligonucleotide sequence and labeling (5′−3′)**
cyt b initial PCR		
F1	Forward primer	GTTTGAGCTATTGGTACTGTTATATTA
R1	Reverse primer	GAAACACCAAGAGGATTGCTT
cyt b nested PCR		
F2	Forward primer	GGCTATCGGTTTCCTAGGTTATGT
R2	Reverse primer*	TGCCCTATTCAAGGTATAGCACTA
cyt b primers for pyrosequencing		
S1	Sequencing primer	GGACAGATGTCATTATGAG

*Primer labeled with biotin at 5'end.

2.3 PYROSEQUENCING ASSAY FOR *cyt b* ALLELES QUANTIFICATION IN AIRBORNE *PoT*I POPULATION

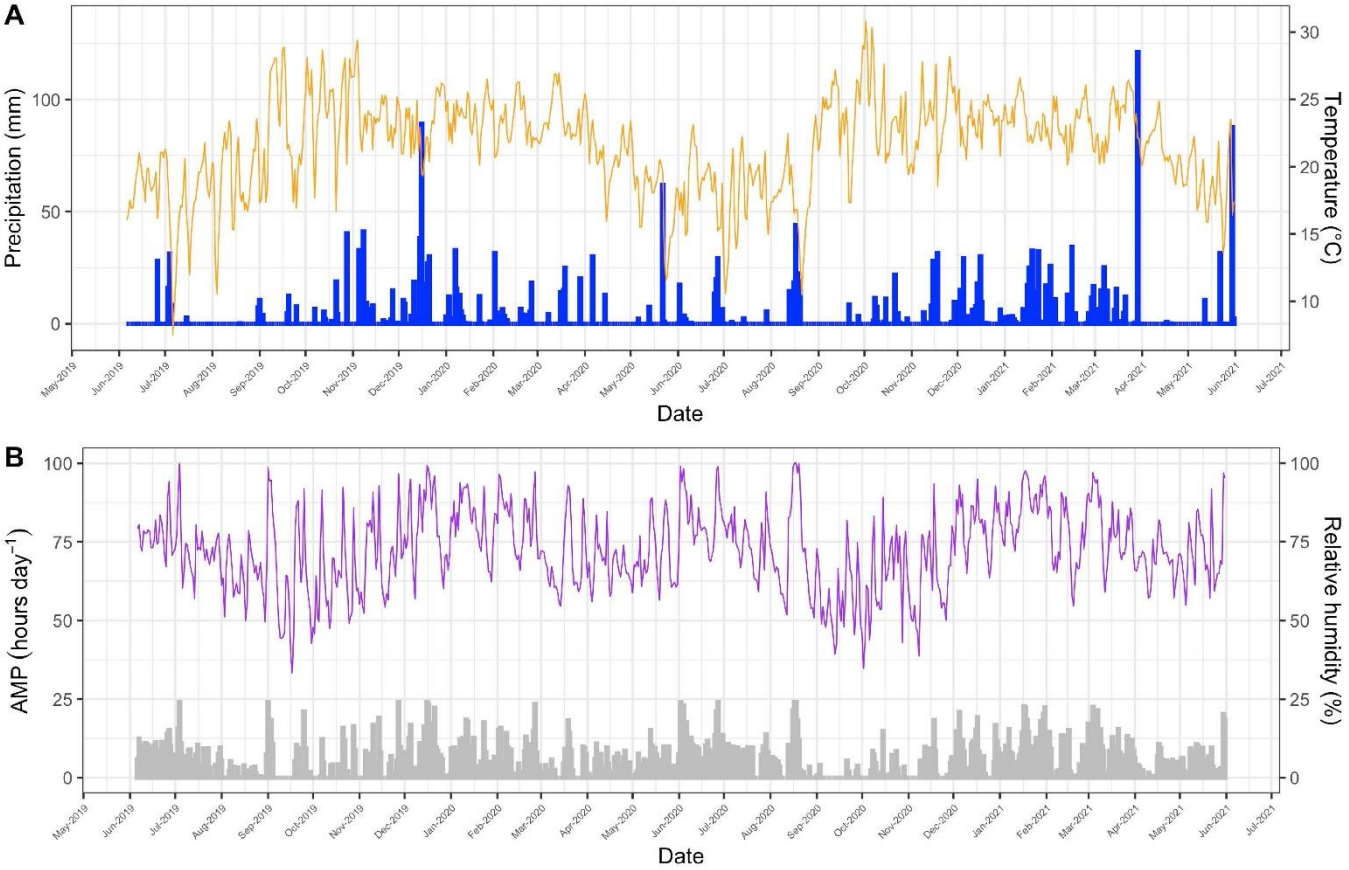
The frequency of cyt *b* A143 allele in *PoT*I, associated with QoI-resistance (G-to-C nucleotide exchange at position 54 of the nested PCR amplicon or at position 700 from the GenBank accession number AY245424 sequence resulting in the amino acid substitution of glycine by alanine at position 143 of the protein (Castroagudín *et al.*, 2015), was determined using a SNP detection pyrosequencing assay. Prior to pyrosequencing, an initial PCR and a subsequent a second PCR (nested-PCR) assay with amplicons from the first reactions were performed to amplify the *cyt b* gene

fragment which contained the target sequence. The amplification reaction of the initial PCR targeting a 305 bp *cyt b* fragment was performed in a total reaction volume of 25 µL containing 20 ng of DNA template, 100 µM of dNTPs, 0.2 µM of each primer (Table 1), 0.5 U of Taq DNA Polymerase (GoTaq, Promega, Madison, Wisconsin, USA) and 1X GoTaq buffer (3 mM $MgCl_2$). A negative control (PCR mixture without DNA) was included in all PCR experiments. The amplification reaction was carried out in a thermocycler as follows: initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. For the nested PCR, targeting a 101 bp fragment, the following protocol was used, with 15 µl of 1:500 dilution of the product from the initial PCR reaction: 100 µM of dNTPs, 0.2 µM of each nested PCR primer (Table 1), 0.5 U of Taq DNA Polymerase (GoTaq, Promega, USA) and 1X GoTaq buffer (3 mM $MgCl_2$, Promega). The amplification reaction was carried out in a thermocycler as follows: initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Pyrosequencing of the biotinylated nested PCR products was performed on a PyroMark Q48 instrument (Qiagen, Hilden, Mettmann, Germany) using TCGTGCTA as dispensation order and C/GTGCTACAGTTATTACTAATCTTATT as sequence to analyze. The pyrosequencing reagent kit (Qiagen) containing binding buffer, annealing buffer, dNTPs, substrate mixture (adenosine 5' phosphosulfate and luciferin), and enzyme mixture (DNA polymerase, ATP sulfurylase and luciferase) were loaded to the cartridges with set volumes according to the Pyromark software. The sequencing primer was added to the cartridge in a predetermined volume; 10 µL of each nested PCR product was loaded to each well of a PyroMark Q48 Disc in triplicate and 3 µL of PyroMark Q48 Magnetic beads (Qiagen) was added to each reaction. *PoT*I isolates with and without cyt b G143A were included as controls. The PyroMark Q48 Autoprep Software was used to quantify the underlying G to C mutation as indicated by the peak heights of the target *cyt b* gene sequence. Final data on the frequency of QoI-resistant A143 alleles are based on the mean of three technical replicate reactions.

2.4 PEARSON CORRELATION ANALYSIS AMONG WEATHER VARIABLES AND THE AMOUNT OF *PoT*I DNA DETECTED IN AIR SAMPLES USING A REAL TIME qPCR ASSAY

Weather data were obtained from the IDR Paraná Experimental Station which was located at short distance from the air sampler. Maximum, minimum and average temperatures (°C), rainfall (mm), leaf wetness duration (hour.day^{-1}), maximum, minimum and average relative humidity (%) and wind speed (m.s^{-1}) were recorded daily for two years (Figure 2).

Figure 2. Daily (A) rainfall (mm, orange lines) and average temperature (°C, dark gray bars), (B) leaf wetness period (hours day^{-1}, light gray bars) and average relative humidity (%, purple lines) in Londrina, PR, at the IDR - Paraná Experimental Station, from June 2019 to June 2021.



To test the correlation between weather variables and the amount detected of *PoT*I DNA from airborne inoculum, data on daily rainfall and leaf wetness period accumulated during 30, 15, 10 and 5 day-periods before the detection event, generating the variables Rainfall 30, 15, 10 and 5 and LW 30, 15, 10 and 5, were used. We also determined the optimal temperature index (number of days within the optimum

temperature for wheat blast disease development, ranging from 22°C to 28°C (ALVES; FERNANDES, 2006; CARDOSO; REIS; MOREIRA, 2008), accumulated in 30, 15, 10 and 5 day-periods. In addition, we also used relative humidity daily values reflecting the optimal relative humidity index (number of days in which the relative humidity was ≥ 85%, which is considered optimum for fungal sporulation (ALVES; FERNANDES, 2006; CARDOSO; REIS; MOREIRA, 2008), accumulated in 30, 15, 10 and 5 day-periods. The Pearson correlation analyses were conducted for four-time windows (May and June; June and July; July and August; August and September) spanning heading, flowering and grain filling stages, according to the distinct regional wheat sowing dates (National Program for Agricultural Zoning based on Climate Risk for Paraná State, ZARC – PR (MAPA, 2022). We also included a period completely outside of the wheat cropping (January to April) to check for correlations with weather variables.

2.5 DESCRIPTIVE REPRESENTATION OF TIME SERIES AND STATISTICAL ANALYSES

Time series of the daily amounts of *PoT*l DNA detected in aerosol samples using qPCR were summarized using the R software packages [46], *dplyr*, *lubridate*, *scale*s, *gridExtr*a, *ggthemes, ggplot2* and the functions *geom_line*, *geom_poin*t and *facet_wra*p.

Pearson correlation analysis ($p \leq 0.05$) among weather variables and the amount of *PoT*l DNA was conducted using the *Stats and Performance Analytics* packages from *R* software and the *R studio* interface (R development core team, 2020).

3 RESULTS

3.1 DETECTION OF *PoT*l IN AEROSOL SAMPLES USING qPCR

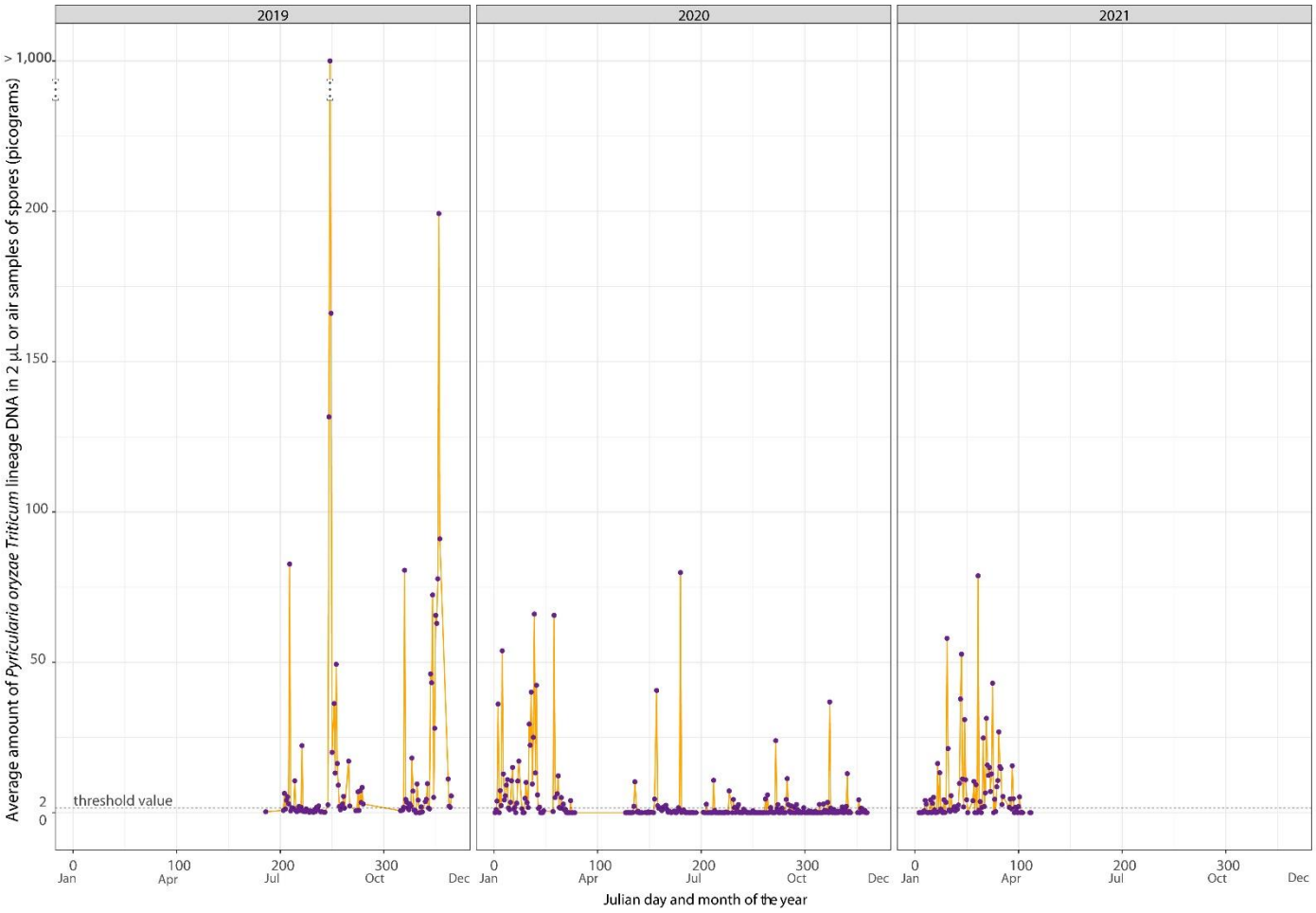
Based on the results of calibration curve samples that were tested in each run, in which 0.2 pg was sometimes detected whereas 2 pg always was detected within 35 cycles of qPCR, we used 2 pg as detection threshold. Using this detection threshold, *PoT*l target DNA was detected in 167 out of the 480 samples (35 %) taken and tested during the period 5 July 2019 till 14 April 2021 (Figure 3). The average amount of *PoT*l DNA in 2 µl of DNA sample was 8.9 pg. The highest amount of the fungus DNA detected in the entire two-year sampling period was from 5 September 2019 (1022 ± 13 pg), while less than 200 pg of target DNA was detected on all other days.

From July to September 2019, which was the period coinciding with wheat cropping in Londrina region, the average daily amount of fungal DNA detected was 28.7 pg. High peaks of the *PoT*l DNA were continuously detected off-season, from December 2019 until late March 2020 (average of 15.2 pg), with only a few days below the detection threshold.

The average amount of fungal DNA detected daily from May to September 2020 during wheat cropping was 1.8 pg, which was considerably lower than the 2019 cropping season (the mean of 2019 minus 2020 equals 26.9; $t = 2.2654$, with $df = 188$, two-tailed *p-value* = 0.0246*). Later in both early and late June 2020, which fell right within the annual wheat cropping season in Paraná State, two isolated higher peaks of *PoT*l DNA were detected (equal to 40.7 and 79.9 pg). In the following off-season, high peaks of the *PoT*l DNA were also continuously detected, from December 2020 until late March 2021 (average of 17.5 pg). The average amount of *PoT*l DNA detected on both previous (2020) and posterior (2021) off-seasons was significantly higher than the average amount detected in the 2020 growing season (the mean of both 2020 and 2021 off-seasons minus the 2020 in-season 2020 amount = -14.6; $t = 2.1973$, with $df = 316$, two-tailed *p-value* = 0.0287*).

A medium peak (36.8 pg) of *PoT*l DNA was detected in November 2020 followed by relatively lower peaks afterward. Similarly, in the 2020 off-season, several peaks of *PoT*l DNA from airborne spores samples were detected between late January to late March 2021 (such peaks varied from 26.9 to 78.8 pg). Therefore, there was a consistent trend of higher peaks of *PoT*l DNA detected during two consecutive wheat off-seasons (in the summer of 2019/20 and 2020/21).

Figure 3. Average daily amounts of *Po*T*I* DNA detected by qPCR in aerosols sampled for 12 h with a high-volume cyclone air sampler at Londrina, Paraná, from 5 July 2019 till 14 April 2021**.** Average daily amount of target DNA was determined from the results obtained after testing 2 µl of sample in duplicate.



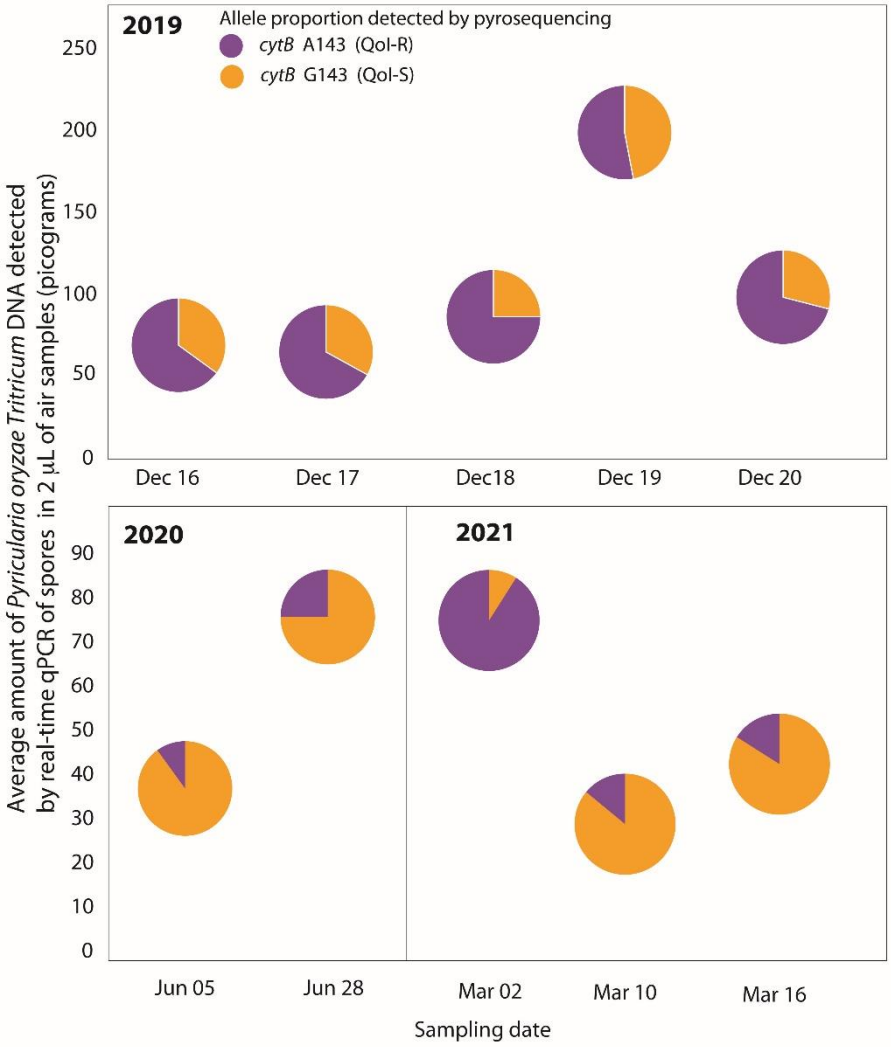
3.2 PYROSEQUENCING ASSAY FOR CYTB ALLELES QUANTIFICATION IN AIRBORNE SPORE SAMPLES OF *PoT*L

The partial *cyt b* gene fragment representing QoI-resistant (A143) and/or QoI-sensitive (G143) alleles was PCR-amplified and pyrosequenced from ten *PoT*I positive aerosol samples that were collected at different times during the two year monitoring period (Figure 4).

QoI-resistant A143 alleles were detected in all ten air samples tested but there were differences in frequencies (Figure 4). Five *PoT*I aerosol populations sampled during December 2019 showed an uniform QoI-R frequency between 52.5 and 74.5 %, whereas lower frequencies of 10.1 and 24.6 % were recorded for two populations

sampled during June 2020 and contrasting frequencies between 14.1 and 91.4 % were measured in three samples collected in March 2021.

Figure 4. Quantitative detection of cytochrome b G143A in *PoT*l aerosol populations sampled daily in Londrina (PR, Brazil) using PCR and SNP detection pyrosequencing assays. Two µl of DNA of each air sample was tested.



3.3 PEARSON CORRELATION ANALYSIS AMONG WEATHER VARIABLES AND THE AMOUNT OF POTL DNA IN AIR SAMPLES

In the first period encompassing heading, flowering and grain filling stages (from May to June), significant correlation was observed between the amount of *PoT*l DNA detected and the following six weather variables: daily rainfall accumulated for 15 days prior to the detection event (*dpde*) (r = 0.28, *p* = 0.1) and for 5 *dpde* (r = 0.34, *p*

= 0.05), the optimal relative humidity index (number of days with relative humidity ≥ 85%) accumulated for 5 *dpde* (r = 0,34, *p* = 0.05), the leaf wetness accumulated for 30 *dpde* (r = 0.24, *p* = 0.1), and the wind speed (r = 0.30, *p* = 0.05); the highest correlation was observed with leaf wetness accumulated for 30 *dpde* (r = 0.40, *p* = 0.01) (Figure 5).

Only two weather variables were correlated with the amount of *PoT*l DNA detected in air samples from the second period (June to July): rainfall accumulated for 5 *dpde* (r = 0.26, *p* = 0.1), and wind speed (r = 0.28, *p* = 0.05). No significant correlation with weather variables was detected at the third period (from July to August). In the fourth period (from August to September) there was significant correlation between the amount of *PoT*l DNA detected and the optimal relativity humidity index accumulated for 5 *dpde* (r = 0.25, *p* = 0.01), the accumulated leaf wetness for 10 *dpde* (r = 0.17, *p* = 0.1) and 5 *dpde* (r = 0.28, *p* = 0.01), and the wind speed (r = 0.24, *p* = 0.05).

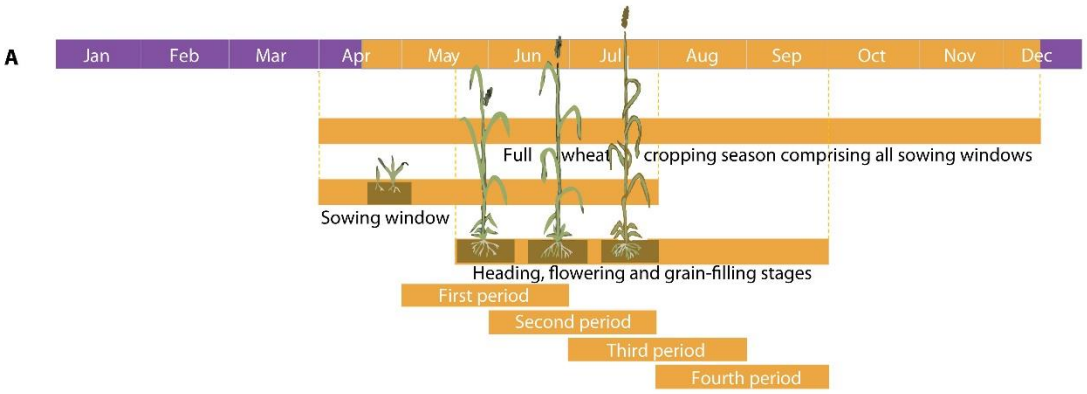
Correlations between the amount of *PoT*l DNA detected and weather variables were also observed in the period outside of the wheat growing season, from January to April. Although the r values for these correlations were relatively lower, this was the period with more weather variables correlated with the molecular detection levels of the pathogen. Positive significant correlation was observed with the accumulated rainfall for 30 *dpde* (r = 0.13, *p* = 0.1) and 5 *dpde* (r = 0.18, *p* = 0.05). The amount of pathogen´s DNA correlated with the optimal relative humidity index accumulated for 30 *dpde* (r = 0.31, *p* = 0.001), 15 *dpde* (r = 0.13, *p* = 0.1), 10 *dpde* (r = 0.20, *p* = 0.05) and 5 *dpde* (r = 0.28, *p* = 0.001). Significant correlation was observed also with accumulated daily leaf wetness for30 *dpde* (r = 0.22, *p* = 0.01), 15 *dpde* (r = 0.14, *p* = 0.1), 10 *dpde* (r = 0.15, *p* = 0.1) or 5 *dpde* (r = 0.23, *p* = 0.01).

There was no correlation between the optimal temperature index (number of days with temperatures ranging from 22°C to 28°C) accumulated for any of the periods and the amount of fungal DNA detected.

4 DISCUSSION

Using a high-volumetric cyclone air sampler positioned in a major wheat cropping area in Paraná State, coupled with a qPCR assay for pathogen detection, our first aim was to directly measure *PoT*I target DNA representing airborne conidia continuously released from 2019 and 2021, both within and off the wheat growing - season, in Londrina, PR.

Figure 5. A. Correlations were determined for two-month data periods comprising heading, flowering, and grain filling stages as defined based on the regional sowing window [45]. **B.** Pearson correlation coefficient (r) between *PoT*I DNA (pg) present in DNA of a daily air sample (2 µl tested) and weather variables accumulated 5, 10, 15, and 30 days prior to spore release detection event. Rainfall, optimal relative humidity index (days with RH ≥ 85%), leaf wetness period (LW), and wind speed (WS) at 2 meters. Significance levels (*p* values) at ***= 0.001; **= 0.01; *= 0.05; ˙= 0.1.



Pearson correlation coefficients (*r*)

B

Two-month periods comprising wheat heading, flowering and grain-filling stages according to the regional sowing window

Weather variables / Days prior to the detection event (*dpde*)	Accumulated rainfall period*(mm.day^{-1})			Optimal relative humidity index (days with RH ≥ 85%)				Accumulated leaf wetness periods period*(hours.day^{-1})				Wind speed (m.s^{-1})
	Rainfall 30	Rainfall 15	Rainfall 5	RH ≥ 85% 30	RH ≥ 85% 15	RH ≥ 85% 10	RH ≥ 85% 5	LW 30	LW 15	LW 10	LW 5	WS
Average amount of *Pyricularia oryzae Triticum* lineage DNA in 2 µL or air samples of spores (picograms)												
First period: May and June												
	-	0.28 ˙	0.34 *	-	-	-	0.34 *	0.24 ˙	-	-	0.40 **	0.30 *
Second period: June and July												
	-	-	0.26 ˙	-	-	-	-	-	-	-	-	0.28 *
Third period: July and August												
	-	-	-	-	-	-	-	-	-	-	-	-
Fourth period: August and September												
	-	-	-	-	-	-	0.25 **	-	-	0.17 ˙	0.28 **	0.24 *
Period outside the cropping season												
January to April												
	0.13 ˙	-	0.18 *	0.31 ***	0.15 ˙	0.20 *	0.28 ***	0,22 **	0.14 ˙	0.15 ˙	0.23 **	-

’The direct detection of *PoT*l’s airborne inoculum density using an automated continuous air sampling system, on a regional scale, may in combination with a molecular detection be useful in providing more accurate predictions of the risks of severe wheat blast epidemics, even before it occurs (LUO *et al.*, 2007), as it has been documented for other pathosystems (CARDOSO; REIS; MOREIRA, 2008; VAN DER HEYDEN *et al.*, 2021). Knowledge on airborne inoculum for wheat blast could optimize fungicide spray decision making as part of an integrated disease management program, allowing reduction of unnecessary sprays and lower the environmental impact (WEST *et al.*, 2008).

In our study, *PoT*l inoculum was consistently detected during the 2019 and 2020 wheat cropping seasons, but amounts varied significantly between these two cropping seasons, with higher amounts detected in 2019. However, high peaks of *PoT*l DNA were also continuously detected in both 2020 and 2021 off-seasons, with an average of 16.42 pg mL^{-1}, which was even significantly higher than the amount detected within the 2020 cropping season. Assuming 0.042 pg of DNA corresponds to approximately 1 spore and 194 m^3 air was sampled daily for 12 h, the highest level of spores recorded daily for the 12 h sampling period was 6259 spores.m^{-3} on 5 September 2019, with all other samples having daily numbers equal or well below 1219 spores.m^{-3} based on a DNA sample volume of 100 µl. These numbers are comparable to *PoT*l spore numbers recorded in air samples collected with a self-made PVC pipe wind vane spore trap near wheat fields in Passo Fundo (Rio Grande do Sul state, Brazil) using spore counting (DANELLI *et al.*, 2019). Danelli *et al*. (2019) also recorded high amounts of *PoT*l airborne inoculum in the off-season period from February to March.

With the lack of regional wheat cultivation in the off-season during summer, from December to March (according to the National Program for Agricultural Zoning based on Climate Risk for Paraná State, ZARC – PR (Ministério da Agricultura Pecuária e Abastecimento - MAPA, 2022), the detection of *PoT*l airborne inoculum indicates that the pathogen is continuously and successfully reproducing in other grass hosts and being air dispersed. In fact, *PoT*l can survive between growing seasons in over 12 invasive grass host species (CASTROAGUDÍN *et al*., 2015), and may contribute to the initial inoculum for the onset of wheat blast disease every cropping season. Besides the invasive grass species, important forage crops species such as signal grass (*U. brizantha*, the country's most extensively cultivated grass pasture for cattle grazing) and perennial ryegrass (*Lolium perenne*), are important susceptible secondary hosts

for *PoT*I from which fungal conidia is constantly and abundantly wind-dispersed over short to medium distances (CASTROAGUDÍN *et al.*, 2015; MACIEL *et al.*, 2022). Therefore, our aerobiology data corroborates the assertion that off-season non-wheat hosts play an important role in the early onset of the wheat blast, which can keep a year's long bridge of fungal inoculum in between wheat cropping seasons.

The second objective of our study was to establish the prevalence of QoI-R cyt b A143 alleles in *PoT*I aerosol populations. By a combination of nested PCR and pyrosequencing, we were able to detect A143 alleles in all 10 *PoT*I positive PCR samples that were selected from different periods of our monitoring studies. Five *PoT*I aerosol populations sampled during 16-20 December 2019 showed an uniform QoI-R frequency between 52.5 and 74.5 %, indicating that inoculum is originating from the same source during this period, whereas lower frequencies of 10.1 and 24.6 % were recorded for two populations sampled on 5 and 28 June 2020. The aerosol population sampled on 2 March 2021 showed the highest frequency of QoI resistance alleles, 91 %, whereas the other two samples collected during March 2021 showed contrasting frequencies of 16 and 14 % for 10 and 16 March 2021, respectively. Because the *cyt b* sequence is very similar between *PoT*I and *PoO*I, the nested PCR Pyrosequencing assay is not be able to distinguish the two lineages. We, therefore, also carried out a quantitative SYBR Green real-time PCR assay using primers Pot2a-L2 and Pot2a-R2 which will detect both lineages as described by Pieck *et al*. (2017). By comparing the results of the two assays it was clear that *PoO*I was either absent or present in such limited amounts that it would not have an impact on the recorded QoI-R allele frequencies (data not shown).

Despite the seasonal fluctuations detected in the prevalence of *cyt b* allele for QoI resistance in *PoT*I's airborne inoculum, it was clear from our study that QoI-R strains were prompt to reemerge and dominate the wheat blast fungal population along the cropping season. This ability for re-emergence has been attributed to an inherited competitive advantage associated with the *PoT*I QoI-R strains (Dorigan, 2019) although competitive disadvantage has been reported for cyt b G143A allele carrying QoI-R strains from fungal species such as *Zymoseptoria tritici* and the sister species *P. oryzae Lolium* lineage (MA; UDDIN, 2009; CHEN *et al.*, 2016; HAGERTY; MUNDT, 2016).

Because of the widespread prevalence of resistance to QoI fungicides in field populations of *PoT*l across the major wheat cropping areas from central and southern Brazil reported in recent years, corroborated by our current aerobiology study in Londrina, PR, spraying multiple QoI fungicide based applications should be avoided as an anti-resistance precautionary strategy (Ishii; Hollomon, 2015). The intensive long-term use of QoI fungicides managing wheat diseases has kept a non-stop selection pressure favoring the prevalence of QoI-R *PoT*l strains in the wider environment (CASTROAGUDÍN *et al*., 2015; OLIVEIRA *et al*., 2015; CERESINI *et al*., 2018; VICENTINI *et al*., 2022a).

A similar scenario can occur with the recently-introduced site-specific systemic high risk SDHI fungicide fluxapyroxad, because SDHI-resistant baseline populations of *PoT*l have been detected even before the local labeling of these actives for the management of wheat diseases in Brazil and persisted in current populations of the pathogen (VICENTINI *et al*., 2022a). The SDHI-resistance in *PoT*l has not been associated with single point mutations in the target genes *sdh-B*, *C* or *D*, but rather to a more complex energy-dependent efflux pump mechanism typical of multidrug resistance (VICENTINI *et al*., 2022a; VICENTINI *et al*., 2022b).

In fact, *in vivo* fungicide sensitivity testing of *PoT*l isolates under controlled environment has shown moderate to high levels of resistance to multiple fungicides groups, with blast control efficacy as lower as 3.3% for the QoI azoxystrobin, 31.3% for the QoI pyrachlostrobin (QoI), 31.0% for the SDHI fluxapyroxad, and 51.0% for the DMI epoxiconazole (CAZÓN *et al*., 2022). The multiple fungicide resistance detected in this *in vivo* assay (CAZÓN *et al*., 2022) was probably due to enhanced efflux pump activity in *PoT*l (VICENTINI *et al*., 2022b). Although the impact of this resistance mechanism detected both *in vitro* (VICENTINI *et al*., 2022a; VICENTINI *et al*., 2022b) and *in vivo* (CAZÓN *et al*., 2022) assays might be less than expected for disease control in the field, a meta-analysis from 42 field trials over 9 years (2012 to 2020) indicated average control efficacy of QoI and DMI fungicides ranging from as low as 43% to a maximum of 58% (ASCARI *et al*., 2021). Given this poorer performance and lower profitability of fungicide sprays (ASCARI *et al*., 2021), there is an urgent need for the adoption of integrated wheat blast management strategies that do not rely so heavily on fungicides.

Upon further selection of SDHI fungicides it is likely that *PoT*l will evolve target-site as a whole range of mutations in SdhB, C and/or D have already been reported in

cereal pathogens such as *Z. tritici*, *Pyrenophora teres* and *Ramularia collo-cygni* (Mair, *et al*., 2016). For early detection of new mutations at extreme low frequencies, allowing early implementation and monitoring of fungicide resistance management strategies, next generation sequencing techniques such as **Illumina MiSeq** for short reads targeting single SNPs and Oxford Nanopore **MinION** for long reads targeting combinations of SNPs can be used in combination with automated air sampling (PIECZUL 2017; GUTIERREZ VAZQUEZ, 2022).

The third aim of our study was to determine the correlation among weather variables and the amount of *PoT*l DNA in airborne spores samples. We tested the hypothesis that weather variables were correlated with levels of *PoT*l DNA detected in airborne spores samples collected along two consecutive years.

In our study, statistically significant but low correlations were found between the levels of pathogen molecular detection and the weather variables analyzed in four distinct two-months periods (comprising wheat heading, flowering, and grain-filling stages) within the cropping season. These four periods were defined a priori based on the distinct sowing windows established by an official zoning program along the full cropping season (National Program for Agricultural Zoning based on Climate Risk for Paraná State, ZARC – PR (MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO - MAPA, 2022).

For any of the periods examined, except for the third one (Figure 5A and B, period from July to August), a few climatic variables were correlated with the amount of the pathogen's DNA (Figure 5B). However, in the off-season, most of the weather variables were correlated with the amount of pathogen's DNA detected. The optimal relative humidity index accumulated for 30 *dpde* ($r = 0.31$, $p = 0.001$), and accumulated daily leaf wetness for 5 *dpde* ($r = 0.23$, $p = 0.01$) showed the highest correlation in the off-season period. Despite significance, correlations were very low. Therefore, we concluded that a system based on weather variables has very low predictive value for determining levels of airborne *PoT*l inoculum.

This observation does not invalidate the potential of the continuous direct monitoring of *PoT*l airborne inoculum as it can provide a better understanding of the dynamics of airborne inoculum production, release, and dispersal as well as about the pathogen´s epidemics, which can be critical for more accurate predictions of wheat blast risk (FERNANDES *et al*., 2021). If high levels of *PoT*l airborne inoculum coincide with the wheat heading stage it can result in severe ear infection and high yield losses

(z *et al*., 2017; Fernandes *et al*., 2021). Therefore, monitoring *PoT*l inoculum from air samples both within cropping fields and at the regional scale is very important to developing aerobiology-based forecasting models for blast disease incidence (Nicolau, *et al*., 2016). In conclusion, for wheat blast, prior detection of airborne spore levels of the pathogen can prevent major crop yield losses by ensuring that timely and appropriate disease management practices will be adopted.

Funding: This research was funded by the São Paulo Research Foundation, Brazil (FAPESP) through regular research grants to P.C. (2020/07611-9; 2021/03402-9 and 2022/12661-0); in partnership with Fundação Araucária to A.C. (FPS2020011000057); a Newton Fund grant from the Biotechnology and Biological Sciences Research Council (BBSRC) and UK Research and Innovation (UKRI), with FAPESP as partner institution in Brazil to P.C. (2017/50456-1; 2018/21197-0) and to S.M. (postdoctoral scholarship 2019/12509-1), CNPq (Brazilian National Council for Scientific and Technological Development), grant/award number: Pq-1D 313825/2018-1 and Pq-1C 311895/2022-0; CAPES (Coordination for the Improvement of Higher Level Personnel), Grant/Award Numbers: CAPES/Pro-equipment Program 775202/2012, CAPES/AUXPE Program 88881.593505/2020-01—UNESP Ilha Solteira Campus, CAPES PrInt/UNESP 41/2017 - 88887.570719/2020-00. B.F. received funding from the Newton Fund through grant BB/S018867/2 awarded by BBSRC under the BBSRC-FAPESP AMR and Insecticide Pest Resistance in Livestock and Agriculture Programme at NIAB - National Institute of Agricultural Botany, Cambrigde, UK and Rothamsted Research, Harpenden, UK. S.V. and L.K. were supported by Ph.D. scholarships from CAPES studentship Program 001. L.K. was supported by a studentship from FAPESP (2022/09787-2). The article processing charges were supported mostly by São Paulo State University's Vice-Presidency for Research (PROPE) special grant for publication (Public Call 10/2022) and partially by funds from the Paraná Rural Development Institute, IDR—Paraná/IAPAR.

Informed Consent Statement: The Brazilian Ministry of Environment**/**National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SisGen, issued the Certificates # A59E163 and A100786 authorizing the scientific activities associated with the collection of botanical and fungal material from the wheat agroecosystem in the Cerrado´s biome and access to the genetic diversity of *Pyricularia* species.

Acknowledgments: We acknowledge Rothamsted Research, UK from donating four Cyclone spore samplers purchased from Agri Samplers Ltd., which were then incorporated by UNESP for the realization of this study.

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CONCLUSION

In conclusion, the current status of multiple fungicide resistance in populations of the wheat blast pathogen requires adoption of immediate and accessible anti-resistance strategies, undertaken at regional and national level. Such measures will be useful to prolong the shelf life of existing fungicides (azoles and QoIs) and new actives (e.g. SDHIs) entering the market. Fungicide risk disease management programmes based on integrated disease management need to be developed based on real-time up-to-date disease surveillance and fungicide resistance molecular monitoring systems, such as this one we described in our study. Practical and simple alternatives, which could include the development and distribution of airborne inoculum samplers will provide valuable tools for further detection of the pathogen and could be used in practical alert and risk monitoring networks, associated with alert prediction systems already existing in Brazil. In addition, the data can be available to farmers and field professionals, by a web portal for knowledge transfer on disease surveillance and fungicide resistance monitoring.

APPENDIX

1. Sampling of symptomatic wheat spikes in Minas Gerais (A) and (B) and São Paulo, Brazil (C), (D), (E) and (F).

A)

B)

C)

D)

E)

F)

2. Sampling of symptomatic wheat spikes at IDR (A) and (B), wheat fields of commercial areas (C) and (D) in Londrina Paraná State, Brazil. Symptomatic wheat spike (E). Isolations of *PoT*l in potato dextrose agar (PDA) medium amended with discriminatory doses of DMI and QoI fungicides (F).

A)

B)

C)

D)

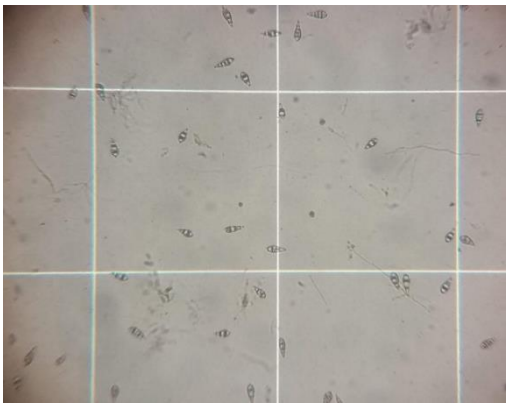
E)

F)

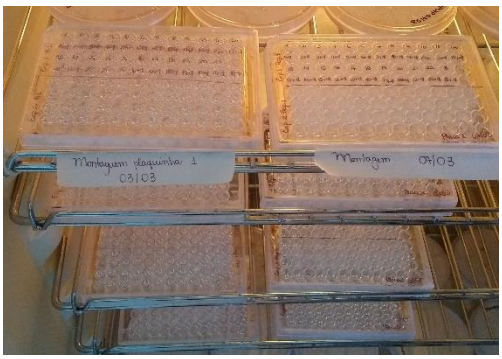
Lab work experiments: *PoT*l conidia counting (A), *PoT*l conidia (B), sensitivity assay using microplate (C) and Petri dishes (D). Agarose gel electrophoresis of *SDH* gene amplification (E).

A)

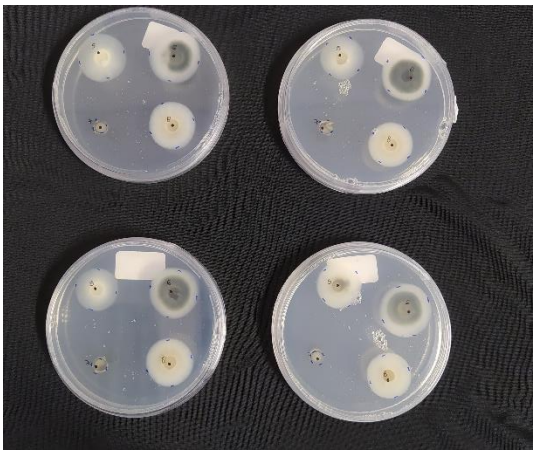
B)



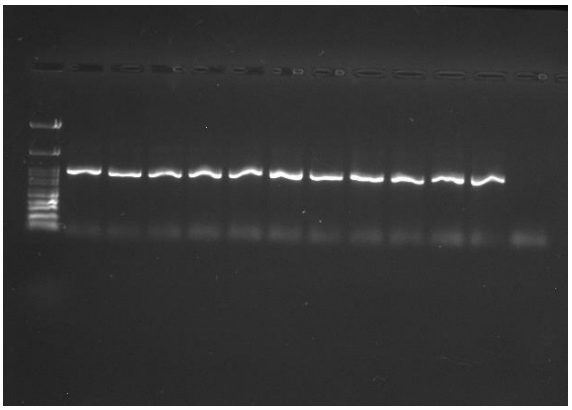
C)



D)

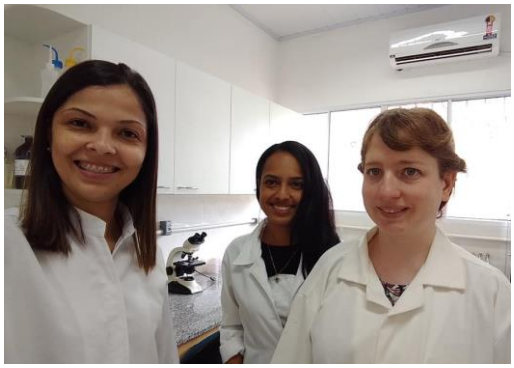


E)



4. Research group at Molecular Plant Pathology Lab - UNESP (A), (B), (C) and (D). High-volume Cyclone aerosol spore sampler (E) and meteorological Station at IDR Paraná Londrina, Paraná, Brazil.

A)



B)

C)

D)

E)

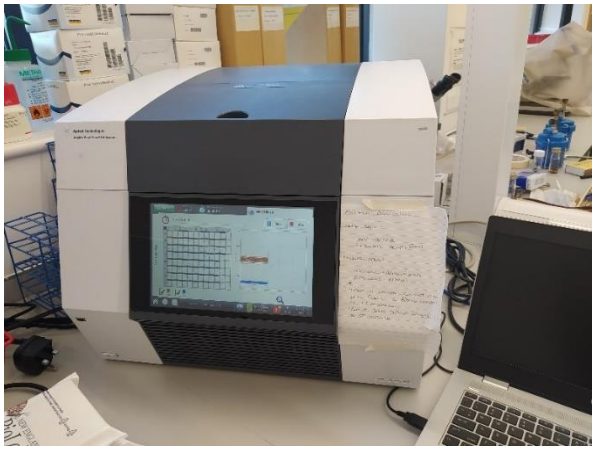
F)

5.National Institute Agricultural of Botany NIAB, Cambridge, UK (A), Rothamsted Research, Harpenden, UK (B). Real-time qPCR analysis at NIAB (C) and (D). Research group at NIAB (E) and (F).

A)

B)

C)



D)

E)

F)