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CÂMPUS DE ILHA SOLTEIRA**

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**PREVALENCE OF RESISTANCE TO SDHI FUNGICIDES IN POPULATIONS OF
THE BLACK AND YELLOW SIGATOKA PATHOGENS FROM BANANAS IN
SOUTHEASTERN BRAZIL**

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**PREVALENCE OF RESISTANCE TO SDHI FUNGICIDES IN
POPULATIONS OF THE BLACK AND YELLOW SIGATOKA
PATHOGENS FROM BANANAS IN SOUTHEASTERN BRAZIL**

Tese apresentada à Faculdade de Engenharia de
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Dedico!

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“Ninguém nasce odiando o outro pela cor de sua pele, ou por sua origem, ou sua religião. Para odiar as pessoas precisam aprender, e se elas aprendem a odiar, podem ser ensinadas a amar”.

(Da autobiografia "O longo caminho para a liberdade", Nelson Mandela 1994).

RESUMO GERAL

O objetivo do primeiro capítulo deste estudo foi desenvolver e validar um ensaio digital de sensibilidade a fungicidas baseado na resazurina, denominado COL-assay, para patógenos fúngicos vegetais dos gêneros *Mycosphaerella* e *Pyricularia*. O ensaio proposto foi baseado em estimativas colorimétricas de redução de resazurina, que foi usada como um indicador metabólico da atividade de respiração fúngica em culturas de microplacas. Os patógenos de Sigatoka amarela e negra e o patógeno de brusone do trigo foram utilizados como modelos fúngicos. Foi comparado o ensaio de detecção espectrofotométrica clássico (SPEC-assay) com o COL-assay proposto, com base na análise de imagens digitais das culturas em microplacas capturadas com câmeras de celular em um transiluminador caseiro. Foi observado que o COL-assay teve precisão semelhante ao SPEC-assay, ou seja, resultou em categorias de sensibilidade a fungicidas semelhantes para isolados fúngicos resistentes ou sensíveis e alta precisão. Como a metodologia completa do COL-assay e a do transiluminador caseiro foram compartilhadas, o ensaio provavelmente será adotado como um meio acessível de monitorar a resistência a fungicidas de populações de importantes patógenos fúngicos vegetais como *M. fijiensis*, *M. musicola* e *P. oryzae* linhagens Triticum e Oryzae. Baseado no ensaio de detecção espectrofotométrica clássico (SPEC-assay) mais uso de resazurina, no segundo capítulo deste estudo, detectamos que a resistência aos fungicidas SDHI é generalizada em populações dos patógenos da Sigatoka negra e amarela em plantações de banana no sudeste do Brasil. O complexo de doenças da Sigatoka negra e amarela (CDS), causado por *Mycosphaerella fijiensis* (Mf) ou *Mycosphaerella musicola* (Mm), respectivamente, é uma das doenças de manchas foliares fúngicas mais destrutivas que afetam os campos comerciais de banana em todo o mundo, levando a perdas significativas de rendimento. No Brasil, os fungicidas de sítio específico SDHI rotulados para o manejo de BSDC desde 2014 apresentam um alto risco de surgimento de resistência se forem implantados intensivamente e isoladamente ou em misturas com outros fungicidas de alto risco, como recomendado por mais de oito anos. Para preencher uma lacuna de informações atual sobre a ocorrência e prevalência de resistência aos fungicidas SDHI, este estudo determinou os níveis de sensibilidade ao boscalida e fluxapiroxade em quatro populações dos patógenos CDS amostrados em 2020 de três regiões geográficas distintas com sistemas de manejo de fungicidas contrastantes. Num total de 67 isolados, a prevalência de resistência aos fungicidas SDHI detectada foi de 94,0% para boscalida e 59,7% para fluxapiroxade. Apenas 1,5% dos isolados foram sensíveis a ambos os fungicidas. Também

determinamos a associação entre níveis de sensibilidade e mudanças nos genes-alvo de fungicidas correspondentes (*SdhB*, *C* e *D*). Os valores de EC₅₀ do boscalida e do fluxapiroxade foram mais altos na população *Mf* SPVR-CI com manejo intensivo de fungicidas, seguida por *Mm* SPNW-C e *Mm* MGN-C. Entre os 10 isolados de *Mf* e 57 de *Mm* sequenciados, apenas uma (*SdhC* N55D) e duas (*SdhB* E196Q e *SdhD* N66K) alterações no local-alvo do Sdh foram detectadas, respectivamente. Um mecanismo de bomba de efluxo também foi detectado em *Mf* conferindo resistência ao fluxapiroxade. Como a resistência aos fungicidas SDHI já é prevalente, indicando emergência simultânea em todas as três regiões geográficas, esses resultados destacam a importância de limitar a implantação de fungicidas SDHI para o manejo de CDS como uma estratégia fundamental de manejo de resistência.

Palavras-chave: patógenos fúngicos foliares; resistência a fungicidas; fungicidas de alto risco; QoI; DMI; SDHI; fenotipagem de resistência antimicrobiana; estratégias inteligentes anti-resistência; decisão de pulverização baseada em vigilância; limitação de pulverizações de fungicidas; controle químico; *SdhB*; *SdhC*; *SdhD*; resistência a fungicidas; Ec50; resazurina.

GENERAL ABSTRACT

The objective of the first chapter of this study was to develop and validate a digital fungicide sensitivity assay based on resazurin, called COL-assay, for plant fungal pathogens of the *Mycosphaerella* and *Pyricularia* genera. The proposed assay was based on colorimetric estimates of resazurin reduction, which was used as a metabolic indicator of fungal respiration activity in microplate cultures. Yellow and black Sigatoka pathogens and the wheat blast pathogen were used as fungal models. The classic spectrophotometric detection assay (SPEC-assay) was compared with the proposed COL-assay, based on digital image analysis of microplate cultures captured with cell phone cameras in a homemade transilluminator. It was observed that the COL-assay had similar precision to the SPEC-assay, resulting in similar fungicide sensitivity categories for resistant or sensitive fungal isolates and high precision. Since the complete methodology of the COL-assay and the homemade transilluminator were shared, the assay is likely to be adopted as an affordable means of monitoring fungicide resistance of important plant fungal pathogen populations such as *M. fijiensis*, *M. musicola*, and *P. oryzae* Triticum and Oryzae lineages. Based on the classic spectrophotometric detection assay (SPEC-assay) plus resazurin use, in the second chapter of this study, we detected that resistance to SDHI fungicides is widespread in populations of black and yellow Sigatoka pathogens in banana plantations in southeastern Brazil. The black and yellow Sigatoka disease complex (CDS), caused by *Mycosphaerella fijiensis* (Mf) or *Mycosphaerella musicola* (Mm), respectively, is one of the most destructive fungal leaf spot diseases affecting commercial banana fields worldwide, leading to significant yield losses. In Brazil, SDHI fungicides labeled for BSDC management since 2014 present a high risk of resistance emergence if deployed intensively and in isolation or in mixtures with other high-risk fungicides, as recommended for over eight years. To fill a current information gap on the occurrence and prevalence of resistance to SDHI fungicides, this study determined the levels of sensitivity to boscalid and fluxapyroxad in four CDS pathogen populations sampled in 2020 from three distinct geographical regions with contrasting fungicide management systems. Out of a total of 67 isolates, the prevalence of detected resistance to SDHI fungicides was 94.0% for boscalid and 59.7% for fluxapyroxad. Only 1.5% of isolates were sensitive to both fungicides. We also determined the association between sensitivity levels and changes in corresponding fungicide target genes (*SdhB*, *C*, and *D*). The boscalid and fluxapyroxad EC₅₀ values were highest in the Mf SPVR-CI population with intensive fungicide management, followed by Mm SPNW-C and

Mm MGN-C. The *Mm* SPNW-O population from an organic banana field had the lowest average EC50s. Among the 10 *Mf* and 57 *Mm* isolates sequenced only, one (SdhC N55D) and two (SdhB E196Q and SdhD N66K) Sdh target site alterations, respectively, were detected. An efflux pump mechanism was also detected in *Mf* conferring resistance to fluxapyroxad. As resistance to SDHI fungicides is already prevalent, indicating simultaneous emergence in all three geographical regions, these results highlight the importance of limiting the deployment of SDHI fungicides for CDS management as a fundamental resistance management strategy.

Keywords: fungal foliar pathogens; fungicide resistance; high-risk fungicides; QoI; DMI; SDHI; anti-microbial resistance phenotyping; smart anti-resistance strategies; surveillance-based spraying decision; limiting fungicide sprays; chemical control; SdhB; SdhC; SdhD; fungicide resistance; Ec50; resazurin.

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CAPÍTULO 1. UM ENSAIO COLORIMÉTRICO DE IMAGEM DIGITAL ACURADO, ACESSÍVEL E PRECISO BASEADO EM RESAZURINA PARA A AVALIAÇÃO DO STATUS DE SENSIBILIDADE A FUNGICIDAS DE POPULAÇÕES FÚNGICAS

RESUMO

Este estudo teve como objetivo o desenvolvimento e a validação de um ensaio colorimétrico preciso e acessível à base de resazurina (ensaio COL) para avaliar a sensibilidade de isolados de *Mycosphaerella* e *Pyricularia* à fungicidas. Este ensaio de imagem digital proposto foi baseado em estimativas colorimétricas de redução de resazurina, que foi usada como um indicador metabólico da atividade respiratória fúngica em culturas de microplacas. Como sistemas modelo fúngicos, usamos os patógenos da Sigatoka amarela e negra [*Mycosphaerella musicola* (Mm) e *M. fijiensis* (Mf), respectivamente] e o patógeno da brusone do trigo, *Pyricularia oryzae* linhagem Triticum (PoTl), que foram previamente caracterizados quanto a sensibilidade a fungicidas à QoI, DMI e SDHI. Em seguida, comparamos o ensaio clássico de detecção por espectrofotometria (ensaio SPEC) com o ensaio COL proposto com base na análise de imagens digitais das culturas das microplacas capturadas com câmeras de telefones celulares em um transiluminador artesanal. Qualitativamente, em termos de precisão, houve total correspondência entre os ensaios SPEC e COL de acordo com a EC_{50} fúngica ou as classes de crescimento relativo em fungicidas QoI, SDHI e DMI para ambos os patógenos. Também observamos coeficiente de correlação de Pearson forte a muito forte entre os valores de sensibilidade fungicida do ensaio COL e do ensaio SPEC para o QoI azoxistrobina, o SDHI fluxapiroxade e o DMI tebuconazol. Nossa conclusão foi que o ensaio COL teve uma precisão semelhante ao ensaio SPEC (ou seja, resultou em categorias de sensibilidade a fungicidas semelhantes para isolados fúngicos resistentes ou sensíveis) e alta precisão. Ao compartilhar abertamente a metodologia completa do ensaio COL e os projetos do transiluminador artesanal, prevemos sua adoção por laboratórios mal equipados em todo o país como uma metodologia acessível para monitorar o status de resistência a fungicidas de populações de importantes patógenos fúngicos de plantas, como *M. fijiensis*, *M. musicola* e *P. oryzae* linhagens Triticum e Oryzae.

Palavras-chave: patógenos fúngicos foliares; resistência a fungicidas; fungicidas de alto risco; QoI; DMI; SDHI; fenotipagem de resistência antimicrobiana; estratégias anti-resistência

inteligentes; decisão de pulverização baseada em vigilância; limitar pulverizações de fungicidas.

CHAPTER 1. AN ACCURATE, AFFORDABLE, AND PRECISE RESAZURIN-BASED DIGITAL IMAGING COLORIMETRIC ASSAY FOR THE ASSESSMENT OF FUNGICIDE SENSITIVITY STATUS OF FUNGAL POPULATIONS

ABSTRACT

This study aimed to develop and validate an accurate and affordable colorimetric assay based on resazurin (COL assay) to evaluate the sensitivity of *Mycosphaerella* and *Pyricularia* isolates to fungicides. This proposed digital image assay was based on colorimetric estimates of resazurin reduction, which was used as a metabolic indicator of fungal respiratory activity in microplate cultures. As fungal model systems, we used the yellow and black Sigatoka pathogens [*Mycosphaerella musicola* (*Mm*) and *M. fijiensis* (*Mf*), respectively] and the wheat blast pathogen, *Pyricularia oryzae* Triticum lineage (PoTl), which were previously characterized for sensitivity to QoI, DMI, and SDHI fungicides. Next, we compared the classical spectrophotometric detection assay (SPEC assay) with the proposed COL assay based on digital image analysis of microplate cultures captured with cell phone cameras on a homemade transilluminator. Qualitatively, in terms of accuracy, there was a total correspondence between the SPEC and COL assays according to the fungal EC₅₀ or relative growth classes in QoI, SDHI, and DMI fungicides for both pathogens. We also observed a strong to very strong Pearson correlation coefficient between the fungal sensitivity values of the COL assay and the SPEC assay for the QoI azoxystrobin, the SDHI fluxapyroxad, and the DMI tebuconazole. Our conclusion was that the COL assay had similar accuracy to the SPEC assay (i.e., resulted in similar fungicide sensitivity categories for resistant or sensitive fungal isolates) and high precision. By openly sharing the complete methodology of the COL assay and the homemade transilluminator designs, we anticipate its adoption by poorly equipped laboratories nationwide as an affordable methodology for monitoring the fungicide resistance status of populations of important plant fungal pathogens, such as *M. fijiensis*, *M. musicola*, and *P. oryzae* Triticum and Oryzae lineages.

Keywords: fungal foliar pathogens; fungicide resistance; high-risk fungicides; QoI; DMI; SDHI; antimicrobial resistance phenotyping; smart anti-resistance strategies; surveillance-based spraying decision; limiting fungicide sprays

1 INTRODUCTION

Fungicide sensitivity assessments of fungal pathogen populations are essential for monitoring the of resistance to high-risk site-specific fungicides and reducing its spread in the agroecosystem (CORKLEY; FRAAIJE; HAWKINS, 2022; KLEINKAUF *et al.*, 2013; LUCAS; HAWKINS; FRAAIJE, 2015). This is especially important to prolong the efficacy of recently launched fungicide active molecules for the management of important crop diseases in agricultural fields (CORKLEY; FRAAIJE; HAWKINS, 2022). Classical methods for assessing the fungicides baseline sensitivity of fungal populations are based on determining the half maximal effective fungicide concentration (EC_{50}) () derived either from measurements of mycelial growth or spore germination in artificial culture media. Used for necrotrophic or hemibiotrophic fungi, which are able to grow in artificial medium, amended with different doses of fungicides (CASADO *et al.*, 2018; CASTROAGUDÍN *et al.*, 2015b; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022a). For biotrophs these assays are conducted in planta (MELLO *et al.*, 2021; MÜLLER; STAMMLER; MAY DE MIO, 2021), which is outside of the scope of our study. Most of these methods are laborious, and time and resources-consuming. Alternatively, the assessment of fungicide sensitivity can be performed measuring the respiratory activity of fungal plant pathogens based on the reduction of the metabolic indicator resazurin (RZ) or alamar blue (“ready to use” resazurin dye solution) in microplates (BARUA *et al.*, 2017; COX *et al.*, 2009; VEGA *et al.*, 2012).

The metabolic indicator RZ is a non-toxic dye that acts as an intermediate electron acceptor in the mitochondrial transport chain without interfering with normal electron transferring (PAGE; PAGE; NOEL, 1993). The RZ dye is extensively used for sensitivity assays of fungi, bacteria, mammalian cells, and other organisms to several chemical compounds as an indicator of metabolic activity (MANIA *et al.*, 2010). The reduced form (pink color) of RZ predominates under high respiratory activity while the oxidized form (blue color) is associated with low cellular respiration (O’BRIEN *et al.*, 2000; PRAKASH *et al.*, 2018). Therefore, in these assays, analyses of fungal plant pathogens’ respiratory activity can be performed under optimum growth conditions on microplates by determining both the reduced (resazurin, RD) and the fluorescent oxidized (resorufin, OX) forms of RZ under spectrophotometry (COX *et al.*, 2009) or spectrofluorometry (BALBAIED; MOORE, 2020).

For assessing the fungicide sensitivity status of important plant pathogens fungal populations from the genera *Mycosphaerella* and *Pyricularia*, we aimed to develop a digital

imaging assay, based on colorimetric estimates of resazurin reduction (RD) as a direct indicator of fungal respiration activity derived from growth on liquid cultures in microplates, like the method described by Borra and collaborators (BORRA *et al.*, 2009). As fungal model systems, we used the yellow and black Sigatoka pathogens [*Mycosphaerella musicola* (*Mm*) and *M. fijiensis* (*Mf*), respectively (OLIVEIRA *et al.*, 2022) and the wheat or the rice blast pathogens *Pyricularia oryzae* Triticum lineage (*PoTl*) and *P. oryzae* Oryza lineage (*PoOl*) (CERESINI *et al.*, 2019), which were previously characterized for QoI, DMI, or SDHI fungicides sensitivity (OLIVEIRA *et al.*, 2022; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022a).

The method from Borra and collaborators (BORRA *et al.*, 2009) used digital images obtained with CCD cameras to evaluate the reduction of resazurin or alamarBlue in microplates. These images were decomposed into three sets of grays from the original red (R), green (G), and blue (B) spectra. The intensities of gray tones were obtained with the ImageLab software (Bio-Rad® Laboratories, Hercules, CA, USA) or with the Adobe Photoshop® software (Adobe Inc., San Jose, CA, USA) where the reduced RZ was inferred from images in the G spectrum while the oxidized RZ from images in the R spectrum. As an open source choice, the ReadPlate3.0 plugin (DELFINO, 2020) of the software ImageJ ((SCHINDELIN *et al.*, 2012; SCHNEIDER; RASBAND; ELICEIRI, 2012), available at <http://fiji.sc/> (accessed on 01 April 2022)) enables the automatic determination of the color spectra variation from all 96 wells of a microplate at once, with the possibility of reading in four different filters: green, red, blue, and gray. In fact, ReadPlate3.0/ImageJ has been widely used in many distinct colorimetric assays (COLZANI *et al.*, 2017; DA SILVA *et al.*, 2020; DELFINO, 2020).

The success of a digital imaging assay depends on prior optimization of the fungal culturing conditions, which includes medium composition, medium pH, amount and type of initial inoculum, and incubation time, as well as the optimization for the RZ dye reaction to reflect the fungal respiratory activity (BRITO *et al.*, 2020a; CASTROAGUDÍN *et al.*, 2015b; COX *et al.*, 2009; DORIGAN *et al.*, 2019; GOMES *et al.*, 2013; POLONI *et al.*, 2021; RAMPERSAD, 2012; VEGA *et al.*, 2012; VICENTINI *et al.*, 2022a).

Regarding the culture media, its suitability for measurements of respiratory activity with the RZ dye is fungal dependent. For instance, potato dextrose (PD) medium was considered suitable in fungicide resistance assays of *Monilinia fructicola* to the DMI fenbuconazole based on measurements (COX *et al.*, 2009). However, while PD was unsuitable for RZ-based QoI azoxystrobin sensitivity assays for *Alternaria alternata*, the CM medium was considered more appropriate for that assay (VEGA *et al.*, 2012). This was associated with the

PD medium's high carbohydrates content, which triggered a high secretion of acids by the fungus *A. alternata* metabolism during growth. Because this high acidity condition accelerated RZ reduction, without a buffer adjusting and keeping the medium's pH around neutrality, PD might be unsuitable for RZ-based fungicide sensitivity assays for *A. alternata* (RAMPERSAD, 2012; VEGA *et al.*, 2012).

As for the incubation period, the recommendation is to assess the respiratory activity at its maximum level, which occurs during the log (exponential) phase of the fungal growth (RAMPERSAD, 2012). The few RZ-based fungicide sensitivity assays conducted with plant pathogens so far are based on 24-h incubation assessments (COX *et al.*, 2009; VEGA *et al.*, 2012). However, particularly for *Pyricularia*, the assessment of fungicide sensitivity based on the evaluation of the fungus respiration activity in this short 24-h incubation period could result in very different phenotypes from those obtained previously (CASTROAGUDÍN *et al.*, 2015b; DORIGAN *et al.*, 2019; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022a), making the former relevant data incomparable with any data recently generated. This is because previous assays for QoI, DMI, and SDHI sensitivity for *Pyricularia* were based on fungal mycelial growth from five days incubation on liquid medium in microtiter plates. The same applies to QoI and DMI fungicide sensitivity assays for *Mycosphaerella*, which takes from seven to 10 days incubation (BRITO *et al.*, 2020b; GOMES *et al.*, 2013).

In our study, after the experimental conditions have been optimized, including medium composition and pH, fungal inoculum concentration, incubation time, and conditions, we compared the classical spectrophotometry detection assay (SPEC-assay) with the proposed colorimetric assay (COL-assay) based on analyses of digital images for *Mycosphaerella* and *Pyricularia* previously characterized for sensitivity to the site-specific fungicides.

2 MATERIALS AND METHODS

2.1 Fungal Strains

To carry out this study, we selected 13 isolates of *Mycosphaerella fijiensis* and *M. musicola*, causal agents of black and yellow Sigatoka fungal pathogens, with previous information about their sensitivity status to QoI (OLIVEIRA *et al.*, 2022), DMI, and SDHI fungicides derived from pilot studies in our lab. We also selected 14 isolates from the wheat or rice blast pathogens *Pyricularia oryzae* *Triticum* (PoTl) or *Oryza* (PoOl) lineages, previously classified according to their resistance category to QoI (CASTROAGUDÍN *et al.*, 2015a), DMI (POLONI *et al.*, 2021), and SDHI (VICENTINI *et al.*, 2022a, 2022b) fungicides. These *Mycosphaerella* and *Pyricularia* isolates were selected from our fungal collections spanning 2007, 2017, 2018, and 2019 sampling years from either banana plantations or cereal (wheat or rice) fields from Central-southern Brazil (Table 1).

Table 1 - *Mycosphaerella* and *Pyricularia* isolates selected for this study, their fungicide resistance categories, and doses of QoI, DMI, and SDHI fungicides tested.

Fungicide Group and Active Ingredient	<i>Mycosphaerella</i> Isolates ^{a,b}	Doses of Fungicide Tested ($\mu\text{g}\cdot\text{mL}^{-1}$) ^c	<i>Pyricularia</i> Isolates ^{a,b}	Doses of Fungicide Tested ($\mu\text{g}\cdot\text{mL}^{-1}$) ^c
QoI: Azoxystrobin	<i>Mm</i> ISC3 (S) <i>Mm</i> ISC14 (S) <i>Mf</i> JA3.9a (S) <i>Mm</i> ISC9 (R) <i>Mm</i> ISC64 (R) <i>Mf</i> JA2.24 (R)	0 and 10.0	<i>PoTl</i> 12.1.312 (R) <i>PoTl</i> 18SPK6 (R) <i>PoTl</i> 18MGH19 (R) <i>PoTl</i> 12.1.015 (S) <i>PoO</i> 421 (S) <i>PoO</i> 656 (S) <i>PoTl</i> 12.1.130 (R) <i>PoTl</i> 12.1.183 (R)	0 and 10.0
	<i>Mm</i> ISR47 (S) <i>Mm</i> ISR55 (S) <i>Mf</i> JA2.24 (RS) <i>Mm</i> ISC118 (R) <i>Mf</i> JA3.9a (R) <i>Mf</i> SA6 (R)	0, 0.0066, 0.033, 0.066, 0.132 and 1.320	<i>PoTl</i> 12.1.312 (HR) <i>PoTl</i> 12.1.045i (RS) <i>PoTl</i> 18MGF3 (R) <i>PoTl</i> 18MGH25 (RS) <i>PoTl</i> 12.1.037 (R) <i>PoTl</i> 12.1.299 (S)	0 and 1.0
DMI: Tebuconazole	<i>Mm</i> ISC92 (S) <i>Mm</i> ISR21 (RS) <i>Mf</i> JA2.24 (RS) <i>Mf</i> SA6 (RS) <i>Mm</i> ISR6 (R) <i>Mf</i> JA3.9a (HR)	0, 0.66, 3.3, 6.6, 16.5 and 33.0	<i>PoTl</i> 12.1.312 (S) <i>PoTl</i> 12.1.045i (S) <i>PoTl</i> 18SPK6 (S) <i>PoO</i> 704 (S)	0 and 5.0

^a Fungal species: *Mf* = *Mycosphaerella fijiensis*, *Mm* = *M. musicola*, *PoOl* = *Pyricularia oryzae* Oryza lineage, and *PoTl* = *P. oryzae* Triticum lineage. ^b The fungicide resistance categories: S = sensitivity, RS = reduced sensitivity, R = resistance, HR = high resistance. ^c The fungicide resistance categories for *Mycosphaerella* isolates were inferred based on EC₅₀ values determined in this study for the DMI and SDHI fungicides, while for the QoI fungicide we determined the relative growth based on the discriminatory dose of 10.0 $\mu\text{g}\cdot\text{mL}^{-1}$ (GOMES *et al.*, 2013; OLIVEIRA, 2022). In comparison, the fungicide resistance categories for *Pyricularia* isolates were previously determined for QoI (CASTROAGUDÍN *et al.*, 2015a), DMI (DORIGAN *et al.*, 2019; POLONI *et al.*, 2021), and SDHI (VICENTINI *et al.*, 2022a, 2022b) fungicides, therefore we only experimentally tested the relative growth of the fungus on the discriminatory doses indicated above. **Source:** (By author/self)

2.2 Fungal Inoculum, Microplate Cultures Preparation, and Incubation Conditions for Fungicide Sensitivity Testing

Sensitivity tests to QoI, DMI, and SDHI fungicides were conducted with flat-bottomed 96-well microtiter plates (Kasvi, Parganas North, West Bengal, India) using the mycelial fragments protocol (BRITO *et al.*, 2020c; MONTROYA *et al.*, 2006). Isolates of *M. fijiensis* and *M. musicola* were reactivated on PDA medium (20.7 g·L⁻¹ potato dextrose, 15 g·L⁻¹ agar) supplemented with chloramphenicol and streptomycin (50 µg mL⁻¹ of each). Isolates of *Pyricularia oryzae* Triticum and Oryza lineages were reactivated in oatmeal agar medium (30 g·L⁻¹ oatmeal, 25 g·L⁻¹ agar) supplemented with 100 µg·mL⁻¹ chloramphenicol. After 10 days of growth at 25 °C and 12 h photoperiod, for both *Mycosphaerella* and *Pyricularia*, fungal mycelium fragments were transferred to 1.5 mL microtubes containing 0.5 mL of 0.1 mm diameter glass beads. A total of 1000 µL of distilled water was added to the mixture, which was bead-beatered in a Fast-Prep apparatus for 20 s at speed 4 m·s⁻¹. This beating cycle resulted in a suspension of small mycelial fragments, which were diluted in 10 mL of distilled water. The final concentration of the mycelial fragment's suspension was adjusted to 10⁴ mL⁻¹ based on Neubauer chamber counts. The fungicides tested at distinct doses were mixed with PD medium prepared with 0.025M phosphate buffer, and final pH adjusted to 5.0. The total volume dispensed in each microplate well was 150 µL as follows: 100 µL of PD medium with and without fungicides at distinct doses, 50 µL of fungal propagules suspension. The experimental design was completely randomized with eight replicates and each experiment was replicates once. The microplate with fungal liquid cultures were wrapped in plastic film and incubated at 25 °C in the dark and shaken at 150 rpm for five days for *Pyricularia* or 10 days for *Mycosphaerella*, when the fungal growth reached its maximum.

2.3 Spectrophotometric Assay (SPEC-Assay) for Fungicide Sensitivity Testing Based on Measuring the Reduction of Resazurin, a Metabolic Indicator for Fungal Respiration Activity

After completing the incubation period for maximum fungal growth, 50 µL of resazurin at 160 µM was added to each microplate well to obtain a final concentration of 40 µM (to a final volume of 200 µL) and initial absorbance readings at 569 nm (Abs_{569 nm} at T₀) were taken using a microplate reader device (Multiskan™ FC Microplate Photometer, Thermo Fisher

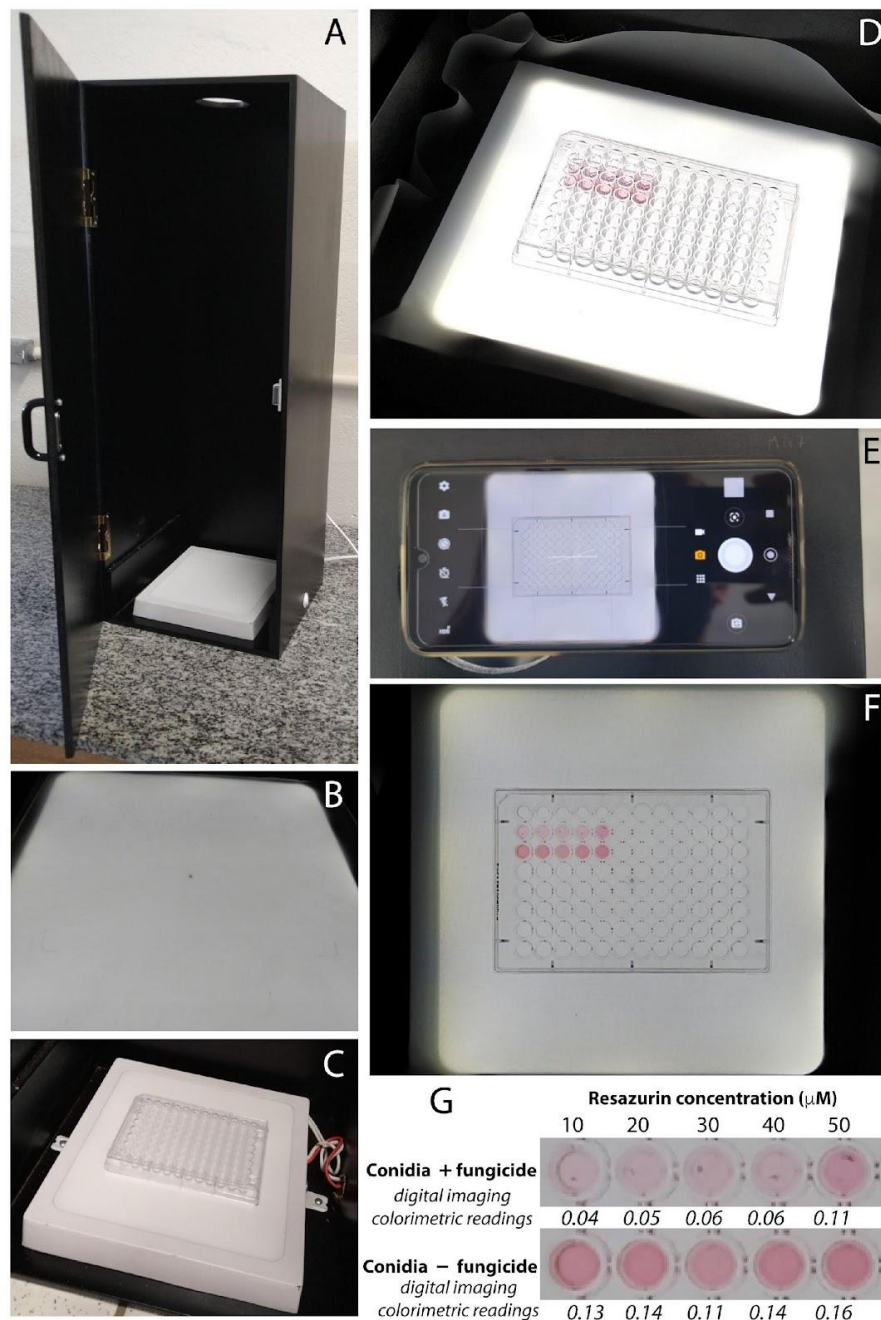
Scientific, Waltham, MA, USA) for spectrophotometric estimates of RZ reduction (SPEC-assay). Subsequently, the microplates were kept at 25 °C under complete dark for a 4 h-reaction-period for *Pyricularia* or a 24 h-reaction-period for *Mycosphaerella*, when final absorbance readings were then taken at the same wavelengths ($Abs_{569\text{ nm}}$ at T₄ or T₂₄).

2.3 Resazurin-Based Colorimetric Assays (COL-Assay) for Fungicide Sensitivity Testing Using a Handmade Trans-Illuminator with Attached Mobile Phone Digital Cameras

A handmade trans-illuminator was built according to Delfino (DELFINO, 2020) (Figures 1 and 2) using 9-mm-thick medium-density wood fiber (MDF), painted in matte black (80 cm high × 30 cm wide × 25 cm deep), with a front door with the same height and width. A 7.5 cm diameter hole at the top of the trans-illuminator was made to attach a mobile phone digital camera. The entire interior of the trans-illuminator was covered with photographic diffuser film (Zcod®, São José, Brazil). A 20 cm × 20 cm × 2.5 cm LED lamp (Vanyluz™, São Paulo, Brazil) was installed inside, at its base. The lamp is activated by a side switch, and the power supply is 110 V. A small mark was made in the center of the LED lamp base (Figure 1B) for correctly positioning the microplate (Figure 1C). Once the light is activated (Figure 1D), the front door is closed, and the mobile phone digital camera is positioned in the top hole (Figure 1E). We applied the camera function that allows the screen to be centered in the microplate image captured, using “guides” (Figure 1E, arrow). The camera settings and the zoom were standardized, so that the images were captured under the same conditions and size (Figure 1F). A technical drawing of the handmade trans-illuminator for microplates is presented in Figure 2.

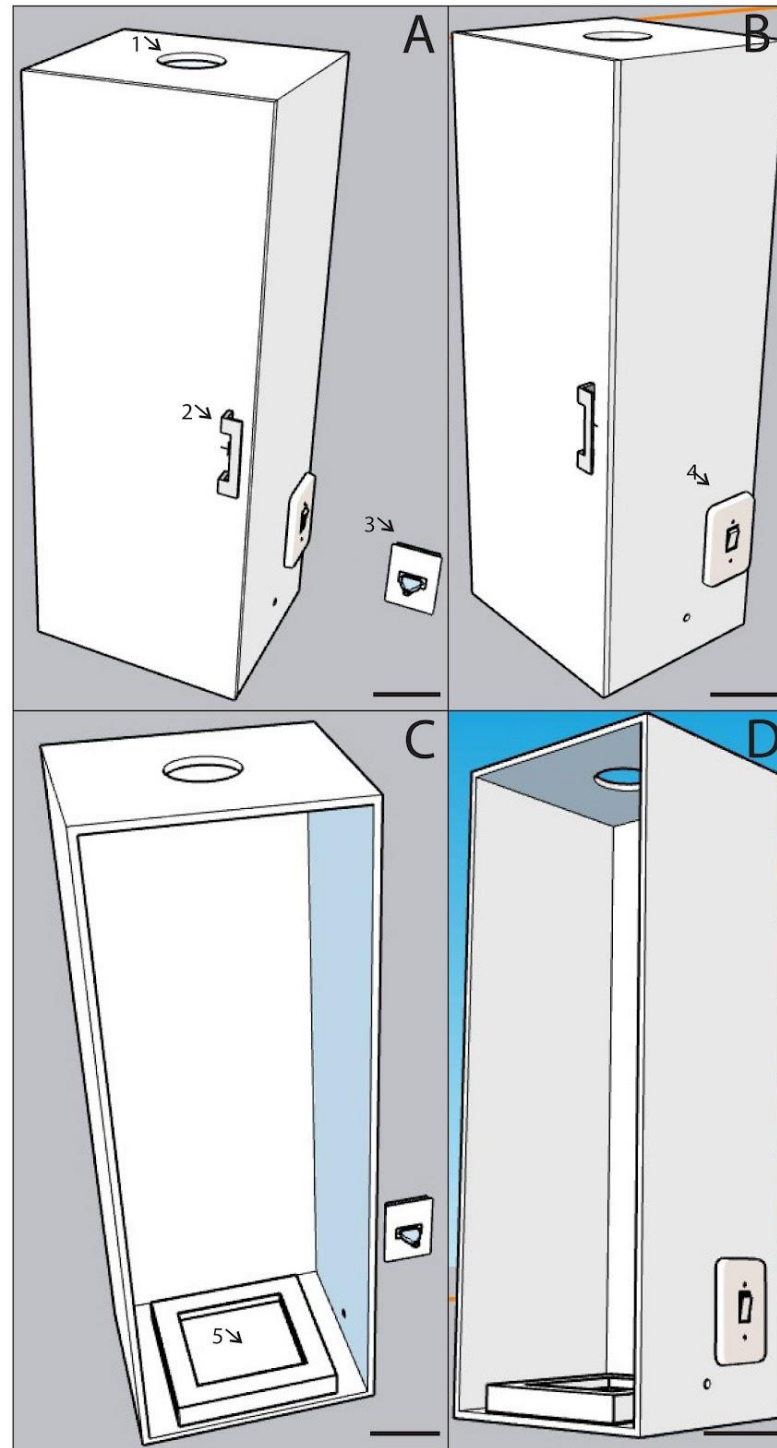
After completing the incubation period defined for the fungal respiration resazurin-based fungicide sensitivity testing for either *Mycosphaerella* or *Pyricularia*, colorimetric estimation of RZ reduction from each individual fungal culture microplate well was determined using the ReadPlate3.0 application from the ImageJ software, with background correction (A_{corr} , Lachine, QC, Canadian), as described by Delfino (DELFINO, 2020).

Figure 1 - (A) Handmade transilluminator for the colorimetric resazurin-based digital-imaging assay (COL-assay) developed by our group for this study. (B) Photography diffuser film covering square led lamp (marked at the center). (C) Microplate positioned above the LED lamp. (D) LED lamp turned on. (E) Positioning of the mobile phone digital camera over the top hole of the transilluminator. (F) Positioning of the experimental microplate at the center of the LED lamp. (G) Digital images data obtained using ReadPlate3.0 plugin from Imagej software and sensitivity analysis on a preliminary test using suspension of mycelia fragments as fungal inoculum from the PoOI isolate 363 growing in PD medium supplemented or not with the fungicide azoxystrobin at $10 \mu\text{g} \cdot \text{mL}^{-1}$. Values shown at the bottom of each individual well are the digital-imaging-estimated colorimetric values and the pink color intensity indicates the differences in fungal respiration.



Source: (By author/self)

Figure 2 - Technical drawing of the transilluminator for the colorimetric resazurin-based digital-imaging assay (COL-assay) developed for capturing fungal respiration activity from mycelial growth on microplates. **(A)** Closed door view. 1: camera top hole. 2: door handle. 3: power plug. **(B)** Side view. 4: light switch. **(C)** Inside view. 5: square led lamp. **(D)** Side with door opened. Scale bars = 10 cm. The transilluminator was made with medium density wood fiber (MDF) 90 mm thick, painted in matte black color, 80 cm high, 30 cm wide, 25 cm deep.

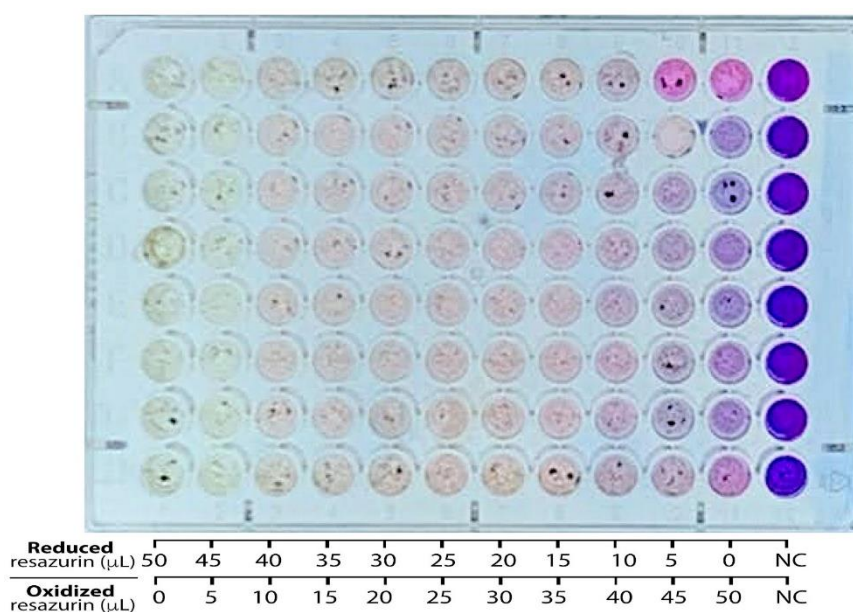


Source: (By author/self)

2.4 Resazurin Oxidation-Reduction Gradient Testing for Substantiating the Assays

This experiment was conducted using an oxidation-reduction gradient resulting from the mixture of completely oxidized (OR, blue color) to completely reduced (RD, pink color) states of RZ, with the purpose of substantiating both spectrophotometric (SPEC-assay) and the digital imaging assay based on colorimetric estimates (COL-assay) of resazurin reduction. The reduced RZ solution was obtained by autoclaving the oxidized RZ for 15 min and allowing it to cool to room temperature in the dark (BORRA *et al.*, 2009. The experiments were conducted in 96-well microplates. In each individual microplate well a total of 100 μL of PD medium prepared with 0.025M phosphate buffer, final pH 5.0 and 50 μL of *M. musicola* or *P. oryzae* Triticum lineage inoculum suspension (10^4 mycelial fragments. mL^{-1} or water as the negative control) were added. After a 5 day-incubation period for *Pyricularia* or a 10 day-incubation period for *Mycosphaerella* at 25 °C in the dark and under shaking at 150 rpm, 50 μL of RZ 160 μM were added to obtain a final concentration of 40 μM RZ in the following proportions of the reduced/oxidized forms (Figure 3).

Figure 3 - Oxidation-reduction gradient obtained with the mixture of the reduced and oxidized forms of the resazurin solution at the final concentration of 40 μM *.* Mixtures of reduced and oxidized forms of RZ and respective volumes added in the microplate wells containing PD medium prepared with 0.025 M phosphate buffer, final pH 5.0 and 50 μL of *M. musicola* or *P. oryzae* Triticum lineage inoculum suspension (10^4 mycelial fragments. mL^{-1} or water as the negative control). RD = reduced resazurin (autoclaved for 15 min). OR = oxidized resazurin (no autoclaving). NC = negative control (no mycelium fragments added).



Source: (By author/self)

Initial absorbance readings at 569 nm ($Abs_{569\text{ nm}}$ at T_0) were taken for the SPEC-assay estimates of RZ reduction, as described earlier in item 2.3. The microplate was also digitally photographed using a mobile phone camera attached to the transilluminator designed for colorimetric (COL-assay) estimation of RZ reduction at T_0 using ReadPlate3.0 from ImageJ as described earlier in item 2.4. Subsequently, the microplates were kept at 25 °C under complete dark for a 4 h-reaction-period for *Pyricularia* or a 24 h-reaction-period for *Mycosphaerella*, when final absorbance readings were taken at the same wavelengths ($Abs_{569\text{ nm}}$ at T_4 or T_{24}) and the microplates were then digitally photographed also at T_4 or T_{24} for colorimetric estimation of RZ reduction.

2.5 Accuracy and Precision of the Resazurin-Based Colorimetric Assay (COL-Assay) Based on a Handmade Trans-Illuminator for Fungicide Sensitivity Testing

The objective of this phase of the study was to compare the classical spectrophotometry detection assay (SPEC-assay) with the proposed colorimetric assay (COL-assay) based on analyses of digital images captured with mobile phone cameras on a handmade trans-illuminator built. We aimed to determine the accuracy and precision of the COL-assay for fungicide sensitivity testing with both *Mycosphaerella* and *Pyricularia* fungal pathogens. For digital images capture we used an iPhone 11 with iOS16 operating system, both from Apple Inc. (Cupertino, CA, USA), with a 12MP wide camera with $f/1.8$ aperture.

The fungicide sensitivity tests were conducted with flat-bottomed 96-well microtiter plates (Kasvi, Parganas North, West Bengal, India) using the mycelial fragments protocol, microplate cultures preparation, and incubation conditions for fungicide sensitivity testing as described earlier in this study in item 2.2. Each microplate well was filled with 50 μL of inoculum suspension and 100 μL of PD broth [20.7 g L^{-1} of potato dextrose (Kasvi), 1 L of distilled water)] amended with different concentrations of the QoI, DMI, or SDHI fungicides as described in Table 1.

We applied both the SPEC- and COL-assays' protocols for fungicide sensitivity testing based on measuring the RZ reduction, as described earlier in items 2.3 and 2.4. For *Mycosphaerella*, The RZ reduction was estimated as follows:

$$RR_{Mycosphaerella} = T_0 - T_{24} (1)$$

where, $RR_{Mycosphaerella}$ = relative reduction of resazurin; T = Absorbance at 569 nm for the SPEC-assay or colorimetric reading for the COL-assay; T_0 = reading at time zero (immediately after adding resazurin to the fungal 10-day-old liquid culture in buffered PD medium); T_{24} = reading at time 24 (24 h after adding resazurin).

For *Pyricularia*, The RZ reduction was estimated as follows:

$$RR_{Pyricularia} = T_0 - T_4(2)$$

where, $RR_{Pyricularia}$ = relative reduction of resazurin; T_0 = reading at time zero (immediately after adding resazurin to the fungal 5-day-old liquid culture in buffered PD medium); T_4 = reading at time 4 (4 h after adding resazurin).

Based on the RZ reduction (RR) estimates we were able to determine the effective fungicide concentration for inhibiting 50% of the fungal respiration activity (EC_{50} , in $\mu\text{g mL}^{-1}$) using the macro ED50 plus v1.0 (VARGAS, 2000) for Excel (Microsoft™, Redmond, WA, USA). This macro uses a logarithmic function for estimating the EC_{50} by a dose-response curve between log (doses) and RR values. We also determined the relative growth (RG) of fungal cultures at discriminatory doses of fungicides as described earlier (Table 1). We calculated the EC_{50} and RG values based on RR determined by both SPEC- and COL-assays.

2.6 Statistical Analysis

Analysis of variance (ANOVA) by the F test and means comparison were performed using the R software with the statistical libraries *agricolae* and *laercio* (R DEVELOPMENT CORE TEAM, 2022).

As a measure of accuracy of the resazurin-based colorimetric assay (COL-assay), we applied the Scott–Knott test (at $\alpha \leq 0.05$ probability) to compare means with the resazurin-based spectrophotometric assay (SPEC-assay). The boxplot figures depicting the correspondence between fungicide resistance categories (based on EC_{50} values or relative growth estimates) as a measure of accuracy of the COL-assay in comparison with the SPEC-assay were built using the R software library *tidyverse* 1.3.1 [33], which included the packages *ggplot2* 3.3.5, *purrr* 0.3.4, *tibble* 3.1.6, *dplyr* 1.0.7, *tidyr* 1.1.4, *stringr* 1.4.0, *readr* 2.1.0, and *forcats* 0.5.1, and the functions *ggplot*, *geom_boxplot*, *stat_summary*, *geom_jitter*, *ggtitle*, *theme*, and *geom_text*.

As a measure of precision of the COL-assay, we built and compared the simple linear and the polynomial (quadratic) regression models to predict the correspondence between the

relative growth or EC_{50} estimates from the resazurin-based spectrophotometric assay at 569 nm (SPEC-assay) on the basis of the COL-assay measurements. The data were randomly split into a training set (77.5% of the data, for building the predictive models) and a test set (22.5%, for evaluating the models), setting a random seed for reproducibility. Next, the *R* function *predict* [specifying the model, the data set, the option `interval = "confidence"`] was used for predicting outcome values and the corresponding 95% confidence interval reflecting the uncertainty around the mean predictions for each regression model. The comparisons among regression models' performance were achieved by analyzing the *RMSE* and the R^2 metrics (BRUCE; BRUCE; GEDECK, 2020). The *RMSE* represents the model prediction error, that is the average difference between the observed outcome values and the predicted outcome values. The R^2 represents the squared correlation between the observed and predicted outcome values. The best model chosen between linear and quadratic was the one with the lowest *RMSE* and the highest R^2 . For depicting a scatter plot containing a regression line and the confidence interval band for the model chosen, we used the *R* package *ggplot2* 3.3.5.

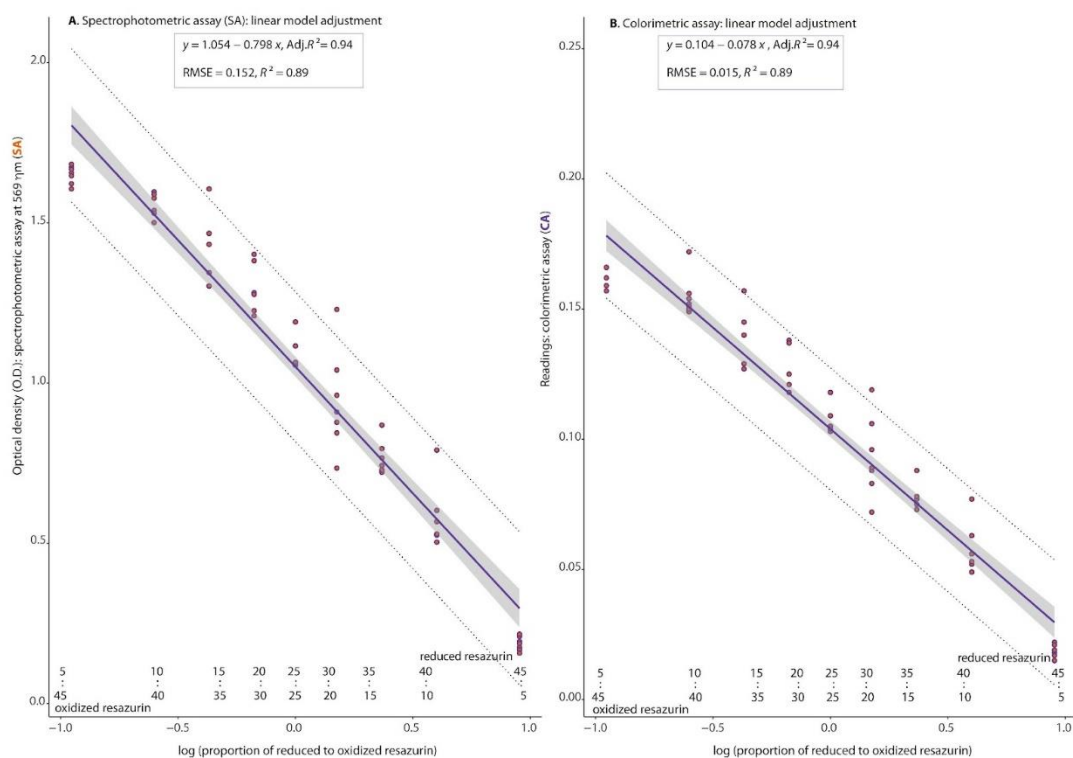
The whole set of colors palette chosen to build all figures with accessibility are color-blind safe and print friendly, using the resources from Color Brewer 2.0 available at the URL <https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3> (accessed on 01 April 2022).

3 RESULTS

3.1 Resazurin Oxidation-Reduction Gradient Testing for Substantiating the Assays

The oxidation-reduction experiment reflected a perfect color gradient spanning the completely oxidized (OR, blue) to the completely reduced (RD, pink) states of RZ, used for calibrating both the spectrophotometric (SPEC-assay) and the digital imaging assay based on colorimetric estimates (COL-assay) of resazurin reduction (Figure 3). This observation was corroborated by the detection of a negatively significant correlation between the proportion of oxidized to reduced RZ and the measurements of absorbance at 569 nm (A) or the colorimetric readings (B) with $p \leq 0.01$ and Adj. $R^2 = 0.94$ (Figure 4).

Figure 4 - Simple linear regression models predicting the correspondence between the optical density (O.D.) estimates from the resazurin-based spectrophotometric assay at 569 nm (SA = SPEC-assay) or the readings from the resazurin-based colorimetric assay (CA = COL-assay) on the basis of the proportion of reduced to oxidized resazurin (RZ) for *Mycosphaerella musicola*.



Source: (By author/self)

3.2 Accuracy and Precision of the Resazurin-Based Colorimetric Assay (COL-Assay) on a Handmade Trans-Illuminator for Fungicide Sensitivity Testing

From the qualitative point of view of the phenotyping, by which we inferred accuracy, the group of fungal isolates associated with the Sigatoka disease complex *M. fijiensis* (JA2.24, JA3.9a, and SA6) and *M. musicola* (ISC92, ISR6, and ISR21) were similarly characterized as resistant or sensitive to QoI or DMI fungicides using either the SPEC-assay (SA) or the COL-assay (CA) (Figure 5A,C). For the SDHI fungicide, in particular (Figure 5B), although the between assays EC_{50} values for fluxapyroxad were significantly different by the Scott–Knott test (at $\alpha \leq 0.05$) for three of the isolates tested, the resistance category was not mistakenly inferred. However, quantitatively, either by the SPEC- or the COL-assay, significant phenotypic differences were detected between resistant and sensitive isolates of *M. fijiensis* and *M. musicola* to all three fungicide groups (Figure 5). This joint observation demonstrated the accuracy of the COL-assay fungicide sensitivity testing.

From the quantitative point of view, by which we measured the precision of the assay, in general, for the three fungicides tested, the EC_{50} or the values of relative growth in discriminatory doses of fungicides for the COL-assay were significantly and positively correlated with the values from the SPEC-assay (with *Adj. R²* varying from 0.76 for the SDHI, 0.81 for the DMI, to 0.99 for the QoI fungicides) (Figure 6A–C), indicating the fair to high precision of the assay.

A similar outcome was observed for the fungicide sensitivity testing conducted with isolates of the wheat or rice blast pathogens (PoTl or PoOl), previously characterized according to their resistance category to QoI (CASTROAGUDÍN *et al.*, 2015a), SDHI (VICENTINI *et al.*, 2022a, 2022b), or DMI (DORIGAN *et al.*, 2019; POLONI *et al.*, 2021) fungicides. Qualitatively, in terms of accuracy, there was full correspondence between the SPEC-assay and the COL-assay according to the fungal relative growth classes on QoI, SDHI, and DMI fungicides (Figure 7A–C) inferred for the group of PoTl (12.1.015, 12.1.037, 12.1.045i, 12.1.130, 12.1.183, 12.1.299, 12.1.312, 18MGH19, 18MGH25, or 18SPK6) and PoOl isolates (421, 656, or 704). In addition, either by the COL-assay or by the SPEC-assay, significant phenotypic differences were detected between resistant and sensitive isolates of PoTl or PoOl to all the three fungicide groups.

Qualitatively, considering the measure of precision of the assay, the values of relative growth in discriminatory doses of fungicides for the COL-assay were significantly and

positively correlated with the values from the SPEC-assay for all three fungicides, fitting to either linear or quadratic models (with *Adj. R*² varying from 0.60 for the DMI, 0.83 for the QoI, to 0.84 for the SDHI) (Figure 8), also indicating the fair to high precision of the assay.

4 DISCUSSION

The main objective of our study was the development of an accurate, affordable, and precise resazurin-based digital imaging colorimetric assay for the assessment of fungicide sensitivity status of populations of fungal plant pathogens.

Preliminary steps for the optimization of the fungal culture conditions were taken to aid the development of our RZ-based digital imaging colorimetric assay. For instance, the testing of three culture media (PD (CASTROAGUDÍN *et al.*, 2015b; DORIGAN *et al.*, 2019), SN (LESLIE; SUMMERELL, 2008), and V8 medium (HANADA; GASPAROTTO; PEREIRA, 2002), at three different pHs (5.0, 6.0, or 7.0), for RZ-based sensitivity assays to determine QoI, DMI, and SDHI fungicides for *Mycosphaerella* and *Pyricularia*, indicated the suitability of PD medium for both fungal pathogens with an initial pH 5.0 (data not shown). Differing from other assays reported (COX *et al.*, 2009; VEGA *et al.*, 2012), in our RZ-based assay we have chosen to use buffered PD medium prepared with 0.025M phosphate buffer, to avoid significant changes in its pH during fungal growth and when supplementing the media with distinct fungicide concentrations.

Considering the amount of fungal inoculum for our RZ-based fungicide resistance colorimetric assays, we checked the effect of variable concentrations of fungal propagules (for both *Mycosphaerella* and *Pyricularia*). There was a positive correlation between respiratory activity measured as RZ reduction and fungal inoculum concentration (from 0 to 10^5 propagules.mL⁻¹), validating the sensitivity of the assay (data not shown). For the subsequent RZ-based assays conducted in this study, the concentration of 10^4 propagules.mL⁻¹ was chosen as an initial inoculum, which matched previous non-RZ-based fungicide sensitivity assays with these pathogens (BRITO *et al.*, 2020c; CASTROAGUDÍN *et al.*, 2015a; DORIGAN *et al.*, 2019; GOMES *et al.*, 2013; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022a).

As for the incubation period, in our proposed RZ-based fungicide sensitivity assays we kept the same incubation period from five to 10 days, as reported in previous studies with *Pyricularia* and *Mycosphaerella*. We then amended the liquid medium of the fungal growing colonies with the RZ dye and subsequently measured the respiration activity at four to 24 h, for *Pyricularia* or *Mycosphaerella*, respectively, after incubation at 25 °C under completely dark conditions (BRITO *et al.*, 2020c; CASTROAGUDÍN *et al.*, 2015a; DORIGAN *et al.*, 2019; GOMES *et al.*, 2013; POLONI *et al.*, 2021; VICENTINI *et al.*, 2022a).

After the experimental conditions had been optimized, we tested the hypothesis that the colorimetric assay (COL-assay) was as accurate and precise for fungicide sensitivity testing as the spectrophotometric assay (SPEC-assay), under similar conditions. The fungicide sensitivity was assessed based on a direct measure of fungal respiratory activity in distinct fungicide doses, by quantifying the reduction of the metabolic indicator resazurin (RZ), as extensively reported for other systems (BARUA *et al.*, 2017; COX *et al.*, 2009; VEGA *et al.*, 2012). The equivalence between the estimates of optical density values from the SPEC-assay and the readings from the COL-assay with the corresponding levels of RZ reduction (Figure 4) was the first indication that the COL-assay developed here can be useful for fungicide resistance phenotyping.

As model systems, we phenotyped the black and yellow Sigatoka fungal pathogens *M. fijiensis* and *M. musicola* and the wheat or the rice fungal pathogens *P. oryzae* Triticum and *Oryza* lineages. For the COL-assay we built a handmade trans-illuminator for capturing digital images of the experimental 96-well microplates with an attached mobile phone camera (Figures 1 and 2).

The COL-assay required the use of the opensource software Image J “ReadPlate” plugin, which allows the user to define a grid of circular regions superimposed upon the microplate image. A grid is subsequently created by defining the number of rows and columns of the microplate, delimiting the pixel coordinates of each well (e.g., well A1 to H12) and the diameter of each analysis circle (PARKER; WILSON; MACE, 2020).

Similar techniques based on image analysis using ImageJ have been used to evaluate a variety of analytical targets, from residues of organophosphates, as well as forensically and clinically relevant compounds (AMARAL *et al.*, 2021; HWANG *et al.*, 2016; SHAH *et al.*, 2021; TIAN *et al.*, 2009).

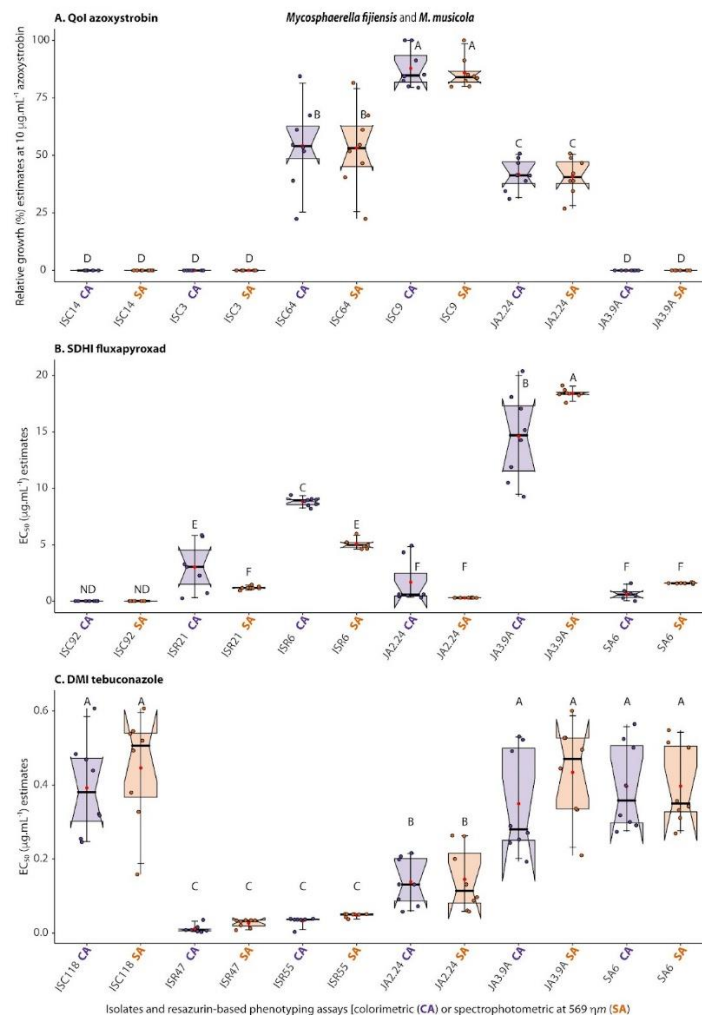
Using previously optimized experimental conditions, which included medium composition, pH, concentration of fungal inoculum, incubation conditions, and period, we then tested the hypothesis that the COL-assay was as accurate and precise as the SPEC-assay for fungicide sensitivity testing at similar conditions.

In general, for *Mycosphaerella*, the COL-assay performed as accurately as the SPEC-assay in cataloging fungal population according to fungicide resistance categories (based on EC₅₀ values or relative growth in discriminatory doses of the active ingredients) to the three major fungicide classes tested: QoI—strobilurin (azoxystrobin), SDHI (fluxapyroxad), and the DMI—triazole (tebuconazole). Therefore, all the *Mycosphaerella* isolates were classified in the exact same resistance or sensitivity category in both methods (Figure 5). The COL-assay also

performed as precisely as the SPEC-assay in determining levels of fungicide resistance as indicated by the correspondence between EC_{50} values or fungal relative fungal growth estimates from the two assays for all the three fungicides classes (Figure 6). In all three cases, the trend line plotted from the distribution of the data pairs from the two assays indicated a positive linear correlation. From a merely statistical point of view (by which correlation coefficients could be classified as weak/moderate/strong/very strong), we observed a very strong correlation coefficient between the COL-assay and the SPEC-assay values for the QoI azoxystrobin ($R^2=0.997$) and strong for fluxapyroxad ($R^2= 0.60$) and tebuconazole ($R^2 = 0.88$) (CALLEGARI-JACQUES, 2003) (Figure 6).

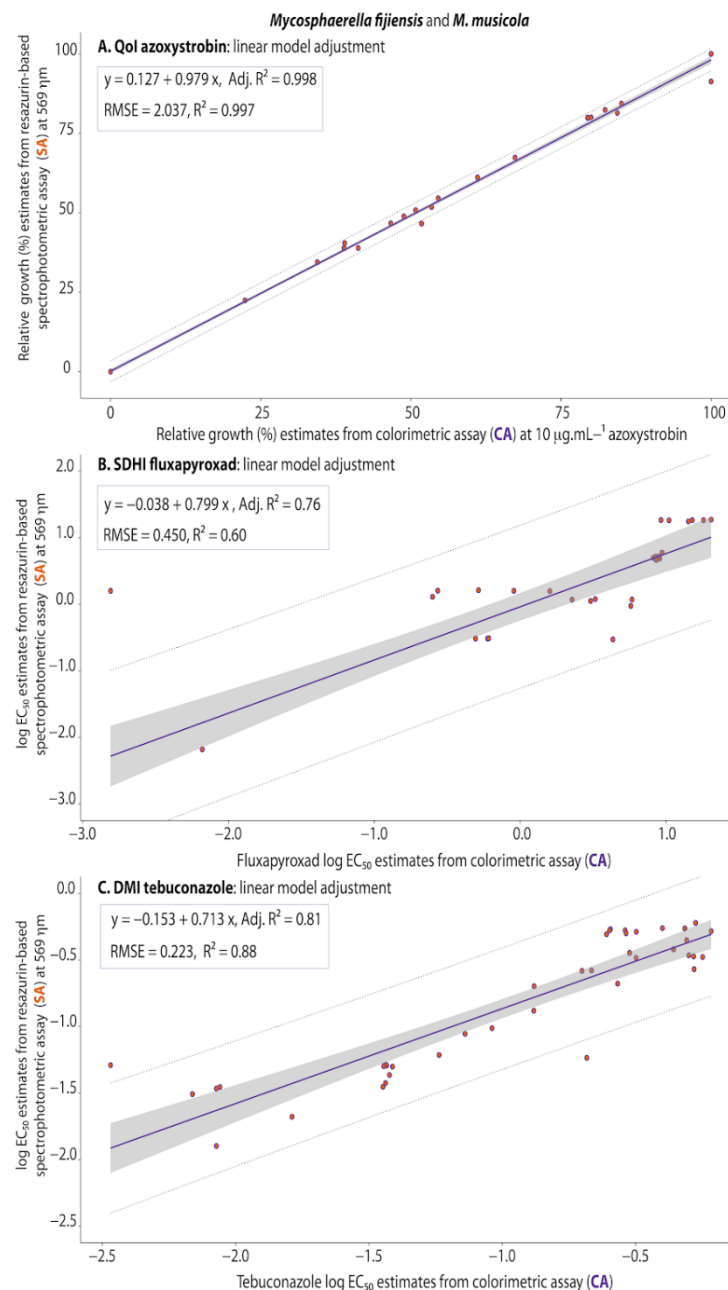
Similar to the *Mycosphaerella* pathogens, correspondence between the COL- and the SPEC-assays was also detected for the resistance categories of the wheat or rice blast *Pyricularia* pathogens *PoTl* and *PoOl* to the QoI azoxystrobin, SDHI fluxapyroxad, and DMI tebuconazole, indicating both accuracy and precision (Figures 7 and 8).

Figure 5 - Correspondence between fungicide resistance categories (based on EC_{50} values or relative growth estimates) as a measure of accuracy of the resazurin-based colorimetric assay (CA = COL-assay, purple boxplots) in comparison with the classical spectrophotometry detection assay (SA = SPEC-assay, orange boxplots) for testing sensitivity of *Mycosphaerella fijiensis* or *M. musicola* to QoI (A), SDHI (B), or DMI (C) fungicides ^{a,b}. ^a The COL-assay was based on analyses of digital images of experimental microplates captured with mobile phone cameras on a handmade trans-illuminator designed for colorimetric estimation of RZ reduction from T0 to T24 using the ReadPlate3.0 in ImageJ developed by Delfino (DELFINO, 2020) as described in item 2.6 from this study. ^b The figure depicts boxplots with the medians represented by black lines across the notches, the average as red circles along the whisker lines, and the jittered data points in purple or orange to avoid data overplotting. The F statistics for treatment effect was significant at least at $p \leq 0.05$. Boxplots with the same letter on top (from A to F) indicate that the means for the particular isolate or resazurin-based phenotyping assay are not significantly different by the Scott–Knott test at $\alpha \leq 0.05$.



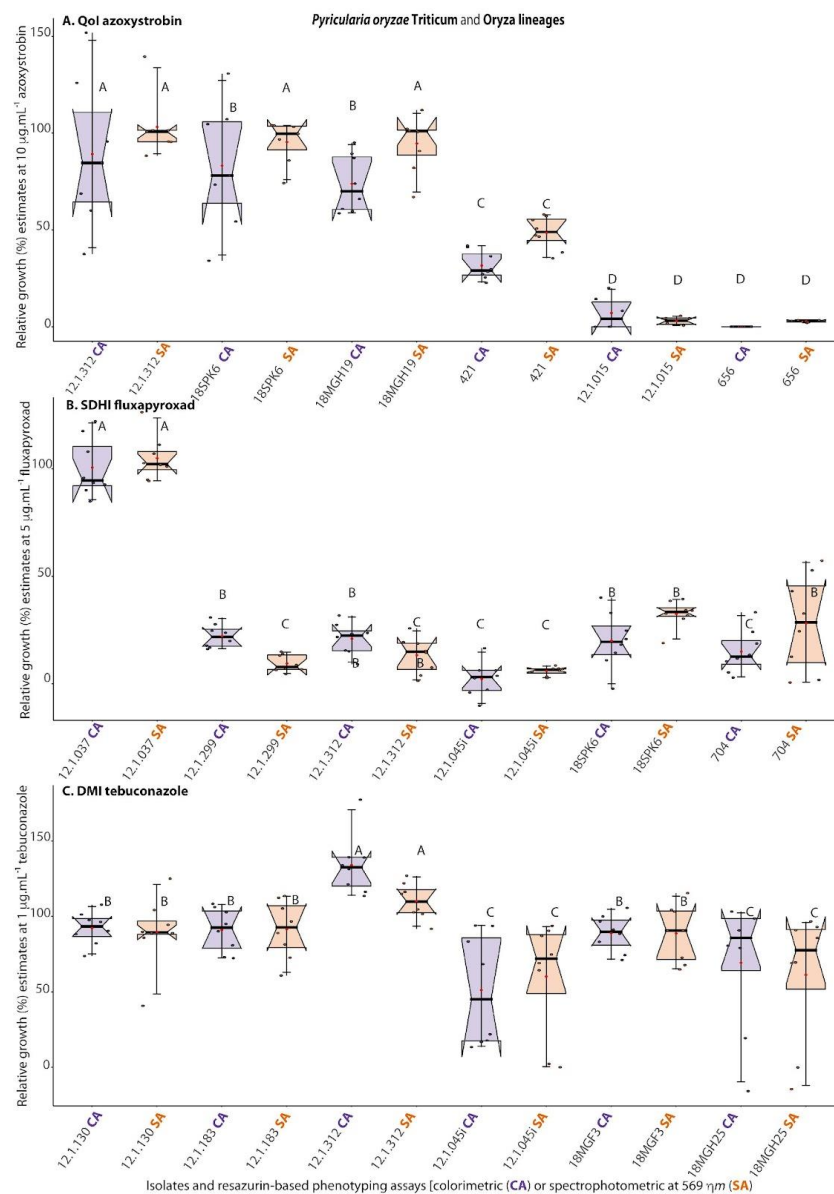
Source: (By author/self)

Figure 6 -Simple linear regression models predicting the correspondence between the log EC_{50} or fungus relative growth estimates from the spectrophotometric assay at 569 nm (SA = SPEC-assay) on the basis of log EC_{50} or relative growth estimates from the colorimetric assay (CA = COL-assay) for QoI (A), SDHI (B), or DMI (C) fungicide sensitivity testing for *Mycosphaerella fijiensis* and *M. musicola*^{a,b}. ^a The rectangle within each figure contains the corresponding linear equations, the adjusted R^2 (Adj. R^2) as a measure of precision of the colorimetric assay, and the model fit parameters (RMSE and R^2). In all three cases the linear regression had the best fit as a predicting model, with the lowest RMSE and the highest R^2 . ^b The regression lines were presented in purple, the confidence interval band of the prediction model in gray and the distribution of the values by orange circles contained within the limits of dotted lines.



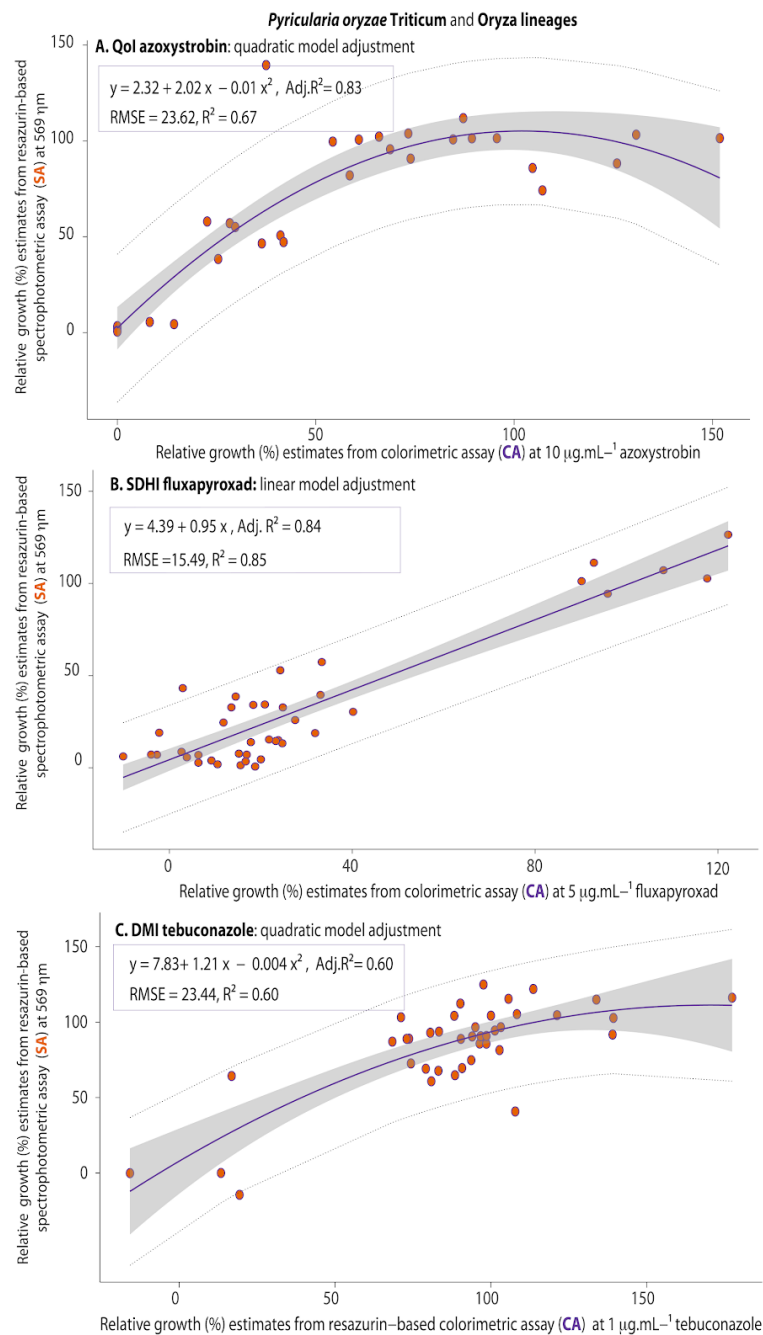
Source: (By author/self)

Figure 7 - Correspondence between fungicide resistance categories (based on relative growth estimates) as a measure of accuracy of the resazurin-based colorimetric assay (COL-assay, purple boxplots) in comparison with the classical spectrophotometry detection assay (SPEC-assay, orange boxplots) for testing sensitivity of *Pyricularia oryzae* Triticum (PoTl) or *P. oryzae* Oryza lineage (PoOl) to QoI, (A), SDHI (B), and DMI (C) fungicides a,b. a The COL-assay was based on analyses of digital images of experimental microplates captured with mobile phone cameras on a handmade trans-illuminator designed for colorimetric estimation of RZ reduction from T0 to T4 using the ReadPlate3.0 in ImageJ developed by Delfino [21], as described in item 2.6 from this study. b The figure depicts boxplots with the medians represented by black lines across the notches, the average as red circles along the whisker lines, and the jittered data points in purple or orange to avoid data overplotting. The F statistics for treatment effect was significant at least at $p \leq 0.05$. Boxplots with the same letter on top (from A to D) indicate that the means for the particular isolate or resazurin-based phenotyping assay are not significantly different by the Scott–Knott test at $\alpha \leq 0.05$.



Source: (By author/self)

Figure 8 - Regression models predicting the correspondence between the fungus relative growth estimates from the SPEC-assay at 569 nm (SA) on the basis of relative growth estimates from the COL-assay (CA) for QoI (A), SDHI (B), and DMI (C) fungicide sensitivity testing for *Pyricularia oryzae* Triticum lineage and *P.oryzae* Oryza lineage ^{a,b}. ^a The rectangle within each figure contains the corresponding linear equations, the adjusted R^2 (Adj. R^2) as a measure of precision of the colorimetric assay, and the model fit parameters (RMSE and R^2). For the QoI (A) and DMI fungicide (C) the quadratic regression had the best fit as a predicting model while for SDHI (B) the linear regression had the best fit, with the lowest RMSE and the highest R^2 . ^b The regression lines were presented in purple, the confidence interval band of the prediction model in gray and the distribution of the values by orange circles contained within the limits of dotted lines.



Source: (By author/self)

Taking all this information together, our study explored the applicability of a digital image colorimetric assay (COL-assay), associated with the measurement of resazurin activity during fungal growth, using a hand-made apparatus for the digital documentation of reactions in fungicide resistance detection experiments for both *Mycosphaerella* and *Pyricularia* pathogens. Since the COL-assay has been shown to be accurate and precise, it is an affordable choice for poorly equipped laboratories with no access to a spectrophotometric microplate reader, and for end users such as extension plant pathologists with little experience in digital image processing. For instance, the COL assay specific costs require the framing of the handmade transilluminator (which includes carpentry: US\$ 50; painting: US\$ 20; lamp, cable and power plug acquisition: US\$ 25; electrician and other services: US\$ 10; totaling: US\$ 105) and the purchase of a cell phone device (US\$ 583), totaling US\$ 688. In comparison, the SPEC assay requires the availability of a microplate spectrophotometer containing the specific 569 and 620 nm filters for the readings. Our model (Multiskan™ FC Microplate Photometer, from Thermo Fisher Scientific, USA) costs US\$ 8070. Therefore, the COL-assay could result in saving up to US\$ 7382 in equipment costs.

By openly sharing its full methodology and the blueprints of the trans-illuminator we foresee its adoption throughout the country as a phenotyping platform for monitoring the fungicide resistance status of populations of several other important necrotrophic or hemibiotrophic fungal plant pathogens, besides *M. fijiensis*, *M. musicola*, and *P. oryzae*. In Brazil, these relevant pathogens include, as examples, *Alternaria alternata* on tangerines (CHITOLINA *et al.*, 2021), *Botrytis cinerea* on strawberries (MAIA *et al.*, 2021), *Colletotrichum accutatum* on apples (MOREIRA *et al.*, 2019), *Colletotrichum truncatum* and *Corynespora cassiicola* on soybeans (BOUFLEUR *et al.*, 2021; DIAS *et al.*, 2019; MELLO *et al.*, 2022), *Lasiodiplodia theobromae* on papaya (LI *et al.*, 2022), *Monilinia fruticola* on stone fruits (DUTRA *et al.*, 2020), *Phyllosticta citricarpa* on citrus (POSSIEDE *et al.*, 2009), and *Ramulariopsis gossypii* and *R. pseudoglycines* on cotton (MATHIONI *et al.*, 2022). This fungicide resistance phenotyping platform will certainly require successful optimization and validation of the COL-assay for such a broad range of pathosystems.

Such a countrywise fungicide resistance detection platform would deliver free access to data on the status of fungicide resistance and guide smart decisions, in real time (i.e., during the ongoing cropping seasons) on reducing or limiting the spraying of high-risk fungicides for which resistance has been detected.

5 CONCLUSIONS

The fungicide sensitivity assay developed here based on colorimetric analyses of digital images of fungal growth on microtiter plates allowed the accurate and precise assessment of fungicide resistance phenotype from several samples of two fungal pathogen species.

The handmade trans-illuminator developed for the colorimetric assays has low costs, thus offering an affordable alternative of large-scale fungicide resistance phenotyping in poorly equipped labs in Brazil.

6 PUBLICATION ADDITIONAL INFORMATION: This study was published in full as an open access article in January 25th 2023.

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6.2 Author Contributions: Conceptualization, P.C.C., S.I.M., T.C.S., and S.N.C.V.; methodology, T.C.S., P.C.C., S.N.C.V., F.G.A.J., T.Y.K.O., and A.G.S.; software, F.G.A.J. and P.C.C.; validation, T.C.S.; formal analysis, T.C.S. and P.C.C.; investigation, T.C.S., F.G.A.J., S.N.C.V., S.I.M., F.S.C.J., T.Y.K.O., and A.G.S.; resources, A.A.P.C., P.C.C., and M.C.G.G.; data curation, T.C.S., S.I.M., and P.C.C.; writing, T.C.S., P.C.C., and S.I.M.; writing—review and editing, T.C.S., S.I.M., S.N.C.V., M.C.G.G., A.A.P.C., R.P.L.J., W.C.J.J., and P.C.C.; visualization, M.C.G.G., R.P.L.J., W.C.J.J., and P.C.C.; supervision, P.C.C. and S.I.M.; project administration, P.C.C.; funding acquisition, A.A.P.C. and P.C.C. All authors have read and agreed to the published version of the manuscript.

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6.4 Data Availability Statement: Upon publication, the phenotypic data presented in this study will be publicly available at Mendeley Data repository at doi: 10.17632/rcjy95gbvg.1

6.5 Informed Consent Statement: The Brazilian Ministry of Environment/National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SisGen, issued the Certificates # A64D0EA and A100786 authorizing the scientific activities associated with the collection of botanical and fungal material from the banana and wheat agroecosystems in the Cerrado's and Atlantic Forest's biomes and access the phenotypic diversity of *Mycosphaerella* and *Pyricularia* species.

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

6.7 Appendix A. Reagents and media for resazurin-based fungicide sensitivity assays
Phosphate buffer at 0.025 M: 2.18 g of K₂HPO₄, 0.68 g of KH₂PO₄, to a final volume of 1 L distilled water. Use this buffer to replace water for media preparation.

Potato dextrose (PD) medium: 27g PD Kasvi[®], 0.05g of Chloramphenicol, 0.05 g of Streptomycin, 0.078g of salicylhydroxamic acid, to a final volume of 1L of 0.025M phosphate buffer.

SN medium: 1 g of KH₂PO₄, 1 g of KNO₃, 0.5 g of MgSO₄·7H₂O, 0.5g of KCl, 0.2 g of glucose, 0.2 g of sucrose, 0.05 g of chloramphenicol, 0.05 g of streptomycin, 0.078 g of salicylhydroxamic acid, to a final volume of 1L of 0.025M phosphate buffer.

V8 medium: 100 mL of V8 juice, 1 g of CaCO₃, 0.05 g of chloramphenicol, 0.05 g of streptomycin, 0.078 g of salicylhydroxamic acid, complete with 0.025 M phosphate buffer to a final volume of 1 L.

NaOH solution at 1 M for pH adjustment of the media: 0.399 g of NaOH in 10 mL of distilled water.

HCl solution at 4M for pH adjustment of the media: Dilute 84 mL of concentrated HCl in water to a final volume of 250 mL.

Note about medium preparation: Use as little NaOH and HCl as possible adding drop by drop in the medium to reach the desired pH. After preparing the medium, adjust the medium's pH before autoclaving. After autoclaving, add the chloramphenicol and salicylhydroxamic acid (SHAM), and adjust the final pH again. These steps are essential

otherwise the medium will have a very dark color after autoclaving, which can interfere with the experimental results.

Resazurin solution: Prepare Resazurin (Sigma-Aldrich®) (RZ) stock solution at 800 μM (= 0.02% w/v): 0.01 g in 50 mL of potassium phosphate buffer. Perform filtration of the stock solution using 0.22 μm filter and syringe.

Prepare RZ working solution: dilute to 160 μM . For example, to prepare 50 mL of 160 μM RZ, add 10 mL of 800 μM RZ stock solution + 40 mL of potassium phosphate buffer.

Potassium phosphate buffer at 0.1 M, pH 7.0: For a final volume of 50 mL mix 0.1 M K_2HPO_4 (0.435 g/25 mL water) + 0.1 M KH_2PO_4 (0.135 g/25 mL water).

Note about preparing working resazurin solutions: RZ solutions should not be exposed to direct light. Preparation and storage can be performed using 50 mL Falcon tubes, wrapped in aluminum foil. Store RZ solutions in the refrigerator at 10 °C (~20 months) or at -20 °C (indefinite time). Heat RZ solution for 10 min in a water bath at 37 °C before use (if stored at -20 °C, thaw it first).

The recommended final RZ concentration is 40 μM . Therefore mix 50 μL RZ solution at 160 μM with 100 μL medium and 50 μL of inoculum suspension, to a final volume of 200 μL .

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CAPÍTULO 2. A RESISTÊNCIA A FUNGICIDAS SDHI É GENERALIZADA EM POPULAÇÕES DE PATÓGENOS DA SIGATOKA NEGRA E AMARELA EM PLANTAÇÕES DE BANANA NO SUDESTE DO BRASIL

RESUMO

As doenças do complexo Sigatoka (SDC) é composta por *Mycosphaerella fijiensis* (*Mf*) e *M. musicola* (*Mm*), as doenças fúngicas de manchas e riscas nas folhas de banana mais destrutivas em plantações comerciais em todo o mundo, causando perdas significativas na produção. No Brasil, os fungicidas SDHI específicos para o controle da SDC rotulados desde 2014 apresentam alto risco para o surgimento de resistência se forem usados intensivamente e isoladamente. Para reduzir o risco, eles têm sido usados em misturas com outros fungicidas de alto risco, conforme recomendado há mais de oito anos. Para preencher uma lacuna de conhecimento atual sobre a ocorrência e prevalência de resistência a fungicidas SDHI, este estudo determinou os níveis de sensibilidade ao boscalid e fluxapyroxad em quatro populações dos patógenos SDC coletados em 2020 de três regiões geográficas distintas com programas fungicidas contrastantes. Resistência fúngica a fungicidas é a capacidade dos fungos de sobreviver ou crescer na presença de concentrações de fungicidas que normalmente seriam letais ou inibitórias para eles. A detecção de resistência em isolados foi realizada por meio de testes de sensibilidade *in vitro* (EC₅₀). Os níveis de resistência podem ser classificados em diferentes categorias, dependendo do grau de resistência observado. A prevalência de resistência nessas populações foi de 59,7 e 94,0% para os SDHIs fluxapyroxad e boscalid, respectivamente. Apenas 1,5% dos isolados foram sensíveis a ambos os fungicidas. Também determinamos a associação entre os níveis de sensibilidade e as alterações correspondentes nos genes que codificam a proteína-alvo do fungicida (*SdhB*, *C* e *D*). Os maiores valores médios de EC₅₀ de isolados para boscalid e fluxapyroxad foram detectados na população *Mf* SPVR-CI, seguida pelas populações *Mm* SPNW-C e *Mm* MGN-C, todas recebendo programas fungicidas intensivos. A população *Mm* SPNW-O, coletada em uma plantação orgânica de banana, apresentou os menores EC₅₀ médios. Entre os 10 isolados de *Mf* e 57 de *Mm* sequenciados, apenas uma (*SdhC* N55D) e duas (*SdhB* E196Q e *SdhD* N66K) alterações no local-alvo do Sdh foram detectadas, respectivamente. Nenhuma das alterações no local-alvo foi associada a sensibilidade reduzida, mas foi detectada resistência múltipla a drogas (MDR), pois a adição de inibidores de bomba de efluxo nos testes *in vitro* aumentou a sensibilidade ao SDHI fluxapyroxad. Um monitoramento adicional de mutações no alvo do Sdh será importante, mas um papel importante para outros mecanismos de resistência, como a presença de vários parálogos do Sdh, também não pode ser descartado. Esses resultados destacam a importância da implementação de estratégias sólidas de manejo de resistência quando fungicidas SDHI são utilizados para o controle do complexo de doenças Sigatoka.

Palavras-chave: controle químico; *SdhB*; *SdhC*; *SdhD*; resistência a fungicidas; Ec₅₀; resazurina.

CHAPTER 2. RESISTANCE TO SDHI FUNGICIDES IS PERVASIVE IN POPULATIONS OF THE BLACK AND YELLOW SIGATOKA PATHOGENS IN BANANA PLANTATIONS FROM SOUTHEASTERN BRAZIL

ABSTRACT

The Sigatoka disease complex (SDC) comprises with *Mycosphaerella fijiensis* (*Mf*) and *M. musicola* (*Mm*) the most destructive fungal leaf streak and spot diseases of commercial banana fields worldwide, leading to significant yield losses. In Brazil, the site-specific SDHI fungicides labeled for SDC management since 2014 present a high risk for the emergence of resistance if deployed intensively and singly. To reduce the risk they have been used in mixtures with other high risk fungicides as recommended for over eight years. To fill a current knowledge gap on the occurrence and prevalence of resistance to SDHI fungicides, this study determined the levels of sensitivity to boscalid and fluxapyroxad in four populations of the SDC pathogens sampled in 2020 from three distinct geographical regions under contrasting fungicide programmes. Fungal resistance to fungicides is the ability of fungi to survive or grow in the presence of concentrations of fungicides that would normally be lethal or inhibitory to them. Detection of resistance in isolates was performed through *in vitro* sensitivity testing (EC_{50}). Resistance levels can be classified into different categories, depending on the degree of resistance observed. The prevalence of resistance in these populations was 59.7 and 94.0% for the SDHIs fluxapyroxad and boscalid, respectively. Only 1.5% of the isolates were sensitive to both fungicides. We also determined the association between sensitivity levels and changes in the corresponding fungicide target protein encoding genes (*SdhB*, *C*, and *D*). The highest average isolate EC_{50} values for boscalid and fluxapyroxad were detected in population *Mf* SPVR-CI, followed by populations *Mm* SPNW-C and *Mm* MGN-C, all receiving intensive fungicide programmes. Population *Mm* SPNW-O, sampled from an organic banana field, had the lowest average EC_{50} s. Among the 10 *Mf* and 57 *Mm* isolates sequenced only, one (*SdhC* N55D) and two (*SdhB* E196Q and *SdhD* N66K) *Sdh* target site alterations, respectively, were detected. None of target site alterations were associated with reduced sensitivity but MDR was detected, as addition of efflux pump inhibitors in the *in vitro* tests increased the sensitivity to the SDHI fluxapyroxad. Further monitoring for *Sdh* target mutations will be important, but an important role for other resistance mechanisms such as presence of multiple *Sdh* paralogs can also not be ruled out. These results highlights the importance of implementing sound anti resistance management strategies when SDHI fungicides are deployed for management of SDC.

Keywords: chemical control; *SdhB*; *SdhC*; *SdhD*; fungicide resistance; EC_{50} ; resazurin.

1 INTRODUCTION

The Sigatoka disease complex (SDC) has been considered the most important fungal leaf streak and leaf spots affecting commercial banana production worldwide for over two decades, causing high yield losses (SOUSA *et al.*, 2022). This disease complex has been associated with four ascomycetous fungi belonging to the genus *Mycosphaerella*: the black Sigatoka leaf streak pathogen *Mycosphaerella fijiensis* (Mf) (syn. *Pseudocercospora fijiensis*); the yellow Sigatoka pathogen *M. musicola* Leach (Mm) (syn: *P. musae*); the leaf spot pathogen *M. thailandica* (syn. *Parapallidocercospora thailandica*), and the eumusae leaf spot pathogen *M. eumusae* (syn. *P. eumusae*) (BRITO *et al.*, 2020; BRITO; FRAAIJE; MILLER, 2015). Black and yellow Sigatoka are considered the two most relevant diseases of the SDC in Brazil (NOMURA *et al.*, 2020; OLIVEIRA *et al.*, 2022; ROCHA; CATÃO; MUNIZ, 2012; SANTOS, 2005).

The leaf symptoms resulting from the infections are mostly unique for each of the individual Sigatoka-like pathogens, except for *M. thailandica*, which has been frequently recovered from typical black Sigatoka leaf streak lesions in Brazil (MALIMPENSA, 2018; OLIVEIRA *et al.*, 2022). However, some reports suggest that Black Sigatoka has been misdiagnosed for the less devastating yellow Sigatoka (GOMES *et al.*, 2013).

In Brazil, Black Sigatoka was first reported in 1998 in the Amazon region (BRITO; FRAAIJE; MILLER, 2015; GASPAROTTO *et al.*, 2000), with the pathogen detected in 17 states since, including São Paulo and Bahia, the two most important banana producing states (RAMOS *et al.*, 2018). Black Sigatoka is regarded as the major constraint to banana production, reducing yield up to 100% (BRITO; FRAAIJE; MILLER, 2015; NOMURA *et al.*, 2020). Yellow Sigatoka, first reported in the Amazon region in 1944, is more widespread and present in all banana-growing regions (GOMES *et al.*, 2018). Yield losses of up to 50% have been reported for yellow Sigatoka (BRITO; FRAAIJE; MILLER, 2015; NOMURA *et al.*, 2020). Invasion of the stomata by the fungus and subsequent colonization of the intercellular spaces causes necrotic damage that reduces the photosynthetic capacity of the leaf, affects the size of the bunch, shortens the green life of the fruits and the time between harvest and ripening (ARZANLOU *et al.*, 2007).

The geographical distribution and prevalence of the SDC in Brazil follows a humidity cline. For instance, the black Sigatoka is considered more prevalent and destructive under conducive weather conditions, which include high humidity (>90%), the presence of leaf

wetness, and temperatures ranging from 26–28 °C (BENDINI, 2012; BENDINI *et al.*, 2013; GHINI *et al.*, 2007; MALIMPENSA, 2018; MORAES; NOMURA, 2020). In banana plantations using the susceptible varieties Prata and Nanica, the incubation period for *Mf* could be as short as 13 to 14 days, whereas under unfavorable conditions the pathogen's incubation period could extend to 35 days (BENDINI, 2012; BENDINI *et al.*, 2013; MORAES; NOMURA, 2020; SANTOS, 2005). For *Mm*, spores germinate 2-3 hours after deposition on banana leaves, if a water film is present or if humidity is high. Relative humidity >73% is sufficient for ascospores release. The optimum temperature for conidial and ascospore germination is approximately 26°C (CORDEIRO, 1997; ROCHA, 2008).

SDC diseases are polycyclic and have a mixed reproductive system. During the growing season several cycles of sexual reproduction are completed, resulting in the release of airborne ascospores that can initiate epidemics as primary inoculum source. Clonal epidemic dispersal over short distance takes place via asexually produced conidia that are spread by rain splash (BELTRÁN-GARCÍA *et al.*, 2014; BURT, 1994). Genetic diversity studies revealed that *Mf* and *Mm* population from Brazil, Mexico, or the Philippines have high genotypic variation and are shaped by gene flow over long distances, indicative of an important role for sexually-produced airborne ascospores (BRITO; FRAAIJE; MILLER, 2015; GOMES *et al.*, 2018; MANZO-SÁNCHEZ *et al.*, 2019; MENDOZA; ARDALES, 2019).

The main strategy for management of SDC has been the intensive and preventive (calendar-based) spraying of protectant or systemic fungicides, or the combination of both (BRITO; FRAAIJE; MILLER, 2015). As varietal resistance is practically absent or only partial in most banana cultivars widely cultivated in the country, under high disease pressure, up to 52 sprays of protectant multi-site fungicides or 26 sprays of systemic site-specific fungicides can be applied annually (BRITO; FRAAIJE; MILLER, 2015). Consequently, the populations of the SDC pathogens are under strong selection pressure for the emergence of fungicide resistance (BRITO *et al.*, 2020; CAÑAS-GUTIÉRREZ *et al.*, 2009; DIAZ-TRUJILLO *et al.*, 2018; GRICE *et al.*, 2013; OLIVEIRA *et al.*, 2022). These systemic site-specific fungicides include the major chemical groups quinone outside inhibitors (QoIs) – strobilurins, Demethylation inhibitors (DMIs) – azoles, and more recently the succinate dehydrogenase inhibitors (SDHIs) – carboxamides (BRITO; FRAAIJE; MILLER, 2015; CHURCHILL, 2011; MALIMPENSA, 2018). Resistance to site-specific fungicides has been, in fact, a recurrent serious problem that limits the ability to control agricultural, medical and veterinary fungal pathogens since these fungicides pose risk for resistance to emerge, especially

for pathogens with high evolutionary potential (FISHER *et al.*, 2018; HAWKINS; FRAAIJE, 2018; LUCAS; HAWKINS; FRAAIJE, 2015; MAIR *et al.*, 2016; MCDONALD; LINDE, 2002).

The new SDHI fungicides (also known as second generation carboxamides), in particular, have been labeled since the 2010's and quickly adopted not only for effectively controlling the BSDC pathogens but also a number of other fungal foliar pathogens from several other crops/commodities such as *Alternaria alternata* on pistachios and peaches, *A. solani* on potatoes, *Botrytis cinerea* on apples, grapes, and strawberries, *Clavibacter* sp. (formerly *Sclerotinia homoeocarpa*) on turfgrass, *Corynespora cassicola* on pumpkins, *Phakopsora pachyrhizi* on soybeans, *Pyrenophora teres* on barley, *Sclerotinia sclerotiorum* on canola, *Venturia inaequalis* on apples, and *Zymoseptoria tritici* on wheat (SANG; LEE, 2020). Perceived as highly effective for disease control, the widespread adoption of the systemic site-specific SDHI fungicides may have led to a high selection pressure for resistance to emerge on populations of such a broad spectrum of fungal crop pathogens (FRAAIJE *et al.*, 2019; KLAPPACH; STAMMLER, 2019; SIEROTZKI; SCALLIET, 2013). In fact, the more specific the mode of action (i.e., the processes impacted and the target sites in the fungal cells), the greater the likelihood of resistance, since a non-synonymous mutation in the target gene changing just a single amino acid in the corresponding protein can result in insensitivity to the chemical molecule (BRENT, 1995; LUCAS; HAWKINS; FRAAIJE, 2015; SIEROTZKI; SCALLIET, 2013).

SDHI fungicides are respiration inhibitors targeting succinate dehydrogenase (Sdh) (succinate:ubiquinone oxidoreductase) or mitochondrial respiratory chain complex II, a heterotetrameric enzyme complex that participates in both the citric acid cycle (SdhA) and the electron transport chain (SdhB, C and D).. The ubiquinone binding site pocket of SDHs is formed by Sdh subunits B, C and D. SDHI binding physically blocks access to the substrate and prevents the oxidation cycle of succinate which is essential for mitochondrial respiration (KLAPPACH; STAMMLER, 2019; SIEROTZKI; SCALLIET, 2013)..

The most common mechanism of resistance to SDHs includes target site mutations leading to amino acid changes in Sdh subunits B, C and/or D, although other mechanisms can also occur, such as multiple copies and/or the overexpression of the target genes and/or overexpression of multidrug efflux pumps (i.e., ATP binding cassette (ABC) or major facilitator superfamily (MFS) transporters that export multiple fungicides from the inside to the outside of the cell, decreasing their intracellular concentration) (AMIRI; ZUNIGA; PERES, 2020;

AYER *et al.*, 2019; DE MICCOLIS ANGELINI *et al.*, 2014; FISHER *et al.*, 2018; FÖRSTER *et al.*, 2022; FRAAIJE *et al.*, 2019; HE *et al.*, 2020; MÜLLER; STAMMLER; MAY DE MIO, 2021; PENG *et al.*, 2021; PRASAD; RAWAL, 2014; REHFUS *et al.*, 2018; VELOUKAS *et al.*, 2014; YAMASHITA; FRAAIJE, 2018).

Currently, the SDHI fungicides labeled for the management of the SDC in banana plantations include boscalid, fluopyram, fluxapyroxad, and isopyrazam (FRAC, 2022). Particularly in Brazil, only a single fungicide co-formulation (Collis, from Basf S.A.) containing the SDHI anilide boscalid and the QoI kresoxim-methyl actives has been labeled by the Ministry of Agriculture, Livestock and Supplies for spraying on bananas since 2014 (MAPA – MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO - BRAZIL, 2023).

FRAC surveillance data on SDHI resistance in SDC pathogens registered to date have shown that a few SDHI-insensitive strains of *Mf* were detected from 2012 to 2017 in Costa Rica, Ecuador, Guatemala, and Colombia in areas where sprays containing boscalid, fluopyram, fluxapyroxad, and/or isopyrazam have been applied in banana plantations (FRAC, 2022). A large knowledge gap still exists in Brazil concerning the development of SDHI resistance in SDC pathogens. If resistance occurs locally, there will also be a lack of information on the molecular mechanisms associated with levels of resistance to SDHIs in populations of the two SDC pathogens. This lack of knowledge about the resistance status of *Mf* and *Mm* to SDHI fungicides can lead to inefficient use of the actives, targeting populations of the SDC pathogens which potentially already have developed resistance to this class of fungicides.

Therefore, in a context of high selection pressure associated with the intensive use of these fungicides on banana plantations, the main objective of our study was to characterize the prevalence and the degree of sensitivity reduction and/or resistance in populations of the SDC pathogens *Mf* and *Mm* to SDHI fungicides in Brazil, as well as to elucidate their resistance mechanisms. To accomplish this objective we determined the sensitivity status of *Mf* and *Mm* populations to the SDHI fungicides boscalid and fluxapyroxad at four sites in Southeastern Brazil with three distinct management systems for controlling SDC: programmes using intensive, reduced or no fungicide spray applications. We also investigated whether site-specific resistance is associated with mutations in genes encoding Sdh subunits B, C and/or D. Since the SDHI fungicides have been used for controlling SDC on bananas in Brazil since 2014, we hypothesized that the selection pressure due to fungicide spraying have selected for resistance in highly variable pathogens populations.

2 MATERIALS AND METHODS

2.1 Population sampling area for the black and yellow Sigatoka pathogens.

Population samples of *Mf* and *Mm* were obtained from infected leaf samples with typical black and yellow Sigatoka symptoms collected in 2020 at four geographical locations in Southeastern Brazil, with three different disease management systems: i: Intensive, from Vale do Ribeira, São Paulo (population SPVR-CI, from Jacupiranga, Registro and Sete Barras counties); ii: Reduced, from northwestern São Paulo (population SPNW-C, from Ilha Solteira county); and from northern Minas Gerais (population MGN-C, from Janaúba county); iii. Organic, with no fungicide (population SPNW-O, from Ilha Solteira). The geographical locations of the population samples are shown in Figure 1.

At the first location in Vale do Ribeira, São Paulo (SPVR-CI), management was based on intensive fungicide spraying with eight to 14 preventive applications of fungicides per year for chemical control of black Sigatoka (MALIMPENSA, 2018; OLIVEIRA *et al.*, 2022). This region represents the largest banana production in São Paulo state and also in Brazil, where the SDC susceptible banana cultivars Prata (*Musa* spp. AAB, also called “Lady Finger” banana) and Nanica (*Musa* spp. AAA, Cavendish subgroup) predominate (MORAES; NOMURA, 2020; NOMURA *et al.*, 2020; OLIVEIRA *et al.*, 2022; RANGEL; PENTEADO; TONET, 2002). At the second site, in northwest of São Paulo (SPNW-C), and the third site, in northern Minas Gerais (MGN-C), fungicide use is reduced, with four to five preventive sprays against Yellow Sigatoka (OLIVEIRA *et al.*, 2022). The SPNW-C field had 40 ha of susceptible variety Maçã (triploid AAB (OLIVEIRA *et al.*, 2022) while in MGN-C, the banana plantation was established with Prata and Nanica varieties (OLIVEIRA *et al.*, 2022). In the last site sampled (SPNW-O) no fungicides were sprayed and consisted of several small family plantations of banana plants from different varieties and ages in Ilha Solteira county (OLIVEIRA *et al.*, 2022).

2.2 Isolation of *Mm* and *Mf*

Fragments of banana leaves with symptoms of black or yellow Sigatoka diseases were placed in paper bags, transported, and stored in a refrigerator until the pathogens were isolated. The leaves were washed with tap water, cut into 2 cm² pieces, and disinfested with 75% ethanol and sterile distilled water for one minute. Leaves were then dried on sterile filter paper and transferred to Petri dishes containing water-agar medium [15 g L⁻¹ agar] amended with the antibiotics chloramphenicol and streptomycin (50 µL mL⁻¹ each) and incubated at 26°C in the dark until sporodochia formed. Using a fine needle and with the aid of a stereomicroscope,

sporodochia were transferred from lesions to Petri dishes containing PDA [20.8 g L⁻¹ potato dextrose (Kasvi, India), 15 g L⁻¹ agar + chloramphenicol and streptomycin (50 µL mL⁻¹ each)] and kept at 26°C in the dark until microcolonies characteristic of *Mf* or *Mm* were observed. Young colonies were transferred to new PDA plates. After 14 days of growth, sterilized filter paper strips were deposited within PDA plates. Subsequently, fungal mycelium discs were transferred onto the plates for fungal colonization of the paper fragments. Plates were then incubated for 10 days at 25 °C in the dark, after which the fungal- mycelium-colonized filter paper strips were transferred to sterile Petri plates, and kept in a flow chamber for three days to dry. (BRITO *et al.*, 2020; GOMES *et al.*, 2018; OLIVEIRA *et al.*, 2022). For long term storage of the isolates obtained were cryopreserved at -20°C in cryotubes containing silica by transferring the sterile filter paper discs colonized by *Mf* or *Mm*.

2.3 Preparation of inoculum for SDHI fungicide sensitivity testing

For the SDHI fungicides sensitivity testing, a total of ten isolates of *Mf* and 57 isolates of *Mm* were screened; 10 *Mf* and 2 *Mm* isolates from SPVR-CI, 21 *Mm* isolates from SPNW-O, 18 *Mm* isolates from SPNW-C, and 16 *Mm* isolates from MGN-C. Mycelial fragments were prepared according to the protocol described by Silva *et al.* 2023. For this, the SDC isolates were reactivated in PDA medium (20.7 g.L⁻¹ potato dextrose, 15 g.L⁻¹ agar) supplemented with chloramphenicol and streptomycin (50 µg mL⁻¹ each). After 15 days of growth at 25°C and 12 hours of photoperiod, fungal mycelia were transferred to 1.5 mL microtubes containing 0.5 mL of 0.1 mm diameter glass beads. A total of 1.0 mL of distilled water was added to the mixture and bead-beated in a fast-prep device for 20 seconds at a speed of 4 m s⁻¹. Bead beating resulted in a suspension of small mycelial fragments that was further diluted with distilled water to total volume of 10 mL. The final concentration of the mycelial fragment suspensions was adjusted to 10⁴ mL⁻¹ based on Neubauer chamber counting.

2.4 SDHI fungicide sensitivity testing

Sensitivity testing was carried out with the SDHIs boscalid and fluxapyroxad. Formulated boscalid (Cantus™, active ingredient at 500g kg⁻¹, Basf S.A., Brazil) was dissolved in deionized water to 0.1 g mL⁻¹ and then diluted in deionized water to obtain the 500 µg mL⁻¹ stock solution. Technical grade fluxapyroxad (PA, Sigma-Aldrich, USA) was dissolved in acetone to 50 mg mL⁻¹ (PA, Sigma-Aldrich, USA), followed by dilution in deionized water to obtain the 500 µg mL⁻¹ stock solution. The final doses of boscalid (BSC) and fluxapyroxad (FLX) tested were 0.0, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 µg mL⁻¹. Salicylhydroxamic acid (SHAM) was added to 0.5 mM PD broth to suppress alternative oxidase (AOX) activity

(SIEROTZKI; SCALLIET, 2013; MA; UDDIN; OLAYA, 2009). The fungicides tested at different doses were mixed with PD medium prepared with 0.025M phosphate buffer and its pH was adjusted to 5.0. Sensitivity tests were performed in 96-well -bottomed microplates (Kasvi, India). The total volume in each well of the microplate was 150 μ L after adding 100 μ L of the PD medium (with and without fungicides at different dosages) and 50 μ L of mycelium suspension. The experimental design was completely randomized with eight replicates and each experiment was repeated once. The microplates containing the liquid fungal cultures were wrapped in plastic and incubated at 25°C in the dark with shaking at 150 rpm for 10 days when the fungal growth reached its maximum. Then 50 μ L of resazurin (RZ) (160 μ M) was added to each well of the microplate to obtain a final concentration of 40 μ M (for a final volume of 200 μ L), and the initial absorbance values at 569 nm (Abs_{569} nm at T_0) were measured using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific™, USA) for spectrophotometric estimates of RZ reduction as indication the of fungal growth and metabolic activity according to Silva *et al.* 2023. Subsequently, the microplates were kept at 25°C in complete darkness for 24 hours of the reaction when the final absorbance values were measured at Abs_{569} nm.

The RZ reduction was estimated as follows:

$RR_{Mycosphaerella} = T_0 - T_{24}$, where:

$RR_{Mycosphaerella}$ = relative reduction of resazurin due to metabolic activity;

T = Absorbance reading at 569 nm;

T_0 = reading at time zero, immediately after adding RZ to the microplate wells

T_{24} = reading time 24 h after adding RZ.

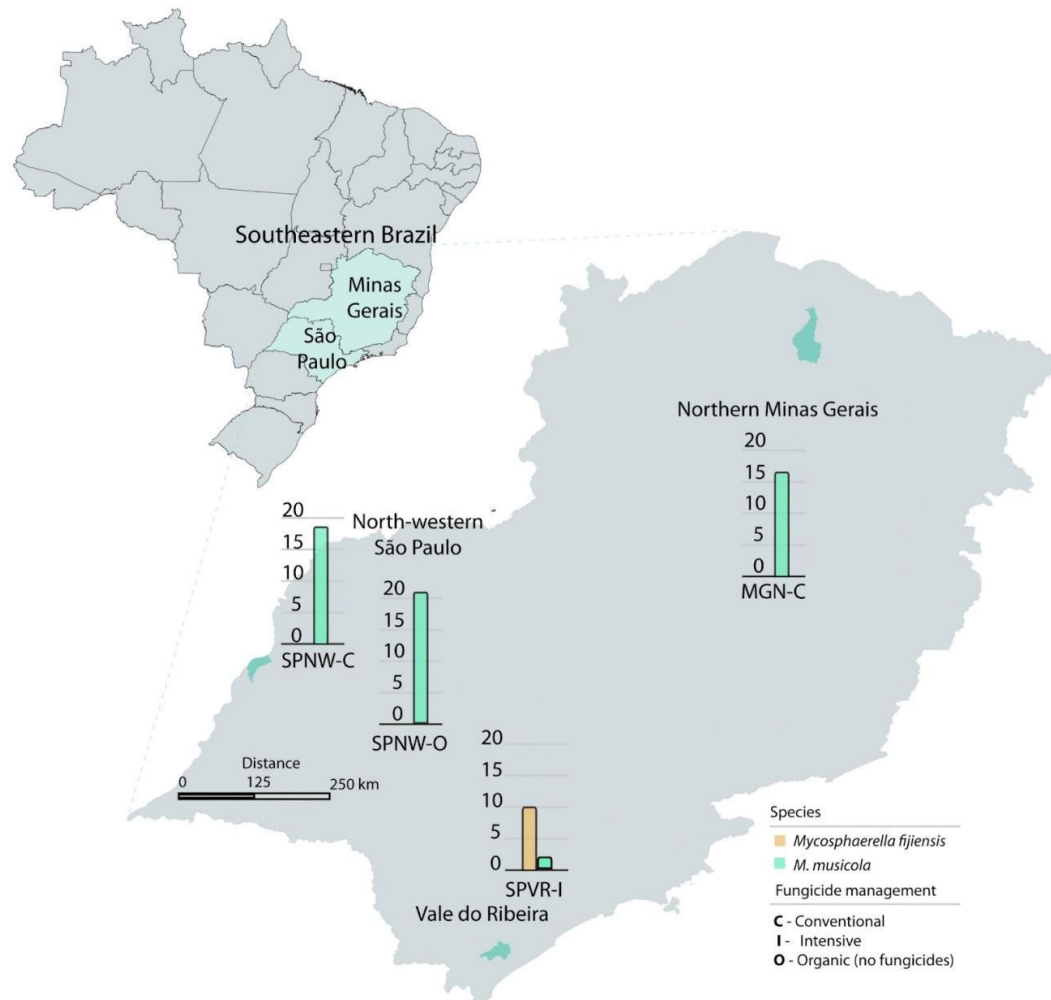
Sensitivity to the two SDHI fungicides was determined as 50% effective concentration to inhibit fungal growth (EC_{50} , in μ g·mL⁻¹), estimated using a dose–response function implemented in the Excel macro ED50plus v1.0 (VARGAS, 2023). For EC_{50} means comparison within each fungicide, the experimental design consisted of complete randomized blocks, with four replicates per treatment and experiments in duplicate. Analysis of variance (ANOVA) and the Scott–Knott test (at 5% probability) for means comparison were performed in the *R* environment using the statistical packages *agricolae* and *ScottKnott* (R DEVELOPMENT

CORE TEAM R: A LANGUAGE AND ENVIRONMENT FOR STATISTICAL COMPUTING, 2023).

The boxplot figures depicting the contrast among fungicide resistance categories, among geographical population of the pathogens, and between groups of isolates of *Mm* and *Mf* per species (based on EC₅₀ values or relative growth estimates) were built using the *R* software library *tidyverse* 1.3.1 (SAN; LEE, 2020), which included the packages *ggplot2* 3.3.5, *purrr* 0.3.4, *tibble* 3.1.6, *dplyr* 1.0.7, *tidyr* 1.1.4, *stringr* 1.4.0, *readr* 2.1.0, and *forcats* 0.5.1, and the functions *ggplot*, *geom_boxplot*, *stat_summary*, *geom_jitter*, *ggtitle*, *theme*, and *geom_text*.

The whole set of colors palette chosen to build all figures with accessibility are color-blind safe and print friendly, using the resources from Color Brewer 2.0 available at the URL <https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3> (accessed on 01 November 2022).

Figure 1 - Population sampling of the black leaf streak (*Mycosphaerella fijiensis*) and yellow Sigatoka (*M. musicola*) pathogens from banana plantations with contrasting fungicide management in Southeastern Brazil during the 2020/21 cropping season. States and counties were colored in green: Ilha Solteira county, from northwestern São Paulo state (SP), Jacupiranga, Registro and Sete Barras counties from Vale do Ribeira (SP), and Janaúba county, from northern Minas Gerais State (MG).



Source: (By author/self)

2.5 Analysis of allelic variation in the SDHI target protein encoding genes *SdhB*, *SdhC*, and *SdhD* in *Mm* and *Mf*

To investigate target site mutations in the *SdhB*, *SdhC*, and *SdhD* genes that could be associated with resistance to SDHI fungicides, all 10 isolates of *Mf* and 57 isolates of *Mm* from four distinct SDC populations PCR amplified and sequenced. PCRs were performed in a volume of 30 μ L containing highly purified distilled water, 50 ng template DNA, 0.3 μ M of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 3 μ L 10 $\times$ PCR reaction buffer PCR and 0.05 U Taq polymerase (Thermo Fisher Scientific, USA). Cycling conditions were as follows:

initial denaturation at 95°C for 5 min, followed by 36 cycles of 94°C for 30 s, 1 min at the specific annealing temperature for each primer pair (Table 1), and at 72°C for 1 min; with a final extension of 72°C for 7 min. PCR products were purified and Sanger sequenced at Macrogen Inc. sequencing facilities, Seoul, South Korea. The DNA sequences were analyzed using the Geneious R 9.0.5 program (Biomatters, New Zealand) to identify mutations, alleles and haplotypes and to distinguish non-synonymous mutations that result in amino acid changes in the deduced protein sequences. For annotation and derivation of protein sequences from the *Sdh* sequences as well as design of primers using Primer3 implemented in the Geneious software (Table 1) we used the following sequences from NCBI/Genbank® as reference: *Mf* NW_006921538 and XM_007930654 for *MfSdhB*, NW_006921536 and XM_007928933 for *MfSdhC* and NW_006921535 and XM_007927648 for *MfSdhD*; LFZO01000506 for *MmSdhB*, LFZO0100037 for *MmSdhC*; LFZO01000522 for *MmSdhD*. The experimentally generated sequences will be deposited in GenBank®. These sequences were derived from *Mf* strains CIRAD86 and *Mm* isolate CBS 116634, originating from Cameroon and Cuba, respectively, and isolated before 2008 (ARZANLOU *et al.*, 2008).

Table 1 - pecific primers designed for this study for PCR amplifications and for assessment of variation in the SDHI binding pocket forming regions from different Sdh subunit encoding genes in *Mycosphaerella fijiensis* (*Mf*) and *Mycosphaerella musicola* (*Mm*)

Target ^a	Primers ^b	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
<i>MfSdhB</i>	SdhB_Mf_F42	TTCTGCTTCACCACGTCTCC	1164	55.0
	SdhB_Mf_R1205	TGTGAGTCTGCCTATCATGA		
<i>MfSdhC</i>	SdhC_Mf_F84	TGTTTGTCTACACCAGCACTG	849	58.0
	SdhC_Mf_R932	AAGCCAAAGTGAGTTGCCCA		
<i>MfSdhD</i>	SdhD_Mf_F92	TCTGTCTTCCCACCTCTCAC	996	58.5
	SdhD_Mf_R1087	GCCACGGGATTGAGCTGTTG		
<i>MmSdhB</i>	SdhB_Mm_F103	CCTCCCCTCTGCTCATTACG	996	59.5
	SdhB_Mm_R1098	CACCACCCCACCACATACC		
<i>MmSdhC</i>	SdhC_Mm_F100	TGTCTGTCTACACCAGCACTG	946	58.0
	SdhC_Mm_R845	CAACCTGCAAACCAAGACCC		
<i>MmSdhD</i>	SdhD_Mm_F153	CCCACCCTGATCTTTTGCAT	899	58.0
	SdhD_Mm_R1051	CCATACCACAAAGCGAGCCA		

^a*Mf* = *Mycosphaerella fijiensis*; *Mm* = *Mycosphaerella musicola*. ^bFor primer design for this study we used Primer3 implemented in the Geneious software), based on the following reference sequences from NCBI/Genbank®: *Mf* NW_006921538 for *MfSdhB*, NW_006921536 for *MfSdhC* and NW_006921535 for *MfSdhD*; *Mm* LFZO01000506 for *MmSdhB*, LFZO0100037 for *MmSdhC*; LFZO01000522 for *MmSdhD*. **Source:** (By author/self)

2.6 Efflux pump inhibition assay

Three *Mf* isolates with different and multiple resistance (i.e., with multidrug resistance (MDR) phenotype) to QoI, DMI and/or SDHI fungicides) according previous studies [SILVA *et al.* 2023, OLIVEIRA, *et al.* 2022 and A. Gomes da Silva, personal information], were selected and retested for this assay giving the following phenotypes: Ja2.24 (QoI R(resistant), DMI MR RS (reduced sensitivity)(moderately resistant), and SDHI ER (extremely resistant)), Ja3.6 (QoI S (sensitive), DMI S to MR depending on DMI tested, SDHI ER), and Ja3.9a (QoI S, DMI MR, SDHI ER). We tested whether two efflux pump inhibitors could revert the MDR phenotype from resistant to sensitive: a) BLT-4 or INF271 [N-(2-methoxyphenyl)-N'-2-naphthalenyl-urea,

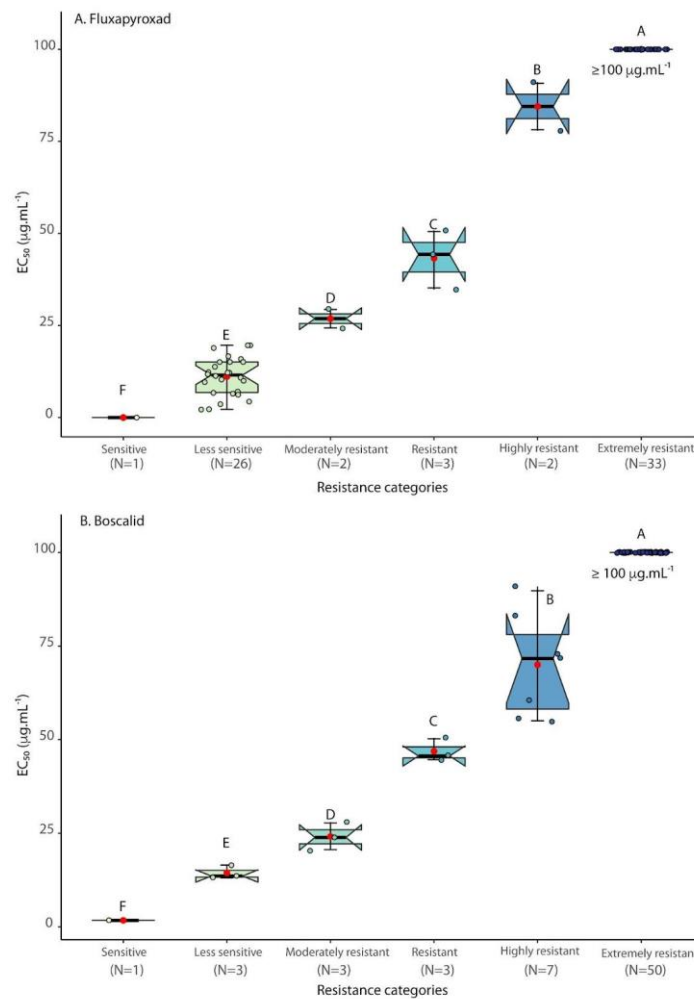
a MFS transporter-type inhibitor] at $0.25 \mu\text{g mL}^{-1}$; and b) verapamil hydrochloride (an ABC transporter-type inhibitor), at $250 \mu\text{g mL}^{-1}$ (LEE *et al.* 2021; LI *et al.* 2022; FERNÁNDEZ-ORTUÑO *et al.* 2017; SIEROTZKI *et al.* 200; VICENTNI *et al.* 2022) The inhibitors were tested individually or in combination. Fungicides were tested at discriminatory doses of $5 \mu\text{g mL}^{-1}$ for boscalid and $3 \mu\text{g mL}^{-1}$ for fluxapyroxad. The assay was performed in 96-well microplates, using the resazurin-based fungicide sensitivity protocol described by Silva *et al.* 2023 and measuring the fungal relative growth using the following experimental treatments: check (with no fungicides or efflux pump inhibitors added); fungicide; BLT-4; verapamil; BLT-4 + verapamil; fungicide + BLT-4; fungicide + verapamil; fungicide + BLT-4 + verapamil. The experimental design consisted of complete randomized blocks, with four reps per treatment. The experiment was repeated once. Analysis of variance (ANOVA) using *F* test and the Scott–Knott test (at 5% probability) for means comparison were performed in the *R* environment using the statistical packages *agricolae* and *ScottKnott*.

3 RESULTS

3.1 SDHI fungicides sensitivity testing

In general, based on the EC_{50} values for the fungicides boscalid and fluxapyroxad, the SDC isolates were classified into the following categories: (i) sensitive (S), with $EC_{50} \leq 2 \mu\text{g mL}^{-1}$, (ii) less sensitive (LS), with $EC_{50} > 2$ and $\leq 10 \mu\text{g mL}^{-1}$, (iii) moderately resistant (MR), with $EC_{50} > 10$ and $\leq 25 \mu\text{g mL}^{-1}$, (iv) resistant (R), with $EC_{50} > 25$ and $\leq 50 \mu\text{g mL}^{-1}$, (v) highly resistant (HR), with $EC_{50} > 50$ and $\leq 100 \mu\text{g mL}^{-1}$, and finally (vi) extremely resistant (ER), with $EC_{50} > 100 \mu\text{g mL}^{-1}$ (Figure 2).

Figure 2 - Estimates of the isolates of *M. fijiensis* and *M. musicola* EC_{50} values for fluxapyroxad (A) and boscalid (B) and corresponding sensitivity and resistance categories. *F* test from ANOVA significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott–Knott test at $p \leq 0.05$.

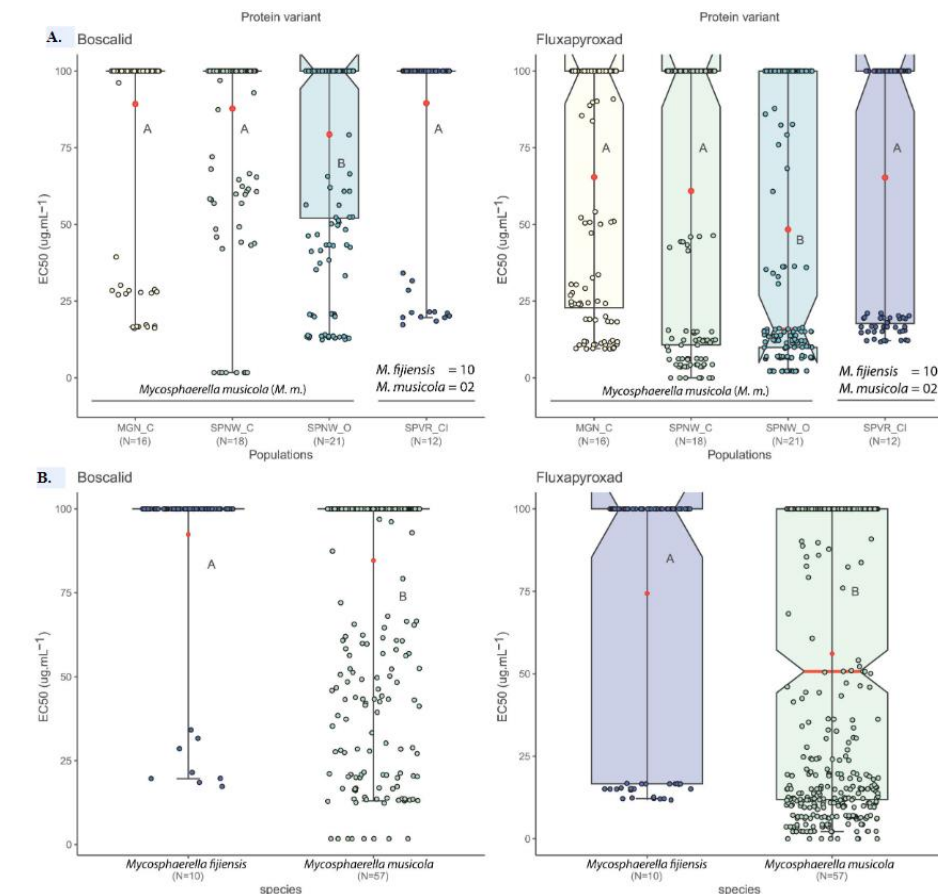


Source: (By author/self)

Reduced sensitivity and resistance to boscalid and fluxapyroxad predominated among the SDC isolates tested. Out of the 67 isolates tested, three (4.5%) and twenty-six (38.8%) were classified as less sensitive (LS) to boscalid and fluxapyroxad, respectively, and only one isolate (1.5%) was classified as sensitive (S) to both fungicides. A total of 63 isolates (94.0%) were insensitive to boscalid, falling into the following categories: three moderately resistant (MR), three resistant (R), seven highly resistant (HR), and 50 extremely resistant (ER). With respect to fluxapyroxad, 40 isolates (59.7%) were insensitive, falling into the following categories: 17 moderately resistant (MR), three resistant (R), one highly resistant (HR), and 35 extremely resistant (ER) (Figure 2).

There were significant differences in the mean EC_{50} values for the SDHI fungicides among geographical populations of the SDC pathogens according to the management system for controlling SDC. Populations SPVR-I, SPNW-C, and MGN-C (with intensive or reduced fungicide inputs) were significantly different from population SPNW-O (with no fungicide use) and all had higher mean EC_{50} values for both boscalid and fluxapyroxad (Figure 3A). In addition, isolates within the less sensitive class (LS) were more common in the SPNW-O population. In contrast, isolates from populations with a history of fungicide use, fell into high and extreme resistance (HR and ER) categories. With the exception of the sensitive isolate, all *Mf* and *Mm* isolates showed positive cross-resistance to both boscalid and fluxapyroxad. The *Mf* isolates showed statistically higher mean EC_{50} values than the *Mm* isolates (Figure 3B).

Figure 3 - A. Contrast between groups of isolates of *Mycosphaerella* species per geographical population of the pathogen, according to EC_{50} of boscalid or fluxapyroxad. B. Contrast between groups of isolates of *Mm* and *Mf* per species, according to EC_{50} of boscalid or fluxapyroxad. The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in different colors to avoid data overplotting. N = number of isolates per population. F test from ANOVA significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$.



Source: (By author/self)

3.2 Detection of mutations in the Sdh genes

All primer combinations designed and used in this study were efficient in amplifying the target *Sdh* sequences and produced fragments of the expected size (Table 1). Complete sequences of the *SdhB*, *SdhC*, and *SdhD* genes were obtained for all the 67 SDC isolates examined. Three non-synonymous substitutions were identified in the *Sdh* genes of the SDC pathogens. Interspecific comparison between the reference *Sdh* amino acid sequences of *Mf* and *Mm* showed a high similarity among the three subunits with only 24 alterations (23

substitutions and one deletion) detected: 10 in the *SdhB* gene (deletion included), eight in the *SdhC*, and six in the *SdhD* (Figure 4).

Mutations in the *SdhB*, *SdhC* and *SdhD* genes from *Mf* and association with SDHI sensitivity

The *SdhB* gene was 961 or 964 base pairs (bp) long, depending on the isolate sequenced, and encoded 299 amino acids (Figure 4). The open reading frame (ORF) was structured into two exons, with an intron of 61 or 64 bp long. The *SdhC* gene was 663 base pairs (bp) long and encoded 184 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two putative introns of 49 and 59 bp in length. The *SdhD* gene was 692 base pairs (bp) long and encoded 188 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two putative introns of 65 and 60 bp in length (Figure 4).

For *Mf*, only one non-synonymous mutations was detected in one of the Sdh target encoding genes. This mutation results in the amino acid substitution N55D (asparagine (AAC) to aspartic acid (GAC) exchange) at codon position 52 of *SdhC* in comparison with the reference sequence XM_007928933. *SdhC*-N52D was detected in all 10 *Mf* isolates from population SPVR-CI. The sensitivity of these isolates ranged from moderately resistant to extremely resistant (Figure 3), with average EC₅₀ values of 65.3 µg mL⁻¹ and 89.5 µg mL⁻¹ for fluxapyroxad and boscalid, respectively. *SdhC*-D52 was present in all *Mm* isolates, including the SDHI sensitive isolate *Mm* ISC92.

3.1.1 Mutations in the *SdhB*, *SdhC* and *SdhD* genes from *Mm*

The *SdhB* fragment was 955 base pairs (bp) long and encoded 300 amino acids (Figure 4). The open reading frame (ORF) was structured into two exons, with a putative intron of 52 bp long. Alignments allowed the identification of a nonsynonymous mutation resulting in E196Q (glutamic acid (GAA) to glutamine (CAA) exchange at codon 196) in *SdhB* from isolate ISR61 (Figure 4). This mutation was only found in this isolate, originating from the SPNW-O population, with ER/HR resistance categories for boscalid and fluxapyroxad fungicides, respectively. With the highest EC₅₀ value, it was the most resistant *Mm* isolate to boscalid and fluxapyroxad (Figure 3).

The *SdhC* fragment was 662 base pairs (bp) long and encoded 184 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two putative introns of 49 and 58 bp in length. No nonsynonymous mutations were identified in this gene (Figure 4).

The *SdhD* fragment was 700 base pairs (bp) long and encoded 188 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two putative introns of 56 and 77 bp in length. The alignment analyses using reference sequences resulted in the identification of one amino acid substitution, N66K (asparagine (AAC) to lysine (AAA) exchange at codon 57). This mutation was only present in a SDHI sensitive isolate Mm ISC92 from the population SPNW-C. It had the lowest average EC_{50} of $1.7 \mu\text{g mL}^{-1}$ and $0 \mu\text{g mL}^{-1}$ for boscalid and fluxapyroxad, respectively (Figure 3).

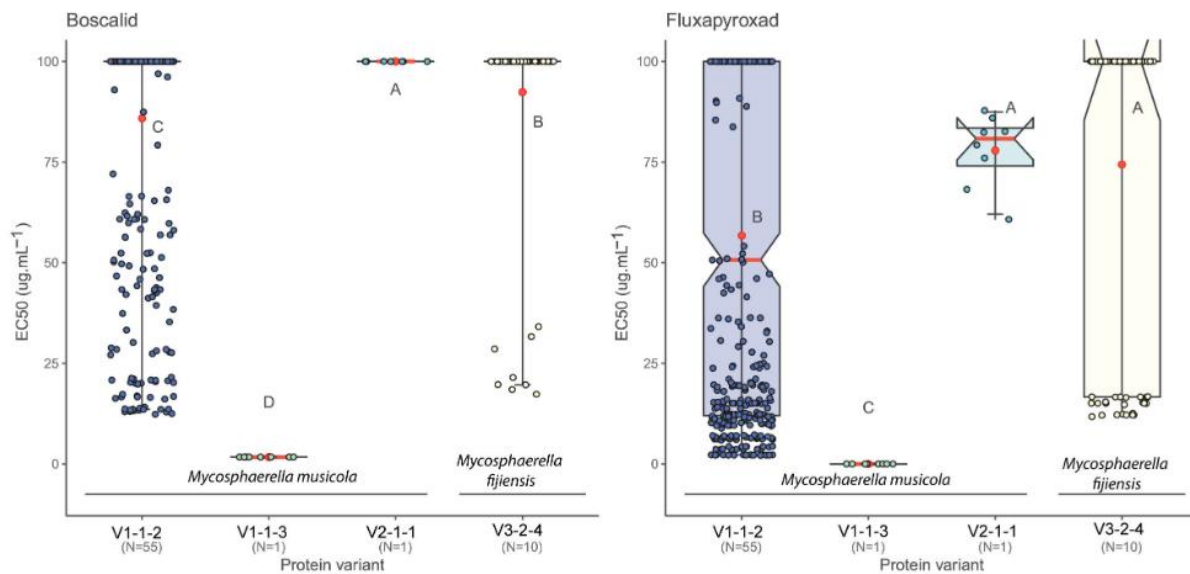
Wild-type Sdh sequences were detected in 55 out of 57 Mm isolates (96.5%), present in all four Mm populations, with EC_{50} values belonging to the less sensitive to extremely resistant categories (Figure 3), with mean EC_{50} values of $58.1 \mu\text{g mL}^{-1}$ and $86.3 \mu\text{g mL}^{-1}$ for fluxapyroxad, and boscalid, respectively.

Figure 4 - Alignment of amino acid sequences translated from the nucleotide sequences of the *SdhB*, *SdhC*, and *SdhD* genes of isolates CALT1 (*Mf*), ISC97 (*Mm*), and ISR61 (*Mm*) to identify nonsynonymous mutations by comparison with the reference sequence of the sensitive isolate ISC92 (*Mm*). Each of these sequences represents a protein variant. The translated NCBI/Genbank® sequences NW_006921536 (*Mf*) and LFZO0100050 (*Mm*) were also aligned for comparison purposes.



Source: (By author/self)

Figure 5 - Contrast among protein variant groups of isolates of *Mycosphaerella fijiensis* and *M. musicola* according to the EC_{50} values for boscalid or fluxapyroxad. The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in different colors to avoid data overplotting. N = number of isolates per population. F test from ANOVA significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$.

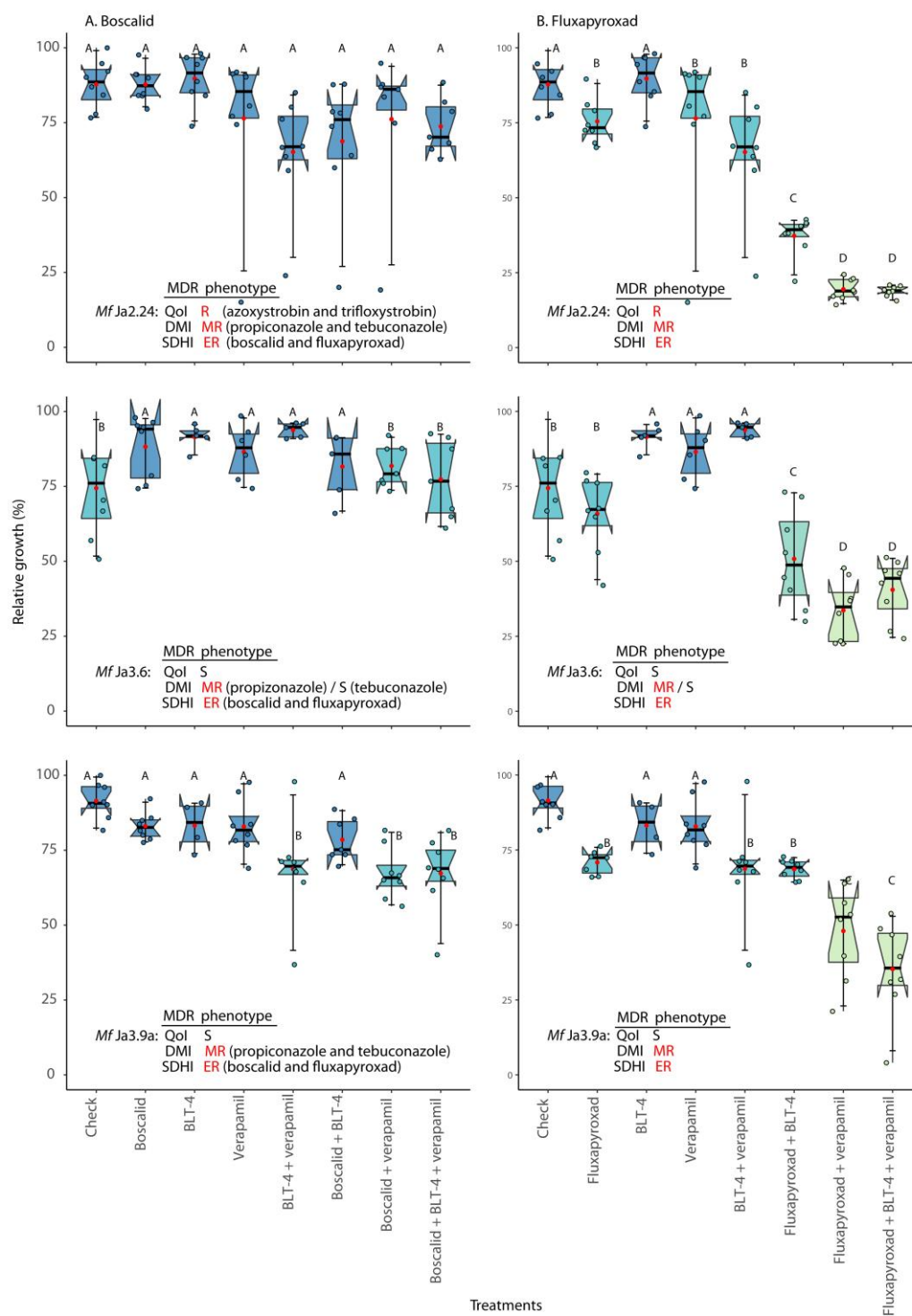


Source: (By author/self)

3.2 Efflux pump inhibition assay

The efflux pump transporter inhibitors BLT-4 and verapamil, in particular, significantly affected the fluxapyroxad sensitivity of the three MDR *Mf* isolates, with an up to 3-fold increase, while the sensitivity of boscalid was not significantly affected (Figure 6).

Figure 6 - Effect of the efflux pump transporters inhibitors BLT-4 and verapamil restoring the sensitivity of MDR resistant isolates of *Mycosphaerella fijiensis* to boscalid and fluxapyroxad.



Source: (By author/self)

4 DISCUSSION

In this study we determined the sensitivity status of *Mf* and *Mm* populations to the SDHI fungicides boscalid and fluxapyroxad at four locations in Southeastern Brazil with distinct management systems for controlling SDC, receiving intensive, reduced or no fungicide inputs. Since the SDHI fungicide boscalid has been sprayed for controlling BSDC on bananas since their introduction in 2014, we hypothesized that the selection pressure due to fungicide spraying selected for resistance in these highly adaptable pathogens populations (BRITO *et al.*, 2020; GOMES *et al.*, 2018; MANZO-SÁNCHEZ *et al.*, 2019). Because fluxapyroxad has not been labeled for the management of SDC yet, it is also important to check for cross resistance as this can inform resistance management strategies.

Because we could not find SDHI-sensitive *Mf* isolates in our sampling, we compared the resistant isolates with the sensitive *Mm* isolate. According to this comparison, the *Mf* isolates were about 65 and 90 times less sensitive than the sensitive *Mm* isolate ISC92. This contrasts with data from the current literature that reported only a few SDHI-insensitive *Mf* strains in banana plantations from Colombia, Costa Rica, Ecuador, and Guatemala, in areas where boscalid, fluopyram, fluxapyroxad, and/or isopyrazam were sprayed from 2012 to 2017 (FRAC, 2022).

Comparing the among populations' resistance factor (RF) for boscalid, which expresses how proportionally high the EC_{50} of the resistant population is in comparison with the sensitive population (highest EC_{50} over the lowest), the insensitivity levels amongst both *Mm* and *Mf* isolates ranged from 1.72 to $> 100 \mu\text{g mL}^{-1}$, with an average resistance factor (RF) of 50.1. In contrast, for fluxapyroxad, these insensitivity values ranged from 0.002 to $100 \mu\text{g mL}^{-1}$, resulting in a $RF = 5,000$, which reflects the fact that fluxapyroxad is intrinsically more active than boscalid.

Using other pathogens as comparison, similar insensitivity levels to boscalid was also observed for isolates of *B. cinerea* from strawberries that stood fungicide concentrations up to $75 \mu\text{g mL}^{-1}$ (HU; FERNÁNDEZ-ORTUÑO; SCHNABEL, 2016) and $RF > 1.612$ (AMIRI; ZUNIGA; PERES, 2020). In *Z. tritici* from wheat, boscalid RF ranged from 0.2 to 10-fold in field strains and to more than 64-fold in laboratory mutants (YAMASHITA; FRAAIJE, 2018). As another example, populations of the wheat blast pathogen *Pyricularia oryzae* Triticum lineage were predominantly resistant to fluxapyroxad, with $RF > 43$ and EC_{50} values ranging from 1.15 (sensitive strains) to $> 50 \mu\text{g mL}^{-1}$ (highly resistant) (VICENTINI *et al.*, 2022a).

Our EC₅₀ values are relatively high in comparison with other fungi but this can be due that mycelium fragments are tested rather than spores, with germination of spores more dependent on energy production from mitochondrial respiration. In addition, the *in vitro* fungicide sensitivity might be less than expected for SDC control in the field, but the EC₅₀ values are still important as baseline sensitivity data to study the adaption of pathogen populations to fungicides in the field over time.

Since resistance to SDHI fungicides was detected in the *Mm* and *Mf* populations, we also investigated the association between the levels of resistance with site-specific mutations in the target genes *SdhB*, *SdhC*, and *SdhD* of the pathogens (SIEROTZKI *et al.*, 2000). In fact, the second important observation of this study is the report of point mutations in the *SdhB* and *SdhD* genes of *M. musicola* that may be associated with resistance to SDHI fungicides.

Most studies on resistance mechanisms to SDHI fungicide in plant pathogens have found target site alterations as the underlying cause (PENG *et al.*, 2021). In our study, three *Sdh* target site alterations were identified in the SDC populations after comparing isolate *Sdh* sequences with those of the reference sequences. All *Mf* isolates, carried *SdhC* N52D, but C-N52D was also detected in all *Mn* isolates, including the SDHI sensitive isolate ISC92 and this part of the *SdhC* is not likely part of the SDHI binding pocket [Scalliet *et al.*]. Variation in the wild-type *SdhC* sequence can also occur among species as shown for *Z. tritici* and the reference sequence for *Mf* originates from an isolate, CIRAD86, from Cameroon. Isolate ISC92 was the only *Mm* isolate that also carried *SdhD* N66K. Comparisons with other fungi show that asparagine at this position is conserved, including *Mf*, but not likely directly involved in SDHI binding (SCALLIET *et al.*, 2012). The third alteration, *SdhB* E196Q, was only found in one isolate, ISR61, having the highest EC₅₀ value for boscalid and fluxapyroxad. Glutamic acid at this position of *SdhB* is well conserved, but not likely directly involved in SDHI binding. We highlight that, to our knowledge, none of these substitutions has been associated with resistance to SDHI fungicides in the SDC pathogens according to the most recent survey conducted by the SDHI Working Group in 2022 (FRAC – FUNGICIDE RESISTANCE ACTION COMMITTEE, 2022). Since this isolate *Mm* ISR61 was sampled from a banana plantation with no fungicide use (SPNW-O), it is plausible that it has been introduced in the area by gene flow from banana farms with history of fungicide use (GOMES *et al.*, 2018).

Point mutations at the target genes leading to protein structural changes due to amino acid substitution in the succinate dehydrogenase has been, in fact, the most common mechanism associated with SDHI resistance in many fungal plant pathogens including: A.

alternata (SdhB-H277Y/R, SdhC-H134R, SdhD-D123E, SdhD-H133R), *B. cinerea* (SdhB-P225L/T/F, SdhB-H272Y/R/L/V, SdhB-N230I, SdhD-H132R), *P. teres* on barley (SdhB-H277Y, SdhC-N75S, SdhC-G79R, SdhC-H134R, SdhC-S135R, SdhD-D124N/E, SdhD-H134R, SdhD-D145G) and *Z. tritici* on wheat (SdhB-N225T, SdhB-R265P, SdhB-T268I, SdhC-T79N/I, SdhC-W80S, SdhC-N86A/S, SdhC-R151M/R/S/T, SdhC H152R) (AVENOT *et al.*, 2008; AVENOT; SELLAM; MICHAILIDES, 2009; DE MICCOLIS ANGELINI *et al.*, 2010, 2014; FRAC – FUNGICIDE RESISTANCE ACTION COMMITTEE, 2022; MIYAMOTO *et al.*, 2009, 2010; SANG; LEE, 2020, 2020; STAMMLER *et al.*, 2011, 2014; VELOUKAS *et al.*, 2011; YIN; KIM; XIAO, 2011).

However, other resistance mechanisms can occur as well, such as overexpression of target genes and the multidrug efflux pumps (FERNÁNDEZ-ORTUÑO *et al.*, 2008; FRAAIJE *et al.*, 2019; PENG *et al.*, 2021; PRASAD; RAWAL, 2014; YAMASHITA; FRAAIJE, 2018). For instance, in *Z. tritici*, strains without target site alterations in the *SdhB*, *C*, or *D* genes were resistant by an efflux pump mechanism and had a 519-bp insert in the *MgMFS1* promote (OMRANE *et al.*, 2015; YAMASHITA; FRAAIJE, 2018). In our study, out of the 63 *Mm* and *Mf* isolates with resistance to SDHI, approximately 77% (N = 49) were also insensitive to the DMIs propiconazole and tebuconazole (OLIVEIRA *et al.*, 2022) and A. G. da Silva, personal information; data not shown). A total of 18.5% of the isolates sampled from these pathogens' populations were also resistant to QoI fungicides (OLIVEIRA *et al.*, 2022). It is plausible that multidrug resistance (MDR) associated with efflux pump mechanisms has emerged in populations of *Mm* and *Mf*, as theoretically predicted and/or reported for other fungal systems (FISHER *et al.*, 2018; HAWKINS; FRAAIJE, 2018; OMRANE *et al.*, 2015; VICENTINI *et al.*, 2022a). In fact, our efflux pump inhibition assay indicated that verapamil and, to a lesser extent, BLT-4, (could lower the sensitivity of *Mf* to fluxapyroxad at least 3-fold (Figure 5). This was an indication that resistance to fluxapyroxad in *Mf*, and likely also in the closely related *Mm*, can pre-exist in field populations due to adaptation to selection pressure by fungicides belonging to different modes of actions due to overexpression of efflux pumps as reported also for *B. cinerea* (VICENTINI *et al.*, 2022b), *P. oryzae* *Triticum* lineage (VICENTINI *et al.*, 2022) and *Z. tritici* (OMRANE *et al.*, 2015; YAMASHITA; FRAAIJE, 2018).

The elucidation of fungicide resistance mechanisms to multiple fungicides in populations of *Mm* and *Mf* from Southeastern Brazil is highly relevant for the decision-making process on fungicide spraying. The decision on fungicide spraying should now consider the

adoption of anti-resistance strategies for SDC management based on integrated disease management practices, since these three groups of fungicide actives (DMIs, QoIs and SDHIs) can already have partly lost their field efficacy due to development of MDR. These anti-resistance strategies could include, for instance, frequent scouting for fungicide resistance in local banana fields to monitor the prevalence and spread of QoI, DMI and SDHI resistant strains of *Mf* and *Mm* based on direct monitoring of airborne inoculum and the prevalence of fungicide resistance alleles, target and non-target site resistance, in aerosol samples of spore (VICENTINI *et al.* 2023^a; VICENTINI *et al.* 2022c). Early detection of airborne spore levels of the pathogens could prevent major crop yield losses by allowing the adoption of timely and appropriate disease management practices.

The current labeling of other SDHI actives for the management of SDC on banana plantations in Brazil, and fluxapyroxad in particular, should be pursued cautiously and only in co-formulations with protectant multisite, low-risk fungicides, since resistance has been detected in *Mf* and *Mm* populations even before the labeling and deployment of this fungicide in banana fields.

5 CONCLUSIONS

In vitro SDHI susceptibility testing of *Mf* and *Mm* populations sampled from banana plantations in different regions from Southeastern Brazil revealed that resistance is omnipresent. Further research is needed to translate the results from the *in vitro* tests into efficacy loss of practical disease control. No target site mutations in *Sdh* genes were associated with reduced sensitivity but MDR was detected as addition of efflux pump inhibitor in the *in vitro* tests increased the sensitivity to the SDDI fluxapyroxad. Further monitoring for *Sdh* target mutations is important, but other resistance mechanisms such as presence of multiple *Sdh* paralogs cannot be ruled out. These results highlights the importance of implementing sound anti resistance management strategies when SDHI fungicides are deployed for management of SDC.

6 PUBLICATION ADDITIONAL INFORMATION

6.1 Author Contributions: Conceptualization, P.C.C. and S.I.M.; methodology, T.C.S., P.C.C.; software, P.C.C.; validation, T.C.S. and F.S.C.J.; formal analysis, T.C.S. and P.C.C.; investigation, T.C.S., S.I.M., F.S.C.J.; M.C.G.G., B.A.F.; resources, P.C.C.; data curation, T.C.S. and P.C.C.; writing— T.C.S., S.I.M. and P.C.C.; writing—review and editing, T.C.S., S.I.M., B.A.F., M.C.G.G., P.C.C.; visualization, M.C.G.G., P.C.C.; supervision, P.C.C., S.I.M.; project administration, P.C.C.; funding acquisition, P.C.C., B.A.F. All authors have read and agreed to the published version of the manuscript.

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6.3 Data Availability Statement: The *sdhB*, *C* and *D* experimental sequence data from *Mf* and *Mm* populations sampled in Southeastern Brazil and that supports the findings of allelic variation in the target genes for SDHI sensitivity will be available at the GenBank/NCBI database. Upon publication, the phenotypic data presented in thi study will be publicly available at the Mendeley Data repository.

6.4 Informed Consent Statement: The Brazilian Ministry of Environment/National System for the Management of Genetic Heritage and Associated Traditional Knowledge—SisGen, issued the Certificates # A64D0EA and A100786 authorizing the scientific activities associated with the collection of botanical and fungal material from the banana agroecosystems

in the Cerrado's and Atlantic Forest's biomes and access to the genetic diversity of *Mycosphaerella* species.

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

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