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ORIGINAL ARTICLE

Multiple resistance of *Colletotrichum truncatum* from soybean to QoI and MBC fungicides in Brazil

Flávia Rogério[1,2] | Renata Rebellato Linhares de Castro[1] | Nelson Sidnei Massola Júnior[1] | Thaís Regina Boufleur[1] | Ricardo Feliciano dos Santos[1]

[1]Department of Plant Pathology and Nematology, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (USP), Piracicaba, SP, Brazil

[2]Departamento de Microbiología y Genética, Instituto de Investigación en Agrobiotecnología (CIALE), Universidad de Salamanca, Villamayor, Salamanca, Spain

Correspondence
Flávia Rogério, Department of Plant Pathology and Nematology, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (USP), Piracicaba, SP 13418-900, Brazil.
Email: flaviarogerio89@gmail.com

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Abstract

Colletotrichum truncatum, the predominant fungal species associated with soybean anthracnose, is responsible for significant losses in this crop. Chemical control via fungicide application is the most effective strategy for the control of soybean foliar diseases. However, the increasing incidence of anthracnose in some regions of Brazil indicates that current chemical control is not effective against anthracnose. In this study, we evaluated the fungicide sensitivity of *C. truncatum* genetic lineages to the fungicides azoxystrobin, thiophanate-methyl, difenoconazole, and fludioxonil using isolates representing two important regions of soybean production in Brazil. We characterized the molecular resistance to the quinone-outside inhibitors (QoI), methyl benzimidazole carbamates (MBC), and demethylation inhibitor (DMI) fungicide groups based on amino acid sequences of the cytochrome b (*cytb*), β-tubulin gene (*β-tub*), and P450 sterol 14a-demethylases (*CYP51*) genes. Multiple resistance of *C. truncatum* isolates to QoI and MBC was observed associated with mutation points in the *β-tub* (E198A and F200Y) and *cytb* (G143A). Alternatively, low EC_{50} values were found for fludioxonil and difenoconazole indicating high efficacy. Analysis of *C. truncatum* genomes revealed two potential DMI targets, *CYP51A* and *CYP51B*, and higher genetic variability in the *CYP51A* gene. A positive correlation was found between genetic differentiation of *C. truncatum* populations and fungicide sensitivity (Student's *t*-test <0.001). To our knowledge, this is the first report of multiple resistance to QoI and MBC fungicides in *C. truncatum* in Brazil.

KEYWORDS
chemical control, disease management, DMI, fungicide resistance, MBC, QoI, soybean

1 | INTRODUCTION

Soybean anthracnose, caused by *Colletotrichum* species, is one of the most important fungal diseases of the crop. While new species have recently been associated with the disease, *Colletotrichum truncatum* is the predominant species and is responsible for significant losses in soybean fields (Boufleur et al., 2021; Shi et al., 2020). In Brazil, grain losses of 90 kg/ha were reported for each 1% increment in anthracnose incidence in commercial soybean fields (Dias et al., 2016). However, under favourable weather conditions characterized by

high temperatures and moisture, the disease can result in total crop losses (EMBRAPA, 2008; Yang & Hartman, 2016).

Since the emergence of Asian soybean rust (*Phakopsora pachyrhizi*), anthracnose has been underestimated in Brazil. Ongoing reports highlighting the increase of soybean anthracnose in the North and Central-West regions suggest that the chemical control program developed for fungal diseases in soybean, mainly focused on rust, has not been effective against anthracnose (Dias et al., 2016). Considering that most of the soybean production in Brazil comes from these regions (CONAB, 2024), where optimal weather conditions for disease development prevail, losses due to anthracnose pose a threat to national production.

Chemical control, which includes seed treatment and fungicide application, remains the most effective strategy for anthracnose management. Numerous commercial products, belonging to different chemical groups, are registered for soybean anthracnose control in the country (AGROFIT, 2021); however, limited information is currently available on their efficacy. The majority of commercial products employed for fungal disease control consist of mixtures of single active ingredients, most of which belong to the chemical group quinone-outside inhibitors (QoI), methyl benzimidazole carbamates (MBC), demethylation inhibitor (DMI), phenylpyrrole (PP), and succinate dehydrogenase inhibitors (SDHI) (FRAC, 2021; Pesqueira et al., 2016).

Increased fungicide use, particularly with repeated applications of molecules with single-site mode of action, may increase selection pressure, favouring the development of resistance. Numerous studies have reported a decline in the sensitivity of *C. truncatum* isolates to QoI, MBC, and DMI across various crops (Chen et al., 2016, 2018; Dias et al., 2016; Poti et al., 2020; Torres-Calzada et al., 2015). In Brazil, studies indicate that the efficacy of anthracnose chemical control in soybean fields in Tocantins State has been suboptimal, with a maximum efficiency of only 41.7% for azoxystrobin (QoI) and cyproconazole (DMI). This suggests that other regions with similar microclimates of humidity and temperature may face similar risks (Dias et al., 2016, 2019).

QoI, MBC, and DMI, extensively employed in agriculture, have specific modes of action, in contrast to multi-site inhibitors that affect a broad spectrum of cellular processes (FRAC, 2021). Site-specific fungicides are conducive to resistance selection, as since a single mutation in the target protein can confer resistance and lead to loss of efficacy (Ma & Michailides, 2005). While various mechanisms can confer fungicide resistance, the majority arise from substitutions in amino acid sequences of the target proteins (Ma & Michailides, 2005; Mair et al., 2016). Molecular studies of resistance in target sites are critical for monitoring fungicide efficacy. They enable the detection of resistant genotypes and facilitate the optimization of their management strategies (Lucas et al., 2015).

QoI fungicides inhibit fungal mitochondrial respiration by binding the ubiquinol-oxidizing (Qo) site of cytochrome *b* (*cytb*), thereby blocking electron transport and preventing ATP production (Bartlett et al., 2002). Three amino acid substitutions – F129L, G137R, and G143A – are linked with QoI resistance, each contributing to different levels of resistance (Gisi et al., 2002; Lucas et al., 2015). MBC fungicides act by inhibiting cell division binding to the beta-tubulin (*β-tub*) gene, preventing microtubule assembly, and disrupting chromosome segregation and migration (Brennan et al., 2007; Downing, 2000). Several target site mutations, mainly in codons E198A/G/K and F200Y, are associated with resistance to MBC fungicides (FRAC, 2021).

DMI resistance disrupts fungal growth by inhibiting the gene cytochrome P450 sterol 14α-demethylase (*CYP51*) in the biosynthesis of sterol (Ziogas & Malandrakis, 2015). The mechanisms of resistance to this group are not fully understood, but three processes have been documented: (i) target-site modification in the gene *CYP51* (Délye et al., 1998), *CYP51* overexpression (Hamamoto et al., 2000), and increased drug efflux pumps (Sanglard et al., 1995). Mutations in the *CYP51* gene appear to be the predominant mechanism (Cools et al., 2013), with various pathogenic fungi, such as *Uncinula necator*, *Blumeria graminis*, *Erysiphe graminis*, and *Candida albicans*, exhibiting mutations in resistant isolates (Délye et al., 1998, 1997; Favre et al., 1999; Wyand & Brown, 2005). *Colletotrichum* species may possess two paralogous *CYP51* genes, displaying distinct levels of sensitivity to DMI (Chen et al., 2020; Wang et al., 2020; Wei et al., 2020). Fludioxonil, a phenylpyrrole (PP) fungicide, has a speculative mechanism of action (FRAC, 2021). Although resistance risk to the group is classified as low to medium, resistance has been identified in other fungal species (Iacomi-Vasilescu et al., 2004; Kanetis et al., 2008). Previous studies demonstrated the efficacy of fludioxonil against *C. acutatum* (Wedge et al., 2007), but information regarding *C. truncatum* remains unavailable.

Fungal plant pathogens frequently exhibit genetically divergent lineages resulting from populational subdivisions caused by distinct factors such as geographic distance and host specialization (James et al., 2006; Soanes et al., 2007). Divergent lineages may employ different mechanisms for causing diseases, developed during the co-evolutionary arms race between fungal populations and their hosts (Plissonneau et al., 2017; Van Oosterhout, 2021). In other words, different populations may harbour varying virulence factors. *C. truncatum* is thought to be an invasive species introduced in Brazil multiple times, leading to the establishment of three genetic lineages dispersed across soybean fields (Rogério et al., 2022, 2019). These lineages exhibit different levels of genetic variation and evidence of sexual recombination, potentially enhancing their adaptation to soybean cultivation.

Given the increase of anthracnose importance in Brazil, this study aimed to investigate the sensitivity of *C. truncatum* isolates from major soybean production regions to four fungicides (azoxystrobin, thiophanate-methyl, difenoconazole, and fludioxonil). Additionally, the study seeks to molecularly characterize isolates with varying levels of sensitivity to these fungicides.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates

Isolates used in this study were collected in 2016 and 2017, from ten soybean commercial fields in two Brazilian regions showing a high incidence of anthracnose (Table 1). Eighteen isolates were

TABLE 1 *Colletotrichum truncatum* isolates used in this study.

Lineage	Isolate	State	GenBank accession number			
			TUB2	*CYTB*	*CYP51A*	*CYP51B*
C1	LFN0169	Mato Grosso	MZ682550	MZ682567	MZ682584	MZ682601
C1	LFN0185	Mato Grosso	MZ682551	MZ682568	MZ682585	MZ682602
C1	LFN0262	Mato Grosso	MZ682556	MZ682573	MZ682590	MZ682607
C1	LFN0309	Goiás	MZ682544	MZ682561	MZ682578	MZ682595
C1	LFN0360	Goiás	MZ682548	MZ682565	MZ682582	MZ682599
C1	LFN0297	Goiás	MZ682542	MZ682559	MZ682576	MZ682593
C1	LFN0346	Goiás	MZ682546	MZ682563	MZ682580	MZ682597
C2	LFN0205	Mato Grosso	MZ682552	MZ682569	MZ682586	MZ682603
C2	LFN0217	Mato Grosso	MZ682553	MZ682570	MZ682587	MZ682604
C2	LFN0248	Mato Grosso	MZ682555	MZ682572	MZ682589	MZ682606
C2	LFN0318	Goiás	MZ682545	MZ682562	MZ682579	MZ682596
C2	LFN0349	Goiás	MZ682547	MZ682564	MZ682581	MZ682598
C2	LFN0288	Goiás	MZ682541	MZ682558	MZ682575	MZ682592
C3	LFN0150	Mato Grosso	MZ682549	MZ682566	MZ682583	MZ682600
C3	LFN0225	Mato Grosso	MZ682554	MZ682571	MZ682588	MZ682605
C3	LFN0268	Mato Grosso	MZ682557	MZ682574	MZ682591	MZ682608
C3	LFN0308	Goiás	MZ682543	MZ682560	MZ682577	MZ682594

previously genotyped by multilocus microsatellite typing and whole-genome sequencing (Rogério et al., 2022, 2019), and they represent the three genetic groups (C1, C2, and C3) detected in those fields.

2.2 | In vitro fungicide sensitivity assays

The sensitivity of *C. truncatum* isolates to fungicides was determined based on mycelial growth inhibition assay fungicide-amended on potato dextrose agar medium (PDA). We used commercial formulations of azoxystrobin (Amistar 500 WG, Syngenta Crop Protection), thiophanate-methyl (Cercobin 700 WP, Ihara), difenoconazole (Score 250 EC, Syngenta Crop Protection), and fludioxonil (Maxim 25, Syngenta Crop Protection). These fungicides were selected based on the active ingredients registered for controlling soybean anthracnose in Brazil (AGROFIT, 2021).

Based on preliminary assays, we observed that *C. truncatum* isolates showed intense mycelial growth, with an effective concentration to inhibit 50% of the mycelial growth (EC_{50}) higher than 100 μg/mL for azoxystrobin and thiophanate-methyl fungicides. In this way, we used single discriminatory doses of 100 μg/mL to distinguish between resistant and sensitive isolates for these fungicides (Moreira et al., 2019). Isolates that showed a percentage of mycelial growth inhibition higher than 50% were classified as resistant. Five-millimetre-diameter mycelial plugs were taken from actively growing 7-day-old colonies on PDA and transferred to PDA plates amended with the fungicide concentration of 0 and 100 μg/mL. Plates were incubated at 25°C under constant light for 5 days. Each fungicide-isolate combination and control plate (i.e., plates onto non-amended PDA) were replicated three times and experiments were performed twice. The diameter of each colony was used to calculate the percentage of mycelium inhibition (MGI). MGI was obtained using the formula: MGI = ((*C*-FT/*C*)*100), where MGI is the mycelial growth inhibition, *C* is the control treatment colony diameter, and FT is the fungicide treatment colony diameter. EC_{50} values for the percentage of mycelial growth inhibition were calculated using linear regression analysis between MGI and the log_{10}-transformed fungicide concentration.

The sensitivity of *C. truncatum* isolates to the difenoconazole and fludioxonil was also determined by mycelia growth assays. Mycelia plugs were placed upside down onto PDA dishes amended with difenoconazole at 0, 0.01, 0.1, and 10 μg active ingredient (a.i.)/mL; and Fludioxonil at 0, 0.001, 0.01, 0.1, and 1 μg active ingredient (a.i.)/mL. The experiment was performed following the methodology described above. A regression analysis based on the percentage of mycelial growth inhibition was performed to estimate the EC_{50} value for these fungicides. The experiment was performed twice, and the combined data demonstrated that variances were homogeneous according to *F*-test ($P < 0.05$).

2.3 | Molecular characterization of fungicides target genes

To investigate point mutations in the *cytb*, *β-tub*, and *CYP51* genes, we used genomic data available from all isolates (Rogério et al., 2022). The BLASTn tool (Altschul et al., 1990) was used to retrieve the gene

sequences related to resistance to the fungicides from the genomes. The *cytb* and *β-tub* genes were retrieved from genomes using as query sequences the strain *C. truncatum* CMES1059 (GenBank accession number MK163913.1 and MK188497, respectively). For DMI, we investigated the presence of the paralogs *CYP51A* and *CYP51B*, as well as the point mutations on them. Therefore, we used the strain *C. truncatum* CtRR131 as query sequences (GenBank accession number MG799553.1 and MG799552, respectively). Predicted amino acid sequences along the DNA sequences obtained were performed using Expasy Bioinformatics Resource Portal and aligned using MEGA11 software (Kumar et al., 2016).

2.4 | Phylogenetic analysis

The deduced amino acid sequences of *CYP51* paralogs genes were used to investigate the phylogenetic relationship between isolates. A phylogenetic tree was constructed based on the concatenated alignment of *CYP51* sequences generated in this study, in addition to *CYP51* homologues from several *Colletotrichum* species and other closely related ascomycete fungi, including *Saccharomyces cerevisiae* as outgroup (GenBank accession XP003713527.1). Multiple alignments were performed using MAFFT v. 7.490 (Katoh et al., 2002) implemented in Geneious 8.1.4., (http://www.geneious.com). The phylogenetic analysis was conducted by the maximum likelihood (ML) method using the JTT matrix-based model.

3 | RESULTS

3.1 | Fungicide sensitivity in vitro assays

The isolates were tested using a single dose of 100 μg/mL of azoxystrobin and thiophanate-methyl, which effectively distinguished between resistant from sensitive isolates for both fungicides. Notably, all isolates tested were resistant to azoxystrobin. Conversely, only the isolates LFN0217 (lineage C2) and LFN0225 (Lineage C3) exhibited sensitivity to thiophanate-methyl. In contrast, all isolates exhibited sensitivity to difenoconazole and fludioxonil, with EC_{50} values ranging from 0.06 to 0.61 μg/mL (mean of 0.17 μg/mL) to difenoconazole and 0.21 e 2.97 μg/mL (mean of 0.84 μg/mL) to fludioxonil (Table 2).

3.2 | Molecular characterization of fungicide resistance mutations

The nucleotide sequences translated of the *cytb* gene from 17 *C. truncatum* isolates revealed a substitution from glycine (G) to alanine (A) at codon 143 in all isolates analysed (Figure 1). This mutation is extensively well documented in the literature as conferring resistance to QoI fungicides. As observed in vitro sensitivity assay, these isolates were classified as resistant, and the presence of this mutation at the molecular level further supported the identified resistance.

TABLE 2 Sensitivity of *Colletotrichum truncatum* isolates from soybean in Brazil to fludioxonil and difenoconazole fungicides.

		Mean EC_{50} values (μg/mL)		Mycelial growth inhibition[a]	
Lineage	Isolate	Fludioxonil	Difenoconazole	Azoxystrobin	Thiophanate-methyl
C1	LFN0297	0.078	1.244	22%	20%
C1	LFN0346	0.211	1.422	3.10%	17.06%
C1	LFN0360	0.178	1.523	0%	16%
C1	LFN0309	0.081	1.035	15%	14.50%
C1	LFN0169	0.089	1.150	0.50%	17%
C1	LFN0185	0.464	2.968	0%	1.50%
C1	LFN0262	0.081	0.526	6.25%	21%
C2	LFN0318	0.079	1.245	3.20%	16.50%
C2	LFN0217	0.145	0.440	5%	65%
C2	LFN0248	0.055	0.233	5.50%	20%
C2	LFN0205	0.083	0.221	6%	6.50%
C2	LFN0288	0.088	0.668	7.20%	20%%
C2	LFN0349	0.182	0.312	2.20%	25%
C3	LFN0150	0.607	0.227	4.50%	30%
C3	LFN0308	0.053	0.650	9.30%	17.50%
C3	LFN0268	0.131	0.212	5%	30%
C3	LFN0225	0.371	0.283	0.50%	88%

[a]Mycelial growth inhibition (%) of *C. truncatum* isolates by azoxystrobin and thiophanate-methyl at 100 μg/mL, using the discriminatory dose method.

Isolate	*In vitro* sensibiliy	129														137														143							
CMES1059[1]	Sensitive	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	G	A	T	V	I	T	N	L
LFN0297	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0346	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0360	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0309	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0169	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0185	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0262	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0318	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0217	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0248	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0205	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0288	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0349	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0150	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0308	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0268	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L
LFN0225	Resistant	G	I	G	F	L	G	Y	V	L	P	Y	G	Q	M	S	L	-	A	A	T	V	I	T	N	L

[1]Amino acid sequence of *C. truncatum* strain CMES1059 (GenBank accession nº MK163913.1).

FIGURE 1 Aligned amino acid sequences of partial cytochrome b gene (codons 126 to 146) of *Colletotrichum truncatum* isolates from soybean. The mutation associated with QoI resistance was observed at codon 143. Amino acids: A, alanine; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Analysis of *β-tub* gene sequences revealed mutations at codons 198 and 200, which confer resistance to MBC fungicides, confirming the sensitivity obtained in vitro tests. The isolates LFN0346, LFN360, LFN390, LFN169, LFN185, LFN318, LFN248, LFN205, LFN349, LFN150, and LFN308 showed substitutions from glutamic (E) to alanine (A) at codon 198 (E198A), while the isolates LFN0297, LFN262, LFN288, and LFN268 showed substitutions from phenylalanine (F) to tyrosine (Y) at codon 200 (F200Y). In contrast, the isolates LFN217 (lineage C2) and LFN225 (lineage C3), identified as sensitive to thiophanate-methyl in the in vitro tests, did not exhibit any of these mutations (Figure 2).

3.3 | Phylogenetic inference of *CYP51* gene

Analysis of *C. truncatum* genomes revealed two paralogous *CYP51* genes, *CYP51A* and *CYP51B*, that putatively encode the protein P450 sterol 14a-demethylases (Figure 3). The deduced 512 amino-acid protein encoded by the 1539 bp coding sequence from *CYP51A* and the 526 amino-acid protein encoded by the 1578 bp coding sequence from *CYP51B* were analysed regarding the presence of mutations.

The substrate recognition sites (SRS) in *CYP51* genes are very conserved in filamentous fungi, and the amino acid alterations occurring around the azole-binding site of the enzyme affect its affinity, and they are commonly investigated in DMI resistance (Han et al., 2010; Mellado et al., 2001). The alignment of sequences from *C. truncatum* isolates and *Aspergillus fumigatus*, here used as reference (GenBank accession number XP_752137.1), revealed eight variations in amino acid sequences, present in 3 SRS, in the form of E105D (SRS1), D253Q, D280E (SRS4), L391V, K484C, K484S, P501A(SRS6) and P501T (SRS6) for *CYP51A* gene (Figure S1). For *CYP51B* no variations were detected between isolates.

4 | DISCUSSION

The recent increase in soybean anthracnose incidence in certain regions of Brazil indicates the ineffectiveness of current chemical management strategies employed for fungal disease control against anthracnose. While chemical control stands as the primary approach to managing anthracnose, limited information regarding its efficacy is available. Here, we reported in vitro resistance of *C. truncatum* isolates to azoxystrobin and thiophanate-methyl associated with point mutations in the *cytb* (G143A) and *β-tub* (E198A and F200Y) genes. Multiple resistance to QoI and MBC, characterized by the same mutations, has recently been reported in *Corynespora cassiicola*, another significant soybean fungus in Brazil (de Mello et al., 2022). Alternatively, fludioxonil and difenoconazole demonstrated high efficacy, with low EC_{50} values. Our investigation also revealed the presence of two *CYP51* paralogous (*CYP51A* and *CYP51B*) and higher genetic variability in the *CYP51A* gene. A slight correlation between the genetic differentiation of *C. truncatum* populations and fungicide sensitivity was observed. Furthermore, difenoconazole EC_{50} values for lineage C1 were found to be statistically different from other lineages (Student's *t*-test <0.001).

All isolates exhibited in vitro resistance to azoxystrobin (QoI) and thiophanate-methyl (MBC) fungicides at a single discriminatory dose of 100 μg/mL. These phenotypic responses were supported at the molecular level through the analysis of the *cytb* gene, revealing the presence of G143A mutation in all isolates. QoI resistance

Isolate	*In vitro* sensibilty	190								198		200			
LFN0297	resistant	H	Q	L	V	E	N	S	D	E	T	Y	C	I	D
LFN0346	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0360	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0309	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0169	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0185	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0262	resistant	H	Q	L	V	E	N	S	D	E	T	Y	C	I	D
LFN0318	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0217	sensitive	H	Q	L	V	E	N	S	D	E	T	F	C	I	D
LFN0248	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0205	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0288	resistant	H	Q	L	V	E	N	S	D	E	T	Y	C	I	D
LFN0349	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0150	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0308	resistant	H	Q	L	V	E	N	S	D	A	T	F	C	I	D
LFN0268	resistant	H	Q	L	V	E	N	S	D	E	T	Y	C	I	D
LFN0225	sensitive	H	Q	L	V	E	N	S	D	E	T	F	C	I	D

FIGURE 2 Aligned amino acid sequences of partial beta-tubulin gene (codons 190 to 200) of *Colletotrichum truncatum* from soybean. Highlighted in black are mutations at codon E198A and F200 linked to MBC resistance. Amino acids: A, alanine; D, acid aspartic; E, acid glutamic; F, phenylalanine; H, histidine; I, isoleucine; L, leucine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine.

has been documented in various *Colletotrichum* species, including *C. graminicola*, *C. siamense*, *C. acutatum*, and *C. cereale* (Avila-Adame et al., 2003; Chechi et al., 2019; Forcelini et al., 2016; Hu et al., 2015; Young et al., 2010). For *C. truncatum*, isolates highly resistant to azoxystrobin have been previously reported, but the molecular mechanism conferring resistance has not been investigated (Torres-Calzada et al., 2015). To our knowledge, this study represents the first report of G143A mutation associated with QoI resistance in this species.

For thiophanate-methyl, we detected the presence of both resistant and sensitive isolates, with resistance prevailing in the majority (88%) of cases. All resistant isolates exhibited F200Y mutations in the *β-tub* gene. The mutation at codon 198 is commonly found in isolates displaying high levels of MBC resistance, while a mutation at position 200 is correlated with moderate resistance levels (Lucas et al., 2015). MBC fungicide resistance has been previously reported in *C. truncatum* across various crops. A high frequency of isolates resistant to carbendazim was observed in soybean fields in Thailand, with the presence of both mutations (Poti et al., 2020). Isolates from diverse hosts (including pepper, papaya, and physic nut) showed resistance to thiabendazole associated with the E198A mutation (Torres-Calzada et al., 2015). A prior study investigating the efficacy of several fungicides, including carbendazim (a representative molecule of the benzimidazoles class), against soybean anthracnose under natural conditions in Brazil, reported a gradual decline in fungicide effectiveness. (Dias et al., 2016). The elevated risk of MBC resistance in *Colletotrichum* spp. is recognized and should be considered in anthracnose control (Nalumpang et al., 2010; Suwan & Na-Lampang, 2013; Torres-Calzada et al., 2015; Vieira et al., 2017; Wong et al., 2008). Cross-resistance between MBC fungicides is documented in numerous phytopathogenic fungi (Chung et al., 2010; Cunha & Rizzo, 2003; Sun et al., 2010; Wong et al., 2008), posing a potential risk to chemical control, given the extensive historical use of MBCs in soybean fields, either alone or in combination with other fungicide groups such as DMIs and QoIs (Pesqueira et al., 2016).

Regarding sensitivity for difenoconazole belonging to DMI, all isolates demonstrated sensitivity. The low EC_{50} value was consistent with previous studies with difenoconazole in *C. truncatum* (Chen et al., 2018, 2016; Zhang et al., 2017). We detected the presence of two *CYP51* paralogous, but no known mutations associated with DMI resistance were detected. A similar result was found in *C. gloeosporioides* isolates evaluated for difenoconazole and propiconazole (Wang et al., 2020). However, we detected eight variations in amino acid sequences in three substrate recognition sites (SRS) within the *CYP51A* gene. In contrast, no variations were detected in *CYP51B*, in concordance with previous studies noting that variation at this paralogous is uncommon (Brunner et al., 2015; Délye et al., 1997). The *CYP51A* gene is reported to be more relevant to DMI sensitivity, and its higher variability suggests potential adaptation under selection pressure, possibly under positive selection. On the other hand, *CYP51B* performs a more conserved function, with purifying selection likely acting on it (Brunner et al., 2015; Chen et al., 2018). Interestingly, some studies have reported resistance of *C. truncatum* from several hosts (including soybean) to DMI fungicides (Carstens et al., 2017; Chen et al., 2018, 2016; Zhang et al., 2017). Although resistance to DMI was not observed in this study, the presence of

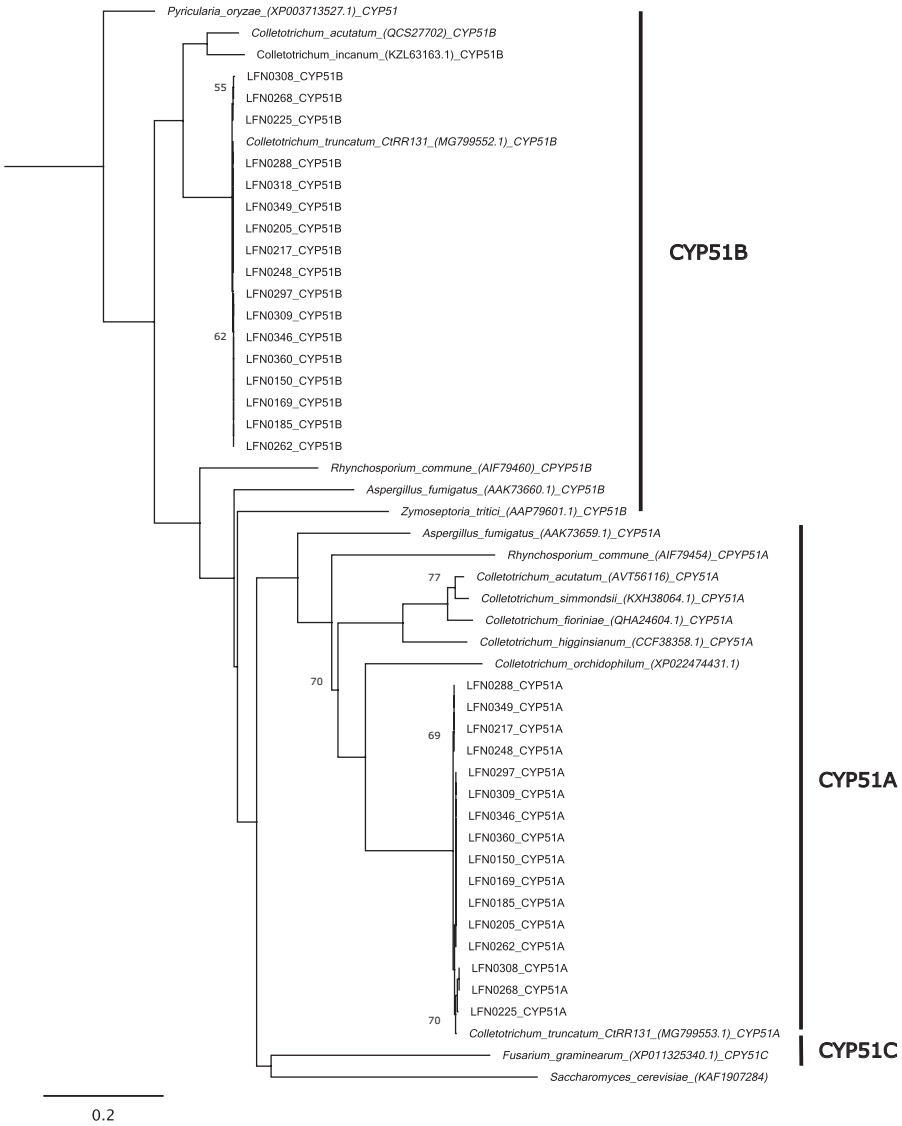

FIGURE 3 Phylogenetic inference of *CYP51* proteins generated by the maximum likelihood method. The deduced amino acid sequences of seventeen *Colletotrichum truncatum* isolates from soybean and other *Colletotrichum* species and fungal species were used in this analysis: CYP51A – *C. truncatum* (strain CtRR131) (GenBank accession no MG799553.1); *C. acutatum* (GenBank accession no AVT56116); *C. simmondsii* (GenBank accession no KXH38064.1); *C. higginsianum* (GenBank accession no CCF38358.1); *C. orchidophilum* (GenBank accession no XP022474431.1); *C. fioriniae* (GenBank accession no QHA24604.1); *Rhynchosporium commune* (GenBank accession no AIF79454). CYP51B – *Aspergillus fumigatus* (GenBank accession no AAK73659.1); *C. truncatum* (strain CtRR131) (GenBank accession no MG799552.1); *C. acutatum* (GenBank accession no QCS27702); *C. incanum* (GenBank accession no KZL63163.1); *Rhynchosporium commune* (GenBank accession no AIF79460); *Aspergillus fumigatus* (GenBank accession no AAK73660.1); *Zymoseptoria tritici* (GenBank accession no AAP79601.1); CYP51C – *Fusarium graminearum* (GenBank accession no XP011325340.1); CYP51 – *Pyricularia oryzae* (GenBank accession no XP003713527.1); *Saccharomyces cerevisiae* (GenBank accession no KAF1907284). Support values below 80 are shown on the nodes.

high nucleotide variability in the *CYP51A* gene may suggest ongoing selective pressure, posing a potential risk for the development of resistance in the future.

Similar to difenoconazole, all isolates exhibited sensitivity to fludioxonil. This fungicide has been used for many years as a seed coating to control plant pathogenic fungi and has a relatively low risk of resistance development (Bersching & Jacob, 2021; Kuang et al., 2011; Walker & Leroux, 2015). In studies with other species of *Colletotrichum* species, fludioxonil has demonstrated efficacy against anthracnose (Chen et al., 2016; Gao et al., 2018). For *C. truncatum*, a similar result was found when isolates from different hosts were evaluated (Torres-Calzada et al., 2015). However, this study used a fludioxonil + cyprodinil mix, which did not enable to determine whether one of the two active ingredients is the most active in the mix or whether there is some synergy between them.

According to our findings, the genetic differentiation of *C. truncatum* populations did not have a notable impact on fungicide sensibility to the fungicides investigated. Despite our expectation that lineage C3, heavily affected by genetic introgression from other lineages and enriched with secreted protein-encoding genes acquired

through such genetic exchanges, might exhibit higher virulence factors, including potential mutation conferring fungicide resistance (Rogério et al., 2022), our results did not support this hypothesis. In other words, the genetic makeup of *C. truncatum* populations currently present in the soybean fields is not related to distinct phenotypic responses to fungicides. However, we did observe a significant difference (Student's *t*-test <0.001) in EC_{50} values to difenoconazole within lineage C1. The correlation between population structure and phenotypes poses a challenge in evolutionary genetics studies, given that virulence factors are often associated with single genes, whereas subdivisional populational affects the entire genome, making it challenging to visualize such signals. In conclusion, for the first time in Brazil, our study reveals multiple resistance of *C. truncatum* to QoI and DMI fungicides which are widely used in the soybean fields.

AUTHOR CONTRIBUTIONS

Flávia Rogério: Conceived the study, designed the project, performed analyses, and wrote the manuscript. Renata Rebellato Linhares de Castro: Performed analyses and reviewed the manuscript. Nelson Sidnei Massola Júnior: Conceived the study, designed the project, and reviewed the manuscript. Thaís Regina Boufleur: Performed analyses and reviewed the manuscript. Ricardo Feliciano dos Santos: Performed analyses and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jph.13341.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/, reference number PopSet: 2315449176.

ORCID

Flávia Rogério https://orcid.org/0000-0001-7801-5112
Thaís Regina Boufleur https://orcid.org/0000-0002-6357-0823

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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