

RESSALVA

Atendendo a solicitação do autor, o
arquivo completo desta tese só será
disponibilizado somente a partir de
17/03/2025.

**UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE ENGENHARIA
CÂMPUS DE ILHA SOLTEIRA**

TATIANE CARLA SILVA

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

Ilha Solteira
2023

PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

TATIANE CARLA SILVA

**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
Ilha Solteira – UNESP como parte dos requisitos
para obtenção do título de Doutorado em
Agronomia.

Paulo César Ceresini
Orientador

Silvino Intra Moreira
Coorientador

FICHA CATALOGRÁFICA
Desenvolvido pelo Serviço Técnico de Biblioteca e Documentação

S586p Silva, Tatiane Carla.
Prevalence of resistance to SDHI fungicides in populations of the Black and Yellow Sigatoka pathogens from bananas in southeastern Brazil / Tatiane Carla Silva. -- Ilha Solteira: [s.n.], 2023
87 f. : il.

Tese (doutorado) - Universidade Estadual Paulista. Faculdade de Engenharia de Ilha Solteira. Especialidade: Sistemas de Produção, 2023

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

1. Fungal foliar pathogens. 2. Fenotipagem de resistência antimicrobiana. 3. Anti-microbial resistance phenotyping. 4. Fungicide resistance. 5. Ec50. 6. Resazurin.


Raiane da Silva Santos

Supervisora Técnica de Seção
Seção Técnica de Referência, Atendimento ao usuário e Documentação
Diretoria Técnica de Biblioteca e Documentação
CIB/8 - 9999



UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Ilha Solteira

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Prevalence of resistance to SDHI fungicides in populations of the Black and Yellow Sigatoka pathogens from bananas in Southeastern Brazil

AUTORA: TATIANE CARLA SILVA

ORIENTADOR: PAULO CEZAR CERESINI

COORIENTADOR: SILVINO INTRA MOREIRA

Aprovada como parte das exigências para obtenção do Título de Doutora em AGRONOMIA, área:
Sistemas de Produção pela Comissão Examinadora:

Documento assinado digitalmente
 PAULO CEZAR CERESINI
Data: 17/03/2023 17:51:58-0300
Verifique em <https://validar.itl.gov.br>

Prof. Dr. PAULO CEZAR CERESINI (Participação Presencial)
Departamento de Fitossanidade, Engenharia Rural e Solos / Faculdade de Engenharia de Ilha Solteira - UNESP

Profª. Drª. MARIA CÂNDIDA DE GODOY GASPAROTO (Participação Remota)  MARIA CANDIDA DE GODOY GASPAROTO
Departamento de Engenharia Agrônômica / Cursos de Engenharia Agrônômica e Engenharia de Registro
Data: 17/03/2023 17:45:25-0300
Verifique em <https://validar.itl.gov.br>

Prof. Dr. SAMI JORGE MICHEREFF (Participação Remota)  SAMI JORGE MICHEREFF
Centro de Ciências Agrárias e da Biodiversidade / Universidade Federal do Cariri - I
Data: 17/03/2023 17:29:45-0300
Verifique em <https://validar.itl.gov.br>

Prof. Dr. EDSON AMPELIO POZZA (Participação Remota)  EDSON AMPELIO POZZA
Departamento de fitopatologia / Universidade Federal de Lavras - UFLA
Data: 17/03/2023 17:36:14-0300
Verifique em <https://validar.itl.gov.br>

Pesquisador Dr. LUADIR GASPAROTTO (Participação Presencial)  LUADIR GASPAROTTO
Laboratório de Fitopatologia / EMBRAPA, Amazônia Ocidental, Manaus, AM
Data: 17/03/2023 17:49:54-0300
Verifique em <https://validar.itl.gov.br>

Ilha Solteira, 17 de março de 2023

DEDICATÓRIA

Esta tese é dedicada a todos os meus professores, desde a minha alfabetização. Vocês são portais do conhecimento e os pilares da educação.

Dedico!

AGRADECIMENTOS

Agradeço a Deus e Nossa Senhora Aparecida, por me proteger durante toda essa caminhada.

Aos meus pais, por todo exemplo, apoio e carinho durante todos esses anos. Quero deixar claro que a base para essa realização foram vocês. Eu jamais chegaria aqui sem o apoio desse casal, que não tiveram oportunidade de estudar em suas vidas, mas sempre me entusiasmou a estudar e trabalhar muito. Meu pai levanta todos os dias entre 3 e 4h da manhã para trabalhar, sempre será o meu maior exemplo de esforço. Minha mãe, todos os dias pela manhã, conversa com suas plantinhas. É o meu maior exemplo de simplicidade. Eu prometo que vocês ainda irão se orgulhar de mim.

Às minhas irmãs, Elaine, Eliane e Tuane (*in memorian*), cunhados (Rogério e Jonas) e sobrinhos (Daniel, Gabriel, Alik, Samuel, Lorenzo e Manuela) por todo amor nos reencontros, quando volto pra casa.

Ao meu esposo Rafael e meu filho Gael, pela paciência pelos momentos que tive que estar ausente.

Agradeço aos parentes e amigos, que torcem por mim. Em especial, minha amiga Shirlei.

Agradeço aos amigos verdadeiros que fiz em Ilha Solteira: Tamiris, Karina, Mairê, Biscoito, Fabíola, Bruna, Mel, Imara, Janderson, Lídia, Maila, Gláucia e Katy.

Agradeço ao professor Dr. Paulo César Ceresini, pela orientação, amizade e por me dar liberdade de expressar as minhas ideias e posicionamento no decorrer desse trabalho.

Agradeço aos colegas de laboratório e todos os colaboradores envolvidos nesse trabalho.

Agradeço ao presidente Luiz Inácio Lula da Silva, por apoiar os programas de distribuição de renda e educação, que fez com que pessoas como eu, que saí de uma pequena cidade no interior de Minas Gerais pudesse sonhar mais alto e se tornar doutora.

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

“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.

LIST OF FIGURES

CHAPTER 1. AN ACCURATE, AFFORDABLE, AND PRECISE RESAZURIN-BASED DIGITAL IMAGING COLORIMETRIC ASSAY FOR THE ASSESSMENT OF FUNGICIDE SENSITIVITY STATUS OF FUNGAL POPULATIONS	20
---	----

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 PoI 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. 28

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. 29

Figure 3 - Oxidation-reduction gradient obtained with the mixture of the reduced and oxidized forms of the resazurin solution at the final concentration of $40 \mu\text{M}$. * 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 $\cdot \text{mL}^{-1}$ 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). 30

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*. 34

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 $\alpha \leq 0.05$ 40

Figure 6 -Simple linear regression models predicting the correspondence between the log EC₅₀ or fungus relative growth estimates from the spectrophotometric assay at 569 nm (SA = SPEC-assay) on the basis of log EC₅₀ 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..... 41

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..... 42

CHAPTER 2. RESISTANCE TO SDHI FUNGICIDES IS PERVASIVE IN POPULATIONS OF THE BLACK AND YELLOW SIGATOKA PATHOGENS IN BANANA PLANTATIONS FROM SOUTHEASTERN BRAZIL..... 55

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). 64

Figure 2 - Estimates of the isolates of *M. fijiensis* and *M. musicola* EC₅₀ 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$68

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).....69

There were significant differences in the mean EC₅₀ 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 EC₅₀ 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 EC₅₀ values than the *Mm* isolates (Figure 3B).69

Figure 3 - A. Contrast between groups of isolates of *Mycosphaerella* species per geographical population of the pathogen, according to EC₅₀ of boscalid or fluxapyroxad. B. Contrast between groups of isolates of *Mm* and *Mf* per species, according to EC₅₀ 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$70

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.....72

.....72

Figure 5 - Contrast among protein variant groups of isolates of *Mycosphaerella fijiensis* and *M. musicola* according to the EC₅₀ 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 $p \leq 0.05$ 73

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. 74

LIST OF TABLES

CHAPTER 1. AN ACCURATE, AFFORDABLE, AND PRECISE RESAZURIN-BASED DIGITAL IMAGING COLORIMETRIC ASSAY FOR THE ASSESSMENT OF FUNGICIDE SENSITIVITY STATUS OF FUNGAL POPULATIONS.....20

Table 1 - *Mycosphaerella* and *Pyricularia* isolates selected for this study, their fungicide resistance categories, and doses of QoI, DMI, and SDHI fungicides tested..... 25

CHAPTER 2. RESISTANCE TO SDHI FUNGICIDES IS PERVASIVE IN POPULATIONS OF THE BLACK AND YELLOW SIGATOKA PATHOGENS IN BANANA PLANTATIONS FROM SOUTHEASTERN BRAZIL.....55

Table 1 - Specific primers designed for this study for PCR amplifications and for assessment of variation at the *Sdh*'s genes in *Mycosphaerella fijiensis* and *Mycosphaerella musicola*.....66

TABLE OF CONTENTS

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.....	18
RESUMO.....	18
CHAPTER 1. AN ACCURATE, AFFORDABLE, AND PRECISE RESAZURIN-BASED DIGITAL IMAGING COLORIMETRIC ASSAY FOR THE ASSESSMENT OF FUNGICIDE SENSITIVITY STATUS OF FUNGAL POPULATIONS.....	20
ABSTRACT	20
Keywords:	20
2 MATERIALS AND METHODS	24
2.1 Fungal Strains	24
2.2 Fungal Inoculum, Microplate Cultures Preparation, and Incubation Conditions for Fungicide Sensitivity Testing	26
2.3 Spectrophotometric Assay (SPEC-Assay) for Fungicide Sensitivity Testing Based on Measuring the Reduction of Resazurin, a Metabolic Indicator for Fungal Respiration Activity	26
2.3 Resazurin-Based Colorimetric Assays (COL-Assay) for Fungicide Sensitivity Testing Using a Handmade Trans-Illuminator with Attached Mobile Phone Digital Cameras	27
2.4 Resazurin Oxidation-Reduction Gradient Testing for Substantiating the Assays	29
2.5 Accuracy and Precision of the Resazurin-Based Colorimetric Assay (COL-Assay) Based on a Handmade Trans-Illuminator for Fungicide Sensitivity Testing	31
2.6 Statistical Analysis.....	32
3 RESULTS.....	34
3.1 Resazurin Oxidation-Reduction Gradient Testing for Substantiating the Assays	34
3.2 Accuracy and Precision of the Resazurin-Based Colorimetric Assay (COL-Assay) on a Handmade Trans-Illuminator for Fungicide Sensitivity Testing	34
4 DISCUSSION	37
5 CONCLUSIONS	45
6 PUBLICATION ADDITIONAL INFORMATION.....	46
6.1 Citation:	46
6.2 Author Contributions:	46

6.3	Funding:	46
6.4	Data Availability Statement:	47
6.5	Informed Consent Statement	47
6.6	Conflicts of Interest	47
6.7	Appendix A. Reagents and media for resazurin-based fungicide sensitivity assays	47
7	REFERENCES.....	49
 CAPÍTULO 2. A RESISTÊNCIA A FUNGICIDAS SDHI É GENERALIZADA EM POPULAÇÕES DE PATÓGENOS DA SIGATOKA NEGRA E AMARELA EM PLANTACÕES DE BANANA NO SUDESTE DO BRASIL		
	RESUMO.....	54
	Palavras-chave:	54
 CHAPTER 2. RESISTANCE TO SDHI FUNGICIDES IS PERVASIVE IN POPULATIONS OF THE BLACK AND YELLOW SIGATOKA PATHOGENS IN BANANA PLANTATIONS FROM SOUTHEASTERN BRAZIL.....		
	ABSTRACT	55
	Keywords:	55
1	INTRODUCTION.....	56
2	MATERIALS AND METHODS	60
2.1	Population sampling area for the black and yellow Sigatoka pathogens.....	60
2.2	Isolation of Mm and Mf.....	60
2.3	Preparation of inoculum for SDHI fungicide sensitivity testing	61
2.4	SDHI fungicide sensitivity testing	61
2.5	Analysis of allelic variation in the SDHI target protein encoding genes SdhB, SdhC, and SdhD in Mm and Mf	64
2.6	Efflux pump inhibition assay	66
3	RESULTS.....	68
3.1	SDHI fungicides sensitivity testing	68
3.2	Detection of mutations in the Sdh genes	70
3.1.1	Mutations in the <i>SdhB</i> , <i>SdhC</i> and <i>SdhD</i> genes from <i>Mm</i>	71
3.2	Efflux pump inhibition assay	73
4	DISCUSSION	75
5	CONCLUSIONS	79
6	PUBLICATION ADDITIONAL INFORMATION.....	80

6.1	Author Contributions:	80
6.2	Funding:	80
6.3	Data Availability Statement:	80
6.4	Informed Consent Statement:	80
6.5	Conflicts of Interest	81
7	REFERENCES.....	82

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.

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.

6.2 Funding: This research was funded by FAPESP (São Paulo Research Foundation, Brazil), Grant/Award Numbers: 2017/50456-1, 2018/21197-0, 2019/12509-1, 2020/07611-9, and 2021/03402-9; CNPq (Brazilian National Council for Scientific and Technological Development), Grant/Award Number: Pq-1D 313825/2018-1; CAPES (Coordination for the Improvement of Higher Level Personnel), Grant/Award Numbers: CAPES/Pro-equipment Program 775202/2012, CAPES/AUXPE Program 88881.593505/2020-01—UNESP Ilha Solteira Campus, CAPES PrInt Program/UNESP, CAPES studentship Program 001); Newton Fund /BBSRC (Biotechnology and Biological Sciences Research Council, United Kingdom), Grant/Award Number: BB/S018867/2 awarded by BBSRC under the BBSRC-FAPESP Antimicrobial Resistance and Insecticide Pest Resistance in Livestock and Agriculture Program. The article processing charges were supported in full by São Paulo State University's Vice-Presidency for Research (PROPE) special grant for publication (Public Call 10/2022).

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.

REFERENCES

- AMIRI, A.; ZUNIGA, A. I.; PERES, N. A. Mutations in the membrane-anchored SdhC subunit affect fitness and sensitivity to succinate dehydrogenase inhibitors in *Botrytis cinerea* populations from multiple hosts. **Phytopathology®**, Ithaca, v. 110, n. 2, p. 327–335, 2020.
- ARZANLOU, M. *et al.* Molecular diagnostics for the sigatoka disease complex of banana. **Phytopathology**, Ithaca, v. 97, n. 9, p. 1112–1118, set. 2007.
- AVENOT, H. F. *et al.* Characterization of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. **Phytopathology®**, Ithaca, v. 98, n. 6, p. 736–742, jun. 2008.
- AVENOT, H.; SELLAM, A.; MICHAILIDES, T. Characterization of mutations in the membrane-anchored subunits AaSDHC and AaSDHD of succinate dehydrogenase from *Alternaria alternata* isolates conferring field resistance to the fungicide boscalid. **Plant Pathology**, Oxford, v. 58, n. 6, p. 1134–1143, dez. 2009.
- AYER, K. M. *et al.* Characterization of the *VisdhC* and *VisdhD* genes in *Venturia inaequalis*, and sensitivity to fluxapyroxad, pydiflumetofen, inpyrfluxam, and benzovindiflupyr. **Plant Disease**, St. Paul, v. 103, n. 6, p. 1092–1100, 2019.
- BELTRÁN-GARCÍA, M. J. *et al.* Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen *Mycosphaerella fijiensis* in black Sigatoka disease of bananas. **PLOS ONE**, San Francisco, v. 9, n. 3, p. e91616, 2014.
- BENDINI, H. N. **Processamento digital de imagens para inferência de risco de doença fúngica da bananicultura**. Master's Thesis—São Carlos, SP, Brazil: Federal University of São Carlos (UFSCar), 2012.
- BENDINI, H. N. *et al.* Análise de risco da ocorrência de Sigatoka-negra baseada em modelos polinomiais: um estudo de caso. **Tropical Plant Pathology**, Viçosa, v. 38, n. 1, p. 35–43, 2013.
- BRENT, K. J. **Fungicide resistance in crop pathogens: how can it be managed?** Brussels: GIFAP, 1995.
- BRITO, F. S. D. *et al.* Genetic diversity and azole fungicide sensitivity in *Pseudocercospora musae* field populations in Brazil. **Frontiers in Microbiology**, Lausanne, v. 11, 2020.
- BRITO, F. S. D.; FRAAIJE, B.; MILLER, R. N. G. Sigatoka disease complex of banana in Brazil: Management practices and future directions. **Outlooks on pest management**, Buckinghamshire, v. 26, n. 2, p. 78–81, 2015.
- BURT, P. J. A. Windborne dispersal of sigatoka leaf spot pathogens. **Grana**, London, v. 33, n. 2, p. 108–111, 1 abr. 1994.
- CAÑAS-GUTIÉRREZ, G. P. *et al.* Analysis of the *CYP51* gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. **Pest Management Science**, West Sussex, v. 65, n. 8, p. 892–899, 2009.
- CHURCHILL, A. C. L. *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the

challenges of control: *M. fijiensis*, BLS pathogen of banana. **Molecular Plant Pathology**, Oxford, v. 12, n. 4, p. 307–328, maio 2011.

CORDEIRO, Z. J. M. Doenças. Em: ALVES, É. J. (Ed.). **A cultura da banana: Aspectos técnicos, sócio econômicos e agroindustriais**. Cruz das Almas, Bahia, Brazil: Embrapa SPI, CNPMF, 1997. p. 353–408.

CORKLEY, I.; FRAAIJE, B.; HAWKINS, N. Fungicide resistance management: Maximizing the effective life of plant protection products. **Plant Pathology**, Oxford, v. 71, n. 1, p. 150–169, 2022.

DE MICCOLIS ANGELINI, R. M. *et al.* Selection, characterization and genetic analysis of laboratory mutants of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to the fungicide boscalid. **European Journal of Plant Pathology**, London, v. 128, n. 2, p. 185–199, out. 2010.

DE MICCOLIS ANGELINI, R. M. *et al.* Molecular characterisation and detection of resistance to succinate dehydrogenase inhibitor fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*). **Pest Management Science**, West Sussex, v. 70, n. 12, p. 1884–1893, 2014.

DIAZ-TRUJILLO, C. *et al.* A new mechanism for reduced sensitivity to demethylation-inhibitor fungicides in the fungal banana black Sigatoka pathogen *Pseudocercospora fijiensis*. **Molecular Plant Pathology**, Oxford, v. 19, n. 6, p. 1491–1503, 2018.

FERNÁNDEZ-ORTUÑO, D. *et al.* Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. **International Microbiology: The Official Journal of the Spanish Society for Microbiology**, Barcelona, v. 11, n. 1, p. 1–9, 2008.

FERNÁNDEZ-ORTUÑO, D. *et al.* Resistance to the SDHI fungicides boscalid, fluopyram, fluxapyroxad, and penthiopyrad in *Botrytis cinerea* from commercial strawberry fields in Spain. **Plant Disease**, St. Paul, v. 101, n. 7, p. 1306–1313, 2017.

FISHER, M. C. *et al.* Worldwide emergence of resistance to antifungal drugs challenges human health and food security. **Science**, New York, v. 360, n. 6390, p. 739–742, 18 maio 2018.

FÖRSTER, H. *et al.* Mutations in *Sdh* gene subunits confer different cross-resistance patterns to SDHI fungicides in *Alternaria alternata* causing Alternaria leaf spot of almond in California. **Plant Disease**, St. Paul, v. 106, n. 7, p. 1911–1918, 2022.

FRAAIJE, B. *et al.* **Evolution and spread of SDHI-resistant alleles within field populations of *Zymoseptoria tritici* in the UK**. Plant Health 2019. **Anais...** Em: APS ANNUAL MEETING. Cleveland, OH: APS The American Phytopathological Society, 2019. Disponível em: <<https://www.apsnet.org/meetings/annual/meetingarchives/planthealth2019/Documents/Abstracts/aps2019ab709.htm>>. Acesso em: 22 fev. 2023

FRAC. **Recommendations for Bananas**. Disponível em: <<https://www.frac.info/frac-teams/working-groups/banana-group/recommendations-for-bananas>>. Acesso em: 17 jan. 2023.

FRAC – FUNGICIDE RESISTANCE ACTION COMMITTEE. **Recommendations for SDHI fungicides**. Disponível em: <<https://www.frac.info/frac-teams/working-groups/sdhi-fungicides/recommendations-for-sdhi>>. Acesso em: 22 fev. 2023.

GASPAROTTO, L. *et al.* Controle químico da Sigatoka negra da bananeira. I - trifloxistrobin, propiconazole e difenoconazole. **Comunicado Técnico**, v. 7, p. 1, 2000.

GHINI, R. *et al.* Análise de risco das mudanças climáticas globais sobre a sigatoka-negra da bananeira no Brasil. **Fitopatologia Brasileira**, Viçosa, v. 32, n. 3, p. 197–204, 2007.

GOMES, L. I. S. *et al.* *Mycosphaerella musicola* identified as the only pathogen of the Sigatoka disease complex present in Minas Gerais state, Brazil. **Plant Disease**, St. Paul, v. 97, n. 12, p. 1537–1543, 2013.

GOMES, L. I. S. *et al.* Yellow sigatoka epidemics caused by a panmictic population of *Mycosphaerella musicola* in Brazil. **Plant Pathology**, Oxford, v. 67, n. 2, p. 295–302, 2018.

GRICE, K. *et al.* Yellow Sigatoka (*Mycosphaerella musicola*) populations develop resistance to QoI fungicides in Australia. Em: DEHNE, H.-W. *et al.* (Eds.). **Modern Fungicides and Antifungal Compounds**. Spectrum Phytomedizin. Braunschweig: Deutsche Phytomedizinische Gesellschaft, 2013. v. 7p. 269–270.

HAWKINS, N. J.; FRAAIJE, B. A. Fitness Penalties in the Evolution of Fungicide Resistance. **Annual Review of Phytopathology**, Palo Alto, v. 56, p. 339–360, 25 ago. 2018.

HE, L. *et al.* Activity of the novel succinate dehydrogenase inhibitor fungicide pydiflumetofen against SDHI-sensitive and SDHI-resistant isolates of *Botrytis cinerea* and efficacy against gray mold. **Plant Disease**, St. Paul, v. 104, n. 8, p. 2168–2173, 2020.

HU, M.-J.; FERNÁNDEZ-ORTUÑO, D.; SCHNABEL, G. Monitoring resistance to SDHI fungicides in *Botrytis cinerea* from strawberry fields. **Plant Disease**, St. Paul, v. 100, n. 5, p. 959–965, 2016.

KLAPPACH, K.; STAMMLER, G. Resistance of plant pathogens to succinate dehydrogenase inhibitor (SDHI) fungicides (FRAC Code 7). Em: KLAPPACH, K.; STAMMLER, G. (Eds.). **Fungicide Resistance in North America**. Mycology. 2. ed. Saint Paul, MN: APS The American Phytopathological Society, 2019. p. 85–95.

KRETSCHMER, M. *et al.* Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. **PLoS Pathogens**, San Francisco v. 5, n. 12, p. e1000696, 2009.

LEE, J. *et al.* Field assessment of six point-mutations in Sdh subunit genes conferring varying resistance levels to SDHIs in *Clavireedia* spp. **Plant Disease**, St. Paul, v. 105, n. 6, p. 1685–1691, 2021.

LI, X. *et al.* Resistance to pydiflumetofen in *Botrytis cinerea*: risk assessment and detection of point mutations in sdh genes that confer resistance. **Pest Management Science**, West Sussex, v. 78, n. 4, p. 1448–1456, abr. 2022.

LUCAS, J. A.; HAWKINS, N. J.; FRAAIJE, B. A. The evolution of fungicide resistance. Em: **Advances in Applied Microbiology**, New York, [s.l.] Elsevier, 2015. v. 90p. 29–92.

MA, B.; UDDIN, W.; OLAYA, G. Baseline and non-baseline sensitivity of Magnaporthe oryzae isolates from perennial ryegrass to azoxystrobin in the northeastern United States. **Canadian Journal of Plant Pathology**, [s.l.] v. 31, n. 1, p. 57–64, 1 mar. 2009.

MAIR, W. *et al.* Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. **Pest Management Science**, West Sussex, v. 72, n. 8, p. 1449–1459, ago. 2016.

MALIMPENSA, J. R. **Caracterização da resistência a fungicidas em populações de fungos associados a lesões de Sigatokas em bananais do Vale do Ribeira (SP)**. [s.l.] Instituto Biológico, 2018.

MANZO-SÁNCHEZ, G. *et al.* Genetic variability of *Pseudocercospora fijiensis*, the black Sigatoka pathogen of banana (*Musa* spp.) in Mexico. **Plant Pathology**, Oxford, v. 68, n. 3, p. 513–522, 2019.

MAPA – MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO - BRAZIL. **Agrofit - Sistemas de Agrotóxicos Fitossanitários, Coordenação Geral de Agrotóxicos e Afins**. Disponível em: <http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons>. Acesso em: 5 fev. 2023.

MCDONALD, B. A.; LINDE, C. Pathogen population genetics, evolutionary potential, and durable resistance. **Annual Review of Phytopathology**, Palo Alto, v. 40, n. 1, p. 349–379, 2002.

MENDOZA, M. J.; ARDALES, E. Population Structure of the Banana Black Sigatoka Pathogen [*Pseudocercospora fijiensis* (M. Morelet) Deighton] in Luzon Philippines. **Philippine Agricultural Scientist**, Laguna, v. 102, p. 211–219, 1 set. 2019.

MIYAMOTO, T. *et al.* Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. **Plant Pathology**, Oxford, v. 58, n. 6, p. 1144–1151, 2009.

MIYAMOTO, T. *et al.* Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. **Plant Pathology**, Oxford, v. 59, n. 5, p. 873–881, 2010.

MORAES, W. DA S.; NOMURA, E. S. Monitoramento da Sigatoka e controle químico. Em: **Cultivo da bananeira**. Manual Técnico. Campinas, SP, Brazil: Coordenadoria de Assistência Técnica Integral (CATI), 2020. p. 135–144.

MÜLLER, M. A.; STAMMLER, G.; MAY DE MIO, L. L. Multiple resistance to DMI, QoI and SDHI fungicides in field isolates of *Phakopsora pachyrhizi*. **Crop Protection**, Oxford, v. 145, p. 105618, 2021.

NOMURA, E. S. *et al.* Doenças. Em: **Cultivo da bananeira**. Manual Técnico. Campinas, SP, Brazil: Coordenadoria de Assistência Técnica Integral (CATI), 2020. p. 107–134.

OLIVEIRA, T. Y. K. *et al.* Evidence of resistance to QoI fungicides in contemporary populations of *Mycosphaerella fijiensis*, *M. musicola* and *M. thailandica* from banana plantations in southeastern Brazil. **Agronomy**, Basel, v. 12, n. 12, p. 2952, 2022.

OMRANE, S. *et al.* Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. **Environmental Microbiology**, Oxford, v. 17, n. 8, p. 2805–2823, ago. 2015.

ŌMURA, S.; SHIOMI, K. Discovery, chemistry, and chemical biology of microbial products. **Pure and Applied Chemistry**, Oxford, v. 79, n. 4, p. 581–591, 1 jan. 2007.

PENG, J. *et al.* A Method for the Examination of SDHI Fungicide Resistance Mechanisms in Phytopathogenic Fungi Using a Heterologous Expression System in *Sclerotinia sclerotiorum*. **Phytopathology**®, Ithaca, v. 111, n. 5, p. 819–830, maio 2021.

PRASAD, R.; RAWAL, M. K. Efflux pump proteins in antifungal resistance. **Frontiers in Pharmacology**, Lausanne, v. 5, 2014.

R DEVELOPMENT CORE TEAM. **R: A language and environment for statistical computing**. Available at <https://www.R-project.org/>. Disponível em: <<https://www.R-project.org/>>.

RAMOS, J. B. *et al.* First report of black Sigatoka of banana caused by *Mycosphaerella fijiensis* in Bahia, Brazil. **Plant Disease**, St. Paul, v. 102, n. 10, p. 2035–2035, 2018.

RANGEL, A.; PENTEADO, L. A. C.; TONET, R. M. **Cultura da banana**. 2. ed. Campinas, SP, Brazil: Coordenadoria de Assistência Técnica Integral (CATI), 2002.

REHFUS, A. *et al.* Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. **Plant Pathology**, Oxford, v. 67, n. 1, p. 175–180, 2018.

ROCHA, F. S.; CATÃO, H. C. R. M.; MUNIZ, M. F. S. Aspectos diagnósticos entre *Mycosphaerella* spp. da bananeira, distribuição e manejo no Brasil. **Enciclopédia Biosfera**, [s. l.], v. 8, p. 64–84., 2012.

ROCHA, H. S. **Epidemiology of yellow Sigatoka, phenols quantification in banana varieties and phylogenetic analysis of *Mycosphaerella musicola* isolates using microsatellites**. Tese (Doutorado em Agronomia—Lavras, Minas Gerais, Brazil: Universidade Federal de Lavras, 2008.

SANG, H.; LEE, H. B. Molecular Mechanisms of Succinate Dehydrogenase Inhibitor Resistance in Phytopathogenic Fungi. **Research in Plant Disease**, Gangnam-gu, v. 26, n. 1, p. 1–7, 31 mar. 2020.

SANTOS, A. J. Dominância da Sigatoka-negra em bananais do Vale do Ribeira. **Fitopatologia Brasileira**, Viçosa, v. 30, n. Supl., p. 193, 2005.

SCALLIET, G. *et al.* Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen *Mycosphaerella graminicola*. **PloS one**, San Francisco, v.7, n. 4, p. e35429, 2012.

SHI, Y. *et al.* Two adjacent mutations in the conserved domain of SdhB confer various resistance phenotypes to fluopyram in *Corynespora cassiicola*. **Pest Management Science**, West Sussex, v. 77, n. 9, p. 3980–3989, set. 2021.

SIEROTZKI, H. *et al.* Mode of resistance to respiration inhibitors at the *cytochrome bc1* enzyme complex of *Mycosphaerella fijiensis* field isolates. **Pest Management Science**, West Sussex, v. 56, n. 10, p. 833–841, 2000.

SIEROTZKI, H.; SCALLIET, G. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. **Phytopathology**®, Ithaca, v. 103, n. 9, p. 880–887, 2013.

SILVA, T. C. *et al.* An accurate, affordable, and precise resazurin-based digital imaging colorimetric assay for the assessment of fungicide sensitivity status of fungal populations. **Agronomy**, Basel, v. 13, n. 2, p. 343, 25 jan. 2023.

SOUSA, B. C. M. DE *et al.* Bioactivity of Ethanolic Extracts of *Dipteryx punctata* on *Colletotrichum musae*. **Agronomy**, Basel, v. 12, n. 9, p. 2215, set. 2022.

STAMMLER, G. *et al.* Mutations in the target protein conferring resistance to SDHI fungicides. Em: DEHNE, H.-W. *et al.* (Eds.). **Modern fungicides and antifungal compounds**. Spectrum Phytomedizin. Braunschweig: Deutsche Phytomedizinische Gesellschaft, 2011. v. 6p. 195–198.

STAMMLER, G. *et al.* New findings on the development of insensitive isolates of *Pyrenophora teres* towards SDHI fungicides. **Julius-Kühn-Archiv**, Quedlinburg, v. 447, p. 568, 2014.

VARGAS, M. H. **ED50plus v1.0**. Available at: https://archive.org/details/ed50v10_zip. Disponível em: <https://archive.org/details/ed50v10_zip>. Acesso em: 2 mar. 2022.

VELOUKAS, T. *et al.* Detection and molecular characterization of boscalid-resistant *Botrytis cinerea* isolates from strawberry. **Plant Disease**, St. Paul, v. 95, n. 10, p. 1302–1307, 2011.

VELOUKAS, T. *et al.* Fitness and competitive ability of *Botrytis cinerea* field isolates with dual resistance to SDHI and QoI fungicides, associated with several *Sdh* B and the *Cyt* B G143A mutations. **Phytopathology**®, Ithaca, v. 104, n. 4, p. 347–356, 2014.

VICENTINI, S. N. C. *et al.* Monitoring of Brazilian wheat blast field populations reveals resistance to QoI, DMI, and SDHI fungicides. **Plant Pathology**, Oxford, v. 71, n. 2, p. 304–321, 2022a.

VICENTINI, S. N. C. *et al.* Efflux pumps and multidrug-resistance in *Pyricularia oryzae* Triticum lineage. **Agronomy**, Basel, v. 12, n. 9, p. 2068, 2022b.

VICENTINI, S. N. C. *et al.* Can fungal aerobiology tools help tracking fungicide resistance alleles and block the spread of fungicide resistance in the agroecosystem? Em: CANALE, M. C. *et al.* (Eds.). **Proceedings of the VI EpidemioBrasil: Brazilian Workshop of Plant Disease Epidemiology**. Chapecó, SC: Epagri, 2022c. p. 25–26.

VICENTINI, S. N. C. *et al.* Aerobiology of the wheat blast pathogen: inoculum monitoring and detection of fungicide resistant alleles. **Agronomy**, Basel, v. 13, p. (submitted), 2023.

YAMASHITA, M.; FRAAIJE, B. Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. **Pest Management Science**, West Sussex, v. 74, n. 3, p. 672–681, mar. 2018.

YIN, Y. N.; KIM, Y. K.; XIAO, C. L. Molecular characterization of boscalid resistance in field isolates of *Botrytis cinerea* from apple. **Phytopathology**®, Ithaca, v. 101, n. 8, p. 986–995, 2011.