



Article

Resistance to Site-Specific Succinate Dehydrogenase Inhibitor Fungicides Is Pervasive in Populations of Black and Yellow Sigatoka Pathogens in Banana Plantations from Southeastern Brazil

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Abstract: The Sigatoka disease complex (SDC), caused by *Mycosphaerella fijiensis* (Mf) and *M. musicola* (Mm), comprises the most destructive fungal leaf streak and spot diseases of commercial banana crops worldwide. In Brazil, the site-specific succinate dehydrogenase inhibitor (SDHI) fungicides labeled for SDC management since 2014 present a high risk for the emergence of resistance if deployed intensively and solo. Our study determined the levels of sensitivity to boscalid and fluxapyroxad in four populations of the SDC pathogens sampled in 2020 from three distinct geographical regions under contrasting fungicide programs. Resistance, defined as EC₅₀ values exceeding 20 µg mL⁻¹, was prevalent at 59.7% for fluxapyroxad and 94.0% for boscalid. Only 1.5% of isolates exhibited sensitivity to both fungicides. We also assessed the changes in the corresponding fungicide target protein-encoding genes (SdhB, C, and D). None of the target site alterations detected were associated with reduced sensitivity. A second SdhC paralog was also analyzed, but target alterations were not found. However, MDR (multidrug resistance) was detected in a selection of isolates. Further monitoring for Sdh target mutations will be important, but an important role for other resistance mechanisms such as the presence of additional Sdh paralogs and MDR cannot be ruled out. These results highlight the importance of implementing sound anti-resistance management strategies when SDHI fungicides are deployed for the management of SDC.

Keywords: chemical control; fungicide resistance; *Sdh* target site mutations; *SdhC* dispensable paralog; multidrug resistance



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1. Introduction

The Sigatoka disease complex (SDC), covering the most important fungal leaf streak and leaf spot diseases of banana, has been affecting commercial banana production for over two decades, thereby causing high yield losses worldwide [1,2]. This disease complex has been associated with four ascomycetous fungi belonging to the genus *Mycosphaerella*: the black Sigatoka leaf streak pathogen *Mycosphaerella fijiensis* (Mf) (syn. *Pseudocercospora fijiensis*); the yellow Sigatoka pathogen *M. musicola* (Mm) (syn. *P. musae*); the leaf spot pathogen *M. thailandica* (syn. *Parapallidocercospora thailandica*); and the eumusae leaf spot

pathogen *M. eumusae* (syn. *P. eumusae*) [2,3]. Black and yellow Sigatoka are considered the two most relevant diseases of the SDC in Brazil [4–7]. Banana is a vital staple crop for Brazilian agriculture, holding unparalleled importance to the local economy [2,6,7]. Therefore, understanding the impact of SDC is crucial not only for local banana growers but also for ensuring food security on a broader scale. It also underscores the necessity for comprehensive research on management strategies to combat SDC [2,4,6,7].

The leaf symptoms produced by the infections are mostly unique for each of the pathogen species within the SDC, except for *M. thailandica*. This particular species has been frequently recovered from typical black Sigatoka leaf streak lesions in Brazil [7,8]. However, some reports suggest instances of misdiagnosis, where black Sigatoka is confused with the less devastating yellow Sigatoka. This points to the misidentification of the two fungal species, either *Mf* as *Mm* or vice versa [5,6,9].

In Brazil, yellow Sigatoka was first reported in the Amazon region in 1944 and has become more widespread, now present in all banana-growing regions [10]. It causes significant yield losses, with reports of up to a 50% reduction [2,6]. On the other hand, black Sigatoka was initially reported in 1998, also in the Amazon region [2,11]. Since then, the pathogen has been detected in 17 states, including São Paulo and Bahia, the two most important banana-producing states [12]. Black Sigatoka is regarded as the major constraint on banana production, reducing yields up to 100% [2,6]. Invasion of the banana leaf stomata by either *Mm* or *Mf*, and subsequent colonization of the intercellular spaces, causes necrotic damage that reduces the photosynthetic capacity of the leaf, affects the size of the bunch, shortens the green life of the fruits, and shortens the time between harvest and ripening [13].

The geographical distribution and prevalence of the SDC in Brazil follow a humidity cline. For instance, black Sigatoka is considered more prevalent and destructive under conducive weather conditions, which include high humidity (>90%), the presence of leaf wetness, and temperatures ranging from 26–28 °C [8,14–17]. In banana plantations where the susceptible and widely cultivated varieties Prata and Nanica [6,18] are used, the incubation period for *Mf* could be as short as 13 to 14 days, whereas under unfavorable conditions, the pathogen's incubation period could extend to 35 days [4,14,16–18]. For *Mm*, in planta epidemiological studies under controlled conditions indicated that spores germinate 2–3 h after deposition on banana leaves if a water film is present or if humidity is high [19]. Relative humidity >73% is sufficient for ascospore release [19]. The optimum temperature for conidial and ascospore germination is approximately 26 °C [19].

The fungal pathogens associated with the SDC diseases are characterized by a polycyclic reproductive cycle and a mixed reproductive system. Throughout the growing season, several cycles of sexual reproduction are completed, resulting in the release of airborne ascospores that can initiate epidemics as the primary source of inoculum. Epidemic clonal dispersal over short distances occurs through asexually produced conidia spread by rain splash [20,21]. Genetic diversity studies have revealed that *Mf* and *Mm* populations in Brazil, Mexico, and the Philippines exhibit high genotypic variation and are shaped by gene flow over long distances, indicating a crucial role for sexually produced airborne ascospores [2,10,22,23].

The main strategy for the management of SDC has been the intensive and preventive (calendar-based) spraying of protectant or systemic fungicides, or the combination of both [2]. As varietal resistance is practically absent or only partial in most banana cultivars widely cultivated in the country [5,6,18], up to 52 sprays of protectant multi-site fungicides and/or systemic site-specific fungicides are applied annually under high disease pressure, which is very costly [2]. Consequently, the populations of the SDC pathogens are under strong selection pressure for the emergence of fungicide resistance [3,7,24–26]. These systemic site-specific fungicides include the major groups of quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs), and, more recently, succinate dehydrogenase inhibitors (SDHIs) [2,8,27]. Resistance to site-specific fungicides has been, in fact, a recurrent serious problem that limits the ability to control agricultural, medical, and veterinary

fungal pathogens since these fungicides pose a risk for resistance to emerge, especially for pathogens with high evolutionary potential [28–32].

The second generation of SDHI fungicides, starting with the registration of boscalid in 2002, have, due to their broad-spectrum activity, been introduced to control SDC pathogens and a range of other pathogens, including *Alternaria alternata* on pistachios and peaches, *A. solani* on potatoes, *Botrytis cinerea* on apples, grapes, and strawberries, *Claviceps* sp. (formerly *Sclerotinia homoeocarpa*) on turfgrass, *Corynespora cassiicola* on pumpkins, *Phakopsora pachyrhizi* on soybeans, *Pyrenophora teres* on barley, *Sclerotinia sclerotiorum* on canola, *Venturia inaequalis* on apples, and *Zymoseptoria tritici* on wheat [33]. Perceived as highly effective for disease control, the widespread and intensive use of SDHIs may have led to a high selection pressure for resistance to emerge on a single target protein, as shown by the high numbers of resistance cases for a wide range of pathogens worldwide [34–37].

SDHI fungicides are respiration inhibitors targeting succinate dehydrogenase (Sdh) (succinate:ubiquinone oxidoreductase) or mitochondrial respiratory chain complex II, a heterotetrameric enzyme complex that participates in both the citric acid cycle (SdhA) and the electron transport chain (SdhB, C, and D). The ubiquinone binding site pocket of SDHs is formed by Sdh subunits B, C, and D. SDHI binding physically blocks access to the substrate and prevents the oxidation cycle of succinate, which is essential for mitochondrial respiration [35,36].

The most common mechanism of resistance to SDHIs includes target site mutations leading to amino acid changes in Sdh subunits B, C, and/or D, although other mechanisms can also occur, such as the presence of multiple copies/paralogs (such as the SdhC paralog) and/or overexpression of target genes and/or multidrug efflux pumps contributing to a multidrug-resistant (MDR) phenotype. Both ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters that export multiple fungicides from the inside to the outside of the cell, decreasing their intracellular concentration, have been reported to contribute to MDR in a range of fungal pathogens [28,34,38–50].

Currently, the SDHI fungicides labeled for the worldwide management of the SDC in banana plantations include boscalid, fluopyram, fluxapyroxad, and isopyrazam [51,52]. In Brazil, only a single fungicide co-formulation (CollisTM, from BASF, São Paulo, Brazil) containing the SDHI boscalid and the QoI kresoxim-methyl has been labeled by the Ministry of Agriculture, Livestock, and Supplies for control of SDC on bananas since 2014 [53].

FRAC (Fungicide Resistance Action Committee) surveillance data on SDHI resistance in SDC pathogens have shown that a few SDHI-insensitive strains of *Mf* were detected from 2012 to 2017 in Costa Rica, Ecuador, Guatemala, and Colombia in areas where sprays containing boscalid, fluopyram, fluxapyroxad, and/or isopyrazam have been applied in banana plantations [51,52]. A large knowledge gap still exists in Brazil concerning the development of SDHI resistance in SDC pathogens. If resistance occurs locally, there will also be a lack of information on the molecular mechanisms associated with levels of resistance to SDHIs in populations of the two SDC pathogens. This lack of knowledge about the resistance status of *Mf* and *Mm* to SDHI fungicides can lead to inefficient use of the actives, targeting populations of the SDC pathogens that potentially already have developed resistance to this class of fungicides.

Therefore, in a context of high selection pressure associated with the intensive use of fungicides on banana plantations, our study aims to characterize the prevalence and degree of sensitivity reduction and/or resistance in populations of the SDC pathogens, *Mf* and *Mm*, to SDHI fungicides in Brazil. The primary objectives include determining the sensitivity status of *Mf* and *Mm* populations to the SDHI fungicides boscalid and fluxapyroxad using in vitro microtiter plate-based assays.

To accomplish this, we sampled populations at four sites in southeastern Brazil, representing three distinct SDC management systems based on intensive, reduced, or no fungicide spray applications. Our investigation also delved into elucidating resistance mechanisms, particularly examining whether SDHI insensitivity is associated with mutations in genes encoding Sdh subunits B, C, and/or D forming the SDHI binding pocket.

Additionally, for a selection of isolates, we explored the potential role of an additional SdhC paralog, as reported for a subpopulation of *M. graminicola* conferring resistance to a subgroup of SDHIs [49,50]. We also investigated if a multi-drug resistance (MDR)-related mechanism is operating by testing the effects of two efflux pump inhibitors.

Given the extensive use of SDHI fungicides for controlling SDC on bananas in Brazil since 2014, we hypothesized that the selection pressure from fungicide spraying has selected for resistance in highly variable pathogen populations.

2. Materials and Methods

2.1. Sampling Area

The *Mf* and *Mm* populations were obtained from leaf samples displaying characteristic symptoms of black and yellow Sigatoka, collected in 2020 at four distinct geographical locations in southeastern Brazil. These locations correspond to the following three distinct disease management systems: (i) intensive management from Vale do Ribeira, São Paulo (population SPVR-CI, sourced from Jacupiranga, Registro, and Sete Barras counties); (ii) reduced management from northwestern São Paulo (population SPNW-C, obtained from Ilha Solteira county); and (iii) organic management, with no fungicide applications (population SPNW-O, collected from Ilha Solteira). The geographical distribution of these population samples is depicted in Figure 1. It is noteworthy that these isolates represent a subset of the population sample investigated for QoI resistance by Oliveira et al. [7].

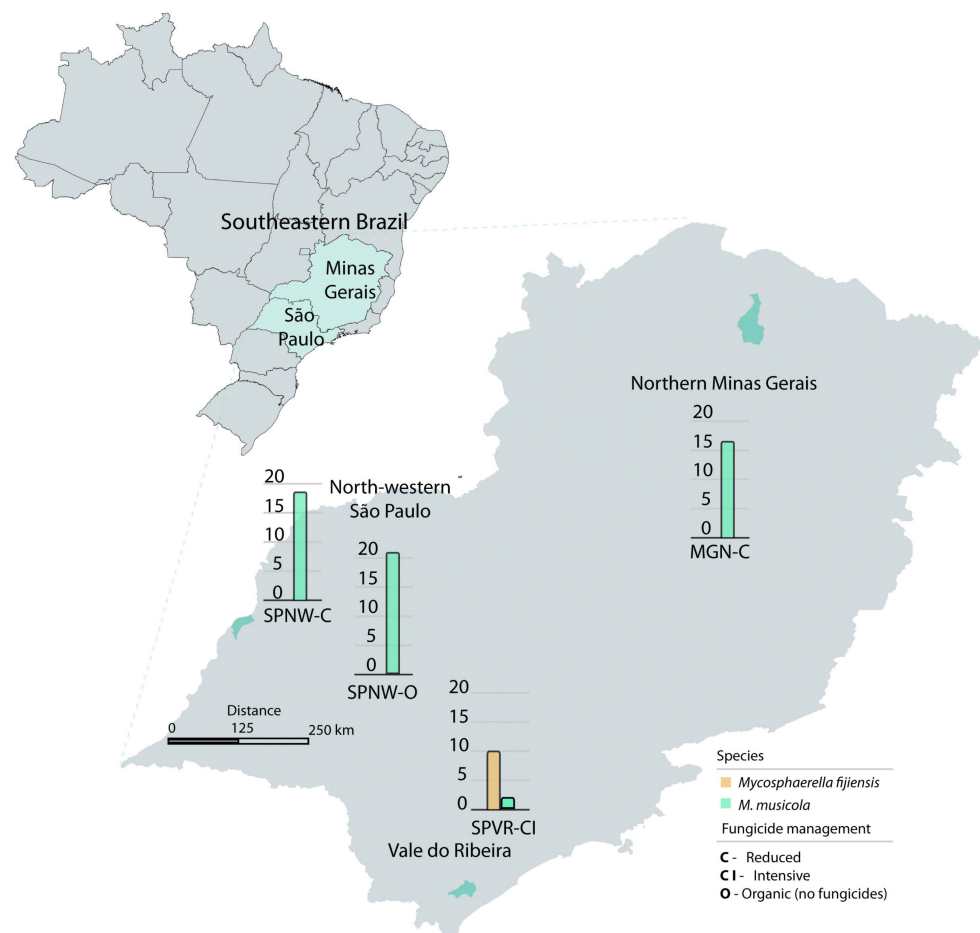


Figure 1. Population sampling of the black leaf streak (*Mycosphaerella fijiensis*) and yellow Sigatoka (*M. musicola*) pathogens from banana plantations with contrasting fungicide management in southeastern Brazil during the 2020/21 cropping season. States and counties were colored green: Ilha Solteira county, from northwestern São Paulo state (SP), Jacupiranga, Registro, and Sete Barras counties from Vale do Ribeira (SP), and Janaúba county, from northern Minas Gerais State (MG) [7].

At the initial location in Vale do Ribeira, São Paulo (SPVR-CI), the management approach relied on intensive fungicide spraying, involving 8 to 14 preventive applications per year for the chemical control of black Sigatoka [7,8]. This region is renowned as the primary hub for banana production in São Paulo state and across Brazil. Here, the prevalent banana cultivars are the SDC-susceptible Prata (*Musa* spp. AAB, commonly known as “Lady Finger” banana) and Nanica (*Musa* spp. AAA, Cavendish subgroup) [6,7,14,54]. At the second site, situated in the northwest of São Paulo (SPNW-C), and the third site, located in northern Minas Gerais (MGN-C), fungicide usage is scaled down to four to five preventive sprays targeting Yellow Sigatoka [7]. The SPNW-C field covers an area of 40 hectares dedicated to the susceptible Maçã variety (triploid AAB) [7]. Conversely, in MGN-C, the banana plantation features Prata and Nanica varieties [7]. In the final sampled site (SPNW-O), no fungicides were applied. This area comprises several small family plantations with banana plants of diverse varieties and ages in Ilha Solteira county [7].

2.2. Pathogen Strain Isolation, Identification, and Storage

Fragments of banana leaves with symptoms of black or yellow Sigatoka diseases were placed in paper bags, transported, and stored in a refrigerator at 10 °C until the pathogens were isolated. The leaves were washed with tap water, cut into 2 cm² pieces, and disinfested with 75% ethanol and sterile distilled water for 1 min. Leaves were then dried on sterile filter paper and transferred to Petri dishes containing water–agar medium (15 g L^{−1} agar) amended with the antibiotics chloramphenicol and streptomycin (at the final concentration of 50 µL mL^{−1} each) and incubated at 26 °C in the dark until sporodochia formed. Using a fine needle and with the aid of a stereomicroscope, sporodochia were transferred from lesions to Petri dishes containing PDA (20.8 g L^{−1} potato dextrose (Kasvi, India), 15 g L^{−1} agar + chloramphenicol, and streptomycin (50 µL mL^{−1} each) and kept at 26 °C in the dark until microcolonies characteristic of *Mf* or *Mm* were observed [7]. Young colonies were transferred to fresh PDA plates. After 14 days of incubation, sterilized filter paper strips were placed onto the PDA plates. Following this, fungal mycelium discs were transferred onto the plates to colonize the paper strips. The plates were then incubated for 10 days at 25 °C in darkness. Subsequently, the fungal-mycelium-colonized filter paper strips were transferred to sterile Petri plates and placed in a flow chamber for three days to dry [3,7,10]. For long-term storage, the isolates obtained were cryopreserved at −20 °C in cryotubes containing silica by transferring the sterile filter paper discs colonized by *Mf* or *Mm*. Initially, *Mycosphaerella* colonies were morphologically identified at the genus level and subsequently molecularly at the species level. In addition to the three SDH genes investigated in our study, we included the analysis of the *cytB* gene from the same set of isolates, as addressed in the study by Oliveira et al. [7].

2.3. Fungicide Sensitivity Evaluation

For the SDHI fungicide sensitivity testing, a total of ten isolates of *Mf* and 57 isolates of *Mm* were screened: 10 *Mf* and 2 *Mm* isolates from SPVR-CI, 21 *Mm* isolates from SPNW-O, 18 *Mm* isolates from SPNW-C, and 16 *Mm* isolates from MGN-C. Mycelial fragments were prepared according to the protocol described by Silva et al. [55]. For this, the SDC isolates were reactivated in PDA medium (20.7 g.L^{−1} potato dextrose, 15 g.L^{−1} agar) supplemented with chloramphenicol and streptomycin (50 µg mL^{−1} each). After 15 days of growth at 25 °C and 12 h of photoperiod, fungal mycelia were transferred to 1.5 mL microtubes containing 0.5 mL of glass beads that are 0.1 mm in diameter. A total of 1.0 mL of distilled water was added to the mixture and bead-beated in a fast-prep device for 20 s at a speed of 4 m s^{−1}. Bead beating resulted in a suspension of small mycelial fragments that were further diluted with distilled water to a total volume of 10 mL. The final concentration of the mycelial fragment suspensions was adjusted to 10⁴ fragments/mL^{−1} based on Neubauer chamber counting.

SDHI Fungicide Sensitivity Testing

Sensitivity testing involved the use of SDHIs boscalid and fluxapyroxad. Formulated boscalid (Cantus™, active ingredient at 500 g kg⁻¹, BASF, São Paulo, Brazil) was dissolved in deionized water to a concentration of 0.1 g mL⁻¹, then further diluted in deionized water to yield a stock solution of 500 µg mL⁻¹. Technical-grade fluxapyroxad (PA, Pure for Analysis, Sigma-Aldrich, St. Louis, MI, USA) was dissolved in acetone to a concentration of 50 mg mL⁻¹, followed by dilution in deionized water to create a stock solution of 500 µg mL⁻¹. The final tested doses for boscalid (BSC) and fluxapyroxad (FLX) were 0.0, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 µg mL⁻¹. Salicylhydroxamic acid (SHAM) was introduced to a 0.5 mM PD medium (20.7 g L⁻¹ potato dextrose) broth to inhibit alternative oxidase (AOX) activity [36,56]. The fungicides tested at different doses were mixed with PD medium prepared with 0.025 M phosphate buffer, and its pH was adjusted to 5.0. Sensitivity tests were performed on 96-well-bottomed microplates (with a flat bottom) (Kasvi, Bangalore, Karnataka, India). The total volume in each well of the microplate was 150 µL after adding 100 µL of the PD medium (with and without fungicides at different dosages) and 50 µL of mycelium suspension. The experimental design was completely randomized with eight replicates, and each experiment was repeated once. The microplates containing the liquid fungal cultures were wrapped in plastic and incubated at 25 °C in the dark with shaking at 150 rpm for 10 days, when the fungal growth reached its maximum. Then, 50 µL of resazurin (160 µM; RZ) were added to each well to obtain a final concentration of 40 µM (final volume of 200 µL), and the initial absorbance values at 569 nm (T₀) were measured using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific™, Waltham, MA, USA) according to Silva et al. [55]. Subsequently, the microplates were kept at 25 °C in complete darkness for 24 h, and the absorbance values were measured at 569 nm (T₂₄). The RZ reduction was estimated as follows [55]:

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

where:

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

T = absorbance reading at 569 nm;

T₀ = reading at time zero, immediately after adding RZ to the microplate wells;

T₂₄ = reading time 24 h after adding RZ.

Sensitivity to the two SDHI fungicides was determined as 50% effective concentration to inhibit fungal growth (EC₅₀, in µg·mL⁻¹), estimated using a dose–response function implemented in the Excel macro ED50plus v1.0 [57]. For EC₅₀, which means comparison within each fungicide, the experimental design consisted of complete randomized blocks with four replicates per treatment and experiments in duplicate. Analysis of variance (ANOVA) and the Scott–Knott test (at 5% probability) for means comparison were performed in the R environment using the statistical packages *agricolae* and *ScottKnott* [58].

Based on the EC₅₀ values for the fungicides boscalid and fluxapyroxad, the SDC isolates were classified into the following categories: (i) sensitive (S), with EC₅₀ ≤ 2 µg mL⁻¹, (ii) less sensitive (LS), with EC₅₀ > 2 and ≤ 10 µg mL⁻¹, (iii) moderately resistant (MR), with EC₅₀ > 10 and ≤ 25 µg mL⁻¹, (iv) resistant (R), with EC₅₀ > 25 and ≤ 50 µg mL⁻¹, (v) highly resistant (HR), with EC₅₀ > 50 and ≤ 100 µg mL⁻¹, and finally (vi) extremely resistant (ER), with EC₅₀ > 100 µg mL⁻¹.

The boxplot figures depicting the contrast among fungicide resistance categories, among geographical populations of the pathogens, and between groups of isolates of *Mm* and *Mf* per species (based on EC₅₀ values or relative growth estimates) were built using the R software library *tidyverse* 1.3.1, which included the packages *ggplot2* 3.3.5, *purrr* 0.3.4, *tibble* 3.1.6, *dplyr* 1.0.7, *tidyr* 1.1.4, *stringr* 1.4.0, *readr* 2.1.0, and *forcats* 0.5.1, and the functions *ggplot*, *geom_boxplot*, *stat_summary*, *geom_jitter*, *ggtitle*, *theme*, and *geom_text* [58].

The whole set of color palettes chosen to build all figures with accessibility are color-blind, safe, and print-friendly, using the resources from Color Brewer 2.0 available at

the URL <https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3> (accessed on 1 November 2022).

2.4. Determination of Mutations in the *SdhB*, *SdhC*, *SdhC2*, and *SdhD* Genes

To investigate target site mutations in the *SdhB*, *SdhC*, *SdhC2*, and *SdhD* genes that could be associated with resistance to SDHI fungicides, we PCR amplified and sequenced all 10 isolates of *Mf* and 57 isolates of *Mm* from four distinct SDC populations. Primers designed using Primer3 implemented in the Geneious Prime software R 9.0.5 version 2023 (Biomatters—Auckland, New Zealand) (Table 1) were based on reference sequences obtained from NCBI/Genbank®, including: *Mf* NW_006921538 and XM_007930654 for *MfSdhB*; NW_006921536 and XM_007928933 for *MfSdhC*; XM_007925990 for a second *SdhC* paralog (*MfSdhC2*); and NW_006921535 and XM_007927648 for *MfSdhD*; LFZO01000506 for *MmSdhB*; LFZO0100037 for *MmSdhC*; LFZO01000210 for a second *SdhC* paralog (*MmSdhC2*); and LFZO01000522 for *MmSdhD*. These sequences were derived from *Mf* strains CIRAD86 and *Mm* isolate CBS 116634, originating from Cameroon and Cuba, respectively, and isolated before 2008 [7,59].

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

Target	Primers	Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature (°C)
<i>MfSdhB</i>	SdhB_Mf_F42	TTCTGCTTCACCACGTCTCC	1164	55.0
	SdhB_Mf_R1205	TGTGAGTCTGCCTATCATGA		
<i>MfSdhC</i>	SdhC_Mf_F84	TGTTTGTCTACACCAGCACTG	849	58.0
	SdhC_Mf_R932	AAGCCAAAGTGAGTTGCCCA		
<i>MfSdhC2</i>	Sdh_altC_Mf_F35	TCGAAGTGATGCAGGATAAGAATC	341	58.4
	Sdh_altC_Mf_R341	AGTGAGCCGAAGATATGCAAG		
<i>MfSdhD</i>	SdhD_Mf_F92	TCTGTCTTCCCACCTCTCAC	996	58.5
	SdhD_Mf_R1087	GCCACGGGATTGAGCTGTTG		
<i>MmSdhB</i>	SdhB_Mm_F103	CCTCCCCCTGCTCATTACG	996	59.5
	SdhB_Mm_R1098	CACCACCCACCACATACC		
<i>MmSdhC</i>	SdhC_Mm_F100	TGTCTGTCTACACCAGCACTG	946	58.0
	SdhC_Mm_R845	CAACCTGCAAACCAAGACCC		
<i>MmSdhC2</i>	Sdh_altC_Mm_620F	CACAATCATTCATAACTCGGCG	845	58.1
	Sdh_altC_Mm_1464R	TCGGAAAGTAGACATCGACAAC		
<i>MmSdhD</i>	SdhD_Mm_F153	CCCACCCTGATCTTTTGCAT	899	58.0
	SdhD_Mm_R1051	CCATACCACAAAGCGAGCCA		

The PCR protocol involved a reaction volume of 30 µL containing highly purified distilled (ultrapure) water, 50 ng template DNA, 0.3 µM of each primer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 3 µL 10 × PCR reaction buffer PCR, and 0.05 U Taq polymerase (Thermo Fisher Scientific, 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 72 °C for 1 min, with a final extension of 72 °C for 7 min. PCR products were purified and Sanger sequenced at MacroGen Inc. sequencing facilities in Seoul, South Korea. To ensure accuracy, two complementary sequences were generated for each Sdh gene.

The obtained DNA sequences were analyzed using the Geneious Prime software R 9.0.5 version 2023 (Biomatters—Auckland, New Zealand) to identify mutations, alleles,

and haplotypes and to distinguish non-synonymous mutations that result in amino acid changes in the deduced protein sequences. The same reference sequences described earlier were used for annotation and derivation of Sdh protein sequences from the experimental sequence data obtained in our study.

2.5. Sensitivity to Efflux Pump Substrates

We tested whether two efflux pump inhibitors could revert the MDR phenotype from resistant to sensitive: (a) BLT-4 or INF271 [N-(2-methoxyphenyl)-N'-2-naphthalenyl-urea, an MFS transporter-type inhibitor] at $0.25 \mu\text{g mL}^{-1}$; and (b) verapamil hydrochloride (an ABC transporter-type inhibitor), at $250 \mu\text{g mL}^{-1}$, [60–64]. The inhibitors were tested individually or in combination. Fungicides were tested at discriminatory doses of $5 \mu\text{g mL}^{-1}$ for boscalid and $3 \mu\text{g mL}^{-1}$ for fluxapyroxad. The assay was performed in 96-well microplates using the resazurin-based fungicide sensitivity protocol described by Silva et al. [55] and measuring the fungal relative growth using the following experimental treatments: check (with no fungicides or efflux pump inhibitors added); fungicide; BLT-4; verapamil; BLT-4 + verapamil; fungicide + BLT-4; fungicide + verapamil; fungicide + BLT-4 + verapamil. The experimental design consisted of complete randomized blocks, with four reps per treatment, in two separate assays: one for Mf and another for Mm isolates.

Three Mf and Mm isolates with different and multiple resistance (i.e., multidrug resistance (MDR) phenotypes) to QoI, DMI, and/or SDHI fungicides, according to previous ([7,55,59] and Personal communications, 2023 [65] and current studies, were selected and retested for this assay, giving the following phenotypes: Mf Ja2.24 (QoI R (resistant), DMI MR (moderately resistant), and SDHI ER (extremely resistant)), Mf Ja3.6 (QoI S (sensitive), DMI S to MR depending on DMI tested, SDHI ER), and Mf Ja3.9a (QoI S, DMI MR, SDHI ER); Mm Ja1.38c (QoI S, DMI ND, and SDHI MR/HR), Mm MG4 (QoI S, DMI RS, and SDHI RS/MR), Mm MG47 (QoI S, DMI RS (with reduced sensitivity)/MR, and SDHI ER). Two Mm isolates with different and multiple sensitivity phenotypes were included in the assay for comparison, as follows: Mm ISC92 (QoI S, DMI RS, and SDHI S), and Mm ISR61 (QoI S, DMI ND (not determined), and SDHI ER).

2.6. Statistical Analysis

The experiments were repeated once. Analysis of variance (ANOVA) using the *F* test and the Scott–Knott test (at 5% probability) for means comparison were performed in the R environment using the statistical packages *agricolae* and *ScottKnott* [58].

3. Results

3.1. SDHI Fungicides Sensitivity Testing

The SDC isolates were grouped based on how well they responded to boscalid and fluxapyroxad fungicides. They fell into categories like sensitive (S) with low responses, less sensitive (LS), moderately resistant (MR), resistant (R), highly resistant (HR), and extremely resistant (ER) based on their EC_{50} values. (Figure 2).

Reduced sensitivity and resistance to boscalid and fluxapyroxad predominated among the 67 SDC isolates tested. Out of the 67 isolates tested, only 1 isolate (1.5%) was classified as sensitive (S) to both fungicides. For boscalid, three isolates (4.5%) were less sensitive (LS), three (4.5%) moderately resistant (MR), three (4.5%) resistant (R), seven (10.4%) highly resistant (HR), and fifty (74.6%) extremely resistant (ER). With respect to fluxapyroxad, 26 isolates (38.8%) were less sensitive (LS), 2 (3.0%) were moderately resistant (MR), 3 (4.5%) were resistant (R), 2 (3.0%) were highly resistant (HR), and 33 (49.3%) were extremely resistant (ER) (Figure 2).

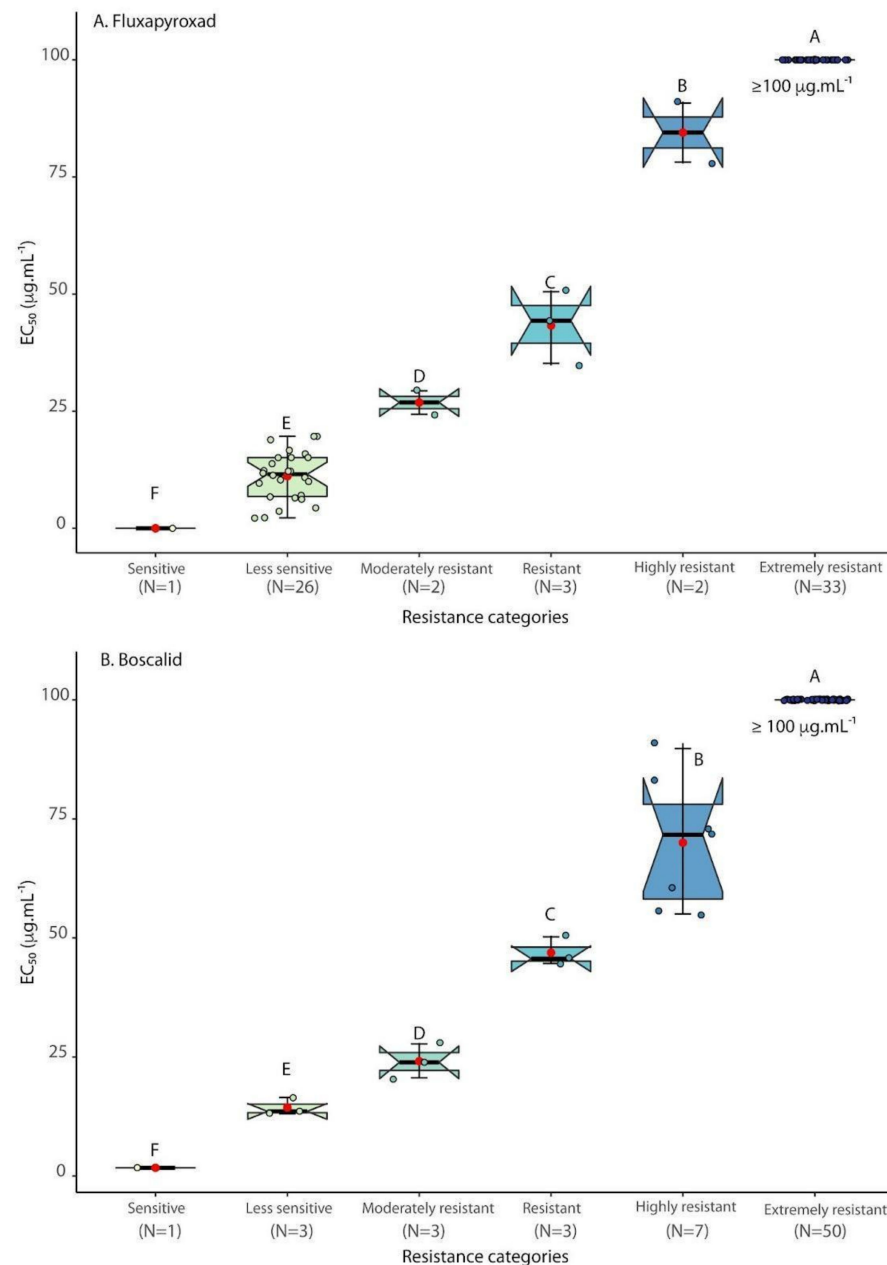


Figure 2. Average fluxapyroxad (**A**) and boscalid (**B**) sensitivities (EC_{50} values in $\mu\text{g mL}^{-1}$) of *M. fijiensis* and *M. musicola* isolates and corresponding resistance categories. The *F* test from ANOVA, significant at $p \leq 0.001$, assesses differences among groups of isolates from distinct resistance categories. Means, indicated by a red dot, followed by the same capital letter, are not significantly different by the Scott–Knott test at $p \leq 0.05$.

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

boscalid and fluxapyroxad. The *Mf* isolates showed statistically higher mean EC_{50} values (mostly $\geq 100 \mu\text{g mL}^{-1}$) than the *Mm* isolates (Figure 3B).

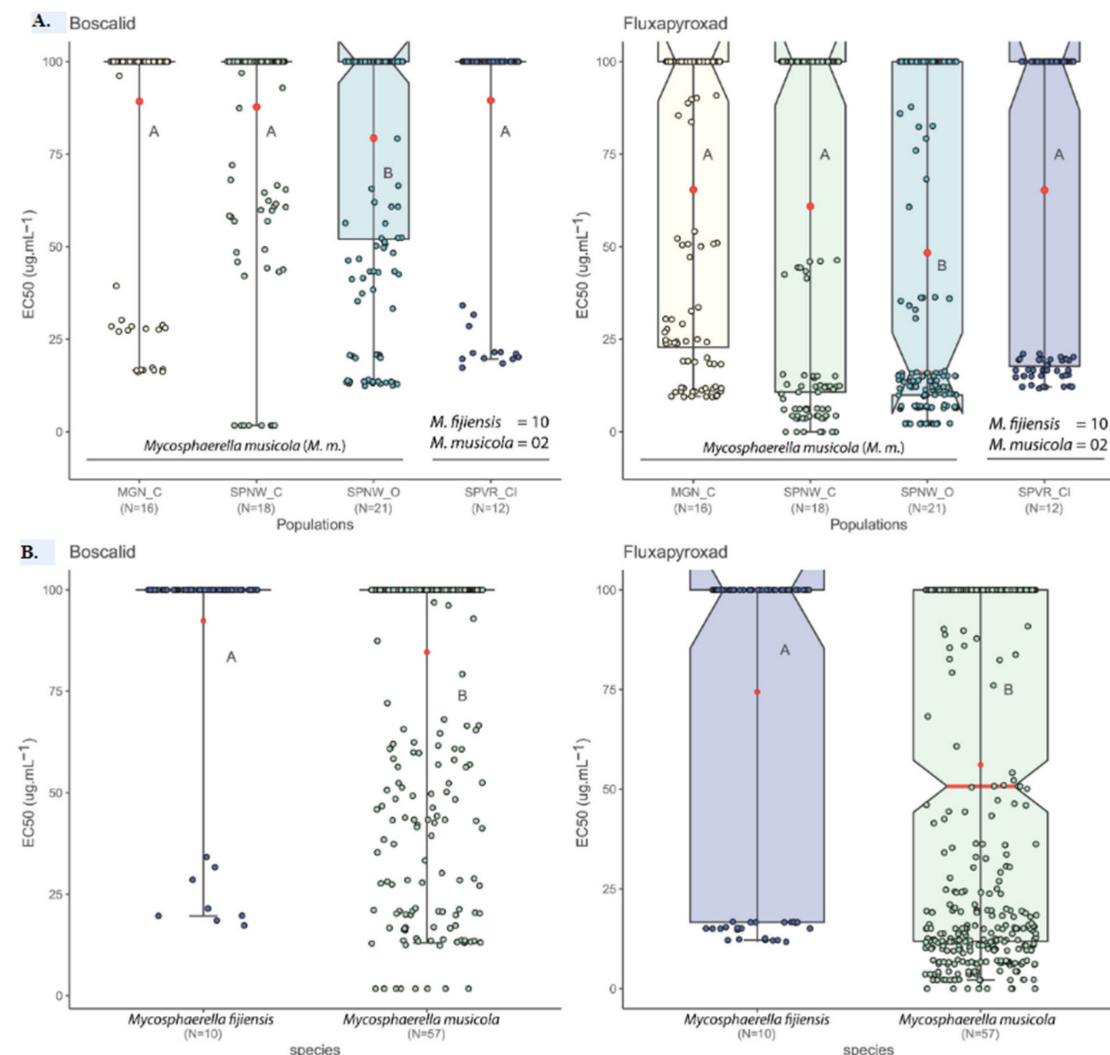


Figure 3. (A) Contrast in SDHI sensitivity between different SDC populations sampled at different locations according to average EC_{50} values ($\mu\text{g mL}^{-1}$) for boscalid and fluxapyroxad. (B) Contrast in SDHI sensitivity between isolates of *Mm* and *Mf* according to EC_{50} values ($\mu\text{g mL}^{-1}$) for boscalid or fluxapyroxad. The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in different colors to avoid data overplotting. The lower whisker covers all the data values from the minimum value up to quartile Q1, that is, the lowest 25% of data values. The upper whisker covers all the data values between quartile Q3 and the maximum value, that is, the highest 25% of data values. N is the number of isolates per population. The F test from ANOVA was significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$.

3.2. Detection of Mutations in the *Sdh* Genes

All primer combinations designed and used in this study were efficient in amplifying the target *Sdh* genes and produced fragments of the expected size (Table 1). Complete sequences of the *SdhB*, *SdhC*, and *SdhD* genes were obtained for all the 67 SDC isolates examined. Three non-synonymous substitutions were identified in the *Sdh* genes of the SDC pathogens (highlighted as red boxes in Figure 4).



Figure 4. Alignment of amino acid sequences translated from the nucleotide sequences of the *SdhB*, *SdhC*, and *SdhD* genes of isolates CALT1 (*Mf*), ISC97 (*Mm*), and ISR61 (*Mm*) to identify nonsynonymous mutations by comparison with the reference sequences NW_006921538 (*Mf*), NW_006921536 (*Mf*), and NW_006921535 (*Mf*) for the *SdhB*, *SdhC*, and *SdhD* genes, respectively.

Interspecific comparison between the reference Sdh amino acid sequences of *Mf* and *Mm* showed a high similarity among the three subunits, with only 24 alterations (23 substitutions and 1 deletion) detected: 10 in the *SdhB* gene (deletion included), 8 in the *SdhC*, and 6 in the *SdhD* (Figure 4).

Each of these sequences represents a protein variant. The translated NCBI/Genbank® sequences LFZO0100506 (*Mm*), LFZO0100371 (*Mm*), and LFZO0100522 (*Mm*) for the *SdhB*, *SdhC*, and *SdhD* genes, respectively, were also aligned for comparison purposes. Sequences from the sensitive isolate ISC92 (*Mm*) were also included. Red boxes indicated amino acid substitution detected in the *SdhB*, *C*, or *D* genes of *Mf* and *Mm* isolates. Green bar indicates identical amino acid at the respective position. Red boxes highlight three non-synonymous substitutions identified in the *Sdh* genes of the SDC pathogens.

3.2.1. Mutations in the *SdhB*, *SdhC*, and *SdhD* Genes from *Mf* and SDHI Sensitivity

The *SdhB* gene was 961 or 964 base pairs (bp) long and encoded 299 amino acids, in addition to the stop codon (Figure 4). The open reading frame (ORF) was structured into two exons, with an intron of 61 (reference sequence *Mf* NW_006921538) or 64 bp long (all *Mf* isolates sampled). The *SdhC* gene was 663 bp long and encoded 184 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two introns

of 49 and 59 bp in length. The *SdhD* gene was 692 bp long and encoded 188 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two introns of 65 and 60 bp in length.

For *Mf*, only one non-synonymous mutation was detected in one of the *Sdh* target-encoding genes. This mutation results in the amino acid substitution N52D (asparagine (AAC) to aspartic acid (GAC) exchange) at codon position 52 of *SdhC* in comparison with the reference sequence XM_007928933. *SdhC*-N52D was detected in all 10 *Mf* isolates from population SPVR-CI. The sensitivity of these isolates ranged from moderately resistant to extremely resistant (Figure 3), with average EC_{50} values of $65.3 \mu\text{g mL}^{-1}$ and $89.5 \mu\text{g mL}^{-1}$ for fluxapyroxad and boscalid, respectively. *SdhC*-D52 was present in all *Mm* isolates, including the SDHI-sensitive isolate *Mm* ISC92.

3.2.2. Mutations in the *SdhB*, *SdhC*, and *SdhD* Genes from *Mm*

The *SdhB* fragment was 955 base pairs (bp) long and encoded 300 amino acids (Figure 4). The open reading frame (ORF) was structured into two exons, with an intron 52 bp long. Alignments allowed the identification of a nonsynonymous mutation resulting in E196Q (glutamic acid (GAA) to glutamine (CAA) exchange at codon 196) in *SdhB* from isolate ISR61 (Figure 4). This mutation was only found in this isolate, originating from the SPNW-O population, with ER/HR resistance categories for boscalid and fluxapyroxad fungicides, respectively. With one of the highest EC_{50} values, ranging from 77.9 to $100 \mu\text{g mL}^{-1}$ for fluxapyroxad and boscalid, respectively, it was one of the most resistant *Mm* isolates. The *SdhC* fragment was 662 bp long and encoded 184 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two introns of 49 and 58 bp in length. No nonsynonymous mutations were identified in this gene (Figure 4). The *SdhD* fragment was 700 bp long and encoded 188 amino acids (Figure 4). The open reading frame (ORF) was divided into three exons, with two introns of 77 and 56 bp in length. The alignment analyses using reference sequences resulted in the identification of one amino acid substitution, N57K (asparagine (AAC) to lysine (AAA) exchange at codon 57). This mutation was only present in the SDHI-sensitive isolate *Mm* ISC92 from the population SPNW-C. It had the lowest average EC_{50} of $1.7 \mu\text{g mL}^{-1}$ and $0.75 \mu\text{g mL}^{-1}$ for boscalid and fluxapyroxad, respectively (Figure 3).

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

3.2.3. Examination of an Additional *SdhC* Paralog from *Mf* and *Mm*

We detected this second *SdhC* paralog in all SDC isolates that we screened ($N = 12$ *Mf* and $N = 37$ *Mm*). The *MfSdhC2* fragment examined was 306 bp long, and a protein sequence of 101 aa was identified based on an original partial reference mRNA transcript of 375 bp (XM_007925990.1 from *P. fijiensis* CIRAD86) and a protein sequence of 125 aa (XP_007924181.1). No nucleotide variation and no amino acid substitutions were detected in the 12 *Mf* *SdhC2* sequences analyzed, represented by the single haplotype *SdhC2* *Mf* CALT1 (Figure 5).

For *Mm*, an 845 bp DNA fragment covering a 629 bp full-length sequence of the second *SdhC* paralog (hypothetical protein AC579_1677 (GenBank KXT11278.1)) was amplified by PCR based on whole genome sequence information (LFZO01000210.1). The open reading frame (ORF) of this second *SdhC* paralog, *MmSdhC2*, was divided into two exons of 116 and 466 bp, separated by one intron of 47 bp in length. No nonsynonymous amino acid substitutions were identified in the *MmSdhC2* sequences from 37 *Mm* isolates, with different levels of insensitivity measured for boscalid and fluxapyroxad (Figure 5).



Figure 5. Alignment of amino acid sequences translated from the nucleotide sequences of the second SdhC paralog (SdhC2) of isolates ISR61 (*Mm*), ISC97 (*Mm*), ISR12 (*Mm*), MG17 (*Mm*), MG57 (*Mm*), ISC92 (*Mm*), and CALT1 (*Mf*) to identify nonsynonymous mutations by comparison with the SdhC and alt-SdhC/SdhC2 reference sequences.

The translated NCBI/Genbank® sequences XP_003850451 (*Zt*) as a general reference sequence, LFZO0100371 (*Mm*) and NW_006921536 (*Mf*) from the *SdhC* gene, and QCI34350 (*Zt*), KXT11278.1 (locus tag AC579_1677, *Mm* genome LFZO01000210), and XM_007925990.1 (*Mf*) from the alt-SdhC/SdhC2 dispensable paralog were also aligned for comparison purposes. Blue boxes indicate positions where SdhC mutations have been reported for *Zt* isolates [33,35,47,66]. The red box highlights the Qp-site amino acid residue likely involved in the differential SDHI sensitivity pattern (residues 84 and 78 in the *Z. tritici* SdhC and alt-SdhC sequences) [50].

All *Mf* and *Mm* isolates showed the presence of an isoleucine residue at position 88 of alt-SdhC, which is equivalent to positions 84 and 78 in the *Z. tritici* SdhC and alt-SdhC sequences, respectively, where alanine is present (see red box in Figure 5). Substitution of alanine by isoleucine at these residues in SdhC and alt-SdhC has been shown to confer resistance to stretched heterocycle-amide SDHIs in *Z. tritici*, such as fluopyran [50].

3.3. Efflux Pump Inhibition Assay

The efflux pump transporter inhibitors BLT-4 and verapamil significantly affected the fluxapyroxad sensitivity of the three MDR *Mf* isolates, with an up to 3-fold increase, while the sensitivity of boscalid was not significantly affected (Figure 6). Similarly, both BLT-4 and verapamil significantly affected the fluxapyroxad sensitivity of the three MDR *Mm* isolates, with an up to three-fold increase (Figure 7). In contrast to MDR *Mf*, verapamil significantly affected the sensitivity of the three MDR *Mm* isolates to boscalid (Figure 7).

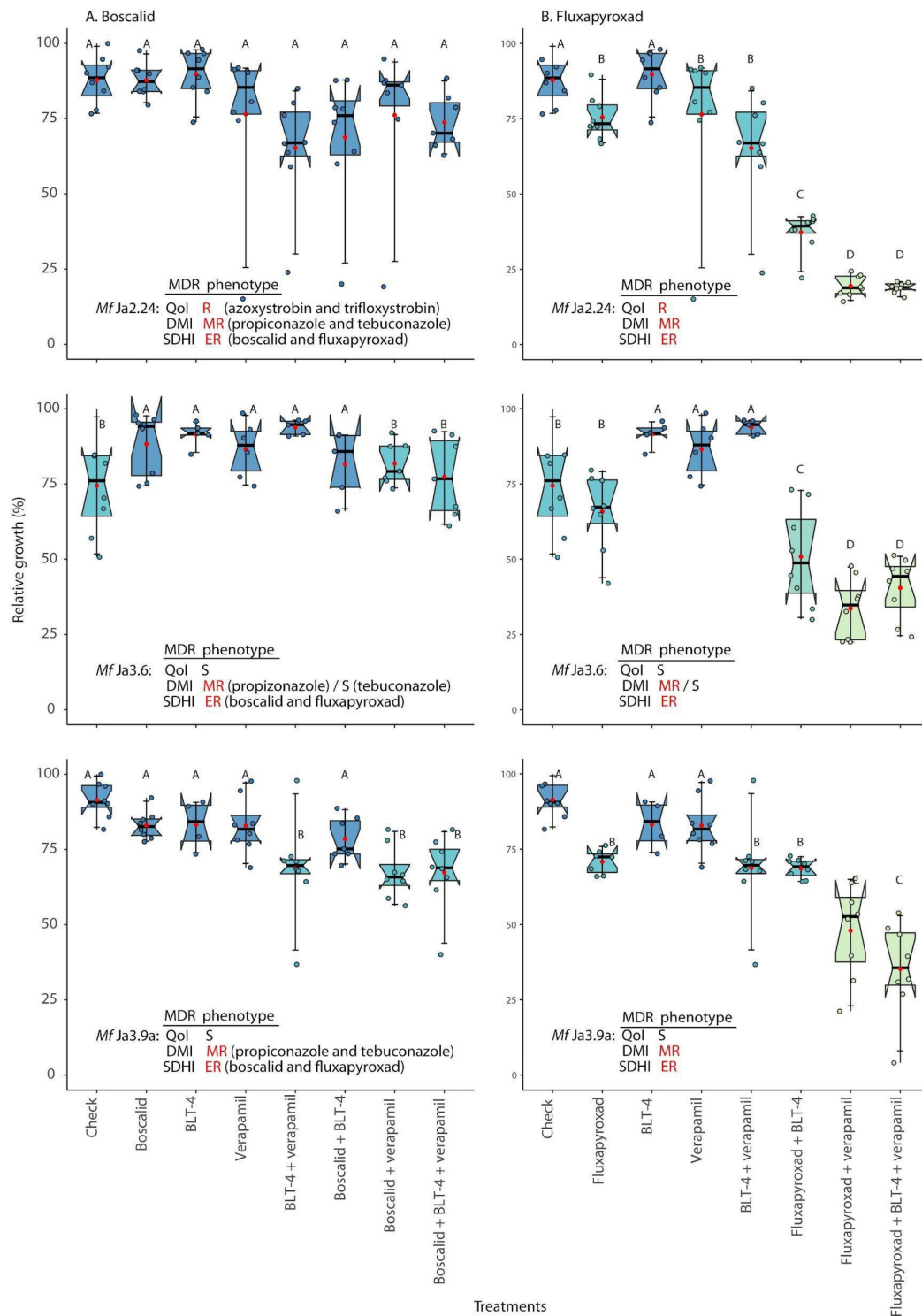


Figure 6. Impact of efflux pump transporter inhibitors BLT-4 and verapamil on reinstating sensitivity in multi-drug-resistant (MDR) isolates of *Mycosphaerella fijiensis* to boscalid and fluxapyroxad. The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in different colors to avoid data overplotting. The lower whisker covers all the data values from the minimum value up to quartile Q1, that is, the lowest 25% of data values. The upper whisker covers all the data values between quartile Q3 and the maximum value, that is, the highest 25% of data values. The F test from ANOVA was significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$.

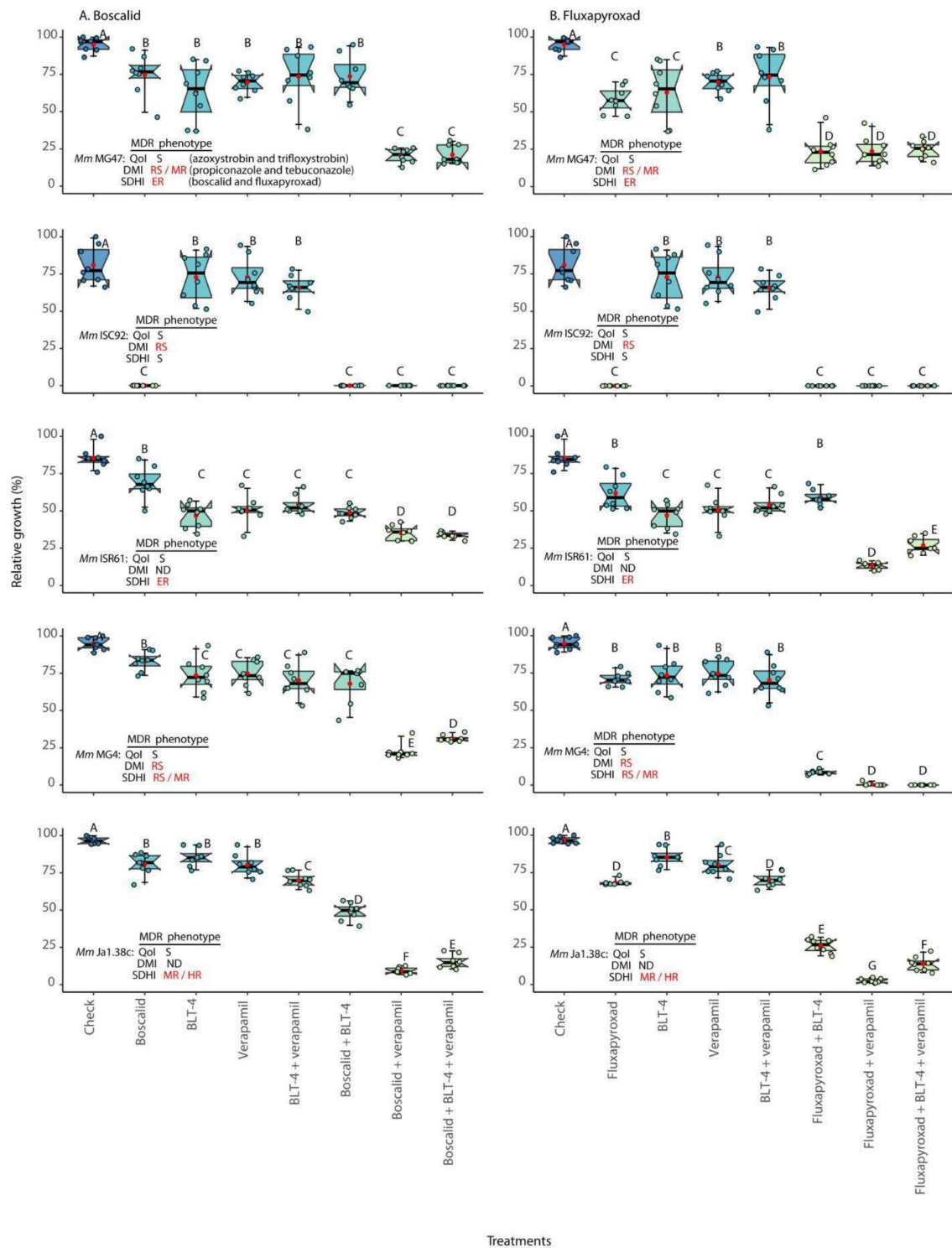


Figure 7. Impact of efflux pump transporter inhibitors BLT-4 and verapamil on reinstating sensitivity in multi-drug-resistant (MDR) isolates of *Mycosphaerella musicola* to boscalid and fluxapyroxad. The figure depicts boxplots with the medians represented by red lines across the notches, the average as red circles along the whisker lines, and the jittered data points in different colors to avoid data overplotting. The lower whisker covers all the data values from the minimum value up to quartile Q1, that is, the lowest 25% of data values. The upper whisker covers all the data values between quartile Q3 and the maximum value, that is, the highest 25% of data values. The F test from ANOVA was significant at $p \leq 0.001$. Means followed by the same capital letter are not significantly different by the Scott-Knott test at $p \leq 0.05$.

4. Discussion

In this study, we determined the in-vitro sensitivity status of *Mf* and *Mm* populations to the SDHI fungicides boscalid and fluxapyroxad at four locations in southeastern Brazil with distinct management systems for controlling SDC, receiving intensive, reduced, or no fungicide inputs. Though in-vitro fungicide sensitivity assays might not give an accurate prediction for the efficacy of SDC control in the field, EC_{50} values are still important as baseline sensitivity data to study the adaptation of pathogen populations to fungicides in the field over time. Considering that the SDHI fungicide boscalid has been sprayed to control SDC on bananas since its introduction in 2014, we hypothesized that continuous fungicide spraying exerted selection pressure on these highly adaptable pathogen populations, leading to the emergence and spread of resistance [3,10,22]. Because fluxapyroxad has not been labeled for the management of SDC yet, it is also important to check for cross-resistance, as this can inform resistance management strategies.

The first important observation of our study was the high prevalence of resistance to the SDHI fungicides boscalid and fluxapyroxad in populations of *Mf* and *Mm*, especially in, but not restricted to, banana fields with intensive fungicide spraying. Using in-vitro sensitivity assays, the prevalence of resistance (i.e., the proportion of individuals with $EC_{50} > 20 \mu\text{g mL}^{-1}$) ranged from 60% (40 out of 67 isolates) to 94.0% ($N = 63$ out of 67 isolates) to fluxapyroxad and boscalid, respectively. Significant fractions of the isolates, 52% for fluxapyroxad and 75% for boscalid, belonged to the extremely resistant (ER) category (Figures 2 and 3). Only a single *Mm* isolate (ISC92) was fully sensitive (EC_{50} values between 0.75 and $1.7 \mu\text{g mL}^{-1}$ for fluxapyroxad and boscalid, respectively). This contrasts with data from the current literature that reported only a few SDHI-insensitive *Mf* strains in banana plantations from Colombia, Costa Rica, Ecuador, and Guatemala, in areas where boscalid, fluopyram, fluxapyroxad, and/or isopyrazam were sprayed from 2012 to 2017 [51,52].

Those EC_{50} values for boscalid and fluxapyroxad were, respectively, 8.0 to 8.7 times higher than the mean EC_{50} for the sensitive *Mm* isolates. Because we could not find boscalid-sensitive *Mf* isolates in our sampling, we compared the resistant *Mf* isolates with the sensitive *Mm* isolates. According to this comparison, the *Mf* isolates were, on average, 8.2 times less sensitive than the sensitive *Mm* isolates. For fluxapyroxad, the mean EC_{50} for the resistant *Mf* isolates was 6.8 times higher than the mean EC_{50} for the three sensitive *Mf* isolates detected.

Comparing the populations' resistance factor (RF) for boscalid, which expresses how proportionally high the EC_{50} of the resistant population is in comparison with the sensitive population (highest EC_{50} over the lowest), the insensitivity levels amongst both *Mm* and *Mf* isolates ranged from 11.3 to $90.6 \mu\text{g mL}^{-1}$, with an average resistance factor (RF) of 8.05. Similarly, for fluxapyroxad, these insensitivity values ranged from 12.4 to $94.7 \mu\text{g mL}^{-1}$, resulting in a RF of 7.60.

Our EC_{50} values for *Mf* and *Mm* were not dissonant in comparison with other fungi. For instance, isolates of *B. cinerea* from strawberries were able to withstand fungicide concentrations up to $75 \mu\text{g mL}^{-1}$ [67] and RF ranging from 3.6 to 18.3 [39]. In *Z. tritici* from wheat, boscalid RF ranged from 0.2 to 10-fold in field strains and to more than 64-fold in laboratory mutants [49]. As another example, populations of the wheat blast pathogen *Pyricularia oryzae* *Triticum* lineage were predominantly resistant to fluxapyroxad, with EC_{50} values ranging from 1.2 (sensitive strains) to $>50 \mu\text{g mL}^{-1}$ (highly resistant) and RF > 43 [68].

Since resistance to SDHI fungicides was detected in the *Mm* and *Mf* populations, we also investigated the association between the levels of resistance in isolates and mutations in the fungicide target protein-encoding genes *SdhB*, *SdhC*, and *SdhD* [69]. Most studies on resistance mechanisms to SDHI fungicide in plant pathogens have found target site alterations as the main underlying cause [45]. Examples of plant pathogens where *Sdh* mutations have been reported both in field isolates and in lab mutants are *A. alternata* (*SdhB*-H277Y/R, *SdhC*-H134R, *SdhD*-D123E, *SdhD*-H133R), *B. cinerea* (*SdhB*-P225L/T/F, *SdhB*-H272Y/R/L/V, *SdhB*-N230I, *SdhD*-H132R), *P. teres* (*SdhB*-H277Y, *SdhC*-N75S, *SdhC*-

G79R, SdhC-H134R, SdhC-S135R, SdhD-D124N/E, SdhD-H134R, SdhD-D145G), and *Z. tritici* (SdhB-N225T, SdhB-R265P, SdhB-T268I, SdhC-T79N/I, SdhC-W80S, SdhC-S83G, SdhC-A84F/I, SdhC-L85P, SdhC-N86A/S, SdhC-R87C, SdhC-V88D, SdhC-H145R, SdhC-R151M/R/S/T, SdhC H152R) [33,41,52,65,70–74].

In our study, three Sdh target site alterations were identified in the SDC populations after comparing *Sdh* sequences from distinct isolates with those from reference sequences. The first alteration, SdhB E196Q (Figure 4), was only found in one isolate, ISR61, having one of the highest EC₅₀ values for boscalid and fluxapyroxad but absent in other isolates with an extreme resistant phenotype (EC₅₀ values $\geq 100 \mu\text{g mL}^{-1}$). Glutamic acid at this position of SdhB is well conserved but not likely directly involved in SDHI binding. Since the isolate *Mm* ISR61 was sampled from a banana plantation with no fungicide use (SPNW-O), it is plausible that it has been introduced in the area by gene flow from banana farms with a history of fungicide use [10]. The second alteration detected was SdhC N52D (Figure 4). All *Mf* isolates carried SdhC N52D, but C-N52D was also detected in all *Mm* isolates, including the SDHI-sensitive isolate ISC92 (Figure 4). In addition, SdhC C-N52D is not likely part of the SDHI binding pocket [75]. Variation in the wild-type SdhC sequence can also occur among species, as shown for *Z. tritici* and the reference sequence for *Mf* originating from an isolate from Cameroon (CIRAD86) (Figure 5). The third alteration, SdhD N57K (Figure 4), was only carried by the isolate *Mm* ISC92. Comparisons with other fungi, including *Mf*, show that asparagine at this position is conserved but not likely directly involved in SDHI binding [75].

The presence of two additional SdhC paralogs was recently reported for the closely related *Z. tritici*. One dispensable paralog, alt-SdhC encoded by *ZtSdhC3*, was present in a subpopulation of *Z. tritici* as a standing genetic variation and was responsible for conferring resistance to SDHIs [49,50]. A second *SdhC* paralog, *SdhC2*, was also detected in all *Mf* and *Mm* isolates examined in our study (Figure 5). This paralog showed the presence of an isoleucine residue at position 88, which is equivalent to positions 78 and 84 of *Z. tritici* alt-SdhC and SdhC, respectively (Figure 5). For *Z. tritici*, the substitution of alanine by isoleucine in both SdhC and alt-SdhC can confer resistance to stretched heterocycle-amide SDHIs (SHA-SDHIs), a subclass of chemically-related SDHIs such as fluopyram and isofetamid [50]. Further research is needed to check if SdhC2 in *Mm* and *Mf* can form a complex with the three other Sdh subunits, leading to a fully functional enzyme, and whether SDHI fungicides can bind to this paralog. No mutations were found in *Mf* or *Mm* SdhC2 (Figure 5), conferring different levels of resistance to boscalid and fluxapyroxad, so SdhC2 does not seem to play a key role in the different levels of SDHI resistance found in these isolates.

However, other resistance mechanisms can occur as well, such as overexpression of target genes and multidrug efflux pumps [34,45,46,49,76]. For instance, in *Z. tritici*, strains without target site alterations in the *SdhB*, *C*, or *D* genes were resistant by an efflux pump mechanism and had a 519-bp insert in the *MgMFS1* promoter [77]. In our study, out of the 63 *Mm* and *Mf* isolates with resistance to SDHI, approximately 77% (N = 49) were also insensitive to the DMIs propiconazole and tebuconazole ([47,55] and Personal communication, 2023 [65]). A total of 18.5% of the isolates sampled from these pathogens' populations were also resistant to QoI fungicides [7]. It is plausible that multidrug resistance (MDR) associated with efflux pump mechanisms has emerged in populations of *Mf* and *Mm*, as theoretically predicted and/or reported for other fungal systems [28,29,68,77]. In fact, our efflux pump inhibition assay indicated that verapamil and, to a lesser extent, BLT-4 could lower the sensitivity of *Mf* and *Mm* to fluxapyroxad at least three-fold (Figures 5 and 6). This was an indication that resistance to fluxapyroxad in *Mf* and also in the closely related species *Mm* can pre-exist in field populations due to adaptation to selection pressure by fungicides belonging to different modes of action due to overexpression of efflux pumps, as reported also for *B. cinerea* [78], *P. oryzae* *Triticum* lineage [79], and *Z. tritici* [77].

The elucidation of fungicide resistance mechanisms to multiple fungicides in populations of *Mf* and *Mm* from southeastern Brazil is highly relevant for the decision-making

process on fungicide spraying. The decision on fungicide spraying should now consider the adoption of anti-resistance strategies for SDC management based on integrated disease management practices, since these three groups of fungicide actives (DMIs, QoIs, and SDHIs) can already have partly lost their field efficacy due to the development of MDR. These anti-resistance strategies could include, for instance, frequent scouting for fungicide resistance in local banana fields to monitor the prevalence and spread of QoI, DMI, and SDHI-resistant strains of *Mf* and *Mm* based on direct monitoring of airborne inoculum and the prevalence of fungicide resistance alleles, target and non-target site resistance, in aerosol samples of spores [80,81]. Early detection of airborne spore levels of the pathogens could prevent major crop yield losses by allowing the adoption of timely and appropriate disease management practices.

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

5. Conclusions

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

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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 Certificates #A64D0EA and A100786 authorizing the scientific activities associated with the collection of botanical and fungal material from the banana agroecosystems in the Cerrado's and Atlantic Forest's biomes and access to the genetic diversity of *Mycosphaerella* species.

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

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