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

Ilha Solteira

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

Tese apresentada à Faculdade de
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Especialidade: Sistemas de Produção.

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“...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* (*PoTI*), 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 *PoTI* era devida ao(s) mecanismo(s) MDR mediados por bomba de efluxo, caracterizando a sensibilidade de isolados de *PoTI* 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 *PoTI* 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 *PoTI* de amostras coletadas do ar também foi determinada para um subconjunto de 10 amostras de DNA positivas para *PoTI*. 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 (*PoTl*), 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 *PoTl* 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). *PoTl* 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 *PoTl* 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.

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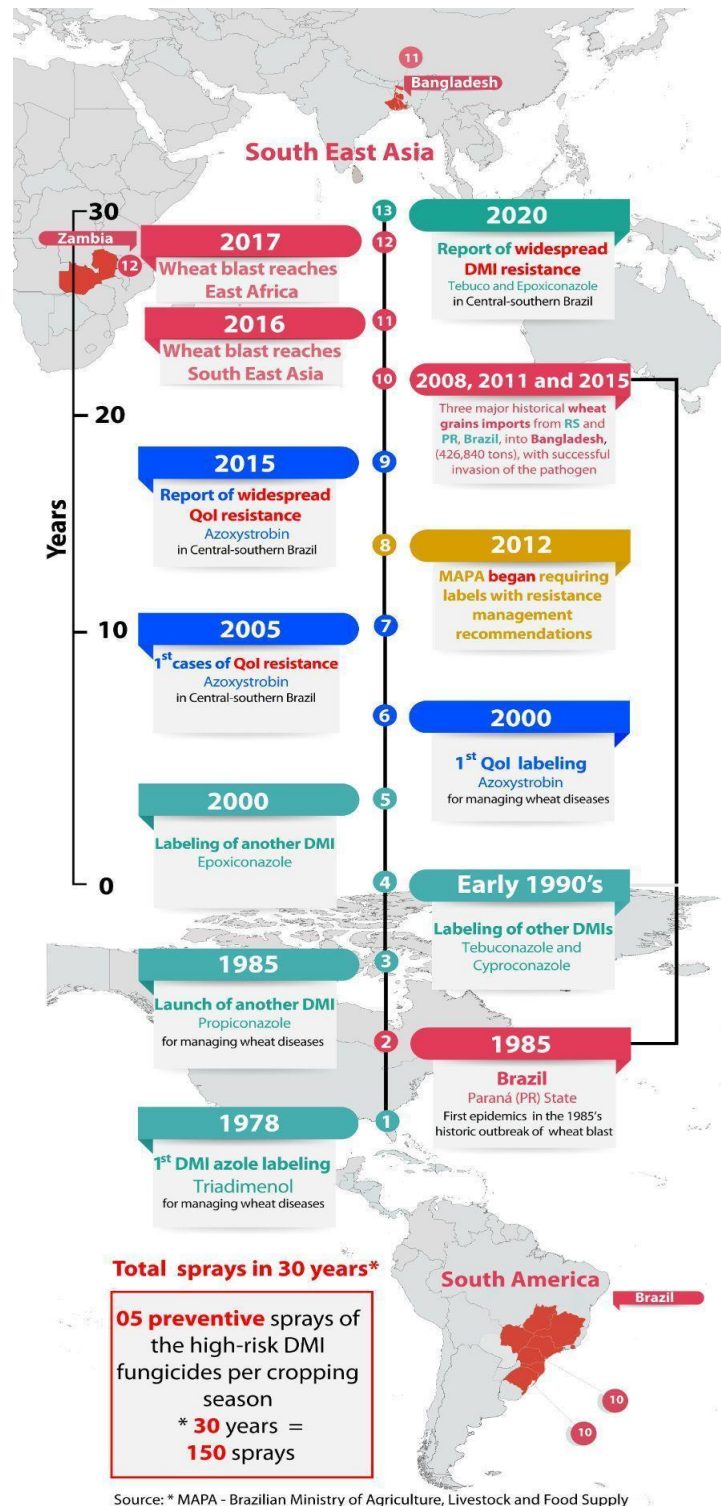
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INTRODUCTION

Wheat production is threatened by recent events of intercontinental expansion of the devastating blast disease, caused by the ascomycetous fungus *Pyricularia oryzae* *Triticum* lineage (*PoTI*). 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 *PoTI* 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 *PoTI* 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 *PoTI* (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 *PoTl* 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 *PoTl* 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, *PoTl* 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 *PoTl* 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 *PoTl* 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 *PoTl* 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 *PoTl* 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 *PoTl*. 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 *PoTl* populations

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

The onset of double fungicide resistance in populations of *PoTI* 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 *PoTI* conidia in the air can be important to develop forecasting models for blast disease (DANELLI *et al.*, 2019), although airborne *PoTI* monitoring studies are still scarce.

This research has three chapters to elucidate the historical and current scenarios of *PoTI* 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 *PoTI* 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 *PoTI* isolates showing reduced sensitivity to SDHI revealed 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 Qol-, DMI- and/or SDHI-resistant isolates of *PoTI* 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=1qehH5zlg6w>). 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 Qol resistant *cytB*-associated allele detected and quantified using pyrosequencing of PCR amplicons from positive samples.

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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.