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

ABIMAEL GOMES DA SILVA

JOINT PLATFORM FOR MONITORING PATHOGEN INOCULUM DYNAMICS AND DETECTING FUNGICIDE RESISTANCE, ENABLING SMART MANAGEMENT OF THE BANANA SIGATOKA DISEASES COMPLEX

Ilha Solteira - SP
2024

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Tese apresentada à Faculdade de Engenharia de
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Área de Concentração: Sistema de Produção

Orientador: Prof. Dr. Paulo Cézar Ceresini

Coorientador: Dr. Silvino Intra Moreira

Coorientadora: Dra. Tatiane Carla Silva

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Grateful!

Quis custodiet ipsos custodes?

GENERAL ABSTRACT

The objective of the first chapter of this study was to determine the current sensitivity levels of *Mycosphaerella fijiensis* (*Mf*) and *M. musicola* (*Mm*) populations to fungicides DMI triazoles. Additionally, allelic variation in the *CYP51* gene was analyzed in populations of these pathogens to identify and characterize major mutations and mechanisms associated with potential reduced sensitivity or resistance. Populations of *Mf* and *Mm* were sampled from commercial banana plantations in Registro, Vale do Ribeira, SP, Ilha Solteira, Northwestern SP, and Janaúba, Minas Gerais. Isolates of *Mf* and *Mm* obtained were identified morphologically and molecularly. Sensitivity to DMI fungicides: propiconazole and tebuconazole, was determined by calculating the 50% inhibitory concentration of mycelial growth (EC_{50}) based on dose-response curves ranging from 0 to 5 µg mL^{-1}. Variation in sensitivity to fungicides was evident, with predominance of reduced sensitivity and resistance phenotypes. Isolates with sensitive phenotypes were not detected. In Vale do Ribeira, where *Mf* predominated and chemical management with DMI fungicides is considered conventional and intensive, resistance levels were higher. Cross-resistance between propiconazole and tebuconazole fungicides was also evident. Several point mutations in the *CYP51* gene, with significantly distinct phenotypic effects, were found in individuals from populations of *Mf* and *Mm*. In this study, we report for the first time in the country the mutations V106D, A446S, Y136F, Y461H, and Y463D in *Mm*. In the second chapter of this study, the application of an automated system for aerosol spore sampling, associated with real-time quantitative PCR (RT-qPCR), was tested to monitor the aerobiology of *Mf* and *Mm* and to detect mutations associated with resistance to QoI (external quinone inhibitors or strobilurins) fungicides in two banana-producing regions in São Paulo State. Total fungal DNA was extracted from the airborne spore samples and it was subsequently analyzed by qPCR to quantify the fluctuation in the prevalence of *Mf* and *Mm* along the year. Specific probes designed for the target-gene *cytB* were then used to detect the prevalence of the fungicide resistance-allele associated with QoI resistance in the spore samples. The RT-qPCR assay accurately detected resistant *Mf* and *Mm* present in the aerobiology samples. This molecular approach can guide the choice and the optimal timing for fungicide spraying. Furthermore, the developed technology represents a significant advancement in disease management as agricultural innovation.

Keywords: chemical control of plant diseases; aerobiology, biological evolution

RESUMO GERAL

O objetivo do primeiro capítulo desse estudo foi determinar os atuais níveis de sensibilidade das populações de *Mycosphaerella fijiensis* (*Mf)* e *M. musicola* (*Mm)* aos fungicidas triazóis DMI. Além disso, a variação alélica no gene CYP51 foi analisada em populações desses patógenos para identificar e caracterizar as principais mutações e mecanismos associados à potencial redução da sensibilidade ou resistência. Populações de *Mf* e *Mm* foram amostradas em plantações comerciais de banana em Registro, Vale do Ribeira, SP, Ilha Solteira, Noroeste de SP, e Janaúba, Minas Gerais. Os isolados de *Mf* e *Mm* obtidos foram identificados morfologicamente e molecularmente. A sensibilidade aos fungicidas DMI: propiconazole e tebuconazole, foi determinada pelo cálculo da concentração inibitória de 50% do crescimento micelial (EC_{50}) com base em curvas dose-resposta variando de 0 a 5 µg mL-1. Foi evidente a variação na sensibilidade aos fungicidas, com predomínio de fenótipos de sensibilidade reduzida e resistência. Isolados com fenótipos sensíveis não foram detectados. No Vale do Ribeira, onde predomina *Mf* e o manejo químico com fungicidas DMI é considerado convencional e intensivo, os níveis de resistência foram maiores. A resistência cruzada entre os fungicidas propiconazole e tebuconazole também foi evidente. Várias mutações pontuais no gene CYP51, com efeitos fenotípicos significativamente distintos, foram encontradas em indivíduos de populações de *Mf* e *Mm*. Neste estudo, relatamos pela primeira vez no país as mutações V106D, A446S, Y136F, Y461H e Y463D em *Mm*. No segundo capítulo deste estudo foi testado a aplicação de um sistema automatizado para amostragem de esporos em aerossol, associado à PCR quantitativo em tempo real (RT-qPCR), para monitorar a aerobiologia de *Mf* e *Mm* e detectar mutação associada à resistência aos fungicidas QoI (estrobirulinas) em duas regiões produtoras de banana em São Paulo. O DNA fúngico total foi extraído das amostras de esporos em aerossol obtidas e subsequentemente analisadas por RT-qPCR para quantificar a flutuação na prevalência de *Mf* e Mm ao longo do ano. Sondas específicas desenhadas para o gene-alvo *cytB* foram então usadas para detectar a prevalência do alelo associado à

resistência a fungicidas QoI em amostras de esporos em aerossol dessas duas plantações de banana. O ensaio de RT-qPCR detectou com acurácia *Mf* e *Mm* resistentes em amostras de aerobiologia. Essa abordagem molecular poderá orientar a escolha e o momento ideal para a pulverização de fungicidas. Além disso, a tecnologia desenvolvida representa um avanço significativo no manejo de doenças em bananeira como inovação agrícola.

Palavras-chave: controle químico de doenças de plantas; aerobiologia, evolução biológica

SUMÁRIO

CHAPTER 1. RESISTANCE TO TRIAZOLES IN POPULATIONS OF *MYCOSPHAERELLA FIJIENSIS* AND *M. MUSICOLA* FROM THE SIGATOKA DISEASE COMPLEX FROM COMMERCIAL BANANA PLANTATIONS IN MINAS GERAIS AND SÃO PAULO, BRAZIL.

ABSTRACT

The sterol demethylation inhibitors (DMIs) are among the most widely used fungicides for controlling black Sigatoka (*Mycosphaerella fijiensis*, *Mf*) and yellow Sigatoka (*Mycosphaerella musicola*, *Mm*) in banana plantations in Brazil. Black Sigatoka is considered more important due to causing yield losses of up to 100% in commercial banana crops under predisposing conditions. In contrast, yellow Sigatoka is important due to its widespread occurrence in the country. This study aimed to determine the current sensitivity levels of *Mf* and *Mm* populations to fungicides DMI triazoles. Additionally, allelic variation in the *CYP51* gene was analyzed in populations of these pathogens to identify and characterize major mutations and mechanisms associated with potential reduced sensitivity or resistance. Populations of *Mf* and *Mm* were sampled from commercial banana plantations in Registro, Vale do Ribeira, SP, Ilha Solteira, Northwestern SP, and Janaúba, Northern Minas Gerais. Isolates of *Mf* and *Mm* obtained were identified morphologically and molecularly. Sensitivity to DMI fungicides propiconazole and tebuconazole was determined by calculating the 50% inhibitory concentration of mycelial growth (EC_{50}) based on dose-response curves ranging from 0 to 5 µg mL^{-1}. Variation in sensitivity to fungicides was evident, with predominance of reduced sensitivity and resistance phenotypes. Isolates with sensitive phenotypes were not detected. In Vale do Ribeira, where *M. fijiensis* predominated and chemical management with DMI fungicides is considered conventional and intensive, resistance levels were higher. Cross-resistance between propiconazole and tebuconazole fungicides was also evident. Several point mutations in the *CYP51* gene, with significantly distinct phenotypic effects, were found in individuals from populations of *M. fijiensis* and *M. musicola*. In this study, we report for the first time in the country the mutations V106D, A446S, Y136F, Y461H, and Y463D in *M. musicola*.

Keywords: propiconazole; tebuconazole; demethylase inhibitor fungicides (DMI); chemical control.

CAPÍTULO 1. RESISTÊNCIA A TRIAZÓIS EM POPULAÇÕES DE MYCOSPHAERELLA FIJIENSIS E M. MUSICOLA DO COMPLEXO DA DOENÇA DE SIGATOKA DE PLANTAÇÕES COMERCIAIS DE BANANEIRA EM MINAS GERAIS E SÃO PAULO, BRASIL.

RESUMO

Os inibidores da desmetilação de esteróis (DMIs) estão entre os fungicidas mais utilizados para o controle da Sigatoka negra (*Mycosphaerella fijiensis*, *Mf*) e da Sigatoka amarela (*Mycosphaerella musicola*, *Mm*) em plantações de banana no Brasil. A Sigatoka Negra é considerada mais importante por causar perdas de rendimento de até 100% nas lavouras comerciais de banana sob condições predisponentes. Em contrapartida, a Sigatoka amarela é importante devido à sua ampla ocorrência no país. Este estudo teve como objetivo determinar os atuais níveis de sensibilidade das populações *Mf* e *Mm* aos fungicidas triazóis DMI. Além disso, a variação alélica no gene CYP51 foi analisada em populações desses patógenos para identificar e caracterizar as principais mutações e mecanismos associados à potencial redução da sensibilidade ou resistência. Populações de *Mf* e *Mm* foram amostradas em plantações comerciais de banana em Registro, Vale do Ribeira, SP, Ilha Solteira, Noroeste de SP, e Janaúba, Norte de Minas Gerais. Os isolados de *Mf* e *Mm* obtidos foram identificados morfologicamente e molecularmente. A sensibilidade aos fungicidas DMI: propiconazole e tebuconazole, foi determinada pelo cálculo da concentração inibitória de 50% do crescimento micelial (CE50) com base em curvas dose-resposta variando de 0 a 5 µg mL-1. Foi evidente a variação na sensibilidade aos fungicidas, com predomínio de fenótipos de sensibilidade reduzida e resistência. Isolados com fenótipos sensíveis não foram detectados. No Vale do Ribeira, onde predomina *M. fijiensis* e o manejo químico com fungicidas DMI é considerado convencional e intensivo, os níveis de resistência foram maiores. A resistência cruzada entre os fungicidas propiconazole e tebuconazole também foi evidente. Várias mutações pontuais no gene CYP51, com efeitos fenotípicos significativamente distintos, foram encontradas em indivíduos de populações de *M. fijiensis* e *M.*

musicola. Neste estudo, relatamos pela primeira vez no país as mutações V106D, A446S, Y136F, Y461H e Y463D em *M. musicola*.

Palavras-chave: propiconazole; tebuconazole; fungicidas inibidores da demetilase (DMI); controle químico.

1. INTRODUCTION

The Banana Sigatoka disease complex (BSDC) encompasses Black Sigatoka (caused by *Mycosphaerella fijiensis*, *Mf*) and Yellow Sigatoka (caused by M. musicola, *Mm*). Black Sigatoka was initially reported in Brazil in 1998 in the Amazon region and has since been detected in 19 states, including São Paulo and Bahia, the country's primary banana-producing states (Brito, 2015; Ferrari et al., 2021; Gasparotto et al., 2000; Ramos et al., 2018; Uchôa et al., 2021). This disease is a significant constraint to banana production, capable of reducing yields by up to 100%. However, there are reports suggesting that Black Sigatoka may have been misidentified as the less devastating Yellow Sigatoka, which was first identified in the Amazon region in 1944 and is widespread across all banana-growing regions of Brazil, causing yield losses of up to 50% (Brito, 2015; Gomes et al., 2013, 2018; Nomura et al., 2020).

The BSDC diseases are polycyclic, with pathogens *Mf* and *Mm* exhibiting a mixed reproductive system involving clonal epidemic dispersal via conidia and cyclic sexual reproduction releasing ascospores (Beltrán-García et al., 2014; Burt, 1994). Populations of *Mf* and *Mm* in Brazil, Mexico, and the Philippines show high genotypic variation due to sexual reproduction and gene flow from distant pathogen migration (Brito, 2015; Gomes et al., 2018; Manzo-Sánchez et al., 2019; Mendoza; Ardales, 2019). Genetic resistance to BSDC is generally absent or partial in most commercial banana cultivars, necessitating disease control strategies primarily based on calendar-based systemic or protectant fungicide sprays (Brito, 2015).

In regions with high disease pressure, extensive fungicide applications, up to 52 sprays of protectant or 26 sprays of systemic fungicides per year, are applied, leading to increased production costs and environmental impact (Brito, 2015; Malimpensa, 2018; Uchôa et al., 2021). Conversely, in the Ribeira Valley of Brazil, Black Sigatoka control involves weekly disease monitoring and frequent fungicide applications,

resulting in 15–20 sprays per year (Uchôa et al., 2021). Control heavily relies on systemic site-specific quinone outside inhibitor (QoI), demethyilation inbibitor (DMI), and the more recently labeled succinate dehydrogenase inhibitors (SDHI) fungicides, but this intensive use of fungicides exerts selective pressure on pathogen populations, leading to the emergence, selection, and spread of fungicide-resistant strains (Cañas-Gutiérrez et al., 2009; Churchill, 2011; Diaz-Trujillo et al., 2018, 2018; GRICE et al., 2013; Oliveira et al., 2022; Silva et al., 2024).

Studies on fungicide resistance in populations of *Mf* and *Mm* from Brazil began to emerge in the past decades. Concerning QoIs, the first surveys in Brazil initially have shown no resistance in *Mf* populations from the Amazon and Ribeira Valley in São Paulo, as well as in *Mm* populations from the Federal District and São Paulo sampled from 2008 to 2018 (Brito, 2015; Gomes et al., 2014; Hanada; Gasparotto; Moreira, 2015). However, recent data indicate varying levels of QoI resistance, with 10.0% and 9.4% of *Mf* and *Mm* isolates, respectively, exhibiting the G143A substitution in CytB, particularly in regions with intensive fungicide use in São Paulo and Minas Gerais (Beltrán-García et al., 2014; Malimpensa, 2018).

Similarly, resistance to SDHI fungicides has been detected in *Mf* and *Mm* populations in Southeastern Brazil sampled in 2020–2021, a few years after its labeling (SILVA et al., 2024). Globally, SDHI fungicides approved for BSDC management in banana plantations include boscalid, fluopyram, fluxapyroxad, and isopyrazam (FRAC – FUNGICIDE RESISTANCE ACTION COMMITTEE, 2022). In Brazil, only one SDHI fungicide co-formulation (Collis™ from BASF) with SDHI boscalid and QoI kresoxim-methyl is labeled for BSDC (MAPA, 2023). Some fungal isolates with resistance to boscalid and fluxapyroxad showed Sdh target site alterations (SdhC N55D, SdhB E196Q, and SdhD K66N) (Silva et al., 2024). None of these substitutions have been associated with SDHI fungicide resistance in BSDC pathogens, based on the latest survey conducted by the SDHI Working Group in 2022 (FRAC – Fungicide Resistance Action Committee, 2022). Continued monitoring for mutations and other resistance mechanisms like multiple Sdh paralogs is critical for effective disease control strategies.

Regarding resistance to DMI fungicides, there is evidence of reduced sensitivity in *Mf* populations since 2008–2009 (Gomes et al., 2014) and (Hanada; Gasparotto; Moreira, 2015). The DMI fungicides, introduced in the 1980s, are among the most widely used for controlling Sigatoka diseases (Brito et al., 2020; Chong et al., 2021). DMIs inhibit the biosynthesis of ergosterol, an essential component of the fungal cell membrane (Yang et al., 2009), targeting the enzyme 14α-demethylase encoded by the *CYP51* gene, a member of the cytochrome P450 family (Zhan; STefanato; MCDonald, 2006). Given the frequent spray of DMI fungicides in banana plantations, the resulting selection pressure may lead to increased frequency of new *Mf* and *Mm* genotypes with reduced sensitivity to these fungicides. Continuous monitoring and detailed investigation into resistance evolution and spread in favorable agroecosystems are crucial for effective disease management and sustainable control strategies (Aguirre, 2016; Corkley; Fraaije; Hawkins, 2022; Gasparotto et al., 2000; Martínez-Bolaños et al., 2012; Moraes; Nomura, 2020).

Mechanisms of resistance to DMI involving *CYP51* mutations have been reported in *Mf* and *Mm* both elsewhere and in Brazil (BRITO et al., 2020; CAÑAS-GUTIÉRREZ et al., 2009), with CYP51 overexpression linked to tandem repeats in *Mf* (DIAZ-TRUJILLO et al., 2018). In Brazil, specific *CYP51* mutations like G462D, Y463H in *Mf* (Malimpensa 2018) and A381G, Y461N, Y463H in *Mm* (Brito, 2015; Brito et al., 2020; Malimpensa, 2018) have been associated with DMI resistance in insensitive strains of the pathogens from the Federal District (Central Western), and Ribeira Valley region in São Paulo (Southeastern Brazil).

Our study aims to provide updated insights into DMI resistance prevalence and mechanisms in *Mycosphaerella* species associated with BSDC, contributing to more sustainable approaches for disease control.

Therefore, based on these premises, the main objectives or our study were: i) to determine the current sensitivity levels of populations of *Mf* and *Mm* from banana plantations in the Ribeira Valley, in Northwestern São Paulo, and in Northern Minas Gerais (Southeastern Brazil) to DMI fungicides; and ii) to determine the variation in the *CYP51* gene to identify key mutations and and to characterize the mechanisms associated with resistance to DMI fungicides locally.

2. MATERIALS AND METHODS

2.1. Sampling of diseased plants and isolation of the fungal pathogens

Diseased plants were obtained from commercial banana plantations from Northwest São Paulo (populations designated SPNW-C and SPNW-O), from Vale do Ribeira region, also from São Paulo (SPVR-CI), and from Northern Minas Gerais (MGN-C), Brazil. These geographic populations were associated with distinct disease management systems: (i) intensive management (population SPVR-CI, sampled from Jacupiranga, Registro, and Sete Barras counties, in the Ribeira Valley); (ii) reduced management (population SPNW-C, obtained from Ilha Solteira county, in Northwest São Paulo, and MGN-C, sampled from Janaúba, Northern Minas Gerais); and (iii) organic management, with no fungicide applications (population SPNW-O, also obtained from Ilha Solteira). The spatial distribution of these population samples is illustrated in **Figure 1**.

In Ribeira Valley (SPVR-CI), the management strategy involved intensive fungicide spraying, with 8 to 14 preventive applications annually for chemical control of black Sigatoka (Malimpensa, 2018; Oliveira et al., 2022). This region is renowned as a major center for banana production in São Paulo state and Brazil. The predominant banana cultivars there are the BSDC-susceptible Prata (*Musa* spp. AAB, commonly known as "Lady Finger" banana) and Nanica (*Musa* spp. AAA, Cavendish subgroup) (Moraes; Nomura, 2020; Nomura et al., 2020; Oliveira et al., 2022; Rangel; Penteado; Tonet, 2002). In the second geographic population, located in northwestern São Paulo (SPNW-C), and the third site, in northern Minas Gerais (MGN-C), fungicide use is reduced to four to five preventive sprays targeting Yellow Sigatoka (Oliveira, 2022). The SPNW-C area encompasses 40 hectares dedicated to the susceptible Maçã variety (triploid AAB) (Oliveira, 2022). Conversely, in MGN-C, the banana plantation comprises Prata and Nanica varieties (Oliveira, 2022). At the final sampled site (SPNW-O), no fungicides were used. This area includes several small family plantations with banana plants of various varieties and ages in Ilha Solteira county (Oliveira, 2022).

Fragments (approximately 20 × 20 cm) of banana leaves showing symptoms of either yellow Sigatoka or black Sigatoka diseases from different ages and cultivars

were collected. At each location, sampling points consisted of 50 m^2 areas where approximately five plants were sampled. The leaf fragments were placed in paper bags and transported to the Molecular Plant Pathology Lab at UNESP, Ilha Solteira Campus. Samples were refrigerated until pathogen isolation.

Pathogen isolation followed the methodology of (Brito et al., 2020), with modifications. Leaves with apparent disease symptoms were rinsed with running water. Samples taken from the leaves, including the lesion area and its marginal parts, were disinfected with a 75% ethanol solution and then rinsed with distilled water to remove excess alcohol, approximately for a minute each. Subsequently, they were dried on sterilized filter paper and incubated in Petri dishes containing water agar medium (15 g L^{-1} agar supplemented with 50 µL mL^{-1} chloramphenicol and streptomycin). Fungal mycelial growth was analyzed under a stereoscopic microscope to distinguish characteristic sporodochia of *Mf* and *Mm*. Direct isolation was performed by transferring conidia from the sporodochia formed in water agar medium to plates containing potato dextrose agar (PDA: 20.8 g L^{-1} potato dextrose and 15 g L^{-1} agar) using streaking technique, followed by incubation for five days at 25 °C under a 12 h photoperiod (Gomes et al., 2013). Colonies were purified by transferring pure microcolonies of *Mf* or *Mm* isolates to new PDA plates and incubating for five days under the same conditions.

For long-term preservation of isolates, sterilized 0.5 cm^2 filter paper fragments were subsequently transferred onto the surfaces of growing fungal colonies, maintained under incubation at 25 °C under a 12 h photoperiod until complete colonization of the fragments. Filter paper fragments colonized by fungal mycelial growth were transferred to Petri dishes and dried under sterile conditions in a laminar flow hood for 24 h. Subsequently, these colonized and dried paper fragments were transferred to cryotubes with silica gel and cryopreserved at -20 °C freezer. These isolates constitute a subset of the population examined for QoI resistance by (Oliveira, 2022) and for SDHI resistance by (Silva et al., 2024).

2.2. Molecular identification of species

For the molecular identification of the two target species, the isolates were reactivated on PDA medium and grown for 10 days at 25 °C under 12 h photoperiod. Approximately 1 cm^2 of mycelial culture per isolate was then lyophilized for 48 hours. The lyophilized mycelium was used for DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions. The quantification of extracted DNA was performed using a NanoDrop® 2000c spectrophotometer (Thermo Fisher Scientific, USA) to achieve final dilutions at a c-oncentration of 100 ng μL^{-1}.

Specific PCR primers were used for the molecular identification of *Mf* (CYP51_Pfijien_F1 / CYP51_Pfijien_R1) and *Mm* (CYP51A_Mm_F523 / CYP51A_Mm_R2457), based on the amplification of the *CYP51* gene (Table 1). Polymerase chain reaction (PCR) reactions were performed using a ProFlex thermal cycler (Applied Biosystems, USA). Reactions were prepared in a total volume of 25 µL, consisting of 1 × PCR buffer (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, 60 µM dNTPs, 0.2 µM of each primer, 1.5 U Taq DNA polymerase (Invitrogen-Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 100 ng of genomic DNA. The PCR conditions for Mf were as follows: initial denaturation at 95 °C for 5 minutes, followed by 36 amplification cycles with temperatures of 94 °C for 30 seconds, 58 °C for 60 seconds, and 72 °C for 60 seconds; with a final extension at 72 °C for 7 minutes. For Mm, the conditions were similar, with the only difference in the annealing temperature, which was 59 °C. The resulting amplicons were analyzed by agarose gel electrophoresis, and the species were identified based on the size of the fragments obtained. Positive identification of *Mf* was confirmed by amplification of a 1700 bp fragment, while for *Mm*, a 1900 bp fragment was amplified. Positive controls for both species and negative controls using DNA extracted from the basidiomycete fungus *Rhizoctonia solani* AG-1 IA and the ascomycete fungus *Pyricularia oryzae Triticum* lineage, available in our laboratory, were included. Additionally, species identification was confirmed using species-specific primers designed for the *CYP51* gene of *Mf* and *Mm*.

2.3. Analysis of allelic variation of the CYP51 gene

To assess allelic variation of the *CYP51* gene in the *Mf* and *Mm* isolates, specific primers targeting the *CYP51* gene were used for PCR amplification. We conducted PCR amplification and sequencing of a total of nine isolates of *Mf* and 42 isolates of *Mm* from the four BSDC populations sampled. The primers used in this study (Table 1) were either the ones used in other studies (Chong et al., 2019) or designed specifically for this study. These particular primers were designed using Primer3 software within Geneious Prime version R 9.0.5 (Biomatters, Auckland, New Zealand). These primers also cover the promoter region of the CYP51 gene, allowing the presence of repetitive elements within the region to be verified, indicated as another mechanism associated with resistance to DMI through upregulation of gene expression (Chong et al., 2021). Reference sequences used for primer design were obtained from NCBI/GenBank® or from a specific publication (Chong et al., 2019), which included: genomic scaffold sequence NW_006921538 from strain CIRAD86; EF581093.1; Bo_1, CaM10_6, and CaM10_21 (Chong et al., 2019) for the *Mf*CYP51; MF521833 and MF521834 for the *Mm*CYP51. These sequences were derived from *Mf* strains CIRAD86, Bo_1, CaM10-6, and CaM10_21, originating from Cameroon, Philippines, or Costa Rica; and from *Mm* isolates 2SJA and 9SJA, from Brazil, and all isolated before 2019 (Brito et al., 2020; Chong et al., 2019).

Table 1: Specific primers used for PCR amplification aiming at species identification, and for Sanger sequencing to determine allelic variation in the *CYP51* gene in *Mycospharella fijiensis* and *M. musicola**

Primers (source)**	Sequence (5' - 3')	Species	Annealing temperature (°C)
CYP51_Pfijien_F1 (CHONG et al., 2019)	AAGGTCATATCGCAGG	*Mf*	56
CYP5_1Mf592F (S)	TGAATGGAAAGCTCAAGGACGT	*Mf*	58
CYP51_Mf1765R (S)	ACTTCTCACTTGGGTTCTCGTC	*Mf*	58
CYP51_Pfijien_R1 (CHONG et al., 2019)	GAATGTTATCGTGTGACA	*Mf*	56
CYP51A_Mm_F523	ATCGACATCACCACCGCTG	*Mm*	59
CYP51A_Mm_R1554 (S)	TGTCCTCCTCTTTCTCTCTCCAGA	*Mm*	59
CYP51A_Mm_F1369 (S)	TTGCCGATCTCTACCACGAC	*Mm*	59
CYP51A_Mm_R2427-2	CGCATGACAGATAAAGAGGAAAGC	*Mm*	59
CYP51_Prom_1800_F	GAA CGA GTT TCC AGG TTG CTG	*Mm*	58
CYP51_Prom_2082_R	GCA GTT TGT GTG AAA GCA GGG	*Mm*	58

*Since the *CYP51* gene is relatively long in both pathogens, multiple pairs of primers were used (two pairs for *Mf* and three pairs for *Mm*) to fully cover the gene and the promoter region. **Unless otherwise indicated, the primers were specifically designed for this study.

The PCR reactions for amplifying and sequencing the *CYP51* gene were carried out in a final volume of 30 µL, containing water, 50 ng of total DNA, 0.3 µM of each primer, 0.2 mM of each dNTP, 2.5 mM of MgCl2, 3 µL of 10× buffer, and 0.5 U of Taq DNA Polymerase (Sigma-Aldrich, USA). Amplifications were performed using a ProFlex thermal cycler (Applied Biosystems, USA) with an 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 (see tables 1), and 72°C for 1 min; with a final extension at 72°C for 7 min.

PCR products were purified and Sanger sequenced at the sequencing facilities of Macrogen Inc. in Seoul, South Korea. To ensure accuracy, four or six complementary sequences were generated for each *CYP51* gene fragment amplified for *Mf* or *Mm*, respectively, which included internal primers (Table 1). DNA sequences were analyzed and aligned using Geneious R 9.0.5 software (Biomatters, New Zealand) to identify alleles, haplotypes, and distinguish non-synonymous mutations leading to amino acid changes in inferred protein sequences. The same reference sequences described previously were used for annotation and derivation of CYP51 protein sequences from the experimental sequence data obtained in our study. The aminoacid substitutions in the protein variants of the CYP51 detected were depicted as lollipops graphs built using the R software libraries *ggplot2*, *dplyr*, *hrbrthemes*, *data.table*, *tidyverse*, *readxl*, *glue*, *ggtext*, and *ggrepel*, and the functions *geom_segment, ggtitle*, *geom_point,* and *geom_text_repel* (R DEVELOPMENT CORE TEAM, 2022).

2.4. Phenotypic assessment of in vitro sensitivity to DMI triazole fungicides and determination of EC_{50}

or 10 days on PDA at 25°C

For the phenotyping of sensitivity to DMI triazole fungicides, a total of nine isolates of *Mf* and 42 isolates of *Mm* were selected: nine isolates of *Mf* from SPVR-CI,

15 isolates of *Mm* from MGN-C, 13 isolates of *Mm* from SPNW-C, and 14 isolates of *Mm* from SPNW-O.

Sensitivity testing involved the use of DMI fungicides propiconazole and tebuconazole. Formulated propiconazole (Tilt™ EC, active ingredient at 250 g L^{-1}, Syngenta S.A., Brazil) was diluted in deionized water to obtain a stock solution of 25 µg mL^{-1}. Formulated tebuconazole (Riza™ 200 EC, active ingredient at 200 g L^{-1}, FMC Química do Brasil Ltda., Brazil) was diluted to obtain a stock solution of 20 µg mL^{-1}. The final tested doses for propiconazole and tebuconazole were 0, 0.001, 0.01, 0.05, 0.1, 1, and 5 µg mL^{-1}. The fungicides tested at different doses were mixed with PDA medium supplemented with chloramphenicol and streptomycin, both at a concentration of 50 µL mL^{-1}. Sensitivity tests were performed in 90 mm diameter Petri plates.

Mycelial fragment suspensions for the fungicide sensitivity testing were obtained from the fungal colonies grown for 10 days on PDA at 25°C, under dark. Mycelial samples with an area of 1 cm^2 were collected and macerated in crucibles using ceramic pistils under sterile conditions in a laminar flow hood. The samples were then resuspended in 5 mL of sterilized deionized water containing 0.05 mL^{-1} of Tween. Subsequently, 5 µL of the inoculum suspension was carefully pipetted onto 0.5 cm diameter sterile filter paper discs, positioned on top of PDA medium containing different fungicide concentrations. The 90 mm diameter plates were sealed with plastic film to prevent drying and external contamination and incubated for 15 days at 25°C and 12 h photoperiod.

2.5. Experimental design, statistical analyses and data depiction

The experimental design consisted of complete randomized blocks with four replicates per treatment and experiments in duplicate. Sensitivity to the two DMI fungicides was assessed by determining the effective concentration of 50% to inhibit fungal growth (EC_{50}, in $\mu g \cdot mL^{-1}$), estimated using a dose–response function implemented in the Excel macro ED50plus v1.0 (Vargas, 2000). For hypothesis testing, isolates were grouped by the CYP51 protein variants detected, by geographical populations of the pathogens, and also by species. 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, 2022). We also classified the individual isolates according to their sensitivity to the DMI fungicides propiconazole and tebuconazole using phenotypic classes described previously (Aguirre, 2016; Cañas-Gutiérrez et al., 2009) (Table 2). We also determined the correlation between the EC_{50} values for propiconazole and tebuconazole to look for evidence of cross resistance between the two fungicides.

Table 2. Sensitivity classes for phenotypic classification of *Mycosphaerella fijiensis* and *M. musicola isolates from BSDC* to the DMI fungicides propiconazole and tebuconazole based on EC_{50} values.

Phenotypic classes	EC_{50} intervals (µg mL^{-1})	
	DMI fungicides	
	Propiconazole	Tebuconazole
Sensitiveness (S)	≤ 0.005	≤ 0.02
Reduced sensitivity (RS)	> 0.005 to 0.05	> 0.02 to 0.2
Moderate resistance (MR)	> 0.05 to 1	> 0.2 to 2
Resistance (R)	> 1	> 2

The boxplot figures depicting the contrast among the *CYP51* protein variants detected, among geographical populations of the pathogens, and between isolates grouped by species were built using the R software library tidyverse 1.3.1, which included the packages *ggplot*, purrr, *tibble*, *dplyr*, *tidyr*, *stringr*, *readr*, and *forcats* , and the functions *ggplot*, *geom_boxplot*, *stat_summary*, *geom_jitte*r, *ggtitle*, *theme*, and *geom_text* (R Development Core Team, 2022). The whole set of color palettes chosen to build figures with accessibility features included color-blind safe, and print-friendly colors, using the resources from Color Brewer 2.0 (URL: https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3, accessed on 1 November 2023).

3. RESULTS

3.1. Analysis of allelic variation of the CYP51 gene

In the present study, we identified a total of 51 isolates of the BSDC pathogens, comprising 42 from *Mm* and nine from *Mf*. A total of eight protein variants of the *CYP51* gene were detected among these isolates. The species *Mm* exhibited the highest diversity of protein variants of the *CYP51* gene (N = 5). The CYP51 A variant from *Mm* was the most frequent in the sample (N=31), while variants B to E were detected at considerably lower frequencies (N ≤ 5). As for the species *Mf*, three variants of the CYP51 gene were detected, with CYP51 F being the most frequent (N=5), while variants G and H occurred at lower frequencies (N ≤ 3) (Figure 3).

These isolates exhibited at least one mutation in the *CYP51* gene that resulted in an amino acid change, except for Y136F-SRS (substrate recognizing site). For the Y136F substitution, the wild-type Y136 codon is TAC at position 405 bp of the CYP51 gene while the altered codons for Y136F are TTC or TTT (Chong et al., 2019). The most frequent CYP51 substitutions detected in *Mm* isolates was V106D (N=42) and A446S (N=42) while the substitution T18I (N=9) was the most frequent in *Mf* isolates. Additionally, less frequent substitutions such as Y136F-SRS (N=2), Y461H (N=3), and Y461N (N=1) were found in *Mm*, whereas CYP51 Y461D (N=3) and G462D (N=1) were observed in *Mf*. The CYP51 Y463D mutation was found in five and six isolates of both *Mf* and *Mm* (Figure 2).

No repetitive elements were found inserted in the promoter region the *CYP51* gene of *Mm* (N=42 isolates), while in *Mf* (N=9 isolates) we detected the insertion of one single element, which was also present in the sequence of the sensitive strain *Mf* Bo-1 used as standard. In contrast, the *CYP51* sequence from the resistant strain *Mf* Ca10-21 harbored six repetitive elements in the promoter region, associated with the DMI resistance mechanism previously described for the pathogen (Diaz-Trujillo et al., 2018).

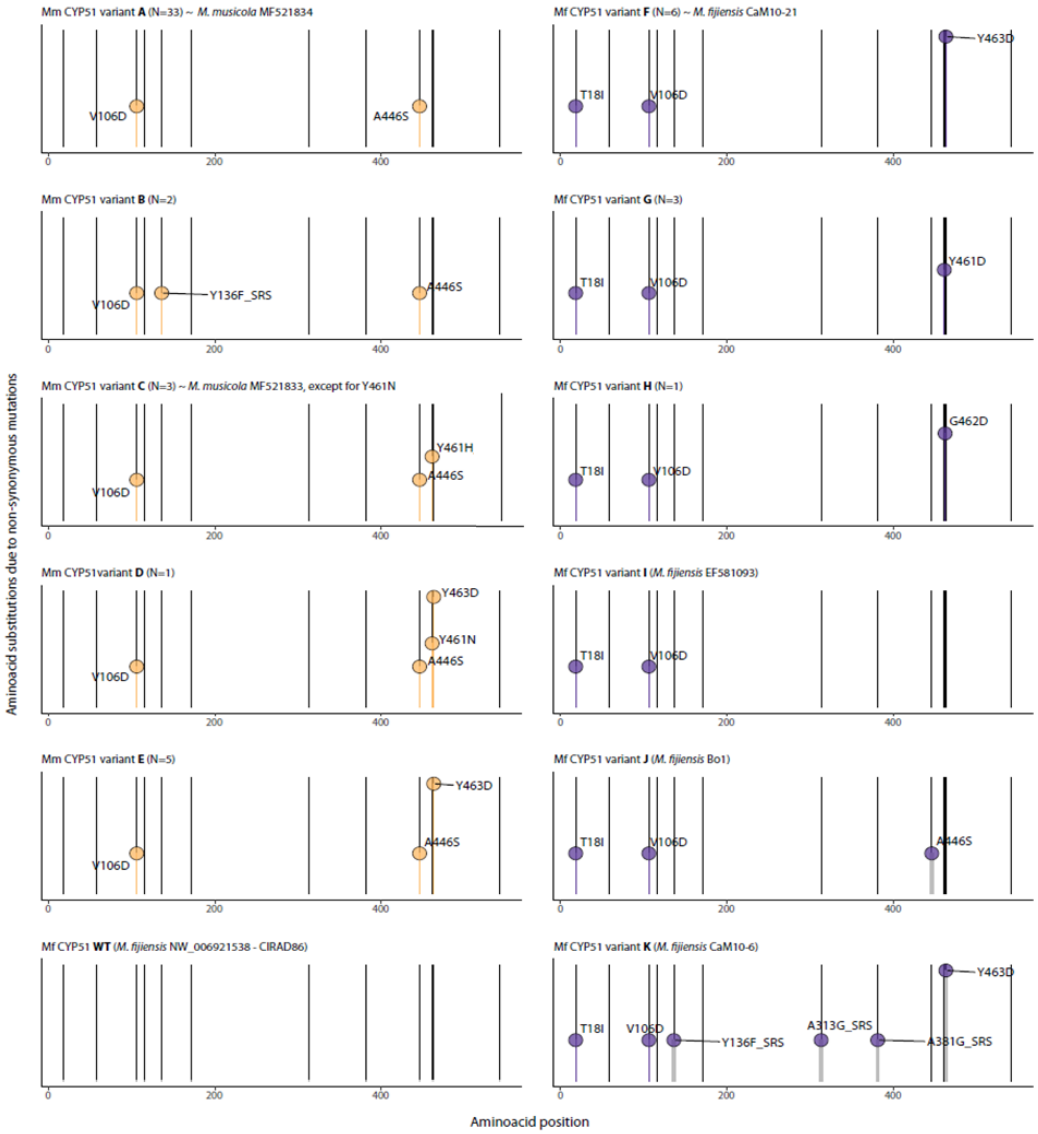


Figura 1. Lollipop depiction of non-synonymous amino acid substitutions conferred by mutations in the *CYP51* gene of isolates of *Mycosphaerella musicola* (at left) and *Mycosphaerella fijiensis* (at right) sampled from four geographical populations from distinct fungicide management systems in Minas Gerais and São Paulo, Brazil*

*CYP51 protein variants A to E were detected in isolates of *Mm*, while variants F, G and H were observed in *Mf* isolates. Orange lollipops indicate the amino-acid substitutions in the inferred CYP51 protein sequence from *Mm* isolates while purple lollipops are substitutions in the protein sequence from *Mf* isolates. When the protein variant detected has shown similarity with reference sequences from the GenBank/NCBI, the sequence code is indicated in the respective variant (e.g., CYP51 variant A was similar to *Mm* MF521834; the variant F was similar to the sequence from *Mf* CaM10-21). CYP51 WT and variants I, J and K were not detected in our sampling.

3.2. Phenotypic assessment of in vitro sensitivity to DMI triazole fungicides and determination of EC_{50}

The phenotyping of *Mf* and *Mm* isolates regarding sensitivity to the DMI fungicides propiconazole and tebuconazole indicated a very low frequency of sensitivity to both fungicides. Most isolates were classified as exhibiting reduced sensitivity (72.6 to 78.4%) to moderate resistance (25.5 to 21.6%) for propiconazole or tebuconazole, respectively. Only 1.9% of the isolates (N=1/51) was sensitive to tebuconazole. There were significant differences among isolates according to their EC_{50} for either propiconazole or tebuconazole (Table 3).

Based on EC_{50} values, there were significant differences among the eight CYP51 protein variants detected in the population sampling of the BSDC pathogens (Table 3, Figure 3 A and B). EC_{50} from variant G from *Mf* was significantly higher for both DMI fungicides ($EC_{50\ propiconazole}$ = 0.43 ± 0.30; $EC_{50\ tebuconazole}$ = 0.89 ± 0.03), followed by variants F ($EC_{50\ propiconazole}$ = 0.29 ± 0.31; $EC_{50\ tebuconazole}$ = 0.73 ± 0.48) and H ($EC_{50\ propiconazole}$ = 0.27 ± 0.001; $EC_{50\ tebuconazole}$ = 0.86 ± 0.001), also from *Mf*. Altogether, these three CYP51 variants from *Mf* had higher EC_{50} than all the variants detected in *Mm* isolates, which ranged from 0.01 to 0.07 for propiconazole and from 0.03 to 0.37 for tebuconazole (Figure 3 A and B).

For propiconazole, the protein variants CYP51 A, B, C, and D of *Mm* were classified as exhibiting reduced sensitivity, while CYP51 E of *Mm*, and CYP51 F, G, and H of *Mf* were classified as showing moderate resistance. Regarding tebuconazole, only the protein variants CYP51 A, B, and C of *Mm* were classified as having reduced sensitivity, while CYP51 D and E of *Mm*, and F, G, and H of *Mf* were classified as exhibiting moderate resistance (Table 2, Figure 3).

We then interpreted the impact of five CYP51 substitutions [Y461D, Y461H, Y461N, G462D, and Y463D) absent in the sensitivity standard isolate *Mf* Bo_1 (protein variant J, carrying T18I, V106D, and A446S substitutions, for comparison (Diaz-Trujillo et al., 2018)] regarding their potential contribution to fungicide resistance based on EC_{50} values and the resistance phenotypic class for tebuconazole and propiconazole fungicides (Figure 2, Table 4).

Table 3. Analysis of variance of the effects of species, geographical populations,protein variants, and isolates in the levels of sensitivity to DMI fungicides propiconazole and tebuconazole in *Mycosphaerella fijiensis* and *M. musicola* from banana plantations in São Paulo and Minas Gerais, Brazil.*

Fungicide: Propiconazole						
Source of variation	**Levels**	**Degrees of freedom (Df)**	**Sum of square (Sum Sq)**	**Mean Sq**	***F* value**	***p* > F**
Isolates	51	50	12.528	0.251	153,703	≤ 2^{-16} ***
Residuals		357	0.001	0.000		
Protein variants	8	7	6.303	0.901	57.86	≤ 2^{-16} ***
Residuals		400	6.226	0.016		
Populations	4	3	5.822	1.941	115.9	≤ 2^{-16} ***
Residuals		404	6.707	0.017		
Species	2	1	5.792	5.792	349.1	≤ 2^{-16} ***
Residuals		406	6.737	0.017		
Fungicide: Tebuconazole						
Source of variation	**Levels**	**Degrees of freedom (Df)**	**Sum of square (Sum Sq)**	**Mean Sq**	***F* value**	***p* > F**
Isolates	51	50	49.07	0,981	16,781	≤ 2^{-16} ***
Residuals		357	0.02	0.0001		
Protein variants	8	7	34.540	4.935	135.7	≤ 2^{-16} ***
Residuals		400	14.550	0.036		
Populations	4	3	29.910	9.969	210.0	≤ 2^{-16} ***
Residuals		404	19.180	0.047		
Species	2	1	29.220	29.218	569.9	≤ 2^{-16} ***
Residuals		406	19.870	0.049		

*The species effect compared *M. fijiensis* (N=9) and *M. musicola* (N=42); the population effect compared four geographical populations MGN-C (N=15), SPNW-C(N=13), SPNW-O (N=14) from *Mm*, and SPVR-CI (N=9) from *Mf*; the protein variant effect compared eight variants [A (N=31), B (N=2), C (N=3), D (N=1), E (N=5), F (N=5), G (N=3), and H (N=1)] detected in the fungal populations sampled; N = number of isolates within each factor.

The following order offers a perspective on each mutation's impact on fungicide resistance, considering associated EC_{50} values (Figure 2, Table 4): i) Y461H, found in *Mm* protein variant D, contributes to reduced sensitivity to both propiconazole and

tebuconazole; EC_{50} values for Y461H ranged from 0.02 to 0.41 µg.mL^{-1}, indicating reduced sensitivity to moderate resistance. ii) Y461D, identified in *Mf* protein variant G, is associated with moderate resistance to both propiconazole (EC_{50} = 0.43 µg.mL^{-1}) and tebuconazole (EC_{50} = 0.89 µg.mL^{-1}). iii) Y461N, present in *Mm* protein variant C, contributes to reduced sensitivity to both propiconazole (EC_{50} = 0.02 µg.mL^{-1}) and tebuconazole (EC_{50} = 0.19 µg.mL^{-1}). iv) G462D, identified in *Mf* protein variant H, is associated with moderate resistance to both propiconazole (EC_{50} = 0.27 µg.mL^{-1}) and tebuconazole (EC_{50} = 0.86 µg.mL^{-1}). v) Y463D, appearing in multiple protein variants (*Mm* D, *Mm* E, *Mf* F), plays a significant role in conferring reduced sensitivity or moderate resistance to both propiconazole and tebuconazole across species, with EC_{50} values associated with this mutation range from 0.02 to 0.37 µg.mL^{-1}.

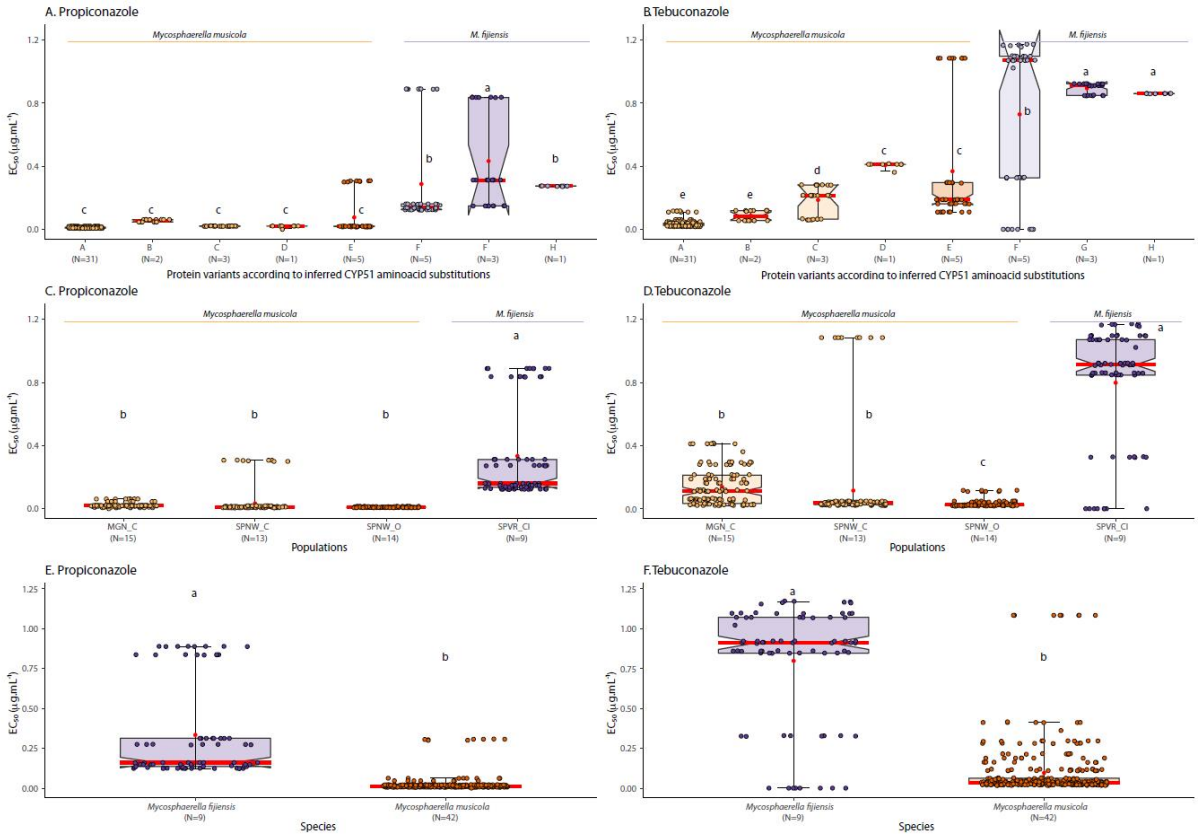


Figure 2. Boxplot comparing the effects of protein variants, geographical populations, and species, in the levels of sensitivity (EC_{50}) to DMI fungicides propiconazole and tebuconazole in *Mycosphaerella fijiensis* and *M. musicola* from banana plantations in São Paulo and Minas Gerais, Brazil.

There were significant differences also among geographical populations of the pathogens (Table 3, Figure 3 C and D). The population SPVR_CI from *Mf* was significantly more resistant to both fungicides than all the other three populations sampled (MGN-C, SPNW-C, SPNW-O). The comparison between species indicated that *Mf* ($EC_{50\ propiconazole}$ = 0.33 ± 0.29; $EC_{50\ tebuconazole}$ = 0.80 ± 0.36) was significantly more resistant than *Mm* ($EC_{50\ propiconazole}$ = 0.02 ± 0.05; $EC_{50\ tebuconazole}$ = 0.10 ± 0.18) for both DMI fungicides (Table 3, Figure 3E and F).

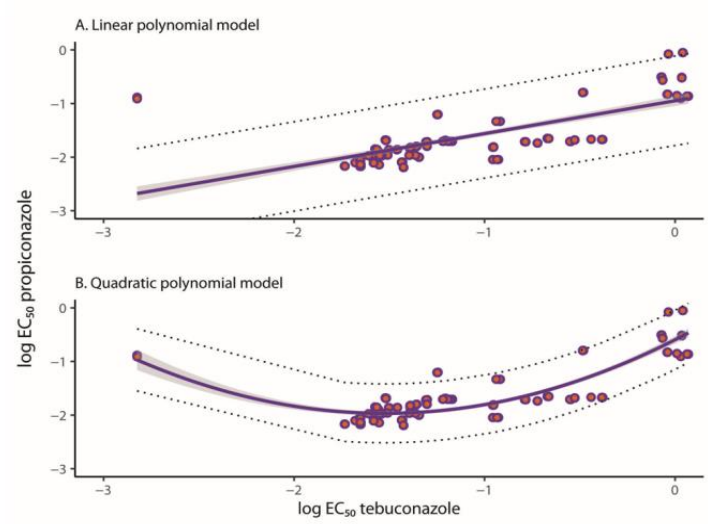
Table 4. Summary outlining five specific substitutions and their corresponding CYP51 protein variants and impact on resistance levels to DMI fungicides in *Mycosphaerella fijiensis* (*Mf*) and *M. musicola* (*Mm*) from the BSDC in Southeastern Brazil.

Species	Protein variant	Substitutions probably associated with resistance*	EC_{50} for propiconazole ($\mu g.mL^{-1}$)	Propiconazole resistance category**	EC_{50} for tebuconazole ($\mu g.mL^{-1}$)	Tebuconazole resistance category**
Mm	A	-	0.01 c	RS	0.03 e	RS
Mm	B	-	0.05 c	RS	0.09 e	RS
Mm	C	Y461N	0.02 c	RS	0.19 d	RS
Mm	D	Y461H, **Y463D**	0.02 c	RS	0.41 c	MR
Mm	E	**Y463D**	0.08 c	MR	0.37 c	MR
Mf	F	**Y463D**	0.29 b	MR	0.73 b	MR
Mf	G	Y461D	0.43 a	MR	0.89 a	MR
Mf	H	G462D	0.27 b	MR	0.86 a	MR

*Resistance categories: RS = reduced sensitivity; MR = moderate resistance; **The sensitivity standard isolate Mf Bo_1 (protein variant J, carrying T18I, V106D, and A446S substitutions) for comparison, did not carry the CYP51 substitutions Y461D, Y461H, Y461N, G462D, and Y463D.

The comparison between the linear and quadratic models in Figure 4 reveals that the quadratic model is more suitable for describing the correlation of DMI fungicides propiconazole and tebuconazole with EC_{50} data from 51 isolates of *Mycosphaerella* spp. This is evidenced by the higher coefficient of determination (R^2) of the quadratic model (0.764) compared to the linear model (0.458), indicating that the quadratic model explains a larger portion of the variability in the data. Additionally,

the root mean square error (*RMSE*) of prediction is lower for the quadratic model (0.225) than for the linear model (0.330), suggesting a better predictive capacity of the quadratic model. These differences indicate that the quadratic model fits the data better, providing a more accurate description of the relationship between DMI fungicides and EC_{50} data from *Mycosphaerella* spp. isolates. Therefore, based on the quadratic model, we detected cross-resistance between propiconazole and tebuconazole for *Mycosphaerella* spp., significant ($p < 0.001$).



Summary of polynomial model fit analyses
Checking linear and quadratic models

Predictors[a]	Linear	C.I.(95%)	p	Quadratic	C.I.(95%)	p
Intercept	-0.95	(-1.04 - -0.85)	***	-0.60	(-0.67 - -0.52)	***
x^1	0.61	(0.54 - 0.69)	***	1.80	(1.67 - 1.93)	***
x^2	...	...	...	0.59	(0.53 - 0.65)	***
F-statistic	$F(1, 315) = 265.84$	...	...	$F(2, 314) = 509.13$	...	...
R^2	0.458	...	...	0.764	...	...
Adjusted R^2	0.456	...	...	0.763	...	...
Observations	N = 317	...	...	N = 317	...	...
...	...	...	...	...	...	...
RMSE of prediction	0.330	...	...	0.225	...	...
Coefficient of determination (R^2)	0.688	...	...	0.845	...	...
Observations	N = 91	...	..	N = 91	...	...

[a] $y = \log EC_{50}$ propiconazole; $x = \log EC_{50}$ tebuconazole
RMSE = Root Mean Square Error is the standard deviation of the residuals (predicted errors)
*** $p < 0.001$

Figure 3. Correlation between the log EC_{50} data for sensitivity of the BSDC pathogens *Mycosphaerella* spp. (N = 51) to the DMI fungicides propiconazole and tebuconazole. The scatter plot illustrates the relationship between propiconazole concentrations (*y*-axis) and tebuconazole concentrations (*x*-axis) and the corresponding EC_{50} values. The purple lines represent the linear regression model (A) and the quadratic regression model (B).

4. DISCUSSION

Resistance to DMI fungicides was prevalent among the populations of *Mf* and *Mm* examined in banana plantations from Southeastern Brazil, with the majority of the isolates exhibiting reduced sensitivity (72.6% to 78.4%) to moderate resistance (21.6% to 25.5%) for propiconazole or tebuconazole. Only 2% of the isolates were sensitive to tebuconazole. This low sensitivity rate of the *Mf* and *Mm* isolates to DMIs

indicates that the frequently sprayed DMI fungicides, within the BSDC management system, exerted a high selection pressure on pathogen populations for resistance (SILVA et al., 2024). This underscores the urgent need to implement integrated disease management strategies aimed at reducing exclusive reliance on fungicides and incorporating less aggressive practices to mitigate resistance development (Ceresini et al., 2024; Silva et al., 2024).

It is important to highlight that all isolates in this study were also analyzed for resistance to QoI and SDHI fungicides, and many exhibited multiple resistance (Oliveira, 2022; Silva et al., 2024). For example, out of the nine *Mf* isolates examined, one (≈11%) showed resistance to the QoI fungicides azoxystrobin and trifloxystrobin (Oliveira et al., 2022), while all demonstrated resistance to SDHI fungicides boscalid and fluxapyroxad (Silva et al., 2024). Regarding *Mm,* among the 42 isolates evaluated, seven (≈17%) exhibited resistance to azoxystrobin and trifloxystrobin. All *Mm* isolates tested showed some degree of resistance to the SDHI fungicides boscalid and fluxapyroxad (Silva et al., 2024).

The disease management system for the BSDC may have contributed to the significant differences among the populations sampled regarding their resistance levels to DMI fungicides. For instance, the *Mf* population from Vale do Ribeira (SPVR_CI) exhibited significantly higher resistance to DMI fungicides compared to all other three sampled populations. It is noteworthy that banana plantations in Vale do Ribeira are subject to more intense fungicide spraying, with 8 to 14 annual preventive applications of fungicides for black Sigatoka control (Oliveira et al., 2022; Silva et al., 2024). In contrast, banana plantations from the other sampled regions, such as northwest São Paulo (SPNW-C) and northern Minas Gerais (MGN_C), are subjected to less intensive fungicide management systems (where fungicide use is reduced to four or five preventive sprays for Sigatoka control), including even no fungicide use (SPNW_O), probably resulting in lower resistance to DMIs. These findings underscore the significant influence of the fungicide management system on the evolution and spread of fungicide resistance within the BSDC (SILVA et al., 2024), while also suggesting the presence of other contributing factors to this complex dynamic. These factors may include specific agricultural practices, genetic

characteristics of local fungal populations, and multifaceted interactions among fungi, host plants, and the surrounding environment (Ceresini et al., 2024).

A total of 28 amino acid mutations have been identified in the CYP51 gene of *M. fijiensis* worldwide, including substitutions such as T18I, A19E, Y58F, I70M, D71E, V106D, V116L, Y136F, K171R, V260L, I264T, A313G, H380N, A381G, R418G, A446S, D460E, D460V, ΔY461, Y461D, Y461N, Y461S, G462A, G462D, Y463D, Y463H, Y463N , and Y463S (AGUIRRE, 2016).

In this study, substitutions identified in *Mf* CYP51 included T18I, V106D, Y461D, G462D, and Y463D. The G462D and Y463H substitutions were previously reported in Southeastern Brazil (Malimpensa, 2018), with Y463H not detected in our sampling.

For *Mm,* substitutions identified in CYP51 included V106D, Y136F, A446S, Y461H, Y461N, and Y463D. Y461N was reported in Central Western Brazil (DF)(Brito, 2015; Brito et al., 2020)), while all other substitutions are reported here for the first time in Brazil. Additionally, A381G and Y463H were reported in a single *Mm* isolate from São Paulo (Malimpensa, 2018) but were not detected in our sampling.

Regarding the former occurrence and geographical distribution of these CYP51 substitutions, the T18I substitution, found in all *Mf* isolates in our study, is widespread globally and documented in Latin America (Aguirre, 2016), now observed in Southeastern Brazil. The V106D substitution, detected in all *Mf* and *Mm* isolates here, has been reported in *Mf* populations from various continents (Aguirre, 2016), now confirmed in Brazil. The Y136F and Y461D substitutions in *Mm* were previously documented in *Mf* from Colombia and other countries (Aguirre, 2016; Cañas-Gutiérrez et al., 2009), now reported for the first time in Brazil. The A446S mutation in *Mm* was previously observed in *Mf* from the Philippines (Aguirre, 2016), newly identified here in Brazil. The Y461H mutation in *Mm* is a novel discovery globally, reported for the first time here but noted in the wheat pathogen *Zymoseptoria tritici* (Cools et al., 2010). Y461N was identified in *Mm* in Brazil (Brito, 2015) and *Mf* in other countries (Aguirre, 2016). The G462D mutation in *Mf* was previously reported in Brazil (Malimpensa, 2018). The Y463D mutation, found in both *Mf* and *Mm*, was described

in *Mf* from Colombia and Brazil (Aguirre, 2016; Brito, 2015; Brito et al., 2020; Cañas-Gutiérrez et al., 2009), now also observed in *Mm* in Brazil.

The analysis of mutation combinations present in the Mf and Mm isolates allowed the identification of eight distinct protein variants of the CYP51 gene. Notably, none of the *Mf* and *M*m isolates in our sampling exhibited the wild-type phenotype without mutations. The identified CYP51 protein variants contained two to four substitutions per isolate (Figure 2). These CYP51 variants were designated as follows: A) V106D and A446S (*Mm*) with 33 isolates; B) V106D, Y136F, and A446S (*Mm*) with two isolates; C) V106D, A446S, and Y461N (*Mm*) with three isolates; D) V106D, A446S, Y461H, and Y463D (*Mm*) with one isolate; E) V106D, A446S, and Y463D (*Mm*) with three isolates; F) T18I, V106D, and Y463D (*Mf)* with five isolates; G) T18I, V106D, and Y461D (*Mf*) with three isolates; H) T18I, V106D, and G462D (*Mf*) with one isolate. These haplotypes highlight significant mutation diversity, revealing a complex genetic landscape within these fungal populations.

Despite the several aminoacid substitutions detected in the inferred *Mf* and *Mm* CYP51 protein, only few had significant phenotypic effects (Figure 2; Table 4): The Y461H and Y461N substitutions, found in *Mm*, contribute to reduced sensitivity to both tebuconazole and propiconazole; the Y463D substitution, detected in multiple protein variants (*Mm* D, *Mm* E, *Mf* F), lead to reduced sensitivity or moderate resistance to both fungicides, with varying EC_{50} values; the Y461D mutation, along with G462D, both in *Mf*, contributes to moderate resistance (MR) to both tebuconazole and propiconazole.

Recently, another mechanism of DMI resistance in *Mf* was identified, involving overexpression of the *CYP51* gene due to the insertion of small repetitive sequences in its promoter region (Diaz-Trujillo et al., 2018). These repetitive sequences were also transmitted across generations of the fungal pathogen (Aguirre, 2016). However, it is important to note that this mechanism was not detected in the C*YP51* gene of the isolates examined in ower study. MDR (multidrug resistance) such as overexpression of target genes and multidrug efflux pumps was detected in a selection of *Mf* and *Mm* isolates examined in this study (Silva et al., 2024).

In summary, the current study represents a significant contribution to understanding the mechanisms, emergence, and spread of DMI fungicide resistance in populations of *Mf* and *Mm* in Brazil. It also underscores the impact of extensive use of chemical fungicides and the associated risks of selecting and dispersing resistant populations of the pathogens (Ceresini et al., 2024; Silva et al., 2024). To mitigate these risks, it is imperative to consider innovative disease management approaches that minimize reliance on preventive fungicide sprays with medium to high resistance risk (Ceresini et al., 2024).

One proposed solution to fungicide resistance involves implementing a smart spraying system that is not based on fixed spray schedules but rather on real-time information about the temporal dynamics of airborne inoculum of these phytopathogens (i.e., aerobiology data) and epidemic risks (Ceresini et al., 2024; Vicentini et al., 2023; West et al., 2008). This system would enable more precise and timely decision-making on when and what to spray. For example, if the presence of fungal inoculum with resistance alleles to major fungicide groups such as DMIs is detected, the system would alternatively recommend spraying only low-resistance risk active ingredients, thereby reducing selective pressure on pathogens and the likelihood of resistance fixation in populations (Ceresini et al., 2024).

This intelligent, data-driven, and evolutionary-adaptive approach guided by aerobiology data of *Mf* and *Mm* would promote more sustainable and effective management of banana Sigatoka complex diseases, helping to preserve fungicide efficacy, mitigate risks associated with fungicide resistance in agroecosystems, and ensure food security for the population (Ceresini et al., 2024; Vicentini et al., 2023).

5. CONCLUSIONS

DMI fungicide resistance prevalence: Widespread resistance to DMI fungicides was observed among *Mf* and *Mm* populations in banana plantations of Southeastern Brazil, with most isolates showing reduced sensitivity (72.6% to 78.4%) to moderate resistance (21.6% to 25.5%) for propiconazole or tebuconazole.

Mutation diversity and resistance association: Mutations like Y461H and Y461N in Mm, and Y461D and G462D in Mf, were associated with reduced sensitivity or moderate resistance to DMIs.

Influence of fungicide management system: Variations in resistance levels were strongly influenced by the intensity of fungicide management practices. The Mf population from Vale do Ribeira exhibited notably higher resistance due to more intense fungicide spraying compared to regions with less intensive fungicide use.

Recommendations for integrated disease management: Urgently implement integrated disease management strategies to reduce reliance solely on fungicides and adopt less aggressive practices to mitigate resistance development. Develop and deploy smart spraying systems guided by real-time aerobiology data to optimize fungicide application and preserve efficacy.

6. FUNDING

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CHAPTER 2. AIRBORNE INOCULUM MONITORING OF *MYCOSPHAERELLA FIJIENSIS* AND *M. MUSICOLA* AND MOLECULAR DETECTION OF STROBILURIN RESISTANCE IN SPORE SAMPLES FROM BANANA PLANTATIONS IN SÃO PAULO STATE

ABSTRACT

The chemical control of Black and Yelllow Sigatoka leaf diseases on bananas, respectively caused by *Mycosphaerella fijiensis* (*Mf*) and *M. musicola* (*Mm*), is relevant due to high yield losses reported in the most susceptible plant varieties and to support more sustainable agricultural practices. However, the intensive spray of fungicides led to the development of resistance and consequent loss of efficacy, demanding new management strategies. This study tested the application of an automated system for airborne spore sampling coupled with real-time quantitative PCR (RT-qPCR) to monitor the aerobiology of *Mf* and *Mm* and to detect mutations associated with resistance to QoI (external quinone inhibitors or strobilurins) fungicides in two banana-producing regions in São Paulo State. Total fungal DNA was extracted from the airborne spore samples and it was subsequently analyzed by qPCR to quantify the fluctuation in the prevalence of *Mf* and *Mm* along the year. Specific probes designed for the target-gene *cytB* were then used to detect the prevalence of the fungicide resistance-allele associated with QoI resistance in the spore samples. The RT-qPCR assay accurately detected resistant *Mf* and *Mm* present in the aerobiology samples. This molecular approach can guide the choice and the optimal timing for fungicide spraying. Furthermore, the developed technology represents a significant advancement in disease management as agricultural innovation.

Keywords: Banana leaf spot diseases. Fungicide resistance. Sigatoka disease complex. Aerobiology monitoring. Disease management innovation.

CAPÍTULO 2. MONITORAMENTO DE INÓCULO AÉREO DE *MYCOSPHAERELLA FIJIENSIS* E *M. MUSICOLA* E DETECÇÃO MOLECULAR DE RESISTÊNCIA À ESTROBILURINA EM AMOSTRAS DE ESPOROS EM PLANTAÇÕES DE BANANEIRA DO ESTADO DE SÃO PAULO

RESUMO

O controle químico das doenças foliares Sigatoka Negra e Amarela em bananeiras, causadas por *Mycosphaerella fijiensis* (*Mf*) e *M. musicola* (*Mm*), respectivamente, é crucial devido às altas perdas de produção em variedades suscetíveis e à falta de variedades resistentes. No entanto, a pulverização intensiva de fungicidas levou ao desenvolvimento de resistência e à consequente perda de eficácia, destacando a necessidade de novas estratégias de manejo. Este estudo testou a aplicação de um sistema automatizado para amostragem de esporos em aerossol, associado à PCR quantitativo em tempo real (RT-qPCR), para monitorar a aerobiologia de *Mf* e *Mm* e detectar mutação associada à resistência aos fungicidas QoI (estrobirulinas) em duas regiões produtoras de banana em São Paulo. O DNA fúngico total foi extraído das amostras de esporos em aerossol obtidas e subsequentemente analisadas por RT-qPCR para quantificar a flutuação na prevalência de *Mf* e Mm ao longo do ano. Sondas específicas desenhadas para o gene-alvo *cytB* foram então usadas para detectar a prevalência do alelo associado à resistência a fungicidas QoI em amostras de esporos em aerossol dessas duas plantações de banana. O ensaio de RT-qPCR detectou com acurácia *Mf* e *Mm* resistentes em amostras de aerobiologia. Essa abordagem molecular poderá orientar a escolha e o momento ideal para a pulverização de fungicidas. Além disso, a tecnologia desenvolvida representa um avanço significativo no manejo de doenças em bananeira como inovação agrícola.

Palavras-chave: Doenças foliares da bananeira. Resistência a fungicidas. Patógenos do complexo de Sigatokas. Monitoramento aerobiológico. Inovação no manejo de doenças.

1. INTRODUCTION

Banana (*Musa* spp.) is a widely cultivated fruit crop consumed worldwide, playing a crucial role in the food security and in the economy of many countries, including Brazil, one of the leading global suppliers (BRITO et al., 2020; BRITO et al., 2015). However, banana yield faces major challenges due to the impact of two severe fungal foliar diseases: the Black Sigatoka, caused by *Mycosphaerella fijiensis* (*Mf*) infection, and the Yellow Sigatoka, caused by *Mycosphaerella musicola* (*Mm*). Black Sigatoka causes the highest yield losses on banana production in Brazil (Brito et al., 2020; Brito et al., 2015).

The negative impact of these two foliar diseases results from severe epidemics, which are mainly associated with the pathogens' ability to produce substantial amounts of spores - both asexual and sexual - during their life cycle. Furthermore, recent studies highlighted the high genetic variation within populations of *Mf* and *Mm*, driven by both sexual reproduction and gene flow. This genetic diversity gives these pathogens a high potential for evolution and adaptation (Brito et al., 2020; Manzo-Sánchez et al., 2019; Mendonza and Ardales, 2019). Symptoms of these diseases include spots and discolorations on banana leaves, primarily affecting young and growing leaves. Yellow Sigatoka begins with mild discolorations, progressing to spots between the leaf's secondary veins, while Black Sigatoka is characterized by initial brown streaks on the leaf's abaxial surface, which later become black streaks with or without a yellow halo. Progressive leaf destruction reduces the plant's photosynthetic capacity, negatively impacting fruit production (Amorim et al., 2016).

Genetic resistance to both Sigatoka diseases is absent or partial in most of the commercial banana cultivars (Churchil, 2011). Therefore, disease control strategies are highly dependent on programmed and frequent calendar-based fungicide sprays (Brito et al., 2015). Contrastingly, in commercial banana plantations from the Ribeira Valley, Brazil, the control of Black Sigatoka is based on weekly monitoring of the disease, which still results in as much as 15–20 fungicide sprays per year, specially under high disease pressure (Uchôa et al. 2021). The systemic site-specifc QoI (external quinone inhibitors, or strobilurins) and DMI (demethylation inhibitors, or triazoles) fungicides are the most frequently sprayed for Sigatokas control (Churchil,

2011). The consequences of the excessive use of fungicides are increased production costs, negative impact on the environment, and high selective pressure on both pathogens' populations, which can lead to emergence, selection and spread of fungicide resistant *Mf* and *Mm* strains (Ceresini et al., 2024; Oliveira et al., 2022).

The effectiveness of chemical control is heavily dependent on the proper timing of fungicide applications (Ceresini et al., 2024; Oliveira et al., 2022). In this context, monitoring the dynamics of *Mf* and *Mm* inocula in banana-producing regions plays a significant role to guide growers' decisions on the optimal timing for fungicide sprays. This research aimed to test the application of an automated system for airborne spore sampling, coupled with real-time quantitative PCR (RT-qPCR), to monitor the aerobiology of *Mf* and *Mm*, and to detect a mutation associated with resistance to QoI fungicides in two banana-producing regions in São Paulo State. Specifically, we employed species specific probes for monitoring the fluctuation in the prevalence of *Mf* and *Mm* in airborne spore samples collected along one year. Subsequently, we targeted the detection of the G428C mutation in the *cyt*B gene (associated with the amino acid substitution G143A) (Oliveira et al., 2022).

If successful, this smart platform could support a disease epidemic risk alert system for Black and Yellow Sigatoka pathogens based on real time inoculum monitoring and fungicide resistance detection, like the one described for wheat blast disease (Ceresini et al., 2024; Vicentini et al., 2023). Local banana growers could then have a choice between adopting the conventional calendar-based, preventive fungicide sprays or the alternative smart inoculum-based disease epidemic risk alert system. We foresee this real time inoculum monitoring platform as a significant contribution towards a sustainable integrated disease management of Sigatoka diseases in São Paulo.

2. MATERIAL AND METHODS

2.1.AEROBIOLOGICAL SAMPLING

Aerobiological samples were collected from two banana plantations in São Paulo State (SP): one in Ilha Solteira municipality (20º25'58" S; 51º20'33" W), in the

Northwest area of SP (SPNW), and another in Registro municipality (24º29'15" S; 47º50'37" W), in the Ribeira Valley (SPRV). Airborne spores were collected daily, 2020 to 20201, in 2 mL tubes using the automated high-volume cyclone system, from Agri Samplers Ltd (Cressex Enterprise Centre, Cressex Business Park, Lincoln Rd. High Wycombe, Buckinghamshire, HP12 3RL, United Kingdom) (VICENTINI et al., 2023). The high-volume cyclone air sampler captured aerosols for 12 h per day, from 6am to 6pm, positioned within the limits of banana plantations at 4.5 m high above the ground level. The airflow was set according to the manufacturer's standard configuration of 270 L min^{-1}. The 64 samples obtained from both areas were organized and stored in a freezer at -20ºC for subsequent DNA extraction (Vicentini et al., 2023).

2.2. DNA EXTRACTION FROM AEROBIOLOGICAL SAMPLES

A modified phenol-chloroform DNA extraction protocol was adopted (Vicentini et al., 2023). This modified DNA extraction protocol consisted of the following steps: a) Adding 0.5 g of sterile glass beads (400 - 455 µm in diameter; Sigma, Saint Louis, Missouri, USA) to the tubes containing samples of airborne spores; b) Grinding of samples in a benchtop Fastprep FP120 (Thermo Fisher, Waltham, MA, USA), a reciprocating device for cell membrane disruption, at a speed of 4 $m.s^{-1}$ for 2 rounds; b) preheating 750 µL of extraction buffer (6 g of polivinilpirrolidona PVP-40; 1.5 g of PVP-360; 3 g of CTAB; 42 mL of 5M NaCl; 6 mL of 0.5M EDTA; and 15 mL of 1M Tris-HCl, pH 8.0, to a final volume of 150 mL) at 65ºC, with the addition of 2 µL of beta-mercaptoethanol; d) adding the buffer to the ground sample and gently mixing by inversion; e) incubating the samples for 45 minutes, with homogenization every 10 minutes; f) adding 450 µL of CIA - chloroform:isoamyl alcohol 24:1, with mixing on a tube shaker for 1 minute and 30 seconds; g) centrifuging the samples at 10,000 rpm, at 20ºC; h) collecting the supernatant and transferring it to a new test tube, followed by the addition of 400 µL of isopropanol (chilled) and 1 µL of the coprecipitant GlycoBlue™ (Sigma Aldrich); i) DNA recovery, with centrifugation of the solution at 10,000 rpm for 5 minutes; j) discarding the supernatant; k) adding 500 µL of 70% ethanol to release the pellet from the tube wall; l) shaking and centrifuging at 12,000 rpm for 5 minutes; m) repeating the step of discarding the 70% ethanol and adding new 500 µL of 70%

ethanol; n) discarding the 70% ethanol and adding 100% ethanol; o) shaking and centrifuging at 12,000 rpm for 5 minutes; p) discarding the 100% ethanol; q) transferring the samples for drying in a laminar flow chamber for 2 hours; r) resuspending the pellet in 40 µL of Tris + 4 µL of RNAse; s) incubating at 37ºC for 30 minutes in a water bath. The extracted DNA from the samples was then quantified using a NanoDrop™ 2000c spectrophotometer.

2.3. DETECTION AND QUANTIFICATION OF THE PREVALENCE OF *M. FIJIENSIS* AND *M. MUSICOLA* IN AEROBIOLOGICAL SAMPLES

Initially, the 64 DNA samples were used for RT-qPCR detection and quantification of the prevalence of the *Mycosphaerella* species associated with the banana Sigatoka complex. Species-specific probes and primers sets were used (Table 1).

Preliminarily, a conventional PCR was performed to amplify a fragment of the β-tubulin gene from the isolates *Mf* JA2.7a and *Mm* ISC14, using species-specific primers (Table 1). The resulting PCR amplicons were used as a DNA template for the standard curves of the subsequent RT-qPCR. PCR reactions were carried out in a final volume of 30 µL containing ultrapure water, 50 ng of total DNA, 0.1 µM of each primer (Table 1), 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 3.0 µL of 10x buffer, and 1 U of Taq DNA Polymerase (Sigma-Aldrich, Saint Louis, MO, USA). Amplifications were performed on a Mastercycler® Nexus thermocycler (Eppendorf®, Hamburg, Germany) using the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, annealing temperature at 55°C for 1 min (Arzanlou et al., 2008), and extension at 72°C for 1 min; with a final extension at 72°C for 5 min.

Positive amplification of the two β-tubulin gene PCR amplicons from *Mf* and *Mm* was confirmed by electrophoresis on 1% agarose gel. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), and stored for further use at the RT-qPCR assay. For quantitative calibration of the RT-qPCR standard curves, a serial dilution of a DNA template of the β-tubulin gene from *Mf* or *Mm* was prepared to obtain the following concentrations in a final reaction volume of 20 µL: 112.5, 56.25, 28.12, 14.06, 7.03, 3.52, and 1.76 pg mL^{-1}.

Table 1. Specific primers and probes used for the detection of *Mycosphaerella fijiensis* and *M. musicola* in aerobiological samples of airborne spores collected using spore samplers installed in two banana plantations from São Paulo, Brazil.

	Stage	**Primers and Probes**	**Sequence (5'-3')**
M. fijiensis	PCR [a]	Mf_b_tub2_130F	AAACCGGCCAGTGTGTATGTAT
		Mf_b_tub2_454_R	TGGGACATACTTGTTGCCAGAG
	qPCR [b]	MFBP - Probe	**HEX**-CTGAGCACGACTGACCACAACGCA-**BHQ1**
		MFBF	CGACACAGCAAGAGCAGCTTC
		MFBRtaq	TTCGAAAGCCTTGGCACTTCAA
M. musicola	PCR [a]	Mm_b_tub2_226F	CCAAATAGGTGCTGCATTCTGG
		Mm_b_tub2_479R	TGGGACATACTTGTTGCCAGAG
	qPCR [b]	FMEP - Probe	**FAM**-CACGTCTGATCTCCAGCTCGAGCGCATG-**BHQ1**
		MMBF	CACACATCAAGAGCAGCACAG
		MMBRtaq	TGGCACTTGGCGGAAGTTTG

[a] These primers were designed specifically for this study for the conventional PCR stage, based on beta-tubulin gene sequences of *M. fijiensis* and *M. musicola* available on GenBank/NCBI. The PCR products generated (324 bp for *Mf* and 253 bp for *Mm*) were used, after purification, for constructing standard detection curves of the pathogens by RT-qPCR. [b] Primers developed by Arzanlou et al. (2008).

Since the probes were labeled with distinct fluorophores (Table 1), RT-qPCR reactions were prepared in multiplex in the same reaction, with both primer pairs and probes for species identification, with a final reaction volume of 20 µL, containing 10 µL of 1X iTaq Probe Master Mix (Bio Rad, Hercules, CA, USA), 1 µL of forward primer at 300 nM, 1 µL of reverse primer at 300 nM, 1 µL of reverse primer at 300 nM, 1 µL of reverse primer at 300 nM, 1 µL of the probe at 150 nM (Table 1), 3 µL of ultrapure deionized water, and 2.5 µL of DNA template from the unknown sample. The reactions were performed using the CFX96 qPCR thermocycler (Bio Rad, Hercules, CA, USA). The following amplification conditions were applied: 2 min at 95°C; (2 - 5 seconds at

95 °C, 15 - 30 seconds at 60 °C) for 40 cycles. Each RT-qPCR reaction was performed in duplicate. Results were analyzed using the Bio Rad CFX Maestro software.

2.4. THE PREVALENCE OF *CYTB* ALLELES OF *M. FIJIENSIS* AND *M. MUSICOLA* IN AEROBIOLOGICAL SAMPLES WITH HIGH CONCENTRATION OF FUNGAL DNA FROM THE PATHOGENS

Aerobiological samples with high amounts of the β-tubulin gene from *Mf* DNA (n = 7) or *Mm* DNA (n = 7) were selected for RT-qPCR detection and quantification of the prevalence of *cyt*B gene alleles that confer either sensitivity (wt G428) or resistance to QoI fungicides (mut G428C, equivalent to the amino acid substitution G143A). New probes for RT-qPCR of the *cyt*B gene of the pathogens and allele-specific primers were designed for this study based on gene sequences available in GenBank/NCBI and generated by Oliveira et al. (2022) for both *Mf* and *Mm* (Table 2, Figure 1).

Initially, DNA samples from two *Mf* isolates (QoI sensitive *Mf* SA14 and QoI resistant *Mf* JA 2.24) and two others from *Mm* isolates (QoI sensitive *Mm* ISR70 and Qo-I resistant *Mm* ISC9) were used in this stage to generate target DNA of the *cytB* gene of *Mf* and *Mm* for subsequent use in the calibration of the standard RT-qPCR curves. For this purpose, conventional PCR was performed with specific primers for the *cyt*B gene of *Mf* or *Mm*, developed for this study, generating PCR amplicons of 398 and 439 bp, respectively, for each species (Table 2, Figure 1). PCR reactions were conducted under the same conditions described previously, with a final reaction volume of 30 µL. Positive amplification of the gene was verified by electrophoresis in 1% agarose gel. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), and stored for subsequent use.

Table 2. Primers and specific probes designed in our study for RT-qPCR detection and quantification of the relative frequencies of the *cytB* gene alleles conferring sensitivity (wt G428) or resistance (*mut G428C*, equivalent to the aminoacid substitution G143A) to QoI fungicides in aerobiological samples of airborne spores from *Mycosphaerella fijiensis* or *M. musicola.*

Organism	Stage	Primers and probes	Sequence (5'-3')
M. fijiensis	PCR[a]	cytB_Mf_89F	CTGGTGTTACTTTAGCCATGCA
		cytB_Mf_468R	CGTCGCCGTAATGTGGTTCA
	qPCR[b]	cytB_Mf_240F_probe	**FAM**-CGTAGGAAGAGGTTTATACTATGGA-**BHQ1**
		cytB_Mf_193F	TTACACTCAAATACTGCCTCAGC
		cytB_Mf_368R_wt_G	ACTGTAGCT**C**CTCATAAAGACA
		cytB_Mf_368R_mut_C	ACTGTAGCT**G**CTCATAAAGACA
M. musicola	PCR[a]	cytB_Mm_1533_F	TAGTTCTAATGATGGCTACCGC
		cytB_Mm_1971_R	GGTGTTTGCATAGGGTTAGCC
	qPCR[b]	cytB_Mm_1662R_probe	**FAM**-AACAGAAAACCCACCTCAAATAA-**BHQ1**
		cytB_Mm_1581F_wt_G	GTCAAATGTCTTTATGAG**G**AGCTACAG
		cytB_Mm_1581F_mut_C	GTCAAATGTCTTTATGAG**C**AGCTACAG
		cytB_Mm_1722_R	GCAGCTAATACGAATGGAAGAACAA

[a] Specific primers were developed for the conventional PCR stage from this study, based on the cytB gene sequences of M. fijiensis and M. musicola, which were previously deposited in the GenBank/NCBI by Oliveira et al. 2022. The resulting PCR products, measuring 398 bp for *M. fijiensis* and 439 bp for *M. musicola*, were purified and subsequently used for constructing standard detection curves of the genes using RT-qPCR technique. [b] Specific probes were also developed for the RT-qPCR stage, ensuring accuracy in pathogen detection and quantification.

For quantitative calibration of the standard RT-qPCR curves, a serial dilution of a DNA template pool of the two isolates of the *cytB* gene of *Mf* or *Mm* was prepared to obtain the following concentrations in a final reaction volume of 20 µL: 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, and 1.9 pg mL^{-1}.

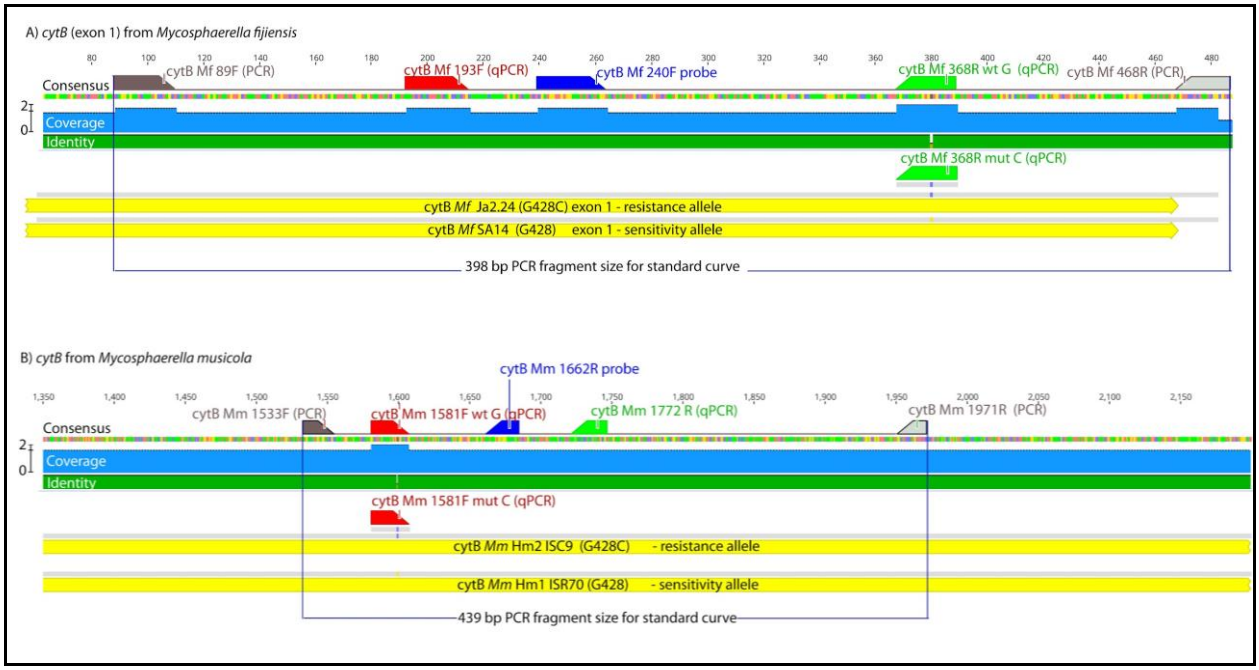

Figure 1. Schematic representation of new primers and probes developed by the authors for the detection and quantification of the relative frequency of *cyt*B gene alleles [conferring sensitivity (wt G428) or resistance to QoI strobilurin fungicides (mut G428C, equivalent to the amino acid substitution G143A)] in aerobiological samples of airbone spores from *Mycosphaerella fijiensis* (A) and *M. musicola* (B).

2.5 DESCRIPTIVE REPRESENTATION OF TEMPORAL SERIES AND STATISTICAL ANALYSIS.

Temporal series of daily quantities of the the β-tubulin gene from *Mf* and *Mm* DNA detected in aerobiological samples using RT-qPCR were summarized in a figure using packages associated with the statistical software R (available online at URL http://www.r-project.org/index.html, accessed on September 25, 2023): *dplyr*, *lubridate*, *scales*, *gridExtra*, *ggthemes*, *ggplot2*, and the functions *geom_line*, *geom_point*, and *facet_wrap*, as described by Vicentini et al. (2023). Frequencies of *cytB* alleles wt G428 and mut G428C determined by RT-qPCR were used to estimate the prevalence of the respective alleles in the samples.

3. RESULTS AND DISCUSSION

Airborne fungal spores are key components for the epidemics of foliar pathosystems such as the Black and Yellow Sigatoka diseases, and spore counts have been used as an essential standard approach to assess and model epidemics of these diseases on bananas (UCHÔA et al., 2021).

The objective of this study was to develop a platform based on an automated spore sampling coupled with RT-qPCR for monitoring airborne inoculum of *Mf* and *Mm*, and to detect the QoI-resistance *cytB* allele in a temporal series of aerobiological samples collected in banana plantations from two distinct producing areas in São Paulo State. This approach is relevant to provide growers with critical information about the dynamics of airborne inoculum to assist in timely and effective decisions in managing Sigatoka diseases in banana plantations.

In the preliminary stage of the study, the use of RT-qPCR probes and primers developed by Arzanlou et al. (2010) based on the β-tubulin gene was highly effective in the specific detection and quantification of *Mf* and *Mm* inoculum in the 64 aerobiological samples analyzed (Figure 2). This approach provided a highly sensitive and selective tool for monitoring the presence and prevalence of these two pathogens in airborne inoculum from both banana plantations.

Therefore, we were able to describe the temporal dynamics of *Mf* and *Mm* inoculum in the banana plantations sampled from Ilha Solteira (SPNW) or Registro (SPRV), during 2020 and 2021 (Figure 2). The temporal dynamics revealed fluctuations in the airborne inoculum density of these two pathogens. The populations of *Mm* sampled during 2020 in Ilha Solteira (SPNW), municipality located in the Northwest of São Paulo, which is the driest region in the State, showed an increase in inoculum density (based on higher *Mm* β-tubulin gene concentration detected) around Julian Day 365. At this season of the year (summer), there is an increase in rainfall and temperature (Affonso et al., 2020). Furthermore, *Mm* predominated over *Mf* population in Northeastern São Paulo (Figure 2), which is considered out of the geographical distribution range for the Black Sigatoka pathogen in Brazil (Silva et al., 2024).

In contrast, higher amounts of *Mf* inoculum were detected in the aerobiological samples from the Ribeira Valley region (SPRV) during the entire sampling year. In fact, in the Ribeira Valley, the average humidity is higher and the rainfall is more evenly distributed annually in contrast with Northeastern São Paulo (Affonso et al., 2020; Emiliano et al., 2022). For Black Sigatoka in particular, the disease progress depended directly on the rainfall, besides higher temperature during the wet season, which were considered the two most essential weather conditions for disease outbreaks, consequently leading to higher spore production and release (Amorim et al., 2016; Uchôa et al., 2021). For instance, in the Ribeira Valley wet season, the the rate of disease increase was $r = 0.0003$ while in the dry season $r = 0.0001$ (Uchôa et al., 2021).

The important finding from our study that the prevalence of *Mf* was much higher than *Mm* in the humid and warm Ribeira Valley (a rate approximately 3:1; Figure 2), has been formerly addressed in the literature about the biology of the pathogens (Churchill, 2011; Silva et al., 2024). According to Churchill (2011), within the past 40 years, *Mf* had spread to most banana plantations globally, and effectively replaced the Yellow Sigatoka as the dominant leaf spot disease. This scenario is also plausible for the Ribeira Valley banana agroecosystem (Silva et al., 2024).

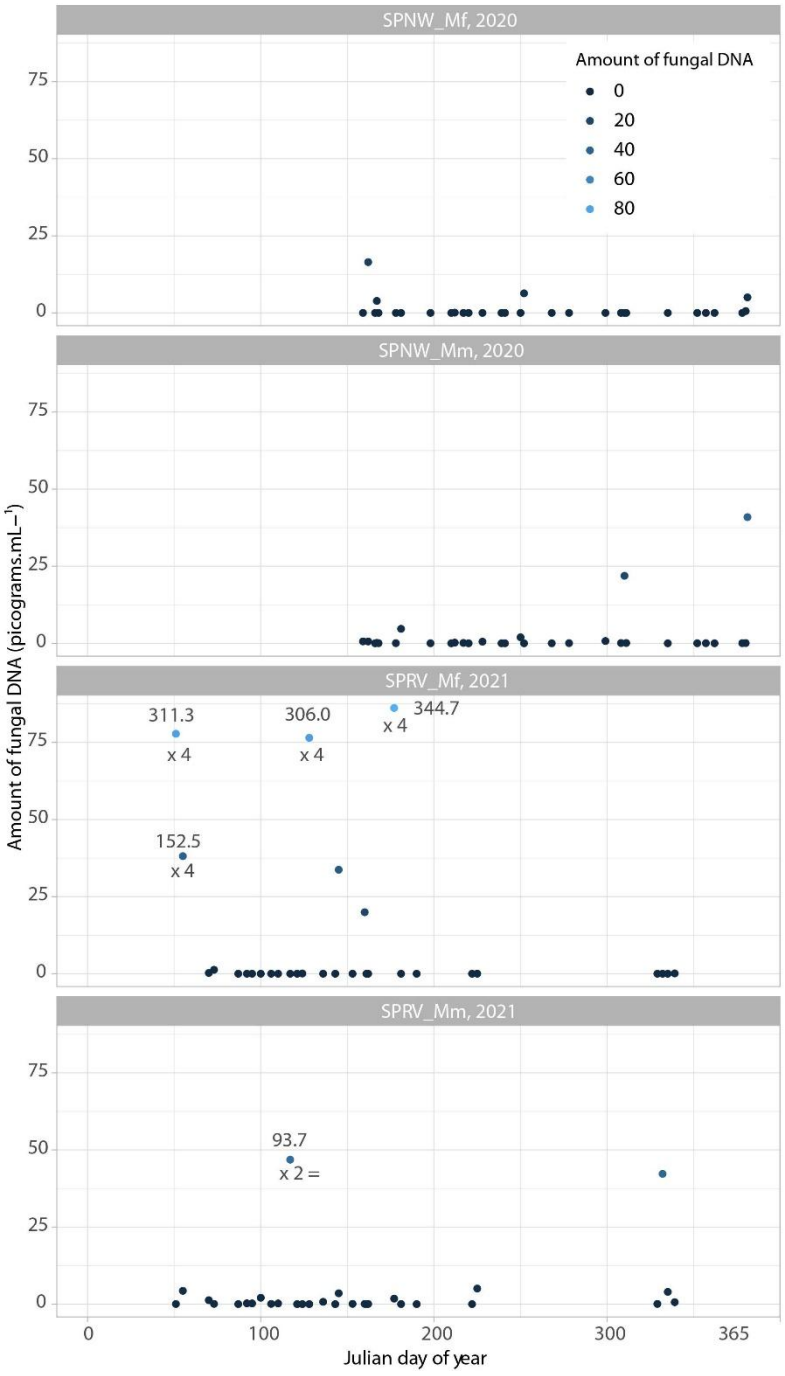

Figure 2. Molecular detection of fungal DNA of the β-tubulin gene in a temporal series of aerobiological samples from *Mf* and *Mm* spores collected during 2020 and 2021 in banana plantations from Ilha Solteira (SPNW) and Registro (SPRV) municipalities.

*Detection by RT-qPCR using specific β-tubulin gene primers and probes for the pathogens. To better visualize the amplitude of values, the scale was fixed at 75 picograms.mL^{-1}, and higher values are indicated numerically, with a multiplicative sign of 2 or 4 times. Peaks of fungal DNA detection are indicated by contrasting colors (> 5 picograms.mL^{-1} in dark blue, and > 90 to picograms.mL^{-1} in lighter shades of blue).

Monitoring inoculum dynamics evaluation over a temporal scale (Figure 2) provides to banana growers a concrete parameter to reduce the number of unnecessary preventive fungicide sprays (the so-called calendar spraying), in the absence of pathogens' inoculum in banana plantations and consequently, unfavorable conditions for the onset of banana Sigatoka epidemics (Ceresini et al., 2024).

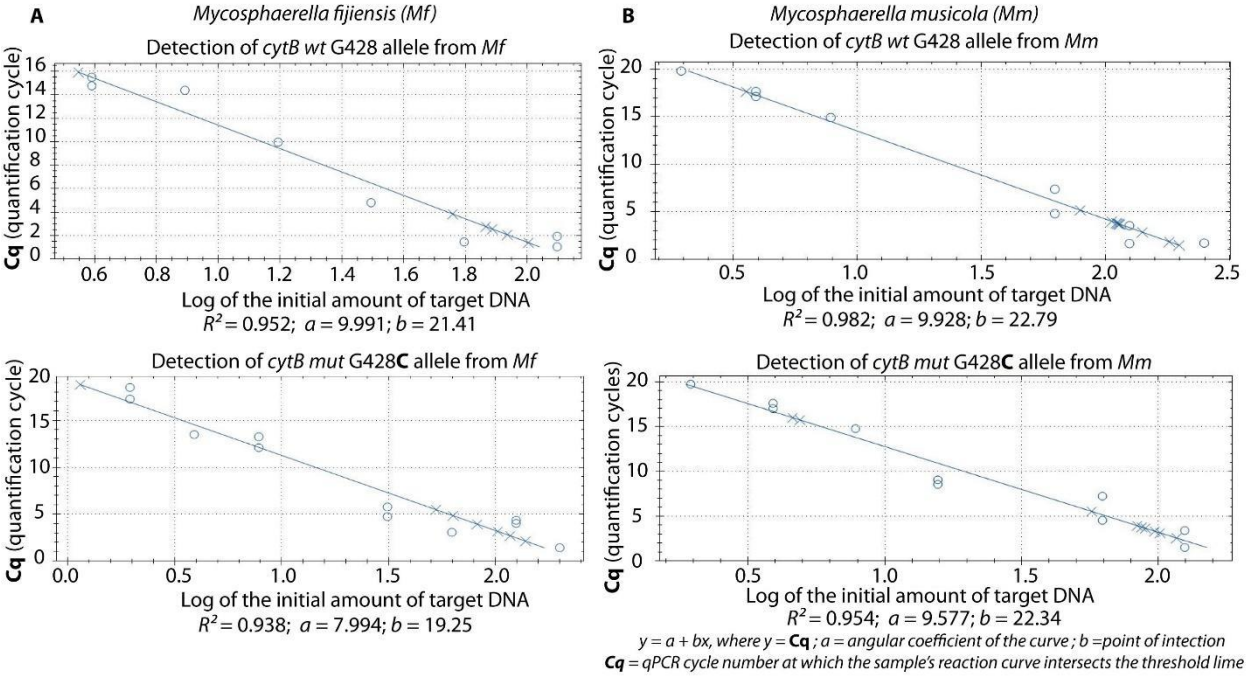

Figure 3. Standard curves of molecular detection and quantification by RT-qPCR of specific *cyt*B gene alleles of (A) *Mycosphaerella fijiensis* (*Mf*) and (B) *M. musicola* (*Mm*).

*Detection by RT-qPCR using probes for the *cytB* gene and allele-specific primers for quantifying the relative frequency of alleles conferring sensitivity (wt G428) or resistance to QoI strobilurin fungicides (mut G428C, equivalent to the amino acid substitution G143A). The Cq represents the number of cycles required for the fluorescence signal to reach the detection threshold. Absolute and relative quantification of nucleic acids in the samples is performed based on this Cq value.

In a subsequent assay of the study, using probes and primers developed by our research group for *Mf* and *Mm cytB* gene, we aimed to detect and quantify either the mutant allele mt G428C (resulting in the amino acid substitution G143A), associated with resistance to QoI fungicides (Oliveira et al., 2023), or the wild type allele wt G428. For this assay, samples of QoI sensitive or QoI resistant *Mf* or *Mm* isolates were used (Figure 3, Table 3). The use of allele-specific RT-qPCR (Figure 3) enabled unequivocal

detection and quantification of the wild-type allele (*cytB* wt G428), or the mutant allele (*cytB* mut G428C).

Subsequently, the platform for detection and quantification of wild-type or mutant *cyt*B alleles was applied to determine the prevalence of sensitivity or resistance to QoI fungicides in aerobiology samples with high concentration of *Mf* or *Mm* the β-tubulin gene (Table 3). In the samples used to detect the prevalence of wild-type or mutant *cyt*B alleles from *Mf*, the mutant allele represented, on average, 35.10 ± 11.67% of the total *cyt*B gene DNA detected, regardless of the sampled region (SPRV or SPNW). In the aerobiology samples used to detect the *cyt*B alleles from *Mm*, the mutant allele was not detected in one of the samples (SPRV 11/29/2021). Therefore, the prevalence of mutant alleles conferring resistance to QoIs averaged 69.31 ± 40.57%, either during periods of intense rainfall (11/06/2020) or during a drought period (06/30/2020). In three samples, the prevalence of the mutant cytB allele G428C was 100%.

From a scientific support standpoint to expand the scope of molecular monitoring of fungicide resistance in populations of other plant pathogens, the technique of RT qPCR targeting the *cyt*B gene has proven effective in identifying mutations associated with fungicide resistance in various other studies. In a recent study conducted by Cherrad et al. (2023), the pathogen *Plasmopara viticola*, responsible for downy mildew in grapevines, developed resistance to strobilurin fungicides due to a G143A mutation in the *cytB* gene. RT qPCR was used to detect the new sequence variant in the *cytB* gene associated with resistance.

Another study with the fungus *Cercospora beticola*, causing leaf spot in sugar beets, RT qPCR TR was used as an effective tool for detecting other variants of the *cytB* gene, associated with substitution such as F129L, G137R, and G143A, all conferring resistance to QoI fungicides, as reported by Birla et al. (2012). In another example of pathosystem, widespread resistance to QoI fungicides in populations of *Podosphaera xanthii* was examined in Spain, RT qPCR played a significant role in detecting the G143A substitution due to a mutation in the *cytB* (Vielba-Fernández et al., 2018). In all these studies, RT qPCR technique contributed to a deeper

understanding of fungicide resistance in different pathogens and providing valuable information for the development of more effective resistance management strategies.

Table 3. Concentration of mutant and wild-type alleles, and percentage prevalence of the mutant *cytB* allele in aerobiology samples of *Mycosphaerella fijiensis* and *M. musicola*.

Samples for detecting *Mf*	Concentration of the cytB wt G428 allele	Concentration of the cytB mut G428C allele	Relative prevalence of the mutant cytB allele (%)	Samples for detecting *Mm*	Concentration of the cytB wt G428 allele	Concentration of the cytB mut G428C allele	Relative prevalence of the mutant *cyt*B allele (%)
	(picograms.mL^{-1})				(picograms.mL^{-1})		
SPRV - 21/02/2021	4.66	1.86	28.54	SPRV - 28/04/2021	2.85	2.32	44.87
SPRV - 25/02/2021	4.33	6.22	58.96	SPRV - 14/08/2021	1.64	4.01	70.97
SPRV - 09/05/2021	10.32	3.67	26.22	SPRV - 29/11/2021	6.48	0.00	0
SPRV - 26/05/2021	1.21	0.63	34.36	SPRV - 02/12/2021	0.00	2.88	100
SPRV - 10/06/2021	4.50	3.08	40.67	SPNW - 30/06/2020	0.00	7.28	100
SPRV - 27/06/2021	8.74	3.80	30.30	SPNW - 06/11/2020	0.00	2.40	100
SPNW - 11/06/2020	14.744	5.35	26.64				

Finally, we postulate that the platform developed by our research group is a strategy with the potential to reduce the selection pressure for pathogen resistance exerted by the continuous use of QoI fungicides. This approach could play a pivotal role in the development of integrated disease management strategies for banana cultivation, contributing to long-term sustainability and productivity. This is because it provides a solid foundation for pathogens' airborne inoculum monitoring with positive implication for epidemics risk prediction (Vicentini et al., 2023; Ceresini et al., 2024).

4. CONCLUSIONS

In summary, the application of an automated system for airborne spore sampling coupled with real-time quantitative PCR (RT-qPCR) to monitor the aerobiology of *Mf* and *Mm* allowed the accurate molecular detection of both pathogens and the quantitation of the prevalence of a *cytB* gene mutation associated with resistance to QoI fungicides.

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