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Evidence of Resistance to QoI Fungicides in Contemporary Populations of *Mycosphaerella fijiensis*, *M. musicola* and *M. thailandica* from Banana Plantations in Southeastern Brazil

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Abstract: Yellow and black Sigatoka, caused by *Mycosphaerella fijiensis* and *M. musicola*, respectively, are the most important worldwide foliar diseases of bananas. Disease control is heavily dependent on intensive fungicide sprays, which increase selection pressure for fungicide resistance in pathogen populations. The primary objective of this study was to assess the level and spread of resistance to quinone-outside inhibitors (QoI—strobilurin) fungicides in populations of both pathogens sampled from banana fields under different fungicide spray regimes in Southeastern Brazil. Secondly, we aimed to investigate when QoI resistance was confirmed if this was associated with the target-site alteration G143A caused by a mutation in the mitochondrial encoded *cytochrome b* gene. QoI resistance was detected in fungicide treated banana fields, while no resistance was detected in the organic banana field. A total of 18.5% of the isolates sampled from the pathogens' populations were resistant to QoI. The newly described *M. thailandica* was also found. It was the second most abundant *Mycosphaerella* species associated with Sigatoka-like leaf spot symptoms in the Ribeira Valley and the highest level of QoI resistance was found for this pathogen. The G143A cytochrome *b* alteration was associated with the resistance to the QoI fungicides azoxystrobin and trifloxystrobin in *M. fijiensis*, *M. musicola* and *M. thailandica* strains. In order to reduce resistance development and maintain the efficacy of QoI fungicides, anti-resistance management strategies based on integrated disease management practices should be implemented to control the Sigatoka disease complex.

Keywords: chemical control; *cytochrome b*; fungicide resistance; quinone-outside inhibitors (QoI); black sigatoka; yellow sigatoka

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1. Introduction

Commercial banana production has been substantially reduced worldwide, for more than two decades, due to the incidence of leaf spots caused by different members of the Sigatoka disease complex. While black Sigatoka is caused by the ascomycetous fungus *Mycosphaerella fijiensis* M. Morelet (*Mf*) (syn. *Pseudocercospora fijiensis* (M. Morelet) Deighton), yellow Sigatoka is associated with *M. musicola* Leach. (*Mm*) (syn. *Pseudocercospora musae* (Zimm.) Deighton) [1–7]. *Mycosphaerella musae* (Speg.) Syd. & P. Syd. [1] can also cause leaf spot, as well as the newly described species *M. thailandica* Crous, Himaman & M. J. Wingf. (*Mt*) (syn. *Parapallidocercospora thailandica* (Crous, Himaman & M.J. Wingfield) Videira & Crous) [1,8], which is highly prevalent in banana plantations from Ribeira Valley, São Paulo,

Brazil [2]. *Mycosphaerella eumusae* Crous & X. Mourichon (*Me*) (syn. *Pseudocercospora eumusae* Crous & Mour), another member of the Sigatoka disease complex, has a considerably lower incidence in Brazil [6].

Sigatoka diseases are polycyclic, in which high numbers of asexual spores (conidia) are produced and released in multiple cycles, resulting in severe epidemics under favorable (wet) weather conditions. The disease cycle also includes sexual spores (ascospores) that are usually produced once a year through sexual reproduction. Therefore, the pathogens' populations are expected to have a mixed reproductive system with occurrence of cyclic sexual reproduction and predominance of epidemic clonal dispersal [9,10]. Recent studies from Brazil, Mexico and the Philippines have revealed that *Mf* and *Mm* populations have high genotypic diversity from both sexual reproduction and genotype flow from long distance migration input, resulting in high evolutionary potential for both pathogens [4,11–13]. Since varietal resistance is practically absent or only partially effective in most widely grown banana cultivars, the intensive and mostly preventive spray application of site-specific systemic fungicides has been the main strategy for the management of the Sigatoka diseases complex, resulting in strong selection pressure for fungicide resistance in the pathogens' populations [4,14,15].

In the late 1990's, quinone outside inhibitors (QoI), also known as strobilurins, a class of systemic fungicides with a new single-site mode of action were introduced to the market [16]. Due to superior efficacy, at that time [17], and broad spectrum activity, they were soon also used for managing black and yellow Sigatoka on bananas in Brazil [18–20]. The QoIs azoxystrobin, pyraclostrobin, trifloxystrobin and kresoxim-methyl were introduced and formulated as straights or in mixtures with azoles, dehydrogenase inhibitors (SDHIs) and/or multi-site inhibitors (mancozeb and chlorothalonil), totaling more than 20 commercial products [16].

The synthetic molecule design of QoIs was based on natural fungicidal metabolite derivatives of β-methoxy acrylic acid, such as strobilurin A, produced by the basidiomycete wood-rotting fungi *Strobilurus tenacellus* [21,22]. The binding site of QoIs is the cytochrome *b* (cyt *b*), a subunit of the cyt bc_1 (complex located inside the mitochondrial membrane. The mode of action is the inhibition of mitochondrial respiration by specifically binding to the oxidation site of quinol (Qo, or ubiquinol) in cyt *b*, blocking the transfer of electrons between cyt *b* and cyt c_1. Therefore, these fungicides prevent the NADH oxidation and the ATP synthesis leading to inhibition of the energy production essential to spore germination and survival [21,23].

Emergence and spread of fungicide resistance rapidly followed the introduction of the QoI fungicides for managing crop diseases worldwide, spanning many distinct pathosystems [24,25]. Because QoI fungicides are classified as a high risk for resistance development [25], their intensive use has resulted in several reports of resistance in many pathosystems, including the Sigatoka complex pathogens [24,26]. For instance, in the early 2000's, after only three years since the QoI azoxystrobin was labeled in Costa Rica, the intensive spraying of this fungicide on banana fields resulted in the early detection of resistance in *Mf* populations [27,28]. QoI-resistant populations of Sigatoka pathogens have been reported in Central and South America, Africa and Australia [15,25,26,29,30], while in Brazil there has been only a single report, from a dissertation, of a few QoI resistant strains [2].

In terms of mechanisms of QoI resistance, of the several *cyt b* mutations that can confer QoI insensitivity, the mutation resulting in the substitution of glycine by alanine at codon 143 (G143A) of cyt *b* has been associated with poor field performance of QoIs in a range of pathogens [25,31]. This target site alteration was also reported in resistant strains of *Mm* from Australia [15], *Mf* from Costa Rica [25,27], and for some *Mf* strains from Brazil [2].

There is a lack of information about the sensitivity status, and frequency distribution of resistance in Brazilian populations of the Sigatoka diseases pathogens to QoI fungicides. In the face of a high selection pressure due to the historical intensive use of QoI fungicides in local banana plantations we hypothesized that resistance to QoI is pervasive in fields with conventional and/or intensive fungicide spraying systems. This lack of information about the sensitivity status of resistance to QoI fungicides is risky as the continuous spraying of QoIs would only increase the frequency of resistant strains in populations of both *Mf* and

Mm, and their spread across distinct fields via gene flow. As a result, it would eventually contribute to the declining efficacy of the QoI fungicides.

Therefore, the objective of this study was to assess the development and spread of resistance to QoI fungicides in populations of *Mf* and *Mm* from bananas in Southeastern Brazil, both phenotypically and genotypically. For hypothesis testing, fields from three contrasting disease management systems were sampled from Vale do Ribeira and Northwestern São Paulo, and from Northern Minas Gerais. If resistance was detected, we also investigated if this was associated with the G143A cyt b target alteration.

2. Materials and Methods

2.1. Yellow and Black Sigatoka Pathogens' Population Sampling

For obtaining *Mm* and *Mf* populations for this study, samples of infected leaves were collected in 2020 from four locations with three contrasting disease management systems: (i) Intensive, in Vale do Ribeira, São Paulo (SPVR-I), from Jacupiranga, Registro and Sete Barras counties (Figures 1 and 2A), and banana varieties Prata (AAB triploid [18], also known as 'lady finger banana') and Nanica (AAA, Cavendish subgroup [23]); (ii) Conventional, in Northeastern São Paulo (SPNW-C), from Ilha Solteira county, var. Maçã (AAB [18]), and in Northern Minas Gerais (MGN-C) from Janaúba county, var. Prata and Nanica; (iii) Organic (without fungicide application), in Northeastern São Paulo (SPNW-O), from Ilha Solteira county, with different cultivars and plant ages.

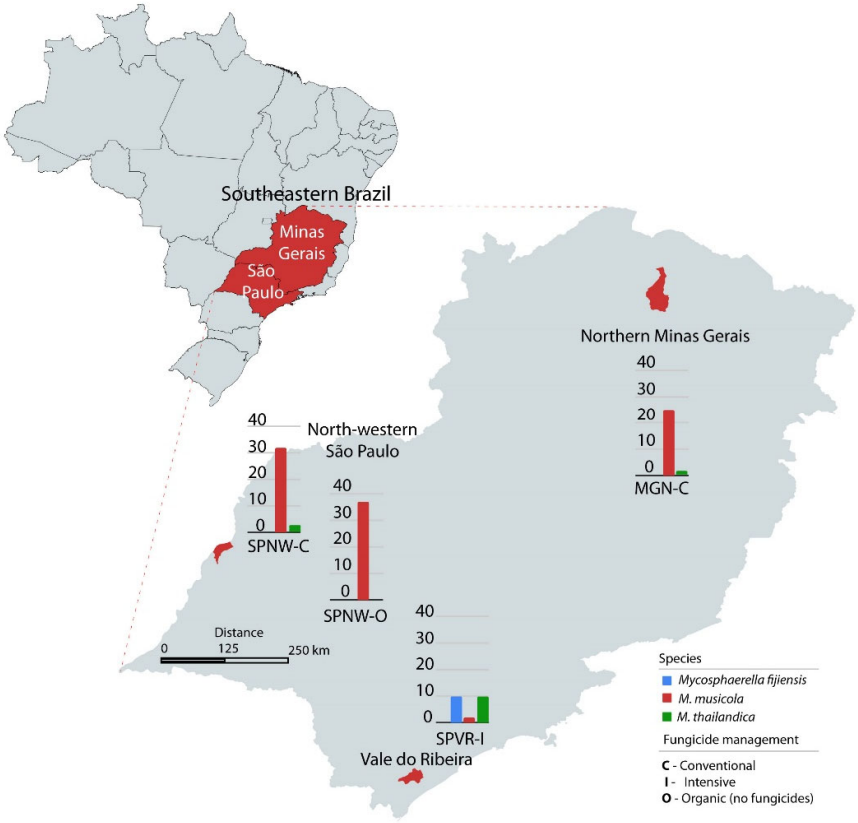

Figure 1. Population sampling of the black leaf streak (*Mycosphaerella fijiensis*), yellow Sigatoka (*M. musicola*), and leaf spot (*M. thailandica*) pathogens from banana plantations with contrasting fungicide management in Southeastern Brazil during the 2020/21 cropping season [a]. [a] States and counties were colored in red: Ilha Solteira county, from Northwestern Sâo Paulo state (SP), Jacupiranga, Registro and Sete Barras counties from Vale do Ribeira (SP), and from Janaúba county, from Minas Gerais State (MG). In addition to *M. fijiensis* and *M. musicola*, the species *M. thailandica* was a prevalent co-inhabitant with the black leaf streak and yellow Sigatoka pathogens in three of the populations sampled.

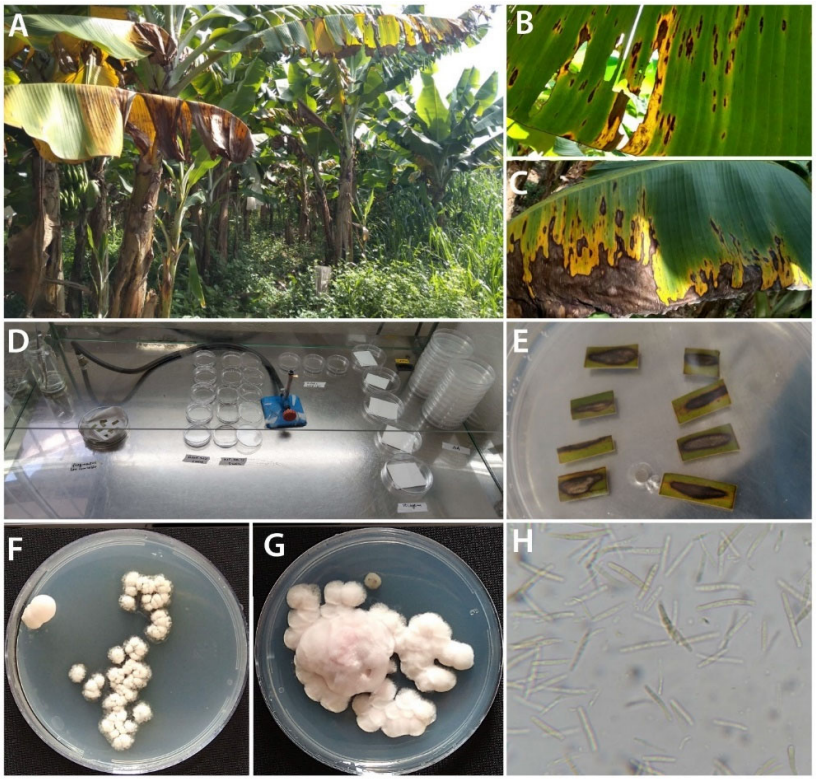

Figure 2. Banana plantation in Vale do Ribeira, SP (**A**). Infected banana leaves showing symptoms of yellow (**B**). and black (**C**). Sigatoka. Material used for indirect isolation of Sigatokas' fungal pathogens (**D**). Fragments of banana leaves used for isolation (**E**). *Mycosphaerella fijiensis* colonies on PDA after 15 (**F**). and 60 (**G**). days incubation at 26 °C. Conidia suspension from a *M. musicola* colony grown for 15 days on PDA (**H**). Source: photos by the authors.

In SPVR-I, the management was based on intensive fungicide sprays, with around 8 to 14 fungicide calendar-based applications per year to control black Sigatoka, mostly because of the extremely predisponent weather conditions for the disease, which includes high humidity (>90%), the presence of leaf wetness and temperatures ranging from 26–28 °C [2,19,32–34]. The Vale do Ribeira region represents the highest banana production both in São Paulo State and Brazil, where the susceptible banana varieties Prata and Nanica predominate [7,19,33]. In plantations from these susceptible banana varieties under the predominant highly favorable weather conditions in the Vale do Ribeira, the pathogens' incubation period could range from as low as 13 to 14 days, whereas under unfavorable weather the incubation period could be extended up to 35 days [7,19,33,34]. Intensive fungicides sprays are usually scheduled to coincide with the continuous emergence of new banana leaves, which are highly susceptible to infection [7]. The SPNW-C and MG-C banana plantations were under reduced fungicide application management, with 4–5 sprays against yellow Sigatoka.

Infected banana leaf fragments with 20 cm^2 of area presenting typical yellow (Figure 2B) or black Sigatoka (Figure 2C) symptoms were sampled from five plants within a 50 m^2 radius, from 20 to 40 points randomly distributed in a field. The samples were packaged in paper bags, transported to the lab and kept in a refrigerator until the isolation of the pathogens.

2.2. *Mycosphaerella* Isolation

Leaf samples were surface cleaned with tap water, cut into 2 cm^2 pieces, and treated with 75% ethanol and sterile distilled water, for one minute each (Figure 2D). The leaf pieces were then dried in sterile filter paper, transferred to Petri dishes with water–agar (15 g L^{-1} agar) medium amended with the antibiotics chloramphenicol and streptomycin (50 μgmL^{-1} each) (Figure 2E), and incubated at 26 °C in the dark until *Mf* or

Mm sporodochia formed and were detected under a stereomicroscope. Using a thin needle, conidia were transferred from the lesions to Petri dishes containing PDA [20.8 g L^{-1} potato-dextrose (Kasvi, India), 15 g L^{-1} agar] amended with antibiotics (as above), streak-plated, and kept at 26 °C in the dark until characteristic *Mycosphaerella* micro-colonies were detected, and typical fungal conidia were observed (Figure 2F–H). Young colonies were transferred to new PDA plates [adapted from 5 and 8]. Long term storage of isolates was performed by initially transferring 0.5 cm^2 pieces of sterilized filter paper to the top of actively growing 14-day-old *Mycosphaerella* colonies on PDA. Subsequently, the pieces of filter paper colonized by the fungus mycelium were transferred to cryotubes with silica gel for cryo-preservation at −20 °C.

2.3. Molecular Identification of the Pathogens

To identify the isolates at the species level, a total of 119 fungal isolates (37 isolates from SPNW-O (ISR strains), 34 isolates from SPNW-C (ISC strains), 26 isolates from MGN-C (MG strains), and 22 isolates from SPVR-I (VR strains)), were firstly analyzed if harboring typical morphological characteristics of *M. fijiensis* or *M. musicola* [1,6]. Secondly, the species were identified based on PCR assay [27,28]. Mycelial fragments with 2 cm^2 diameter colonies grown in PDA media for 10 days at 26 °C in the dark were harvested for lyophilization. Genomic DNA was extracted from lyophilized mycelia using the kit Wizard® Genomic DNA Purification (Promega, Madison, WI), according to the manufacturer's instructions. DNA was quantified in a Nanodrop® 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 50 ηg·μL^{-1} final concentration. The PCR-based species identification was performed using specific primers for *Mf* (ACTR: 5′-GCAATGATCTTGACCTTCAT-3′; MFactF: 5′-CTCATGAAGATCTTGGCTGAG-3′) and *Mm* (MMactF2: 5′-ACGGCCAGGTCATCACT-3′; MMactRb: 5′-GCGCATGGAAACATGA-3′), designed from actin gene sequences [2,30,35]. PCR amplifications were conducted in 15 μL volumes containing ultrapure distilled water, 50 ηg of template DNA, 0.3 μM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1.5 μL of 10× PCR reaction buffer and 0.05 U Taq Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Amplifications were performed in a ProFlex PCR thermal cycler (Applied Biosystems, Waltham, MA, USA), with cycling conditions as follows: initial denaturation at 95 °C for 5 min, followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s, and at 72 °C for 1 min; with a final extension of 72 °C for 7 min. The amplicons were analyzed by agarose gel electrophoresis, where a 500 bp would indicate *Mf*, while a 200 bp fragment would indicate *Mm* identity. Positive *Mf* and *Mm* DNA controls were included in the assay, and negative DNA controls from the basidiomycetous fungus *Rhizoctonia solani* Kühn AG-1 IA, and from the ascomycetous *Pyricularia oryzae* Cavara lineage *Triticum*. To confirm the isolates' identity, we also analyzed the specific amplification of the *Mf cyt b* and *Mm cyt b* genes (Table 1).

Table 1. Specific primers for PCR amplifications, and to evaluate the variation at the *cyt b* gene in *Mycosphaerella fijiensis*, *M. musicola* and *M. thailandica*.

[a] Target gene	Primer	Sequence (5′-3′)	Amplicon Size (bp)	Annealing Temperature (°C)
[b] *Mf cyt b*	cytb_exon1-1_Mf_F2036	CGTCGCCGTAATGTGGTTC	475	56
	cytb_exon1-1_Mf_R2511	GCCGCAACCTTCTAATATTAG		
	cytb_exon2_Mf_F25	CGTGCTTCTGATTCTATTAGGGG	996	56
	cytb_exon2_Mf_R1020	GGCGACTACCAACACAAAT		
[b] *Mm cyt b*	cytb_Mm_F1251	GTTACCTTTGAAACTTCGGATC	963	58.5
	cytb_Mm_R2213	GACTCAACGTGTTTAGCCC		
[c] *Mt cyt b*	cytb_Mt_F2	GAAGCATTTAATTCAGTAGAAC	400	58
	cytb_Mt_R2	CAACTATATCTTGTCCTACTC		

[a] *Mf = Mycosphaerella fijiensis; Mm = Mycosphaerella musicola; Mt = M. thailandica.* [b] primers designed during this study. [c] primers from Malimpensa [2].

Thirteen isolates of *Mycosphaerella* for which the PCR-based species identification failed were identified based on the sequence analyses of the fungal ITS-rDNA region amplified with ITS1 and ITS4 primers [36] with an annealing temperature of 60 °C, using similar PCR conditions as described above. The PCR products were purified and sequenced by the Center for Biological Resources and Genomic Biology (Crebio, UNESP, Jaboticabal, Brazil), using the ABI 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA). DNA sequences were analyzed and aligned using the Geneious R 9.0.5 (Biomatters, Auckland, New Zealand), and compared with the ITS-rDNA sequences from type species of *Mycosphaerella* available at the GenBank/NCBI databases. Based on ITS-rDNA sequence similarity, these isolates (Mt ISC57 and Mt ISC86, from SPNW-C; Mt MG60 from MGN-C; Mt FRA1.21b, Mt JA1.34, Mt JA1.4, Mt JA2.13, Mt JA2.15, Mt JA2.16a, Mt JA2.2p, Mt JA2.3a, Mt JA2.6 and Mt T06 from SPVR-I) were identified as *M. thailandica*.

2.4. QoI Qualitative Fungicide Sensitivity Assays at Discriminatory Dose

One hundred and nineteen *Mycosphaerella* spp. isolates whose molecular species identification has been confirmed from the SPNW-O (N = 37 *Mm* strains), SPNW-C (N = 32 *Mm, 2 Mt*), MGN-C (N = *25 Mm, 1 Mt*) and SPVR-I (N = *10 Mf, 2 Mm, 10 Mt*) populations were used for fungicide sensitivity experiments. The fungicides Priori (QoI-azoxystrobin at 250 g L^{-1}; Syngenta S.A.) and Flint 500 WG (QoI-trifloxystrobin at 500 g Kg^{-1}; Bayer SA) were diluted in deionized water to produce a 500 $\mu g \cdot mL^{-1}$ of stock solutions used in all experiments.

To obtain a suspension of mycelial fragments, a protocol established in this study was used, by which 4 cm^2 of fungal mycelial disks were taken from fungal colonies grown on PDA at 25 °C for 10 days. The fungal mycelial disks were transferred to microtubes containing 0.5 mL of sterile 2 mm glass beads and 1 mL of sterile distilled water. The tubes were sealed and placed in a Fast Prep FP120 Bio 101 (Thermo Savant, Waltham, MA, USA) for 20 s at speed 4 $m \cdot s^{-1}$ to disrupt the mycelial mass and to obtain a suspension of mycelial fragments [14].

The qualitative assay to evaluate the QoI fungicide sensitivity by Sigatoka pathogens was conducted using Petri dishes containing PDA with trifloxystrobin (TRI) or azoxystrobin (AZX) at the discriminatory dose of 10 $\mu g \cdot mL^{-1}$ [29,30], or no fungicide, all amended with salicylhydroxamic acid (SHAM) at 0.5 mM [25,37] and antibiotics as above. The fungal inoculum was prepared using sterile filter paper discs soaked in mycelial fragments suspension, which were then transferred to the assay media. For each isolate and treatment (no fungicide, TRI, and AZX), four plates containing four inoculum discs were used. These experimental plates were sealed, randomly positioned inside the incubator, and kept for 15 days at 26 °C in the dark for colony growth. The entire assay was repeated once.

The two-proportions z-test based on Pearson's Chi-square at $p \leq 0.05$ was used to compare the equality of two observed proportions of QoI resistant isolates from populations of *M. fijiensis*, *M. musicola and M. thailandica* from banana plantations under distinct fungicide management systems [38,39].

Analysis of variance (ANOVA) by the *F* test and means comparison were performed using the R environment with the statistical libraries *agricolae* and *laercio* [39]. For means comparison we applied the Scott–Knott test (at $p \leq 0.05$) to contrast between groups of QoI resistant (QoI-R) and QoI sensitive (QoI-S) isolates of *M. musicola* and *M. fijiensis* per geographical population of the pathogen.

2.5. QoI Quantitative Fungicide Sensitivity Assays Based on EC_{50} Values

A qualitative assay to determine the EC_{50} for TRI and AZX was conducted using six fungicide doses: 0.0, 0.1, 1, 10, 100 and 1000 $\mu g \cdot mL^{-1}$. It consisted of a spectrophotometric assay for fungicide sensitivity testing based on the reduction of resazurin to resorufin, a metabolic indicator for fungal respiration activity [40–42]. Ten isolates, from the three *Mycosphaerella* species detected in our survey, were chosen for this assay: three QoI sensitive (*Mf* CALT1, *Mm* ISC111 and *Mt* ISC86) and seven QoI resistant (*Mf* JA2.24, *Mm* ISC64, *Mm* JA1.37, *Mm* JA1.38c, *Mt* JA2.13, *Mt* JA2.2 and *Mt* JA2.6). A modification of the flat-

bottomed 96-well microtiter plate method was adopted to measure the fungal growth in each fungicide dose [37,43], using the mycelial fragments protocol [4,44]. Mycelium fragments were obtained from *Mycosphaerella* colonies that were grown at 25 °C under 12 h photoperiod for 10 days in PDA supplemented with antibiotics as described above. Fungal mycelial samples from a 10-day old colony from a single agar plate were transferred to 1.5 mL microtubes containing 0.5 mL of 0.1 mm diameter glass beads. A total of 1 mL of distilled water was added to the mixture, and vortexed for two minutes in a Fast-Prep apparatus for 20 s at speed 4 $m \cdot s^{-1}$. The mycelial fragments suspension recovered from the tubes were mixed and pre-diluted to a final volume of 10 mL, counted under a microscope using a Neubauer chamber and adjusted to 10^4 fragments mL^{-1}. Each microplate well was filled with 50 µL of inoculum suspension and 100 µL of PD broth (20.7 $g \cdot L^{-1}$ of potato dextrose (Kasvi, India), prepared with 0.025M phosphate buffer, and final pH adjusted to 5.0) amended with different concentrations of TRI and AZX and in the presence of chloramphenicol and streptomycin (50 $mg \cdot L^{-1}$ each). Salicylhydroxamic acid (SHAM) at 0.5 mM was also added to the PD broth to suppress alternative oxidase (AOX) activity, following the procedures described by Ma et al. [45] and Vicentini et al. [37]. The microplates with fungal liquid cultures were wrapped in plastic and incubated at 25 °C for 10 days under a 12 h photoperiod, when the fungal growth reached its maximum. After incubation, 50 µL of resazurin at 160 µM was added to each microplate well to obtain a final concentration of 40 µM in a final volume of 200 µL. For spectrophotometric estimates of RZ reduction, initial absorbance readings at 569 ηm ($Abs_{569\ \eta m}$ at T_0) were taken using a microplate reader (Multiskan™, FC Microplate Photometer, Thermo Scientific™, Waltham, MA, USA). Subsequently, the microplates were kept at 25 °C under complete darkness for 24 h, when final absorbance readings were then taken at the same wavelengths ($Abs_{569\ \eta m}$ at T_{24}). The RZ reduction was estimated as follows:

$RR_{Mycosphaerella} = T_0 - T_{24}$, where:
$RR_{Mycosphaerella}$ = relative reduction of resazurin;
T = Absorbance reading at 569 ηm;
T_0 = reading at time zero (immediately after adding resazurin to the fungal 10-day-old liquid culture in buffered PD broth);
T_{24} = reading at time 24 (24 h after adding resazurin).

Sensitivity to the two QoI fungicides was determined as 50% effective concentration to inhibit fungal growth (EC_{50}, in $\mu g \cdot mL^{-1}$), estimated using the R package *ec50estimator* coupled with the *drc* library for analyses of dose response curves [46], from multiple isolates data sets. This package also allows for testing the hypothesis on the most fit model function (*fct*) for estimating the EC_{50} by a dose–response curve. Confidence intervals were also estimated.

For EC_{50} means comparison within each fungicide (TRI or AZX), the experimental design consisted of complete randomized blocks, with four replicates per treatment and experiments in duplicate. Analysis of variance (ANOVA) and the Scott–Knott test (at 5% probability) for means comparison were performed in the R environment using the statistical packages *agricolae* and ScottKnott [39]).

2.6. Analysis of Allelic Variation in Target Gene cyt b in Mycosphaerella Populations

To examine variation in the target gene *cyt b* encoding the QoI fungicide binding sites, all 96 *Mm* from populations SPNW-O (N = 37), SPNW-C (N = 32), MGN-C (N = 25), and SPVR-I (N = 2), ten *Mf* isolates from population SPVR-I (N = 10) and 13 *Mt* isolates from populations SPNW- SPNW-C (N = 2), MGN-C (N = 1), and SPVR-I (N = 10) were used for PCR amplifications and further sequencing. PCRs were conducted in 30 µL volume containing ultrapure distilled water, 50 ηg of template DNA, 0.3 µM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 3 µL of 10× PCR reaction buffer and 0.05 U Taq Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 36 cycles of 94 °C for 30 s, 1 min at the specific annealing temperature for each primer pair (Table 1) and at 72 °C for 1 min; with a final extension of 72 °C for 7 min. The PCR products were purified and sequenced by the Crebio (UNESP

Jaboticabal Campus, Jaboticabal, SP, Brazil), using the ABI 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA). DNA sequences were analyzed and aligned using the Geneious R 9.0.5 (Biomatters, Auckland, New Zealand) program to identify alleles, haplotypes and distinguish non-synonymous mutations leading to amino-acid changes in inferred protein sequences. For annotation and inference of protein sequences from the *cyt* b sequences, we included the following reference sequences obtained from the NCBI/GenBank®: NC037198.1 (*Pseudocercospora mori*) and LFZO01000638.1 (*Mm*) for *Mm cyt b*; NC044132, AF343069 and AF343070 (*Mf*) for *Mf cyt b*. There were no sequences from *M. thailandica cyt b* gene available at GenBank for comparison.

3. Results

Our sampling strategy resulted in 96 isolates of *Mm* from all four populations sampled, 10 isolates of *Mf*, only detected in the population SPVR-I, and 13 isolates of *Mt* obtained from populations SPNW-C, MGN-C and SPVR-I (Figure 1, Table 2). The discriminatory dose of azoxystrobin (Figure 3B,E) and trifloxystrobin (Figure 3C,F) allowed to identify the resistant strains among *Mf*, *Mm* and *Mt* isolates samples. From the total of 119 isolates screened, 22 (≈18%) were resistant to both QoI fungicides: from population SPNW-C, isolates *Mm* ISC64 (Figure 3A–C), *Mm* ISC09, *Mm* ISC55a, *Mm* ISC110, *Mm* ISC116, *Mm* ISC117, and Mt *ISC57*; from population MGN-C, isolate *Mm* MG118, and *Mt* MG60; and from population SPVR-I, isolates *Mm* JA1.37, *Mm* JA1.38c, *Mf* JA2.24, *Mt* FRA1.21b, *Mt* JA1.34, *Mt* JA1.4, *Mt* JA2.13, *Mt* JA2.15, *Mt* JA2.16a, *Mt* JA2.2p, *Mt* JA2.3a, *Mt* JA2.6, and *Mt* T06).

Table 2. Distribution of QoI fungicide resistance in populations of *Mycosphaerella musicola*, *M. fijiensis* and *M. thailandica* sampled from four banana plantations with different fungicide management systems [a].

Species, Type of Management with Fungicides and Population	Average Number of Fungicide Sprays per Crop	Number of Isolates with Growth In Culture Medium Supplemented with 0 or 10 µg·mL^{-1} of the QoI Fungicides Azoxystrobin or Trifloxystrobin		
		µg·mL^{-1}		Resistant Isolates (Proportion)
		0	10	
Mycosphaerella musicola				
Organic field				
SPNW-O	0	37	0	0.0000
Conventional fields				
SPNW-C	4	32	6	0.1875
MGN-C	5	25	1	0.0400
Intensive field				
SPVR-I	>10	2	2	1.0000
M. fijiensis				
Intensive field				
SPVR-I	>10	10	1	0.1000
M. thailandica				
Conventional fields				
SPNW-C	4	2	1	0.5000
MGN-C	5	1	1	1.0000
Intensive field				
SPVR-I	>10	10	10	1.0000
Total		119	22	0.1849

[a] SPNW-O (North-western São Paulo, organic field), SPNW-C (North-western São Paulo, with fungicide conventional management system), MGN-C (Northern Minas Gerais, with fungicide conventional management system), and SPVR-I (São Paulo, Vale do Ribeira, fungicide intensive management system).

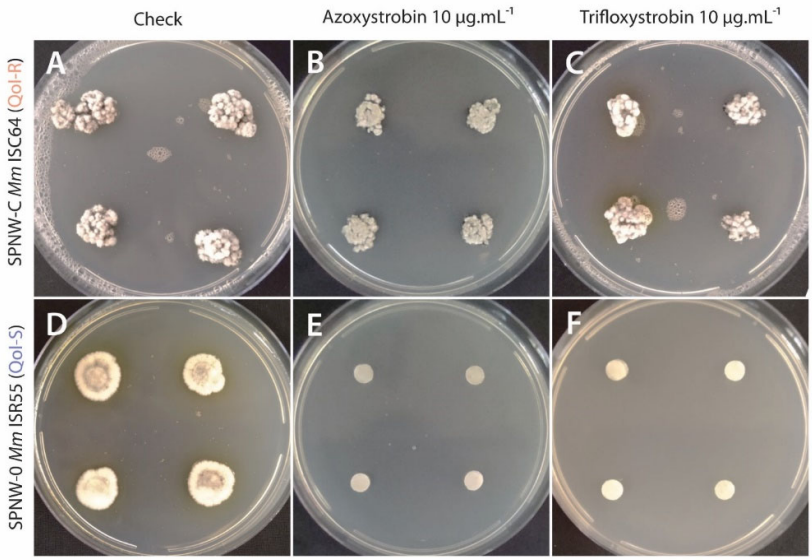

Figure 3. Two isolates of Mycosphaerella musicola with contrasting sensitivity to QoI fungicides [a]. [a] Colonies grown on potato dextrose agar medium without fungicide (**A**,**D**) or amended with azoxystrobin (**B**,**E**) or trifloxystrobin (**C**,**F**) at a discriminatory dose of 10 µg·mL^{-1}; SPNW-C *Mm* isolate ISC64 is resistant to QoI (QoI-R) while SPNW-O *Mm* isolate ISR55 is sensitive to QoI (QoI-S).

The sensitivity assays also revealed cross-resistance to both QoIs fungicides trifloxystrobin and azoxystrobin as all strains showed resistance to both compounds (Table 2). The frequency distribution of resistant and sensitive isolates to QoI fungicides indicated no resistant individuals in the population *Mm* SPNW-O (organic field) (37 strains tested, whereas 7% (two out of 25) were detected in the *Mn* population MGN-C (conventional fungicide management), 20% (seven out of 34) in the population SPNW-C (conventional fungicide management), and 100% (two out of two) in the *Mn* population SPVR-I (intensive fungicide management). For the *Mf* SPVR-I population, one out of ten strains (10%) was resistant to both QoIs tested. In comparison, 50% QoI resistant individuals (one out of two) were detected in the *Mt* population MGN-C, 100% (one out of one) in the population SPNW-C, whereas 100% (10 out of 10) were detected in the *Mt* SPVR-I population.

The two-proportions z-test at $p \leq 0.05$ used to compare the equality of two observed overall proportions of QoI resistant isolates of *Mf*, *Mm* and *Mt* (Table 3) indicated, in fact, that the population SPNW-O (from an organic banana field), with no resistant isolates, was significantly different from populations SPNW-C and SPVR-I (from conventional or intensive management systems), with proportions varying from 20 to 59% of resistant isolates.

Table 3. The two-proportions z-test based on Pearson's Chi-square used to compare the equality of two observed proportions of QoI resistant isolates from populations of *Mycosphaerella musicola*, *M. fijiensis*, and *M. thailandica* from banana plantations under distinct fungicide management [a, b].

	Pearson's Chi-Square Values below the Diagonal and *p* Values above the Diagonal			
Comparisons	**SPNW-O**	**SPNW-C**	**MGN-C**	**SPVR-I**
SPNW-O	-	0.012	0.325	0.000
SPNW-C	6.29	-	0.307	0.008
MGN-C	0.97	1.04	-	0.000
SPVR-I	24.71	7.03	12.34	-

[a] Test statistic with Yates' continuity correction. [b] SPNW-O (North-western São Paulo, organic field), SPNW-C (North-western São Paulo, with fungicide conventional management), MGN-C (Northern Minas Gerais, with fungicide conventional management), and SPVR-I (São Paulo, Vale do Ribeira, fungicide intensive management). Gray shading emphasizes the significant pairwise comparisons between populations.

The contrast between groups of QoI resistant (QoI-R) and QoI sensitive (QoI-S) isolates of *Mycosphaerella* species per geographical population of the pathogen, indicated that only the groups of QoI-R isolates were able to grow at the discriminatory dose of 10 μg·mL^{-1} of azoxystrobin or trifloxystrobin. In addition, on average, the groups of isolates from the populations SPNW-C and SPVR-I were significantly different from the population MGN-C, showing higher relative growth in QoI-fungicide amended medium (Figure 4).

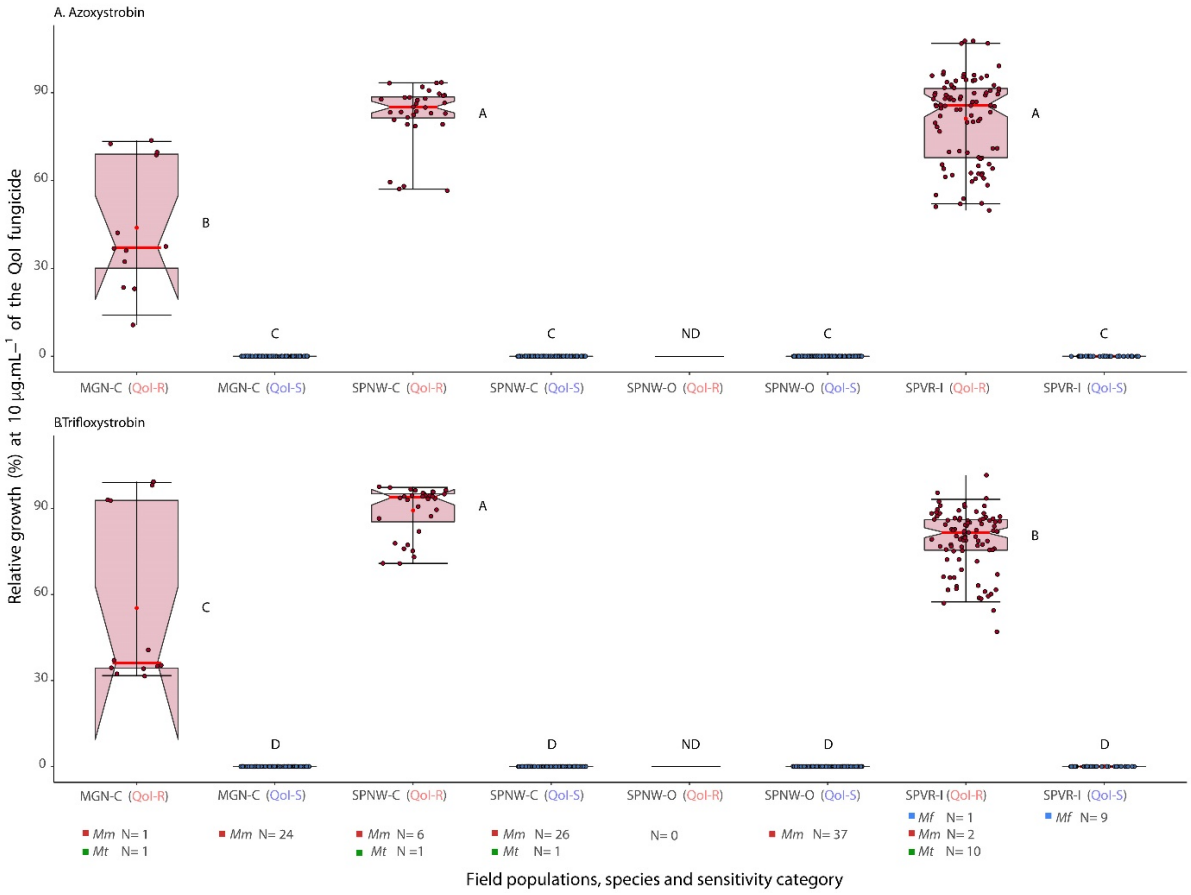

Figure 4. Contrast between groups of QoI resistant (QoI-R) and QoI sensitive (QoI-S) isolates of Mycosphaerella species per geographical population of the pathogen, according to the relative growth at 10 μg·mL^{-1} of azoxystrobin or trifloxystrobin [a,b,c]. [a] The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in dark red or dark blue dots to avoid data overplotting. [b] N = number of QoI-R or QoI-S isolates of *Mf* (blue square in the x axys), *Mm* (red square) and *Mt* (green square) detected per population. [c] F test from ANOVA significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$. N.D. = non-detected.

Based on a QoI quantitative fungicide sensitivity assay, *Mycosphaerella* isolates were grouped into six different sensitivity phenotype classes for TRI or AZX: sensitive (*Mf* CALT1 and *Mt* ISC86, $EC_{50} < 1$ μg·mL^{-1}), less sensitive (*Mm* ISC111, $EC_{50} > 1$ and < 5 μg·mL^{-1}), moderately resistant (*Mm* JA1.37, $EC_{50} > 5$ and < 25 μg·mL^{-1}), resistant (*Mm* ISC64 and *Mm* JA1.38c, $EC_{50} > 25$ and < 75 μg·mL^{-1}), highly resistant (*Mf* JA2.24 and *Mt* JA2.13, $EC_{50} > 75$ and < 150 μg·mL^{-1}), and extremely resistant (*Mt* JA2.2 and *Mt* JA2.6A, $EC_{50} >> 150$ μg·mL^{-1}) (Figure 5).

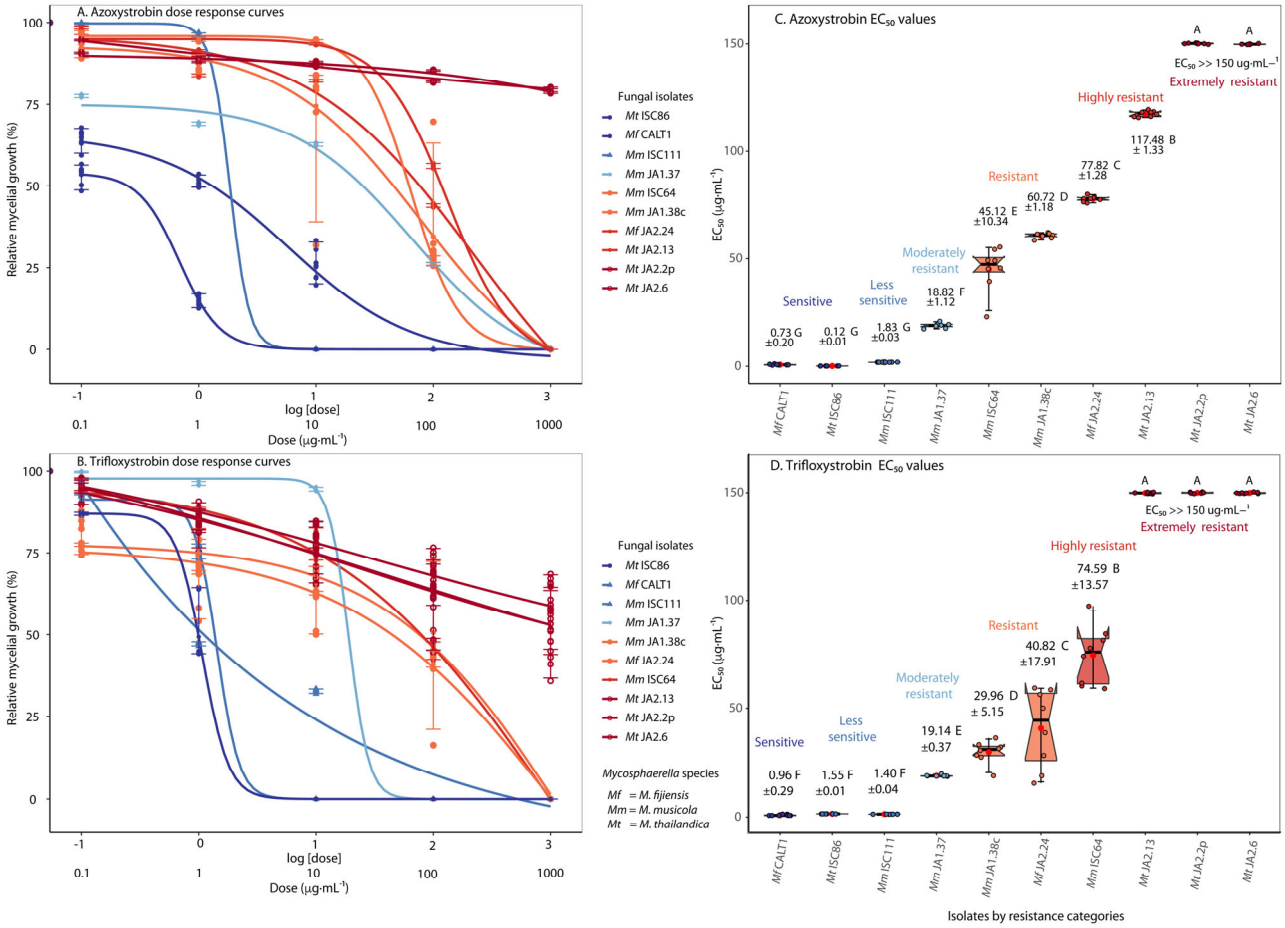

Figure 5. Contrasting in vitro dose–response curves of the relative growth of individual isolates of *Mycosphaerella fijiensis* (Mf), *M. musicola* (*Mm*) and *M. thailandica* (*Mt*) in increasing concentrations of the QoI fungicides azoxystrobin (**A**) and trifloxystrobin (**B**) in PD broth on a resazurin activity microplate assay. Estimates of the isolates' EC_{50} values for azoxystrobin (**C**) and trifloxystrobin (**D**) and corresponding sensitivity and resistance categories [a]. [a] *F* test from ANOVA significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott–Knott test at $p \leq 0.05$.

Analyses of the *cytochrome b* (*cyt b*) partial gene sequences from *Mm*, *Mf* and *Mt* strains showed the known non-synonymous G143A substitution associated with azoxystrobin and trifloxystrobin resistance (QoI-R) in both species (Tables 4 and 5, Figure 6). Considering *M. musicola* in particular (Table 4), 87 *Mm* QoI-sensitive strains (QoI-S), which do not have this mutation were grouped in the *cyt b* haplotype *Hm1*, and nine *Mm* strains (ISC64, ISC55a, ISC09, ISC110, ISC116, ISC117, MG118, JA1.37 and JA1.38c) carrying the G428C mutation leading to the G143A amino acid substitution belongs to the *Hm2*. The *Mm* LFZO01000638.1 *cyt b* sequence used as reference is also *Hm1*. The reference sequence NC037198.1 from *Pseudocercospora mori* presented 20 substitutions, all of them synonymous mutations (*Hm3*). Both these reference strains have no QoI-sensitivity information available (Table 4).

Table 4. Summary of variation at cytochrome b (cyt b) genes from field isolates of Mycosphaerella musicola strains from Brazil with distinct resistance phenotypes to the QoI fungicides azoxystrobin and trifloxystrobin.

Gene	Reference Sequence (GenBank NCBI)	Length of Amplicon Sequence (bp)	[a] Synonymous Mutations: Substitutions (*s*); Deletions (*d*); Insertions (*i*)/Position [a]	Haplo-types detected	Species	Isolates	N	[b] QoI resistance class	Coding region (bp)	Protein length (aa)	Non-Synonymous Mutations Detected /Position	Non-Synonymous a.a. Change/ Position	a.a Change
Mm cyt b	OP715652, OP715653, and OP715654	963 (from 1251 to 2213)	0	*Hm1*	*Mm*	[c] *Mm* populations	87	S	936	312	0	0	
	OP715650, OP715651, and OP715649		0	*Hm2*	*Mm*	[d] ISC9, ISC55a, ISC64, ISC110, ISC116, ISC117, MG118 [e] JA1.37, [e] JA1.38c	9	R			[f] CCT:CGT/1599	[f] G:A/143	*Mm*: Grice et al. [15]
	NC037198.1	1173 (from 1172 to 2344)	20 *s*: A:T/1267; T:A/1312; A:T/1324/T:A/1432; C:G/1450; T:A/1549; A:T/1675;T:A/1816; G:C/1837; A:T/1925; A:T/1957; T:A/1972; C:G/1975; G:C/2018; C:G/2092; T;A/2107; T:A/2156; A:T/2167; A:T/2173; C:G/2194	*Hm3*	*P. mori*	-	1	-	1170	390	0	0	
	LFZO01000638.1		0	*Hm1*	*Mm*	CBS116634	1	-	1173	391	0	0	

[a] The haplotype could still carry other mutations along the non-coding region of the sequence. [b] Fungicide sensitivity phenotype classes: **S** = sensitive, **R** = resistant for both azoxystrobin and trifloxystrobin. [c] *Mm* populations from North-western São Paulo (SPNW) fungicide conventional management system (SPNW-C): ISC isolates; organic plantation (SPNW-O): ISR isolates; and Northern Minas Gerais conventional management system (MGN-C): MG isolates, *Mm* SPVR-I population from a fungicide conventional intensive management system in Ribeira Valley, São Paulo. [d] From SPNW-C and MGN-C populations. [e] From SPVR-I population. [f] Non-synonymous mutations and respective nucleotides substitutions are indicated in red.

Table 5. Summary of variation at cytochrome b (cyt b) genes from field isolates of Mycosphaerella fijiensis strains from Brazil with distinct resistance phenotypes to the QoI fungicides azoxystrobin and trifloxystrobin.

Gene	Reference Sequence (GenBank NCBI)	Length of Amplicon Sequence (bp)	[a] Synonymous Mutations: Substitutions (*s*); Deletions (*d*); Insertions (*i*)/Position [a]	Haplo-Types Detected	Species	Isolates	N	[b] QoI Resistance Class	Coding Region (bp)	Protein Length (aa)	Non-Synonymous Mutations Detected/Position	Non-Synonymous a.a. Change/ Position	Previous Report for a.a. Change
Mf cyt b exon-1	OP734340 toOP734348	475 (2511-2036)	9 *i*: GA(TA)3/2058	*Hf1-1*	*Mf*	[c] SPVR population	9	S	475	151	0	0	-
	OP734339		0	*Hf1-2*	*Mf*	[c] JA2.24	1	R			CCT:CGT/ 1599	G:A/143	*Mf*: Sierotzki et al. [25]
	NC044132	507 (from 2056 to 2562)	2 *d*: 2525 to 2521. 4 *s*: A:T/2521; C:G/2515; A:T/2512; C:G/2509. 8 *i*: GA(TA)3/2058; 2 *i*: (TA)/2521,2522	*Hf1-3*	*Mf*	-	1	-	507	169	0	0	-
	AF343069	459 (2525-2565	0	*Hf1-2*	*Mf*	184.97.1	1	R	459 (−2 gaps)	153	[d] CCT:CGT/ 1599	[d] G:A/143	*Mf*: Sierotzki et al. [25]
	AF343070		0	*Hf1-4*	*Mf*	185.97.3	1	S	459 (−2 gaps)	153	0	0	-
Mf cyt b exon-2	OP734349 toOP734358	996 (from 25 to 1020)	0	*Hf2-1*	*Mf*	[c] SPVR population	10	S	657	219	0	0	-
	...												
	NC044132		0	*Hf2-2*	*Mf*	-	1	-			0	0	-
	AF343069		3 *s*: A:T/431; G:N/481; A:G/808	*Hf2-3*	*Mf*	184.97.1	1	R			0	0	-
	AF343070		3 *s*: A:T/431; G:N/481; A:G/808	*Hf2-4*	*Mf*	185.97.3	1	S			AAA:CAA/788	L:F/68	-

[a] Synonymous mutations in the coding region of the *cyt b* gene. The haplotype could still carry other mutations along the non-coding region of the sequence. [b] Fungicide sensitivity phenotype classes: **S** = sensitive, **R** = resistant for both azoxystrobin and trifloxystrobin. [c] *Mf* SPVR-I population from a fungicide conventional intensive management system in Ribeira Valley, São Paulo. [d] Non-synonymous mutations and respective nucleotides substitutions are indicated in red.

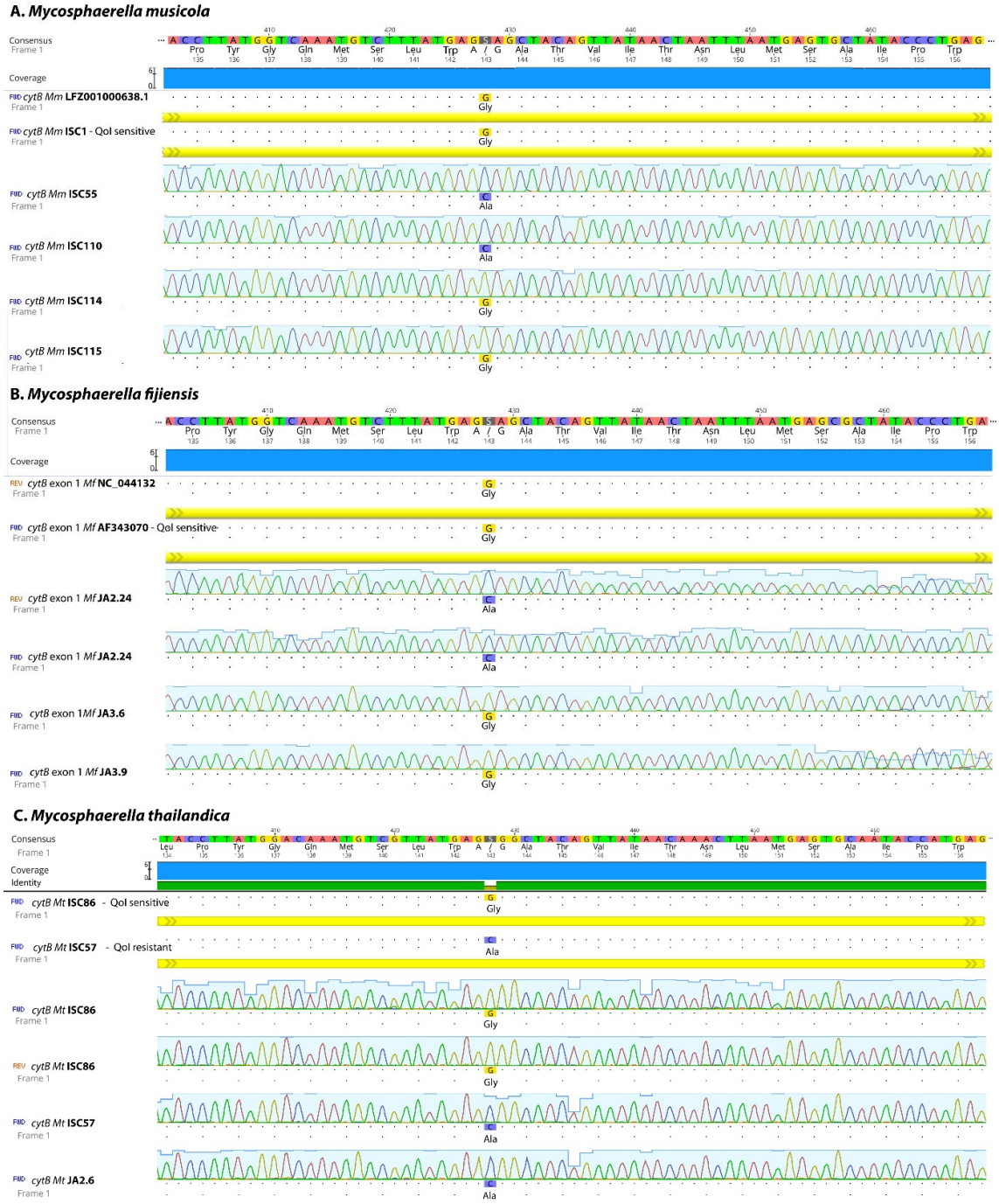

Figure 6. Graphical representation of the part of the *cyt b* gene from *Mycosphaerella musicola* (**A**), *M. fijiensis* (**B**) and *M. thailandica* (**C**) isolates containing the G428C mutation or not, resulting in the Gly143Ala amino acid substitution that confers resistance to QoI fungicides [a, b, c]. [a] Sequences from *M. musicola* aligned with the reference sequences LFZ01000638.1 and with *Mm* ISC1 sensitive to QoIs. [b] Sequences from *M. fijiensis* aligned with the reference sequences NC_044132 and with *Mf* AF343070 sensitive to QoIs. Because no *cyt b* sequences from *Mt* were available in the GenBank/NCBI we were able to align only among the experimental *Mt cyt b* sequences, from both QoI sensitive (ISC86) and resistant (ISC57 and JA2.6) isolates. [c] Fwd = forward sequence; Rev = reverse sequence.

Looking at *M. fijiensis* (Table 5, Figure 6), the first *cyt b Mf* haplotype in exon-1 (*Hf1-1*) is characterized by nine QoI-S strains, without non-synonymous mutation, but presenting an GA(TA)3 insertion at the 2058-bp position, distinct from the *Mf* sensitive reference sequence

AF343070 (*Hf1-4*). Reference sequence NC044132 has no phenotypical data, and belongs to *Hf1-3*, with four non-synonymous substitutions, two deletions, eight insertions GA(TA)3 at the 2058-bp position and two nucleotides TA insertions at 2521 and 2522 positions. The *Mf*-resistant strain JA2.24 (from SPVR-I) carrying the G143A substitution is similar to AF343069 reference, both *Hf1-2*. Four haplotypes of the *Mf cyt b* exon-2 were observed, with none presenting non-synonymous mutations (*Hf2-1*). The reference sequence NC044132 had no change (*Hf2-2*), while both AF343069 (QoI-R) and AF343070 (QoI-S) presented three synonymous substitutions (haplotypes *Hf2-3* and *Hf-2-4*, respectively). This last sequence was different due to an A:C substitution at position 788 (Table 5).

Analyses of the *cyt b* partial gene sequences from QoI resistant *Mt* isolates (*Mt* ISC57 from SPNW-C; *Mt* FRA1.21b, *Mt* JA1.34, *Mt* JA1.4, *Mt* JA2.13, *Mt* JA2.15, *Mt* JA2.16a, *Mt* JA2.2p, *Mt* JA2.3a, *Mt* JA2.6 and *Mt* T06 from SPVR-I) also showed the known non-synonymous G143A substitution associated with QoI resistance (GenBank/NCBI sequences OP796647 to OP796657) (Figure 6). The single QoI sensitive isolate *Mt* ISC86, from SPNW-C, had no mutation in the *cyt b* partial gene (OP796645) (Figure 6).

4. Discussion

Resistance to QoI fungicides linked to the G143A substitution and the corresponding mutation in the *cyt b* gene is a well described mechanism for many plant pathogens, including *Mm* and *Mf* [9,14]. Other substitutions such as the G137R in *Pyrenophora tritici-repentis*, G137S in *Venturia effusa* or the F129L in pathogens such as *Phakopsora pachyrhizi*, *Pyrenophora teres* and *Plasmopara viticola* also confer QoI resistance [47,48], but so far have not been found in either *Mf* or *Mm* [2,25,29,30,49] and are associated with lower levels of resistance. In the present study, a total of 18.5% of all isolates tested (*M. fijiensis*, *M. musicola* and *M. thailandica*) sampled at four different locations in Southern Brazil were QoI-R carrying the G143A substitution in *cyt b*.

Our primary assay for phenotyping sensitivity to QoI fungicides was based on a qualitative approach using fungal mycelial fragments and measuring inhibition of mycelial growth at the discriminatory dose of 10 $\mu g \cdot mL^{-1}$ of azoxystrobin or trifloxystrobin. Similar assays have been used for assessing QoI sensitivity of *Mf* in Costa Rica, where strains carrying *cyt b* G143A alleles showed resistance factors (RFs) > 100-fold [25]. We also applied a quantitative approach on a set of ten *Mycosphaerella* isolates varying in levels of QoI sensitivity, which corroborated the outcome from the qualitative approach (Figure 5). The sensitive isolates showed an EC_{50} range of >0.12 to 2 $\mu g \cdot mL^{-1}$ for azoxystrobin and pyraclostrobin, while resistant isolates showed EC_{50s} > 20 to 150 $\mu g \cdot mL^{-1}$ and extremely resistant isolates an $EC_{50s} \geq 150$ $\mu g \cdot mL^{-1}$ (Figure 5).

This qualitative approach for discriminating sensitivity from resistance to QoI is also strongly supported by the studies from Alfaro [30] who detected *Mf* sensitive isolates, from banana plantations in Costa Rica, with EC_{50} > 0.001–1 $\mu g \cdot mL^{-1}$ for azoxystrobin and pyraclostrobin, while resistant isolates had EC_{50} >10–100 $\mu g \cdot mL^{-1}$ and very resistant isolates ≥ 100 $\mu g \cdot mL^{-1}$.

Although the screening for sensitivity to QoIs with the discriminatory dose of 10 $\mu g \cdot mL^{-1}$ was useful in detecting *Mf*, *Mm* and *Mt* isolates with the G143A mutation in the *cyt b* gene in our study, this approach is not suitable if the goal is to determine moderate sensitivity classes, which may fall within EC_{50} > 1–10 $\mu g \cdot mL^{-1}$, and specially for isolates that did not carry the G143A *cyt b* target site mutation [30]. For such isolates, it is plausible that other target site mutations (e.g., F129L and G137R/S) and/or overexpression of transporters may also be associated with the reduced sensitivity to QoIs, as already reported for other plant pathogens such as *Pyricularia oryzae Triticum* lineage [50] and *Zymoseptoria tritici* [51].

Our study was conducted with current populations of both yellow and black Sigatoka pathogens from banana plantations in Southeastern Brazil, with contrasting fungicide management to control these foliar diseases. Pathogen populations from conventional or intensive use of fungicides (*Mm/Mt* SPNW-C and *Mf/Mm/Mt* SPVR-I) had a higher frequency of resistant isolates than the population with no fungicide input (*Mm* SPNW-

O) (Tables 3–5). The *Mm* and *Mf* resistant isolates from our study were able to grow at 10 μg·mL^{-1} of azoxystrobin or trifloxystrobin. This observation probably reflects the effects of high selection pressure for QoI resistance in the pathogen populations over the years, since no evidence of resistance was formerly detected in populations of the pathogens from Vale do Ribeira sampled as early as 2008 or as recent as 2018 [2,49].

For instance, between 2008 and 2009, the G143A substitution in the *cyt b* gene was not found in a collection of 36 *Mm* isolates sampled from banana plantations in Northern and Central-Western Brazil and from ten *Mf* isolates from Vale do Ribeira, in São Paulo [49]. In 2018, despite the small sample size, Malimpensa [2] reported no QoI resistance nor *cyt b* substitution in *Mf* from Vale do Ribeira, and one *Mm* isolate with the G143A substitution, but with only a reduced sensitivity to strobilurin (at 0.1 μg·mL^{-1} of the fungicide). However, *M. thailandica*, which was the most abundant species in the sampling from Vale do Ribeira, 58.3% of the isolates (7 out of 12 total) carried the G143A substitution and were highly resistant to QoI (at doses higher than 10 μg·mL^{-1}) [2]. Similarly, in our survey, *M. thailandica* was highly prevalent in the population SPVR-I from the same region, representing 45.5% of the isolates obtained (10 out of 22 total), and all these isolates were resistant to QoI fungicides and carried the G143A substitution.

An investigation about the dynamics of QoI resistance in *Mf* populations sampled in Costa Rica from 2000 to 2003, by quantitative PCR detection of the mutation associated with the G143A substitution, indicated frequencies varying from 9% to 78% [27]. The authors concluded that the populations of the pathogen were not structured at scales < 10 m, and wind-born ascospore dispersal have homogenized *Mf* populations distributed on scales from 1 to 10 km. Reduction in QoI fungicide applications on individual farms did not cause a rapid decrease in G143A frequency regardless of their own spray practice, since surrounding farms at regional scale keep the QoI selection pressure favoring the resistant *Mf* populations with G143A mutation. Therefore, evolution of resistance to strobilurins in *Mf* populations was considered to occur regionally, with single individual farms converging on a regional average frequency of G143A mutation, regardless of their own spray system.

Because our pathogen populations were sampled at regional scales varying from a minimum of >10 km (Ilha Solteira, Northwestern São Paulo, Brazil) to a maximum of 750 (Vale do Ribeira, SP)—1200 km (Janaúba, Northern MG, Brazil), the geographical distances might have led pathogen populations to differentiate considering the effects of spray practices on the selection for fungicide resistance.

As a concluding remark, considering that the overall frequency of QoI resistance in three regional populations of the two major Sigatoka pathogens (*Mf* and *Mm*) in Southern Brazil, besides the prevalent *Mt* in Vale do Ribeira, are already at 18.5% on average, anti-resistance strategies should be adopted to avoid their general spread. An important anti-resistance strategy would be to regionally enforce a limit on QoI fungicide sprays to a minimum, and only in mixtures with protectant, multisite, low-risk fungicides to discourage individual decisions to use only systemic, single site, high risk fungicides, given the evidence that the resistant strains of the pathogen could be dispersed long distance from a source of resistant inoculum.

However, from previous studies there was no evidence for rapid selection against the resistant form of *Mf* after removing QoI fungicides from the spray program [27]. No information is available on the fitness cost associated with the G143A substitution in both *Mm* and *Mf* in competition experiments [52]. No such information exists for *Mt* either. In the absence of a fitness penalty, resistance to QoI would persist in the agroecosystem.

Future studies should focus on the development of real time disease surveillance and monitoring of fungicide resistance using real-time PCR or loop-mediated isothermal amplification (LAMP) techniques which can be used for on-the-spot detection to help growers to take smart decisions on spraying fungicides in a timely manner rather than based on calendar systems [53–57]. Ideally, this novel system could contribute to the rational and sustainable management of the Sigatoka diseases complex, by limiting the emergence and spread of fungicide resistance.

5. Conclusions

Overall, a total of 18.5% of isolates of *M. fijiensis*, *M. musicola* and *M. thailandica* sampled from Southern Brazil were resistant to QoI fungicides and carried the G143A substitution in *cyt b*.

Pathogens' populations from banana plantations with conventional or intensive use of fungicides to control Sigatoka diseases (*Mm/Mt* SPNW-C and *Mf/Mm/Mt* SPVR-I) had higher frequency of resistant isolates than the population from fields with no fungicide input (*Mm* SPNW-O).

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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 the genetic diversity of Mycosphaerella species.

Data Availability Statement: The *cyt b* experimental sequencing data from *Mf*, *Mm* and *Mt* populations sampled in Southern Brazil and that supports the findings on allelic variation in the target gene for QoI sensitivity and resistance are available at the GenBank/NCBI database (sequential accession numbers OP734339 to OP734358 for *Mf cyt b* exons 1 and 2; OP715649 to OP715654 for *Mm cyt b*; and OP796645 to OP796657 for *Mt cyt b*). Upon publication, the phenotypic data presented in this study will be publicly available at Mendeley Data repository at doi:10.17632/3xpjnztjpm.1.

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