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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
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PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

TATIANE CARLA SILVA

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Tese apresentada à Faculdade de Engenharia de Ilha Solteira – UNESP como parte dos requisitos para obtenção do título de Doutorado em Agronomia.

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

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 Mandela1994).

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 EC_{50} 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 EC_{50} 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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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 (*PoT*l) and *P. oryzae* Oryza lineage (*PoO*l) (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 suitableness 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 *Myscosphaerella* 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.

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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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