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ORIGINAL ARTICLE

Sensitivity of *Cercospora* spp. from soybean to quinone outside inhibitors and methyl benzimidazole carbamate fungicides in Brazil

Flávia E. de Mello[1] · Valéria S. Lopes-Caitar[2] · Helen Prudente[1] · Sheila A. Xavier-Valencio[1] · Sören Franzenburg[3] · Andreas Mehl[4] · Francismar C. Marcelino-Guimaraes[2] · Joseph-Alexander Verreet[3] · Maria I. Balbi-Peña[1] · Cláudia V. Godoy[2]

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Abstract

Cercospora leaf blight and purple seed stain are caused by *Cercospora kikuchii* and other *Cercospora* spp. The fungus can infect leaves and seeds on soybean, and the disease is controlled with fungicides. In Brazil, the intensive use of fungicides on soybean to control other diseases such as soybean rust has exerted resistance selection pressure on all fungal pathogens that attack in the crop. In this study, we evaluated the sensitivity of 56 *Cercospora* spp. soybean isolates collected during 9 crop seasons in 9 Brazilian states to quinone outside inhibitors (QoI) and methyl benzimidazole carbamate (MBC) fungicides using mycelial growth inhibition in amended media with a discriminatory dose of 10 μg/mL. We also analyzed single polymorphisms in the target genes *cytb* and *β-tubulin* using target genotyping by sequencing by Illumina short reads. Genome-local association was used to correlate the point mutations found in the coding sequence with *in vitro* assays results. For *cytb* gene, it was observed only the G143A mutation in 73% of the isolates. The mutation G143A was present in 97% of the isolates classified as resistant at least for two QoI fungicides. The mutation E198A was present in 71% of the isolates being significantly associated with the reduction control to MBC fungicides. Cross-resistance was observed into QoI (azoxystrobin, picoxystrobin, and pyraclostrobin) and MBC (carbendazim, and thiophanate-methyl) active ingredients fungicides. About 97% of *Cercospora* spp. isolates analyzed possessed the double mutations G143A and E198A and resulting in less sensitive to QoI and MBC fungicides, respectively. All isolates showed homozygous variation in the alternative allele in G143A and E198A mutations. Our results suggest that resistance of the *Cercospora* spp. from soybean to QoI and MBC fungicides has occurred since the 2008 cropping season in Brazil associated with G143A and E198A mutations.

Keywords Cercospora leaf blight · Purple seed stain · *cytb* · *β-Tubulin* · G143A · E198A

Introduction

Soybean (*Glycine max* (L.) Merr.) is the main grain produced in Brazil with a sowing area of 36.7 million hectares in the 2019/20 crop season, representing about 60% of the area with grains during the summer crop season (Conab 2020). The climatic conditions during the cropping season are favorable for the occurrence of diseases, including those caused by *Cercospora* spp. The main *Cercospora* species associated with diseases on soybean are *C. kikuchii* and *C. sojina*. *Cercospora kikuchii* causes Cercospora leaf blight and purple seed stain, whereas *C. sojina* causes frogeye leaf spot (Hartman et al. 2015). Recently, several other *Cercospora* species previously not characterized and reported to infect soybeans are found to be associated with Cercospora leaf blight and purple seed stain (Soares et al. 2015). *Cercospora* spp. is widely disseminated in main Brazilian soybean-producing regions.

In Brazil, Cercospora leaf blight has been controlled with the fungicide applications done for Asian soybean rust, caused

✉ Flávia E. de Mello
flaviamello_89@hotmail.com

[1] Universidade Estadual de Londrina, Londrina, Paraná 86051-970, Brazil

[2] Embrapa Soja, Londrina, Paraná 86001-970, Brazil

[3] University of Kiel, 24118 Kiel, Germany

[4] Bayer CropScience, 40789 Monheim, Germany

by *Phakopsora pachyrhizi* (Godoy et al. 2016). Despite these, in recent years, fungicide failures for Cercospora leaf blight control have become commonplace. A total of 202 fungicides are labeled for soybean to control cercospora in Brazil (AGROFIT 2019). Many of the commonly used fungicides are demethylation inhibitors (DMI), quinone outside inhibitor (QoI), and methyl benzimidazole carbamate (MBC) groups, in pre-mixtures.

The first recommendation of QoI fungicides on soybean in Brazil occurred in 2000. MBC fungicide has been used on soybean since 1997, for powdery mildew control (Recomendações 2000). The use of fungicides in Brazil was intensified in 2001, after the introduction of *P. pachyrhizi.* The average number of fungicides application in soybean increased from 2.4 in 2008 to 3.4 in 2018 (Consórcio Antiferrugem 2019).

Control failure can occur due to the resistance of the fungi to the fungicides. Resistance to QoI group has been detected in several phytopathogenic fungi. The mechanism of QoI resistance involves mutations in the fungi mitochondrial *cytb* gene. The most common mutations are amino acid substitution from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R) (Gisi et al. 2002). These nucleotide changes in *cytb* gene are determined by qualitative and quantitative resistance response resulting in lower sensitivity to QoI fungicides (McGrath 2015). Field resistant populations of *C. kikuchii* (Price et al. 2015), *C. cf. flagellaris* (Albu et al. 2016), *C. sojina* (Mathew et al. 2019; Zhou and Mehl 2020) to QoI fungicides were observed in soybean production regions in the USA. In South America, *C. kikuchii*, *C. nicotianae*, and other several species described in Bolívia (Sautua et al. 2019b) and in Argentina (Sautua et al. 2020) possessed the G143A mutation in *cytb* gene. Recently, resistant *C. beticola* in sugar beet has also been reported in Japan (Kayamori et al. 2020).

MBC chemical group presents a high risk of resistance. Resistance to this group has been reported in many phytopathogenic fungi and usually occurs from specific point mutations in the *β-tubulin* gene that alter the amino acid sequence. These amino acid substitutions have been documented at codons 6, 50, 165, 167, 198, 200, 241, and 257 (Ma and Michailides 2005). The presence of these point mutations in *β-tubulin* gene is associated with qualitative resistance response (McGrath 2015). Resistant populations to MBC were observed in different isolates of *Cercospora* spp. in Japan (Imazaki et al. 2006), the USA (Price et al. 2015), Brazil (Soares et al. 2015), Bolívia (Sautua et al. 2019b), and Argentina (Sautua et al. 2020).

Due to the reported *Cercospora* spp. resistance to QoI and MBC fungicides, we isolated *Cercospora* spp. from soybean lesions of Cercospora leaf blight and purple seed stain in nine Brazilian states to understand the distribution of resistance in soybean-production regions. This study was conducted to determine the *in vitro* sensitivity of *Cercospora* spp. isolates infecting soybean to the QoI fungicides azoxystrobin, picoxystrobin, and pyraclostrobin and to the MBC fungicides carbendazim, and thiophanate-methyl. We also characterized the possible polymorphisms across the target genes to determine the point mutations involved in the QoI and MBC resistance by association with *in vitro* fungicide assay.

Materials and methods

Isolates

Fifty-six isolates of *Cercospora spp*. from soybean were collected in the following Brazilian states: Distrito Federal (DF) ($n = 2$), Goiás (GO) ($n = 7$), Mato Grosso (MT) ($n = 12$), Maranhão (MA) ($n = 3$), Minas Gerais (MG) ($n = 6$), Pará (PA) ($n = 1$), Paraná (PR) ($n = 17$), Tocantins (TO) ($n = 1$), Roraima (RR) ($n = 1$), and unknown origin ($n = 6$) (Fig. 1).

The isolates collected in the cropping seasons of 2015 and 2016 (Table 1) were isolated from soybean seeds with symptoms of purple stain. Isolation was performed directly. Fragments of the fungus mycelium were removed from seeds surface and were transferred in Petri dishes containing potato-dextrose-agar (PDA) culture medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2). The isolates belonging to the Embrapa Soybean Mycological Collection were isolated from the stem (602, 607, 609, 611), pods (610), and leaves (611, 615, 616, 675, 1158, 1160,1185, 1192, 1429, and 1625) (Table 1). *Cercospora* spp. genera were confirmed for each isolate based on colony, morphological characteristics.

In vitro fungicide sensitivity assay

Because of scant sporulation in culture, radial growth assays for assessing percent inhibition were utilized instead of spore germination assays. Since the most common mutations to MBC and QoI lead to a qualitative resistance, a discriminatory dose of 10 μg/mL was chosen to discriminate the isolates. The isolates were cultivated in PDA culture medium at 24 °C under a photoperiod of 12 h/12 h for 7 days. Petri dishes with 9-cm diameter were equally divided into four subunits containing two isolates with two replicates. Each isolate was composed of 4 replicates. Mycelial discs with 4-mm diameter from the edge of the colonies were transferred to Petri dishes containing fungicide-amended PDA media. Petri dishes with PDA without fungicide treatments were used as control.

The following commercial formulations were used: QoI (azoxystrobin (25% a.i.; Priori®, Syngenta Crop Protection Ltd.), picoxystrobin (25% a.i.; Oranis®, DuPont S.A.), and

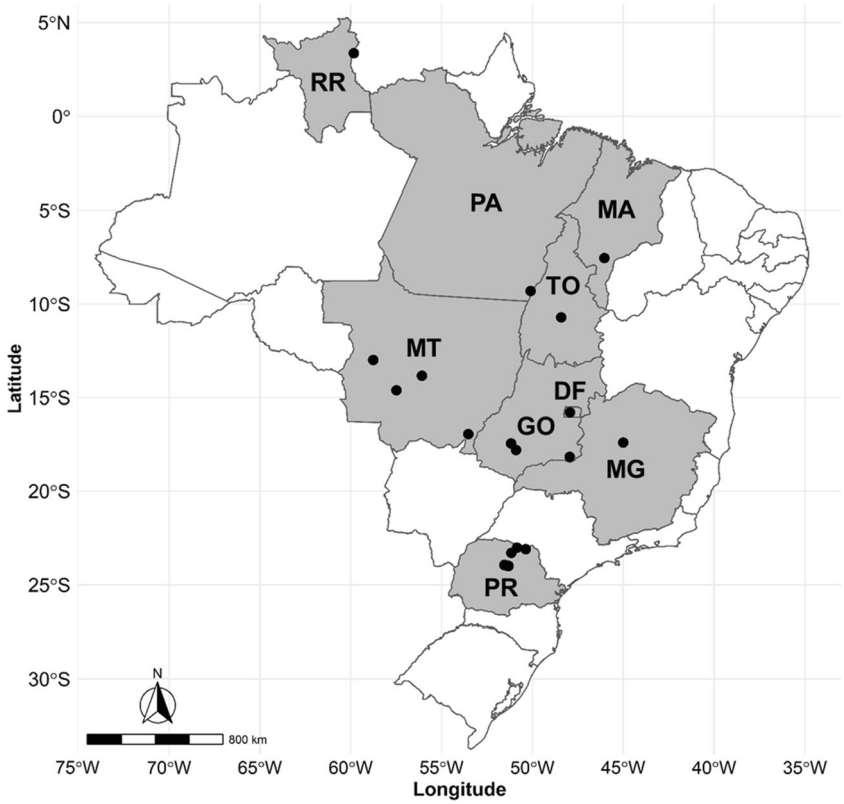

Fig. 1 Geographic location of *Cercospora* spp. collected during 9 soybean crop seasons. The samples were collected in Paraná (PR) ($n = 17$), Minas Gerais (MG) ($n = 6$), Goiás (GO) ($n = 7$), Distrito Federal (DF) ($n = 2$), Mato Grosso (MT) ($n = 12$), Tocantins (TO) ($n = 1$), Pará (PA) ($n = 1$), Maranhão (MA) ($n = 3$), and Roraima (RR) ($n = 1$). In this study, 6 samples had an unknown geographic origin

pyraclostrobin (25% a.i.; Comet®; BASF)) and MBC: (carbendazim (50% a.i.; Bendazol®, Adama Ltd.), thiophanate-methyl (50% a.i.; Cercobin 500 SC®, Iharabras S. A Industries Chemicals)). The fungicides were diluted in sterile distilled water and homogenized in the PDA culture medium to a final concentration of 10 μg/mL and homogenized in the PDA culture medium. The experiment was repeated twice, and the mean mycelia growth was averaged over the repetitions.

Previous research indicates that alternative oxidation (AOX) for QoI occurs in some *Cercospora* spp. and salicylhydroxamic acid (SHAM) may be used to inhibit this alternative pathway (Bradley and Pedersen 2011). SHAM is toxic to isolates of *C. kikuchii in vitro* and an alternative AOX inhibitor, propyl gallate, did not significantly affect radial growth of *C. kikuchii* (Price et al. 2015) and other *Cercospora* species (Sautua et al. 2020). Therefore, AOX inhibitors were not utilized in the experiments. However, future study will be carried out to confirm if AOX inhibitors affect *Cercospora* spp. collected in Brazil.

Plates were incubated at 24 °C ± 2 °C and 12 h/12 h photoperiod for 5 days and the diameter of each colony was measured (orthogonal measurements) for each isolate to determine percentage of control by each fungicide compared to the growth on non-amended media. Isolates with control below 50% were considered resistant and those with control greater than 50% were considered sensitive (Ishii et al. 2009).

Cross-resistance and fungitoxicity effect were analyzed by comparing the percentage of control between fungicides with the same mode of action. The cross-resistance data were submitted to linear regression analysis and the correlation was estimated using *R* squared regression. Analysis and graphical representation were performed using R software version 1.2.1335 (R Core Team 2019) and R package "ggplot2" (Wickham 2016).

Molecular and bioinformatics analysis

For DNA extraction, three mycelium discs of 7 mm were added into potato-dextrose (PD) liquid medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose) during 7 to 10 days, and the mycelial mass was vacuum filtrated. The dried mycelium was frozen in liquid nitrogen, macerated, and stored in a 1.5-mL microcentrifuge tube at − 80 °C. The DNA was extracted following the modified CTAB extraction.

The macerated tissue was homogenized with 1000 μL of extraction buffer (1 M Tris HLC (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, 0.1% β-mercaptoethanol, 200 μL CTAB to 5%) and incubated in a warm bath at 65 °C for 60 min, with shaking and inversion of the microcentrifuge tubes every 15 min. The samples were centrifuged at 6.000 rpm for 10 min. Seven hundred microliters of the supernatant was collected and transferred to a new 1.5-mL microcentrifuge tube. Then 700 μL of phenol:chloroform solution:iso-amyl alcohol (24:24:1) was

Table 1 Isolate code, geographic origin, year of collection, codon position, reference and altered alleles in *cytb* and *β-tubulin* genes, and percentage of control of *Cercospora* spp. isolates using discriminatory doses at 10 μg/mL. Isolates presenting > 50% of control were considered sensitive (S), and < 50% resistant (R)

		QoI								MBC					
Reference position (nt)								428						860	
Reference allele								G (*)[3]						A (*)[3]	
Alter allele								C						C	
Code/origin[1]		(%) of control and classification[2]								(%) of control and classification[2]					
	Year	azoxystrobin		picoxystrobin		pyraclostrobin		G143A	Mutation	carbendazim		thiophanate-methyl		E198A	Mutation
600/PR	1996	70	S	60	S	66	S	*	Wild	82	S	89	S	*	Wild
601/Unk	1996	45	R	38	R	57	S	*	Wild	100	S	100	S	*	Wild
602/PR	1997	22	R	38	R	57	S	*	Wild	100	S	100	S	*	Wild
603/MA	1997	56	S	53	S	73	S	*	Wild	100	S	100	S	*	Wild
604/MT	1998	58	S	57	S	82	S	*	Wild	100	S	100	S	*	Wild
605/MT	1998	44	R	48	R	78	S	*	Wild	100	S	100	S	*	Wild
606/PR	1998	59	S	40	R	52	S	*	Wild	100	S	100	S	*	Wild
607/MA	1999	34	R	41	R	69	S	*	Wild	100	S	100	S	*	Wild
608/PR	1999	51	S	41	R	78	S	*	Wild	100	S	100	S	*	Wild
609/MA	1999	26	R	18	R	61	S	*	Wild	100	S	100	S	*	Wild
611/GO	2001	75	S	59	S	84	S	N.A.	N.A.	100	S	100	S	N.A.	N.A.
612/Unk	2001	40	R	61	S	74	S	*	Wild	100	S	100	S	*	Wild
613/PR	2001	69	S	64	S	75	S	*	Wild	100	S	100	S	*	Wild
615/MT	2001	85	S	56	S	79	S	*	Wild	100	S	100	S	*	Wild
616/PR	2001	46	R	30	R	51	S	*	Wild	100	S	100	S	*	Wild
675/PR	2008	8.1	R	2.6	R	27	R	C	G143A	2.6	R	2.6	R	C	E198A
1158/PR	2013	31	R	9.5	R	33	R	C	G143A	4.2	R	0.6	R	C	E198A
1160/PR	2013	1.4	R	12	R	12	R	C	G143A	12	R	3.6	R	C	E198A
1166/PR	2013	44	R	2.3	R	31	R	C	G143A	6.9	R	34	R	C	E198A
1185/PR	2013	14	R	3.7	R	37	R	C	G143A	1.2	R	0.3	R	C	E198A
1192/TO	2013	24	R	7.6	R	38	R	C	G143A	11	R	0.6	R	C	E198A
1429/RR	2013	22	R	0.6	R	51	S	C	G143A	100	S	68	S	*	Wild
432_1/MT	2015	3.4	R	0.7	R	23	R	C	G143A	0.7	R	0.7	R	C	E198A
432_2/MT	2015	22	R	11	R	25	R	C	G143A	0.3	R	4.9	R	C	E198A
432_3/MT	2015	14	R	0.7	R	33	R	C	G143A	5.9	R	0.7	R	C	E198A
432_4/MT	2015	21	R	8.1	R	29	R	C	G143A	29	R	1.7	R	C	E198A
435_2/MT	2015	14	R	8.3	R	34	R	C	G143A	21	R	1.6	R	C	E198A
435_5/MT	2015	35	R	37	R	36	R	C	G143A	46	R	30	R	C	E198A
435_6/MT	2015	25	R	13	R	38	R	C	G143A	4	R	4	R	C	E198A
469_1/GO	2015	60	S	11	R	79	S	C	G143A	36	R	49	R	C	E198A
469_2/GO	2015	35	R	22	R	53	R	C	G143A	21	R	6.2	R	C	E198A
469_2A/GO	2015	18	R	5.1	R	32	R	C	G143A	1	R	1.7	R	C	E198A
474_1/GO	2015	23	R	19	R	32	R	C	G143A	4.4	R	0.3	R	C	E198A
474_2/GO	2015	40	R	20	R	41	R	C	G143A	15	R	4.7	R	C	E198A
499_1/GO	2015	3	R	9.1	R	35	R	C	G143A	17	R	2.4	R	C	E198A
573_4/MG	2015	13	R	0	R	27	R	C	G143A	0.3	R	0.3	R	C	E198A
576_1/MG	2015	30	R	16	R	35	R	C	G143A	18	R	1.1	R	C	E198A
576_2/MG	2015	31	R	8	R	43	R	C	G143A	9.5	R	6.6	R	C	E198A
576_3/MG	2015	26	R	9.4	R	38	R	C	G143A	0.3	R	4.1	R	C	E198A

Table 1 (continued)

		QoI								MBC					
Reference position (nt)								428						860	
Reference allele								G (*)[3]						A (*)[3]	
Alter allele								C						C	
Code/origin[1]		(%) of control and classification[2]								(%) of control and classification[2]					
	Year	azoxystrobin		picoxystrobin		pyraclostrobin		G143A	Mutation	carbendazim		thiophanate-methyl		E198A	Mutation
576_4/MG	2015	28	R	7	R	26	R	C	G143A	5.4	R	3	R	C	E198A
576_5/MG	2015	26	R	8.9	R	39	R	C	G143A	1.5	R	9.2	R	C	E198A
1625/MT	2016	26	R	19	R	24	R	C	G143A	30	R	2.8	R	C	E198A
1642/DF	2016	2	R	2.4	R	8.2	R	C	G143A	2	R	2	R	C	E198A
1643/DF	2016	38	R	37	R	34	R	C	G143A	7.2	R	24	R	C	E198A
1656/PA	2016	13	R	3.1	R	31	R	C	G143A	4.4	R	4.4	R	C	E198A
1742-1/MT	2016	24	R	20	R	37	R	C	G143A	0.7	R	5.4	R	C	E198A
E4_1/PR	2016	28	R	7.9	R	33	R	C	G143A	0.7	R	2	R	C	E198A
E4_4/PR	2016	14	R	0	R	25	R	C	G143A	8.7	R	18	R	C	E198A
E4_5/PR	2016	2.4	R	2.4	R	22	R	C	G143A	2.4	R	2.4	R	C	E198A
Embrapa_1/PR	2016	1.5	R	0	R	24	R	C	G143A	15	R	0	R	C	E198A
Embrapa_2/PR	2016	19	R	11	R	35	R	C	G143A	13	R	9.4	R	C	E198A
Faxinal_5/PR	2016	8.7	R	12	R	34	R	C	G143A	29	R	4.1	R	C	E198A
G16407_3/Unk	2016	39	R	54	S	68	S	C	G143A	1	R	16	R	C	E198A
G16407_4/Unk	2016	34	R	30	R	47	R	C	G143A	0	R	33	R	C	E198A
515188_1/Unk	Unk	24	R	11	R	32	R	C	G143A	0.3	R	6.4	R	C	E198A
515188_2/Unk	Unk	13	R	1.6	R	31	R	C	G143A	2.9	R	2.9	R	C	E198A

[1] PR samples collected in Paraná, Unk samples had unknown geographic origin, MA samples collected in Maranhão, MT samples collected in Mato Grosso, GO samples collected in Goiás, TO samples collected in Tocantins, RR samples collected in Roraima, MG samples collected in Minas Gerais, DF samples collected in Distrito Federal, PA samples collected in Pará; [2] Classification of *Cercospora* spp. isolates by the percentage of control in amended media with a discriminatory dose of 10 μg/mL by the QoI (azoxystrobin, picoxystrobin, pyraclostrobin) fungicides and MBC (carbendazim and thiophanate-methyl); classification of the *Cercospora* spp. isolates in the categories, (S—sensitive) (%) of control ≥ 50 μg/mL; (R—resistant) (%) of control < 50 μg/mL; [3] (*) absence of mutation; (C) altered allele at position 428 in *cytb* and at position 860 in *β-tubulin* genes, resulting in allele substitution from glycine by alanine at position 143 (G143A) and glutamic acid by alanine at position 198 (E198A), respectively; *N.A.*, not analyzed

added. After further centrifugation at 10,000 rpm for 5 min, 700 μL of the supernatant was transferred to a new microcentrifuge tube. For DNA precipitation, 500 μL of isopropanol was added and then the samples were homogenized in vortex and incubated at − 20 °C for 30 min. The DNA pellet was washed with 70% ethanol and resuspended in 60 μL of mili-q water + 6 μL of sodium acetate (3 M) and 120 μL of 100% ethanol and was stored at 4 °C. The samples were centrifuged at a speed of 14,000 rpm for 5 min. The supernatant was discarded and the pellet was washed again with 70% ethanol and dried. Samples were resuspended in 80 μL of mili-q + RNAse water at the final concentration of 80 μg/mL. DNA quality was assessed using electrophoresis on 1% agarose gel.

The pair of primers for *cytb* (Genbank/EMBL access EF176921.1) and *β-tubulin* (Genbank/EMBL access AY856374.1) were designed based on the sequences for *C. beticola* using the Primer 3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). To achieve this, the primers forward and reverse were designed to cover the maximum size of each analyzed gene. The specificity of the primers was tested *in silico* by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer pairs forward (F) and reverse (R) were described as follows: QoI ((primer QoI_F) 5′- ATGGTTCTTACAAA GCACCTAGAA-3′; (primer QoI_R) 5′- CACCCAAT TGCATCAATAATAAGA-3′); MBC ((primer MBC_F) 5′- CTGCATTCTGGCAGACCAT-3′; (primer MBC_R) 5′- TGAACTGGTCACCGACACG-3′).

PCR reactions for the target genes were prepared to a final volume of 50 μL containing 100 ng DNA from each sample, 1 μM of each primer, 10 μM dNTPs (Thermo Fischer

Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific), and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). The annealing temperatures for *cytb* and *β-tubulin* were 57 °C and 63 °C, respectively, followed by an extension time of 60 s and 35 cycles for amplification. All PCR reactions were performed on the MWG Biotech Inc. Primus 96 thermal cycler. The PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV 360-nm light.

The PCR amplicons products of each gene were pooled by isolate for all samples for each gene were mixed and then purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. The amplicon pools were quantified with the Qubit® (Thermo Fischer Scientific) for a final concentration of 3 ng/μL and sent to the Institute of Clinical Molecular Biology (IKMB) for processing.

The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used to prepare the amplicon library following the manufacturer's instructions. The final 56 libraries were distributed into four lanes on a flow cell for sequencing on an Illumina MiSeq 2500 system, utilizing a 150-bp paired end reads length with an expected coverage of 30X.

The initial base calling, barcodes/adaptors trimming, and quality filtering of the reads generated with the Illumina analysis pipeline (Fastq format) were performed using Trimmomatic 0.36 software (Bolger et al. 2014) and checked by FastQC software (Andrews 2010).

High-quality reads were then aligned, by isolate, to the obtained *Cercospora* spp. reference sequence *cytb* (MT013786.1) and *β-tubulin* (MT013886.1) genes, available in (Sautua et al. 2019a, 2020), using the software bowtie2 (Langmead and Salzberg 2012). The mapped reads were processed using Picard tools version 2.11 (Java, http://broadinstitute.github.io/picard/) to remove duplicate values, and a binary file of the extension bam representing the assembled genome of each sequenced isolate was generated. For SNP/InDel calling, we used Platypus version 0.8.1 (Rimmer et al. 2014) with minimum for three reads coverage. Variants were filtered per mapping depth (< 10); missing data (0.2) and mapping quality (> 30) using Vcftools version 0.1.12b (Danecek et al. 2011) and annotated using SnpEff software. The summary workflow of the *in silico* analysis is available in Fig. 2.

All the point mutations found for *cytb* and *β-tubulin* genes were statistically tested to association with the phenotypic results of *in vitro* fungicide sensitivity assay to QoI and MBC using genome-local association (GLA). The GLA analysis was made using a mixed linear model (MLM). The analysis and graphical associations were performed using R software version 1.2.1335 (R Core Team 2019) and R package for the prediction integrated tool "GAPIT" (Lipka et al. 2012).

Results

In vitro fungicide sensitivity assay

For QoI fungicides, 83.9%, 85.7%, and 69.6% of the isolates were classified as resistant to azoxystrobin, picoxystrobin, and pyraclostrobin, respectively (Table 1). Only sensitive isolates were found in the state of Maranhão.

All wild isolates collected before 2001 were sensitive to the fungicide pyraclostrobin, whereas isolates 601, 602, 605, 607, 609, and 616 were classified as resistant to azoxystrobin and picoxystrobin (Table 1).

The QoI fungicide pyraclostrobin (median = 36.1%) showed higher intrinsic activity to control *Cercospora* spp. than azoxystrobin (median = 25.9%) and picoxystrobin (median = 11.0%) fungicides, respectively (Fig. 3).

For MBC, 71.4% of the isolates were classified as resistant to carbendazim and thiophanate-methyl. Isolates classified as sensitive to the fungicides carbendazim and thiophanate-methyl were collected in 1996 (601), 1997 (602), 1998 (605, 607), 1999 (607, 608, 609), and 2001 (616). These isolates were obtained from plants collected in Paraná, Mato Grosso, and Maranhão states (Table 1).

The isolate 675 from Paraná collected in 2008 was classified as resistant. However, from 39 isolates collected in 2013, 38 were classified as resistant and only the isolate 1429, from Roraima state, was classified as sensitive. Among the two MBC fungicides tested, carbendazim (median = 12.4%) showed higher intrinsic activity against *Cercospora* spp. than thiophanate-methyl (median = 5.4%) (Fig. 3).

Molecular analysis of *cytb* and *β-tubulin* genes

Based on the sequencing results, the gene structure of *Cercospora* spp. for *cytb* and *β-tubulin* genes resulted in a 1665-bp and 2365-bp fragment length, respectively (Fig. 4). The sequencing of the *cytb* and *β-tubulin* genes in *Cercospora* spp. resulted in 114 low impact (synonymous or intronic) mutations. Among these, a total of six and 108 mutations were found for the *cytb* and *β-tubulin* genes, respectively. In addition, eight non-synonymous mutations were identified, of which one mutation was observed for the *cytb* gene and seven mutations for the *β-tubulin* gene.

All samples collected earlier (1996, 1997, 1998, 1999) did not show the G143A mutation. The unique non-synonymous mutation found was G143A. In this mutation, the nucleotide sequence of GGT (wild) was replaced by GCT. This substitution was found in 41 *Cercospora* spp. isolates (Table 1). All the isolates present only a homozygous variant, which was confirmed by Sanger sequencing chromatography wavelength (data not shown). No isolates showed the F129L or G137R mutation.

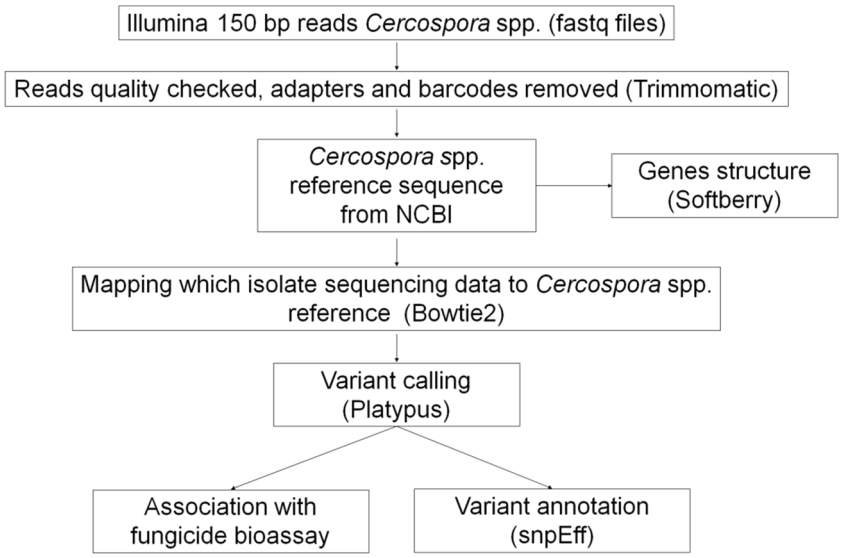

Fig. 2 *In silico* analysis summary workflow for genetic characterization of *cytb* and *β-tubulin* genes from *Cercospora* spp. isolates from soybean

The association of G143A mutation in *cytb* with resistance to QoI based on *in vitro* fungicide test by GLA analysis does not present a significant statistical difference. Despite the *p* value associated with each polymorphisms not crossed the threshold, we still observed the highest association with the three tested QoI fungicides (azoxystrobin (*p* value: 0.14), picoxystrobin (*p* value: 0.17), and pyraclostrobin (*p* value: 0.19)) between the *in vitro* fungicide test and the G143A mutation compared with the other synonymous mutations (Fig. 5).

The G143A mutation was present in the majority of isolates collected after 2008. In this case, of the 41 mutated isolates, 40 isolates were classified as resistant at least for two QoI fungicides. There was a concordance between the isolates which showed the mutation G143A and the isolates classified as resistant in 97% of the samples analyzed. For isolates that did not show the G143A mutation, they were classified as sensitive in at least one of the three QoI fungicides (Table 1). The isolates that showed the G143A mutation, but were classified as sensitive, are the isolates 469_1 and G16407_3. The isolate 469_1 showed 59.9% and 78.7% of control by the fungicides azoxystrobin and pyraclostrobin, respectively. Meanwhile, the isolate G16407_3 showed 54.4% and 68.4% of control when cultivated in amended media the fungicides picoxystrobin and pyraclostrobin, respectively (Table 1).

Sequencing of the *β-tubulin* gene revealed seven non-synonymous mutations at codons M170L, V189I, E198A, N218S, S303A, Q350M, S355D. The mutant isolates showed changes in the nucleotides (ATG/Methionine to TTA/TTG/

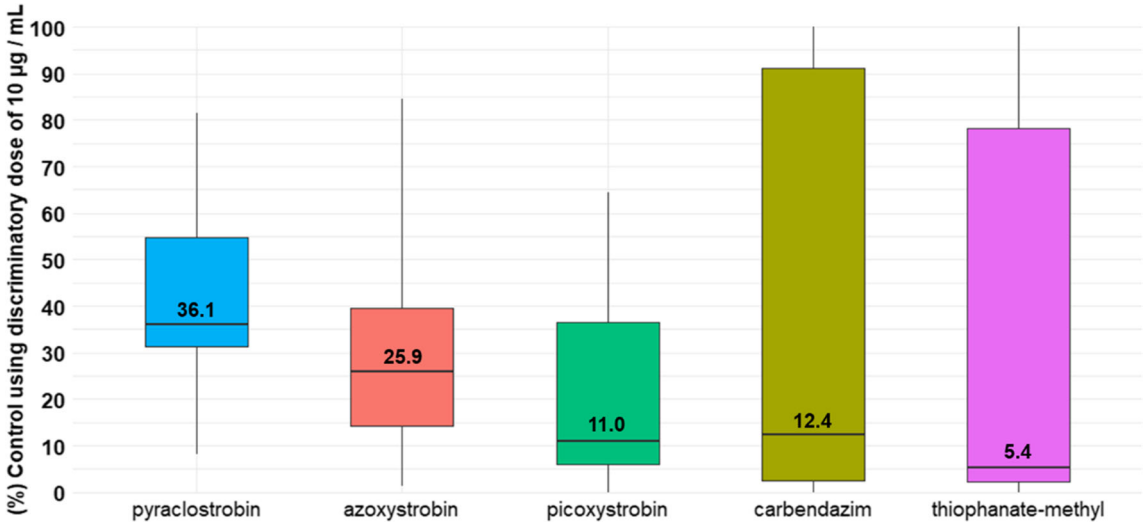

Fig. 3 Median control values of isolates of *Cercospora* spp. to QoI (pyraclostrobin, azoxystrobin, and picoxystrobin) and MBC (carbendazim and thiophanate-methyl) fungicides, using mycelial growth inhibition assay with a discriminatory dose of 10 µg/mL from samples collected in 9 crop season and regions in the main soybean production Brazilian states

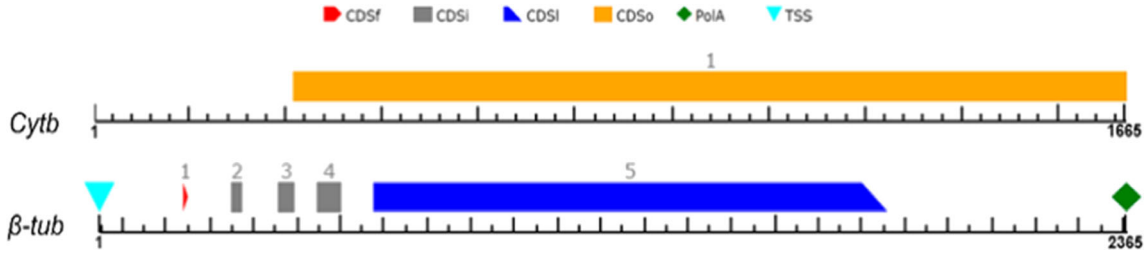

Fig. 4 Prediction of *cytb* and *β-tubulin* genes using genomic DNA of *Cercospora* spp. from soybean. CDSf—First (starting with start codon); CDSi—internal (internal exon), CDSl—last coding segment (ending with stop codon); CDSo: coding sequence, solo—predicted gene with a single exon; PolA—polyadenylation signal sequence (AATAAA), and TSS—position of transcription start (TATA-box position and score)

Leucine at codon 170), (GTC/Valine to ATC/Isoleucine at codon 189), (GAG/Glutamic acid to GCG/Alanine at position 198), (AAC/Asparagine to TCC/Serine at position 218), (AGC/Serine to GCC/Alanine at position 303), (CAG/Glutamine to ATG/Methionine at position 350), and (TCT/Serine to GAG/Aspartic acid at position 355). From these, only the mutation E198A was associated with resistance of *Cercospora* species to MBC fungicides. The M170L, V189I, N218S, S303A, Q350M, and S355D mutations were found only in isolate 1429 collected in the state of Roraima, in the 2013 season. Of these six mutations, all possessed heterozygous variation.

The GLA test of the 108 mutations found in the sequencing in *β-tubulin* gene and the phenotype, detected only the E198A mutation as significantly associated with resistance in the assay results to both MBC fungicides (carbendazim and thiophanate-methyl p value = 0.0004) (Fig. 5).

The E198A mutation was found in 40 isolates, about 71% of the analyzed isolates. For *β-tubulin* gene, all *Cercospora* spp. isolates that showed the E198A mutation were classified as resistant for both MBC fungicides. Only homozygous variations were found in the all *Cercospora* spp. isolates that possessed the E198A mutation (Table 1).

The isolate 675 was the oldest *Cercospora* spp. isolate with the G143A and E198A mutations and it was collected in the state of Paraná in the 2008 crop season (Table 1). In addition, 97% of mutant isolates of *Cercospora* spp. analyzed, presented the double mutations G143A and E198A that confer resistance to QoI and MBC fungicides, respectively.

Cross-resistance and fungitoxicity effect in QoI and MBC fungicides

Cross-resistance among azoxystrobin, picoxystrobin, and pyraclostrobin (QoI) and between carbendazim and thiophanate-methyl (MBC) fungicides was confirmed by high positive linear correlation between mycelial growth inhibition of each compared fungicides (R^2 from 0.8 to 0.96) (Fig. 6).

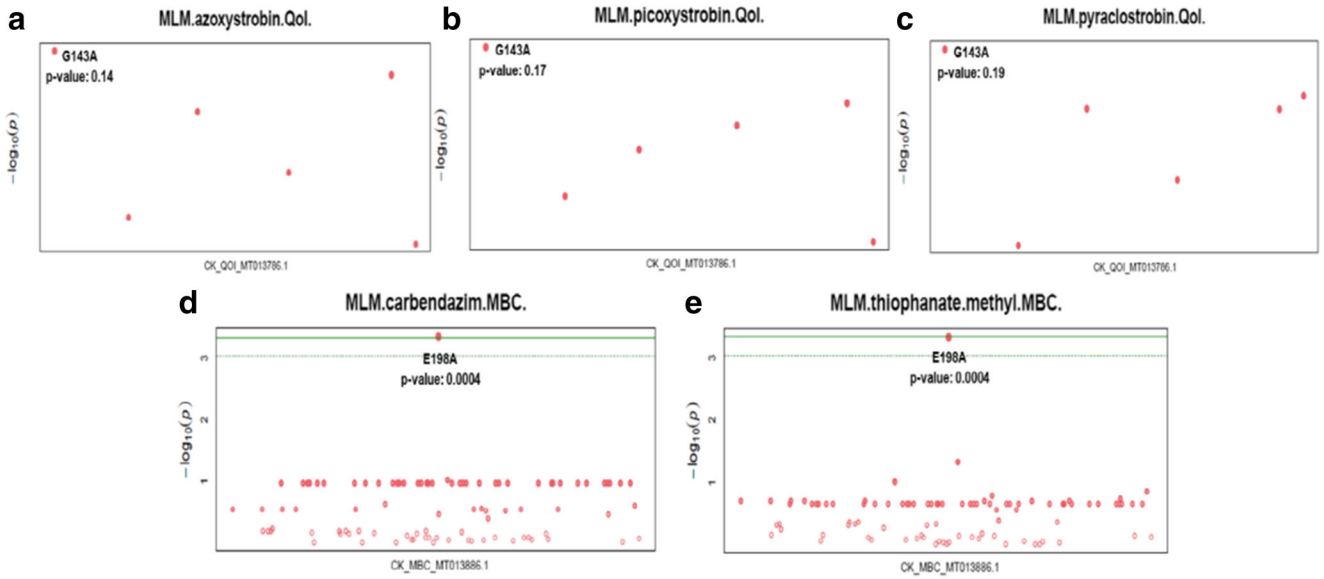

Fig. 5 Manhattan plot and genome-local association parameters of *Cercospora* spp. isolates from soybean for *in vitro* fungicide test to QoI **a**) azoxystrobin, **b**) picoxystrobin, **c**) pyraclostrobin and MBC **d**) carbendazim and **e**) thiophanate-methyl fungicides. The strongest association was found for the G143A mutations and *in vitro* assay test for the three QoI fungicides. Significant associations were correlated for E198A mutation in *β-tubulin* gene and *in vitro* test of mycelial growth inhibition in amended media with a discriminatory dose of 10 μg/mL of carbendazim and thiophanate-methyl. Dots in the graph represent synonym or non-synonymous mutations

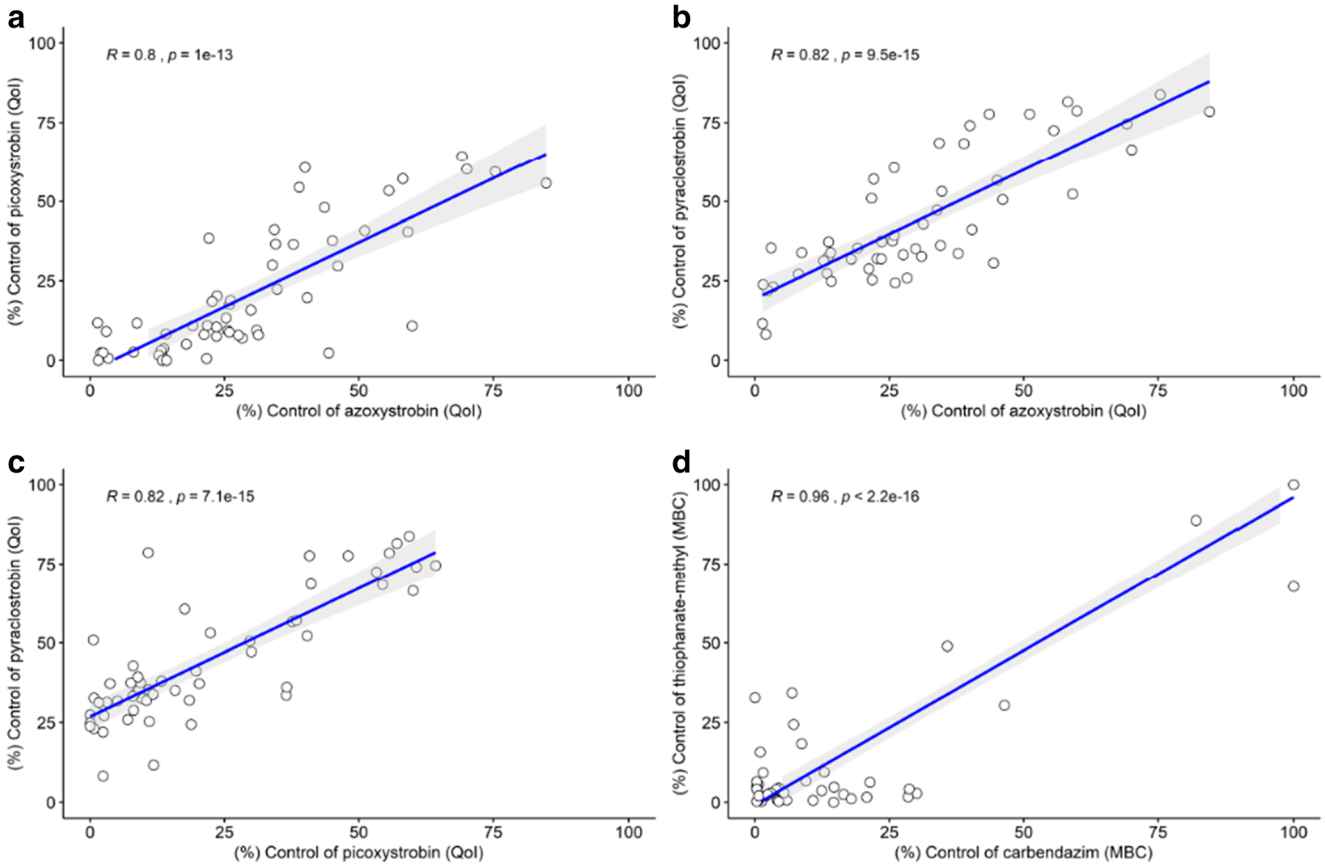

Fig. 6 Cross-resistance between **a**) azoxystrobin vs picoxystrobin, **b**) azoxystrobin vs pyraclostrobin, **c**) picoxystrobin vs pyraclostrobin and **d**) carbendazim vs thiophanate-methyl fungicides of *Cercospora* spp. isolates from soybean fields in Brazil

Among the three QoIs fungicides, pyraclostrobin (median to control wild isolates = 70.6%; median to control mutant isolates = 33.2%) presented a greater fungitoxic action to control wild and mutant *Cercospora* spp. isolates than azoxystrobin (median to control wild isolates = 48.6%; median to control mutant isolates = 22.7%), and picoxystrobin (median to control wild isolates = 44.6%; median to control mutant isolates = 8.9%) fungicides, respectively (Fig. 7).

For MBC group, the fungicide carbendazim (median to control mutant isolates = 4.9%) showed a greater fungitoxic effect to control *Cercospora* spp. isolates that possessed the E198A mutation when compared to thiophanate-methyl (median to control mutant isolates = 3.3%) fungicide. For wild isolates, both fungicides showed the same intrinsic activity (median to control wild isolates = 100%) (Fig. 7).

Discussion

In this study, the discriminatory dose of 10 μg/mL was used to separate between resistant and sensitive isolates to QoI and MBC, showing a concordance with the presence of the mutations. Price et al. (2015) and Sautua et al. (2020) also evaluated *Cercospora* species resistance to fungicides using discriminatory doses. The discriminatory dose is an easier way to classify the isolates, it is less time consuming, saves culture media, space, and it can be applied for a large number of isolates at the same time.

In Brazil, most applications of QoIs fungicides are carried out to control Asian soybean rust, and lower sensitivity of *P. pachyrhizi* to QoIs has been reported since 2013/2014 crop seasons (Klosowski et al. 2016). However, selection pressure occurs for all other fungi that infect the crop. Other fungi have not been frequently monitored since it causes lower losses, although reports of control failure have increased in the last crop seasons.

Isolates of *Cercospora* species resistant to QoI with the G143A mutation have already been reported worldwide. The G143A mutation was reported in *C. sojina* (Zeng et al. 2015; FRAC 2018) and *C. beticola* (Bolton et al. 2013) in the USA and in *C. beticola* in Italy (Birla et al. 2012). Field resistant populations of *C. kikuchii*, *C. cf. flagellaris*, and *C. sojina* to QoI fungicides were observed in soybean production regions in the USA (Price et al. 2015; Albu et al. 2016; Mathew et al. 2019; Zhou and Mehl 2020), Brazil (Soares et al. 2015), Bolívia (Sautua et al. 2019b, 2020), and Argentina (Sautua et al. 2020). In this study, 73% of *Cercospora* spp. isolates showed G143A mutation. Additionally, all isolates collected

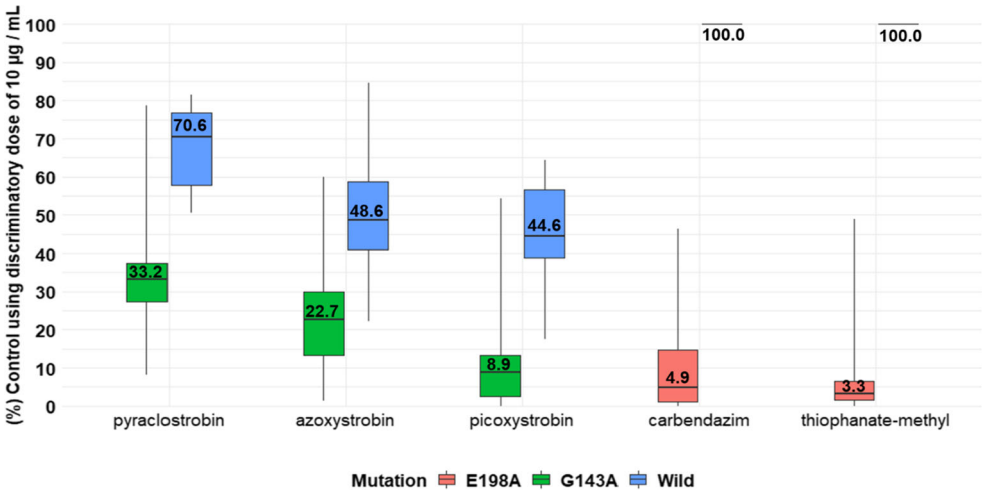

Fig. 7 Percentage of control of *Cercospora* spp. isolates classified as wild (blue) and mutant isolates, which present the G143A (green) and E198A (red) mutations, resulting in resistance to QoI (picoxystrobin, azoxystrobin, and pyraclostrobin) and MBC (carbendazim and thiophanate-methyl) fungicides. Numbers in each box plot represent the median values of control using mycelial growth inhibition in amended media with a discriminatory dose of 10 µg/mL

after 2008 contained the G143A mutation regardless of their geographical origin.

Not all isolates classified as resistant to QoI in this study contained point mutation, while it was found in some sensitive isolates. *Cercospora* spp. isolates sampled in 1996 (601), 1997 (602), 1998 (605, 607), 1999 (607, 608, 609), and 2001 (616) were collected before the intensive use of QoI fungicides in soybean and do not show the G143A mutation, but have variation in sensitivity to the three active ingredients of the QoI group. All isolates of *Cercospora* spp. collected after the 2008 season possessed the G143A mutation and were classified as resistant to at least one of the three fungicides that confers resistance to QoI. Differences in phenotypic classification were also identified in *Cercospora* species from Argentina, which from 65 *Cercospora* species classified as resistant to QoI, the G143A mutation were found in 53 *C.* species (82%) (Sautua et al. 2020).

A possible cause of incompatibility between the assay and phenotype classification is due to the differences of fungitoxicity effect to control *Cercospora* spp. among QoIs fungicides. In this study, pyraclostrobin had a higher fungitoxicity activity in wild and mutant isolates, showing a greater control than azoxystrobin and picoxystrobin, respectively. Despite the differences in intrinsic activity observed into QoI fungicides, our results confirmed the cross-resistance among azoxystrobin × picoxystrobin, picoxystrobin × pyraclostrobin, and azoxystrobin × pyraclostrobin. Cross-resistance were also confirmed for MBC fungicides, carbendazim × thiophanate-methyl. Similar results were found in *C. kikuchii* (Price et al. 2015; Sautua et al. 2019) and in *Corynespora cassiicola* in cucumber in China (Duan et al. 2019).

No statistical significance was found in GLA analysis between the G143A mutation and resistance to QoI, although the resistance selection to QoI is disruptive and the G143A was the unique non-synonymous mutations identified in *cytb* gene. The lack of statistical association in GLA analysis can be related with the low number of mutations found in the gene sequence or the variation of control among QoIs.

The MBC fungicides are not recommended to control Asian soybean rust; however, it was one of the first fungicides indicated to control late-season diseases. Due to excessive applications of MBC in soybean, isolate of *Cercospora* spp. from Brazil resistant to MBC was previously reported by Soares et al. (2015), with a nucleotide sequence substitution from glutamic acid to alanine at codon position 198 (E198A).

In this study, all 40 isolates that were not controlled at the 10 µg/mL dose and were classified as resistant presented the mutation E198A at *β-tubulin* gene. The isolates classified as sensitive to carbendazim and thiophanate-methyl were collected between 1996 and 2001 from Paraná, Mato Grosso, Goiás, and Maranhão states. After 2013, sensitive *Cercospora* spp. isolates were found only in Roraima state.

For the *β-tubulin* gene, all isolates classified *in vitro* assay as resistant to carbendazim and thiophanate-methyl had the E198A mutation. The GLA analysis showed a significant correlation for the E198A mutation and the assays using a discriminatory dose of 10 µg/mL for both MBC fungicides tested (carbendazim and thiophanate-methyl, p value = 0.0004). In this study, homozygous variation was found for all *Cercospora* spp. isolates, which possessed E198A mutation.

Here, we report that about 97% of the 56 *Cercospora* spp. isolates collected since the 2008 season, regardless of their geographical origin sampled in Brazil, showed the G143A and E198A mutations that confer resistance to QoI and MBC fungicides. Additional studies increasing the number and regions sampled and evaluating other groups of fungicides are necessary.

Author contributions CVG, FCMG, MIBP, AM, and JAV planned and designed the experimental work. FEM, HP, SAXV, and SF executed the experiments. FEM and VSLC conducted the data analyses. FEM, VSLC, and CVG wrote the manuscript.

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Data availability Data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) under accession number PRJNA634433.

Compliance with ethical standards

Ethical statement Authors declare that this manuscript have not published elsewhere. All authors read and approved the final version of this manuscript. The authors declare that the present work was developed without any potential conflict of interest, with no human or animal participants.

Conflict of interest The authors declare that they have no conflict of interest.

References

AGROFIT. Consulta de ingredientes ativos 2019. Available at: : http://http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed on: 09 Jan 2020

Albu S, Price T, Doyle V, Padgett B, Schneider R (2016) The G143A mutation is responsible for strobilurin fungicide resistance in *Cercospora* cf. *flagellaris*, a leaf blight and purple seed stain pathogen of Louisiana soybean. Plant Health Progress 17:197–197

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed on: 05 Aug 2020

Birla K, Rivera-Varas V, Secor GA, Khan MF, Bolton MD (2012) Characterization of cytochrome b from European field isolates of *Cercospora beticola* with quinone outside inhibitor resistance. European Journal of Plant Pathology 134:475–488

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120

Bolton MD, Rivera V, Secor G (2013) Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. Pest Management Science 69:35–39

Bradley CA, Pedersen DK (2011) Baseline sensitivity of Cercospora zeae-maydis to quinone outside inhibitor fungicides. Plant Disease 95:189–194

CONAB. Acompanhamento da safra brasileira de grãos, v. 7 - Safra 2019/20, n.4 - Sexto levantamento, janeiro 2020. Available at: https://www.conab.gov.br/info-agro/safras/graos. Accessed on 09 Jan 2020

CONSÓRCIO ANTIFERRUGEM (2019) Custo ferrugem asiática da soja . Available at: http://www.consorcioantiferrugem.net/#/conteudos/view/5. Accessed on 8 Feb 2020

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G (2011) The variant call format and VCFtools. Bioinformatics 27:2156–2158

Duan Y, Xin W, Lu F, Li T, Li M, Wu J, Wang J, Zhou M (2019) Benzimidazole-and QoI-resistance in *Corynespora cassiicola* populations from greenhouse-cultivated cucumber: an emerging problem in China. Pesticide Biochemistry and Physiology 153:95–105

FRAC. List of plant pathogenic organisms resistant to disease control agents. Available at: https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenicorganisms_may2018.pdf?sfvrsn=a2454b9a_2. Accessed on: 08 Jan 2020

Gisi U, Sierotzki H, Cook A, McCaffery A (2002) Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Management Science 58:859–867

Godoy CV, Almeida AMR, Costamilan LM, Meyer MC, Dias WP, Seixas CDS, Soares RM, Henning AA, Yorinori JT, Ferreira LP, Silva JFV (2016) Doenças da soja. In: Amorim L, Rezende JAM, Bergamin Filho A, Camargo LEA (eds) Manual de Fitopatologia. Vol. 2, Doenças das plantas cultivadas, vol 5. Ed. Ceres, São Paulo, pp 657–675

Hartman GL, Rupe JC, Sikora EJ, Domier LL, Davis JA, Steffey KL (eds) (2015) Compendium of soybean diseases and pests. APS Press, The American Phytopathological Society, St. Paul

Imazaki I, Iizumi H, Ishikawa K, Sasahara M, Yasuda N, Koizumi S (2006) Effects of thiophanate-methyl and azoxystrobin on the composition of *Cercospora kikuchii* populations with thiophanate-methyl-resistant strains. Journal of General Plant Pathology 72:292–300

Ishii H, Fountaine J, Chung WH, Kansako M, Nishimura K, Takahashi K, Oshima M (2009) Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. Pest Management Science: formerly Pesticide Science 65:916–922

Kayamori M, Shimizu M, Yamana T, Komatsu T, Minako S, Shinmura A, Sasaki J, Kozawa T, Notsu A, Yasuoka S (2020) First report of QoI resistance in *Cercospora beticola* in sugar beet in Japan. Journal of General Plant Pathology 86:149–153

Klosowski AC, May De Mio LL, Miessner S, Rodrigues R, Stammler G (2016) Detection of the F129L mutation in the cytochrome b gene in *Phakopsora pachyrhizi*. Pest Management Science 72:1211–1215

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nature Methods 9:357–359

Lipka AE, Tian F, Wang Q, Peiffer J, Li M, Bradbury PJ, Zhang Z (2012) GAPIT: genome association and prediction integrated tool. Bioinformatics 28:2397–2399

Ma Z, Michailides TJ (2005) Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection 24:853–863

Mathew FM, Byamukama E, Neves DL, Bradley CA (2019) Resistance to quinone outside inhibitor fungicides conferred by the G143A mutation in *Cercospora sojina* (causal agent of frogeye leaf spot) isolates from South Dakota soybean fields. Plant Health Progress 20:104–105

McGrath MT (2015) Cucurbit powdery mildew in the USA. In: Fungicide resistance in plant pathogens. Springer,Tokyo. p. 401–417

Price PP III, Purvis MA, Cai G, Padgett GB, Robertson CL, Schneider RW, Albu S (2015) Fungicide resistance in *Cercospora kikuchii*, a soybean pathogen. Plant Disease 99:1596–1603

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: URL https://www.R-project.org/. Accessed on: 05 Feb 2020

Recomendações técnicas para a cultura da soja na cultura da soja na região central do Brasil 2000/01 (2000) Embrapa Soja/ Fundação MT, Londrina PR. p. 245

Rimmer A, Phan H, Mathieson I, Iqbal Z, Twigg SR, Wilkie AO, McVean G, Lunter G (2014) Integrating mapping-, assembly-and haplotype-based approaches for calling variants in clinical sequencing applications. Nature Genetics 46:912–918

Sautua, FJ, Gonzalez SA, Doyle VP, Berretta MF, Gordó M, Scandiani MM, Rivarola ML, Fernandez P, Carmona MA (2019a) Draft genome sequence data of *Cercospora kikuchii*, a causal agent of Cercospora leaf blight and purple seed stain of soybeans. Data in Brief 27:104693

Sautua FJ, Searight J, Doyle VP, Price PP III, Scandiani MM, Carmona MA (2019b) The G143A mutation confers azoxystrobin resistance to soybean Cercospora leaf blight in Bolivia. Plant Health Progress 20:2–3

Sautua FJ, Doyle VP, Price PP, Porfiri A, Fernandez P, Scandiani MM, Carmona MA (2020) Fungicide resistance in *Cercospora* species causing cercospora leaf blight and purple seed stain of soybean in Argentina. Plant Pathology 00:1–17

Soares AP, Guillin EA, Borges LL, Da Silva AC, De Almeida ÁM, Grijalba PE, Gottlieb AM, Bluhm BH, De Oliveira LO (2015) More Cercospora species infect soybeans across the Americas than meets the eye. PLoS One 10:e0133495

Wickham H (2016) ggplot2: elegant graphics for data analysis. Available at: https://ggplot2.tidyverse.org. Accessed on 30 July 2020

Zeng F, Arnao E, Zhang G, Olaya G, Wullschleger J, Sierotzki H, Ming R, Bluhm BH, Bond JP, Fakhoury AM, Bradley CA (2015) Characterization of quinone outside inhibitor fungicide resistance in *Cercospora sojina* and development of diagnostic tools for its identification. Plant Disease 99:544–550

Zhou T, Mehl HL (2020) Rapid quantification of the G143A mutation conferring fungicide resistance in Virginia populations of *Cercospora sojina* using pyrosequencing. Crop Protection 127: 104942