



# Fungicide resistance in *Cercospora* species causing cercospora leaf blight and purple seed stain of soybean in Argentina

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## Abstract

*Cercospora* species cause cercospora leaf blight (CLB) and purple seed stain (PSS) on soybean. Because there are few resistant soybean varieties available, CLB/PSS management relies heavily upon fungicide applications. Sensitivity of 62 Argentinian *Cercospora* isolates to demethylation inhibitor (DMI), methyl benzimidazole carbamate (MBC), quinone outside inhibitor (QoI), succinate dehydrogenase inhibitor (SDHI) fungicides, and mancozeb was determined in this study. All isolates were sensitive to difenoconazole, epoxiconazole, prothioconazole, tebuconazole, and cyproconazole ( $EC_{50}$  values ranged from 0.006 to 2.4  $\mu\text{g/ml}$ ). In contrast, 51% of the tested isolates were sensitive ( $EC_{50}$  values ranged from 0.003 to 0.2  $\mu\text{g/ml}$ ), and 49% were highly resistant ( $EC_{50} > 100 \mu\text{g/ml}$ ) to carbendazim. Interestingly, all isolates were completely resistant to azoxystrobin, trifloxystrobin, and pyraclostrobin, and insensitive to boscalid, fluxapyroxad, and pydiflumetofen ( $EC_{50} > 100 \mu\text{g/ml}$ ). The G143A mutation was detected in 82% (53) of the QoI-resistant isolates and the E198A mutation in 97% (31) of the carbendazim-resistant isolates. No apparent resistance mutations were detected in the succinate dehydrogenase genes (subunits *sdhB*, *sdhC*, and *sdhD*). Mancozeb completely inhibited mycelial growth of the isolates evaluated at a concentration of 100  $\mu\text{g/ml}$ . All Argentinian *Cercospora* isolates were sensitive to the DMI fungicides tested, but we report for the first time resistance to QoI and MBC fungicides. Mechanism(s) other than fungicide target-site modification may be responsible for resistance of *Cercospora* to QoI and MBC fungicides. Moreover, based on our results and on the recent introduction of SDHI fungicides on soybean in Argentina, *Cercospora* species causing CLB/PSS are insensitive (naturally resistant) to SDHI fungicides. Insensitivity must be confirmed under field conditions.

## KEYWORDS

*Cercospora* spp., DMI, E198A, G143A, QoI, SDHI

## 1 | INTRODUCTION

Cercospora leaf blight (CLB) and purple seed stain (PSS), caused by *Cercospora kikuchii*, are important fungal diseases of soybean (*Glycine max*) worldwide (Ward *et al.*, 2015). PSS symptoms manifest

on infected seed as light to dark-purple discolorations that can cover part or the whole surface of the seed. CLB symptoms in the field appear as a purplish bronze blight in the upper third of the canopy during the reproductive growth stages of soybean (Ward *et al.*, 2015). CLB and PSS are part of the late season soybean disease (LSD)

complex, which is characterized by latent infections (Carmona *et al.*, 2015).

In Argentina, the average damage caused by LSD to yield is estimated at 10%, but can reach up to 30% (Carmona *et al.*, 2017a). However, because of the difficulty and complexity of assessing yield and economic losses caused only by CLB and PSS under field conditions, there are still no accurate estimates of yield losses in Argentina. In the USA, the average economic losses due to CLB/PSS across 28 states were estimated at \$77.8 million per year between 1996 to 2016 (Bandara *et al.*, 2020).

It is thought that *C. kikuchii* was introduced into South America from Asia, most probably Japan, through seed (Imazaki *et al.*, 2006a). This proposed route of introduction is based on amplified fragment length polymorphism analysis of a collection of isolates from Japan and South America (mainly Argentina and some from Brazil). The first molecular and phylogenetic identification of *C. kikuchii* isolated from CLB/PSS symptoms in Argentina came from Groenewald *et al.* (2013). However, over the past few years additional studies using multilocus phylogenetic analyses of cercosporoid fungi isolated from soybean in Argentina, Brazil, and the USA have confirmed that CLB and PSS are associated with several cryptic species (Soares *et al.*, 2015; Albu *et al.*, 2016a). In South America, *C. kikuchii* and *C. cf. sigesbeckiae* have been reported to be associated with these diseases. Recently, *C. cf. nicotianae* was identified as one of several species in association with CLB in Bolivia (Sautua *et al.*, 2019a), and has been confirmed for the first time to be a causal agent of CLB on soybean (Sautua *et al.*, 2020). However, these studies focused on a limited number of isolates not representative of the major soybean-growing regions in Argentina, one of the largest soybean-producing countries in South America. Therefore, sampling a greater number of isolates from different regions of Argentina to confirm the identity of the CLB/PSS-causing species is critical.

So far, there are no LSD-resistant soybean varieties available, and crop rotation, seed treatment, and fungicide application are the only available control measures to combat these diseases (Carmona *et al.*, 2015). In Argentina, estimates indicate that approximately half of the area planted to soybean annually (on average 17 million hectares) is treated with fungicides at least once per crop season (authors' unpublished data). Mixtures of two or three fungicide active ingredients (a.i.) from demethylation inhibitor (DMI, FRAC Code 3), quinone outside inhibitor (QoI, FRAC Code 11), and succinate dehydrogenase inhibitor (SDHI, FRAC Code 7) mode of action (MoA) groups are commonly used as commercial fungicide formulations sprayed during the reproductive growth stages of soybean (Carmona *et al.*, 2011, 2017a). In Argentina, QoI fungicides in mixtures with DMI fungicides (QoI + DMI) have been applied in soybean cultivation since 2002 (Carmona *et al.*, 2011). In comparison, methyl benzimidazole carbamates (MBC), mainly carbendazim, have been used in soybean crops over the last 30 years as the sole a.i. Lastly, SDHI fungicides were introduced to the market in 2015 as part of a triple mixture QoI + DMI + SDHI (Carmona *et al.*, 2017b).

In Argentina there are no previous studies on fungicide sensitivity of *Cercospora* isolates causing CLB/PSS, except a preliminary

study that determined that one single *C. kikuchii* isolate was sensitive ( $EC_{50} < 1 \mu\text{g/ml}$ ) to four QoI + DMI commercial mixtures (Carmona *et al.*, 2017b). In the USA, Price *et al.* (2015) reported QoI and MBC resistance in *Cercospora* isolates from Louisiana. These researchers reported that QoI fungicides were first used in Louisiana in 2000 and CLB-control failures began to appear in 2005, and were generalized from 2012 onwards. Afterwards, Albu *et al.* (2016b) reported that the G143A mutation in the cytochrome b gene was the cause of QoI-resistance in *C. cf. flagellaris* isolates from Louisiana. Sautua *et al.* (2019a) reported that this mutation conferred resistance to azoxystrobin in Bolivia. These are the only reports on mutations that confer resistance to QoI fungicides in this pathosystem. Dixon *et al.* (2020) reported that mutations F129L and G143A conferred resistance to azoxystrobin in *C. nicotianae*, the causal agent of frog-eye leaf spot of tobacco. Imazaki *et al.* (2006b) reported on the E198A mutation in the  $\beta$ -tubulin gene as responsible for the resistance to thiophanate-methyl (MBC fungicide) in *C. kikuchii* observed in Japan. There are no additional studies on mutations or mechanisms that confer resistance to fungicides in *C. kikuchii* infecting soybean crops.

Similarly, CLB-control failures have been observed over the last 6 years in many soybean fields throughout Argentina. In these field plots, CLB intensity remained high even after being sprayed with MBC fungicides or QoI + DMI or QoI + DMI + SDHI mixtures. These observations motivated the present study to: (a) collect samples of soybean leaves and seeds displaying CLB and PSS symptoms distributed throughout Argentina and isolate cercosporoid pathogens; (b) determine the identity of the isolates using a multilocus phylogenetic approach; (c) determine the sensitivity or resistance of *Cercospora* isolates to DMI, MBC, QoI, and SDHI fungicides; and (d) identify the mutations associated with field resistance.

## 2 | MATERIALS AND METHODS

### 2.1 | Fungal isolates

Isolates of *Cercospora* spp. were obtained from soybean leaves and seeds with symptoms sampled from 2014 to 2019 from commercial soybean fields throughout Argentina and Santa Cruz, Bolivia. Samples were taken at random from different fields. Additionally, isolates of *Cercospora* spp. were obtained from *Amaranthus hybridus* with symptoms and from symptomless *Araujia hortorum* leaves sampled in 2019 from Bragado and San Andrés de Giles, Buenos Aires province, respectively. *A. hybridus* and *A. hortorum* leaves were sampled from soybean fields and adjacent urban areas, respectively, to examine whether these species were alternative hosts for the pathogen, and also to explore their sensitivity to fungicides. The total samples in Argentina were divided into three regions: north-west, centre, and south-east, and within each area, isolates were chosen at random. The north-east (two isolates) and west (three isolates) regions were not included as they were not equally represented. Only one isolate per seed or leaf was selected. Fungal isolation was performed according to the method

described by Price *et al.* (2015). Isolates were maintained on potato dextrose agar (PDA; Britania) at 25°C with a 16 hr light:8 hr dark cycle and preserved with three different storage methods: (a) in cryovials with sterile water at 4°C, (b) on PDA slants covered with sterile mineral oil at room temperature, and (c) in 20% glycerol at -40°C. In Argentina, *Cercospora* isolates had been exposed mainly to carbendazim and QoI + DMI mixtures, whereas in Bolivia they had been exposed to QoI, DMI, SDHI, MBC, and more recently to multisite fungicides such as mancozeb. Thus, for the present study, 62 Argentinian and 3 Bolivian isolates from different locations were chosen to represent surveyed areas equitably (Table S1). Of the total samples from Bolivia, an isolate of each species previously identified (Sautua *et al.*, 2019a) was chosen at random. Thus, in total, the present study included 65 isolates for the *in vitro* fungicide sensitivity tests and the exploration of mutations that were associated with the observed resistance phenotypes. Of these 65 isolates, a subset of 42 isolates was chosen to perform a phylogenetic analysis to determine the species identity (Table S2).

## 2.2 | Pathogenicity tests

To confirm pathogenicity on soybean, we carried out pathogenicity tests in greenhouse assays with each isolate. The 65 isolates were inoculated onto 11 soybean varieties (DM3810, DM4214, DM4612, DM4615, DM4670, DM4915, NS4309, NS4611, NS4619iPRO, NA5009, and NA5019iPRO) that represented the most common cultivars grown in the Argentinian Pampas region. The methodology was carried out according to Sautua *et al.* (2020). Conidial suspensions ( $10^3$  conidia/ml) from each isolate grown on soybean leaf decoction agar (Sautua *et al.*, 2020) were sprayed on whole plants at the beginning seed stage (R5 stage; Fehr and Caviness, 1977). Spore suspensions were prepared by collecting spores from 7-day-old colonies in sterile water, with 1 ml Tween 20 (0.2%) added to facilitate the dispersal of conidia. Leaves sprayed with sterile distilled water plus Tween 20 (0.2%) served as the control. After inoculation, plants were kept in moist chambers for 24 hr. Following this initial period, plants were maintained in the greenhouse at  $24 \pm 3^\circ\text{C}$ . The plants were closely observed on a daily basis for the detection of symptoms. This procedure was carried out until physiological maturity. The experiment was repeated twice. After detection of symptoms in inoculated leaves, these were incubated and the pathogen was reisolated. The reisolated isolates were compared with the original isolates to confirm the completion of Koch's postulates.

## 2.3 | DNA extraction

For DNA extraction, liquid cultures of isolates were initiated by placing small pieces of mycelium into 250 ml flasks containing 50 ml potato dextrose broth, prepared according to a standard recipe (U.S.

Food and Drug Administration, 2001). The initial mycelium was carefully scraped with a sterile needle from the aerial mycelia of cultures growing on PDA plates. Liquid cultures were incubated at 24°C for 3 days in the dark with constant shaking provided by a standard shaker. After incubation, mycelia were recovered with a strainer and approximately 600 mg transferred to a sterile 1.5 ml microcentrifuge tube. Genomic DNA was extracted following the Dellaporta protocol (Dellaporta *et al.*, 1983). Quantification and quality control of extracted DNA was performed with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and diluted to a working concentration of 12 ng/ $\mu\text{l}$ .

## 2.4 | Isolate identification

### 2.4.1 | *actA*, *cmdA*, *his3*, *tef1*, and *gapdh* amplification and sequencing

Forty-two isolates were chosen to be characterized by sequencing portions of five nuclear loci: actin (*actA*), calmodulin (*cmdA*), histone 3 (*his3*), translation elongation factor 1- $\alpha$  (*tef1*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; Table S2), and aligning them with data from 379 other isolates published by Groenewald *et al.* (2013) and Bakhshi *et al.* (2018; Table S3, see Section 2.4.2). Fragments of *actA*, *cmdA*, *his3*, *tef1*, and *gapdh* genes were amplified from genomic DNA with the primers described in Table 1. The protocols and PCR conditions were those previously described (see references in Table 1). Additional primers were designed using Primer3 v. 4.1.0 (Untergasser *et al.*, 2012) for either improving amplification or sequencing of *cmdA* of a few isolates. PCR products were purified using the Wizard PCR and Gel Cleanup System (Promega) and sequencing services were performed by Macrogen Inc. ([www.macrogen.com](http://www.macrogen.com)). Sequencing reactions were carried out with the same primers as in the PCR amplifications.

### 2.4.2 | Model testing, phylogenetic analysis, and species assignment

Sequences were edited and contigs assembled in Geneious v. 8.1.3 (<https://www.geneious.com>). Each locus was aligned using iterative refinement methods implemented in MAFFT v. 7 (Katoh *et al.*, 2019) with the G-INS-I algorithm, a 200 PAM/k = 2 scoring matrix, gap penalty of 1.53, and offset of 0.0. Individual locus alignments were concatenated into a multilocus alignment with data from previous studies. Sequence data of the nuclear ribosomal internal transcribed spacers (nrITS), *actA*, *cmdA*, *his3*, and *tef1* published by Groenewald *et al.* (2013) along with data of *actA*, *cmdA*, *his3*, *tef1*, and *gapdh* from Bakhshi *et al.* (2018) were integrated with the data generated for this study. *Septoria provencialis* CPC\_12226 was included as an out-group. The final alignment lengths and number of parsimony informative characters (PIC)

TABLE 1 List of primers used in this study

Locus	Primer	Sequence (5'-3')	Product size (bp)	Annealing (°C)	Amino acids	Nucleotide length (bp)	Region sequenced <sup>a</sup>	No. introns	Reference
Nuclear									
Actin ( <i>actA</i> )	ACT-512F	ATGTGCAAGCGCGGTTTCGC	300	61	375	1,553	304–530	5	Carbone and Kohn (1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT							
Calmodulin ( <i>cmdA</i> )	CAL-228F	GAGTTCAAGGAGCCTTCTCC	500	55	149	754	172–471	4	Carbone and Kohn (1999)
	CAL-737R	CATCTTCTGGCCCATATGG							
	CAL2Rd	TGRTNGCCTCDGGATCATCTC	500	47.5, 50.8, 54.9			172–600		Groenewald et al. (2013)
	CM008504-1_F	CTCATCATCGTTGGATCGTG	591	58			1–500		Present study
	CM008504-1_R	CCTTGAACCTCGGAGACTTGC							
	CM008504-2_F	ACATCATGGTACGGCGCAAT	550	58			50–500		Present study
	CM008504-2_R	CACTGATGAAGCCGTTGTTG							
Glyceraldehyde-3-phosphate dehydrogenase ( <i>gapdh</i> )	Gpd1-LM	ATTGGCCGATCGTCTTCCGCAA	1,010	53	338	1,281	190–1,103	4	Carbone and Kohn (1999)
	Gpd2-LM	CCCACTCGTTGTCGTACCA							
Histone H3 ( <i>his3</i> )	CYL H3F	AGTCCACTGTGGCAAG	400	47	136	646	218–604	2	Crous et al. (2004)
	CYL H3R	AGCTGGATGTCCTTGGACTG							
Translation elongation factor 1- $\alpha$ ( <i>tref1</i> )	EF1-728F	CATCGAGAAGTTCGAGAAGG	350	58	459	1,866	240–545	5	Carbone and Kohn (1999)
	EF1-986R	TACTTGAAGGAAACCCCTTACC							
Sterol 14 $\alpha$ -demethylase cytochrome P450 ( <i>cyp51</i> )	CYP51FS	GTGTTGGGCAAGGACGTCG	1,353	54	543	1,632	433–1,569	0	Nikou et al. (2009)
	CYP51RS	CTCTCCCACTTCACAACAGC							
	<i>cyp51_432bp_F</i>	TGTTGAAACCCGTACGATGGA	795	60			1–650		Present study
	<i>cyp51_432bp_R</i>	AGGTGCAATCGAATTTGCTG							
	<i>cyp51_63bp_F</i>	AAGCTGTCGCAGAGGAGAAG	704	60			1,376–1,632		Present study
	<i>cyp51_63bp_R</i>	GGCGAAGAATAACCGATTCA							
$\beta$ -tubulin ( <i>tub2</i> )	tub_1F	ACTCGTTGCTGATTGGCTCT	1,938	53	447	1,611	1–1,611	4	Present study
	tub_1R	TTTGGGTCAACAGGGAAAAAG							
	Tub-F1	GGTCATTACACTGAGGGTGC	850	56			620–1,505		Imazaki et al. (2006b)
	Tub-R1	GACAAGATCGTTCATGTTGGACTC							
	tub_inter-1F	CAAGGTCTCCATGAAGGA					1–644		Present study
	tub_inter-1R	ATACCGGCACCCAGTACCAC					1,276–1,611		

(Continues)

TABLE 1 (Continued)

Locus	Primer	Sequence (5'-3')	Product size (bp)	Annealing (°C)	Amino acids	Nucleotide length (bp)	Region sequenced <sup>a</sup>	No. introns	Reference
Succinate dehydrogenase subunit B ( <i>sdhB</i> )	sdhB_1F	GCCGAGCAICTGGTGATTT	1,255	55	300	1,012/1,015	1-1,015	2	Present study
	sdhB_1R	TCGCATTTCTAACGCAGTTG							
	sdhB_seq1F	ACATTCGGAAGAAGTTGC	670	55			400-1,015		Present study
	sdhB_seq1R	TGCTTGTAGAACTGGGTC							
Succinate dehydrogenase subunit C ( <i>sdhC</i> )	sdhC_1F	GCGGACAAAGAAAACCTCAGC	1,166	50	183	881	1-881	2	Present study
	sdhC_1R	GCATGGGCTCTACGAGAAAAG							
Succinate dehydrogenase subunit D ( <i>sdhD</i> )	sdhD_1F	CGATCCAACAAGCACTGGTA	1,413	53	204	775	1-775	2	Present study
	sdhD_1R	AGTGTGCACGTCCTCAAAATTGA							
Mitochondrial									
Cytochrome b ( <i>cytb</i> )	Cytb-F2	GGAAGAGGCTTACTACTATGG	700	49	386	1,161	334-1,020	0	Imazaki et al. (2006a)
	Cytb-R1	CGTGATTAGCACCTAATTGC							
	660_Cbcytb	GACACAGGGTTATCACATCA	668	58			1-228		Birila et al. (2012)
	661_Cbcytb	AAGAAGAAAAATGCTGAAGCTG							
	714_Cbcytb	AGAACATATTATGAGAGAC	734	58			229-886		Birila et al. (2012)
	715_Cbcytb	CTGTGAAAGGCATAGTTAGT							
	658_Cbcytb	AATTACGTTGTGGCCAAATCC	519	58			606-1,161		Birila et al. (2012)
	659_Cbcytb	CAACCGGTATTATTGAGGATTT							

<sup>a</sup>Start and end positions of the sequenced fragment relative to the entire gene sequences of *Cercospora kikuchii* reference isolate ARG\_18\_001 sliced from the genome assembly (Sautua et al., 2019b).

were as follows: concatenated, 3,354 sites (626 PIC); nrITS, 652 sites (14 PIC); *actA*, 234 sites (72 PIC); *cmdA*, 465 sites (103 PIC); *his3*, 400 sites (90 PIC); *tef1*, 684 sites (142 PIC); *gapdh*, 919 sites (205 PIC). The maximum-likelihood phylogeny for each gene and the concatenated alignment were estimated using the parallel AVX version of RAxML v. 8.2.12 (Stamatakis, 2014) specifying the GTR model of nucleotide evolution and a  $\Gamma$  distribution on rate heterogeneity across sites. Bipartition support was estimated with 1,000 bootstrap replicates for each analysis (mpirun raxmlHPC-MPI-AVX -f a -x 12345 -p 12345 -# 1000 -m GTRGAMMA). Prior to Bayesian inference, the best-fit model of nucleotide evolution and best partitioning scheme across alignments was estimated in Partition Finder 2 (Lanfear *et al.*, 2016), specifying the greedy algorithm (Lanfear *et al.*, 2012) with linked branch lengths and using the corrected Akaike information criterion for selecting the best-fit model among the set of models considered for MrBayes. The models selected were SYM + I +  $\Gamma$  for nrITS and GTR + I +  $\Gamma$  for *actA*, *cmdA*, *his3*, *tef1*, and *gapdh*. The joint posterior distribution of parameter values and tree topologies for the concatenated, partitioned multilocus dataset was estimated in MrBayes v. 3.2.6 (Ronquist *et al.*, 2012) with four replicate Markov chain Monte Carlo (MCMC) runs, four Metropolis-coupled chains per run (one cold and three heated), and 2.5 million generations per run with a sampling frequency of 1,000. Convergence was determined by observing a standard deviation of split frequencies below 0.1 and effective sample sizes (ESS) above 200 for most parameters, discarding the first 50% of samples as burn-in. The majority-rule consensus phylogeny was computed using SumTrees v. 4.3.0 in Dendropy v. 4.3.0 (Sukumaran and Holder, 2010). The bipartitions with a posterior probability equal to or greater than 0.85 were mapped, if present, to the maximum-likelihood phylogeny alongside the maximum-likelihood bootstrap support values greater than 50%. Species assignments were based on the results of the concatenated phylogenetic analyses and comparisons with each single-locus phylogenetic analysis. In order to assess the sensitivity of our results to missing data for *gapdh* and nrITS, we ran phylogenetic analyses that both included and excluded *gapdh* as well as including and excluding nrITS in the concatenated analyses and running multiple single-gene analyses. There was remarkable congruence across the analyses and no strongly supported conflict, suggesting that the missing data do not affect the phylogenetic results in a significant way.

## 2.5 | Chemicals

Five DMI (cyproconazole, difenoconazole, epoxiconazole, prothioconazole, and tebuconazole), three QoI (azoxystrobin, pyraclostrobin, and trifloxystrobin), one SDHI (boscalid), and one MBC (carbendazim) were used for sensitivity tests as technical-grade fungicides (>97% active). These fungicides were provided by Agrofina S.A. and Nova S.A. Additionally, the SDHI fungicides fluxapyroxad (Sistiva, 33.3 g a.i./100 ml, BASF Argentina S.A.) and pydiflumetofen (Miravis, 20 g

a.i./100 ml SC, Syngenta Agro S.A.), and a multisite fungicide, mancozeb (Manzate Pro Stick, 75 g a.i./kg DF, UPL Argentina S.A.), were included as their commercial formulation.

## 2.6 | In vitro fungicide sensitivity testing

Because of scant sporulation in culture and following Price *et al.* (2015), mycelial radial growth inhibition instead of spore germination assays were used for determining fungicide sensitivity. In order to calculate the effective fungicide concentration to inhibit 50% of fungal radial growth ( $EC_{50}$ ) for each isolate  $\times$  fungicide combination, all fungicides were tested on isolates 1–35 (Table S1). This set of isolates included 32 isolates from Argentina and 3 isolates from Bolivia. The Bolivian isolates were included for comparative purposes because these strains were isolated from soybean fields that received a significantly higher number of fungicide applications and are resistant to QoI fungicides (Sautua *et al.*, 2019a). PDA plates were amended with serial dilutions of each technical and commercial grade fungicide at 0.001, 0.01, 0.1, 1, 10 and 100  $\mu$ g/ml. Stock solutions of technical grade formulations of each fungicide were prepared at concentrations of 10 mg/ml and serially diluted in acetone. An accurate volume of 20 ml PDA was measured with a Falcon tube and dispensed into each  $9 \times 1.5$  cm Petri dish. There were four replicates (Petri dishes) per concentration. As initial inoculum, 6 mm-diameter mycelial discs were cut from the margins of 7-day-old stock cultures of each isolate actively growing on PDA using a #3 corkborer, inverted, and transferred to amended PDA. Petri dishes were incubated under a 16 hr fluorescent and near-ultraviolet/8 hr darkness cycle at  $24 \pm 1^\circ\text{C}$ . Non-fungicide-amended PDA served as the control. After incubation for 7 days, colony diameter was measured twice using a digital caliper. Each experiment was conducted using a randomized complete block design with incubator shelves serving as blocks. There were four replicates per isolate.

In a second set of experiments, discriminatory doses of each fungicide were used on isolates 36–65 (Table S1) in order to detect fungicide resistance. Because there are no previous studies in Argentina and there is only one previous study in the USA (Price *et al.*, 2015), discriminatory doses of 10 and 100  $\mu$ g/ml were selected based on preliminary research and according to  $EC_{50}$  values calculated from isolates 1 to 35 (Table S1). These experiments were conducted as described above. Isolates that were not inhibited at 100  $\mu$ g/ml were considered completely resistant.

Both types of experiments ( $EC_{50}$  and discriminatory dose to detect sensitivity) were repeated once for cyproconazole, difenoconazole, epoxiconazole, prothioconazole, tebuconazole, carbendazim, and mancozeb, and twice for azoxystrobin, pyraclostrobin, trifloxystrobin, boscalid, fluxapyroxad, and pydiflumetofen.

## 2.7 | Determination of alternative respiration

Some fungi possess the ability to use an alternative oxidation (AOX) pathway (Ziogas *et al.*, 1997); however, it is not known how



frequently this process may occur spontaneously in vivo. It has also been documented that the in vitro growth of QoI-resistant isolates is inhibited only in the presence of AOX inhibitors. Nevertheless, Price *et al.* (2015) found that propyl gallate (an AOX inhibitor) did not significantly affect radial growth of *Cercospora* isolates from Louisiana, USA. In order to determine whether an AOX pathway was present in *Cercospora* isolates chosen for the present study, the effect of salicylhydroxamic acid (SHAM, 99% active; Alfa Aesar) was examined on six *Cercospora* isolates in a preliminary experiment. Isolates 1, 8, 9, 10, 31, and 32 (Table S1) were selected at random for this purpose. Serial dilutions of SHAM were made in ethanol (95%). Afterwards, PDA was amended with SHAM at 0, 30, 60, 100, and 200 µg/ml. The experiment was conducted as described in Section 2.6. Radial growth of *Cercospora* was significantly reduced by SHAM at 200 µg/ml (80% on average) but not at 60 µg/ml (<20% on average). Thus, assays including isolates 1–35 were conducted as in Section 2.6 to compare the effect of azoxystrobin on mycelial growth with and without the addition of SHAM to the media. SHAM was either added to a final concentration of 60 µg/ml or omitted for each concentration of fungicide. The experiment was repeated once.

## 2.8 | Sequence analysis of *cytb*, *tub2*, *cyp51*, and *sdh* genes

In order to assess DNA sequence variation between fungicide-sensitive and -resistant isolates, we amplified and sequenced fragments of the mitochondrial cytochrome b (*cytb*) gene and the nuclear β-tubulin (*tub2*) gene using PCR for 64 of the 65 randomly chosen individuals and sequenced the complete genome of the *C. kikuchii* reference isolate ARG\_18\_001 (Sautua *et al.*, 2019b). We also amplified and sequenced fragments of the sterol 14α-demethylase cytochrome P450 (*cyp51*) gene for 36 isolates (isolates 1–36, Table S1). Additionally, for some randomly chosen isolates, we amplified and sequenced the complete sequence of *cytb*, *tub2*, and *cyp51*. Furthermore, we amplified and sequenced the complete sequence of genes encoding the B, C, and D subunits of the SDHI fungicide-targeted succinate dehydrogenase enzyme (*sdhB*, *sdhC*, and *sdhD*) where mutations associated with SDHI resistance had been previously described in different fungi (Sierotzki and Scalliet, 2013). The summary of the loci sequenced for each isolate and used in both the phylogenetic and the mutation analyses is shown in Table S2. Primers and PCR conditions used in the present study are described in Table 1. New primers were designed for *cyp51*, *tub2*, *sdhB*, *sdhC*, and *sdhD* using Primer3 v. 4.1.0 (Untergasser *et al.*, 2012). Sequencing services were performed as described previously. Multiple sequence alignment was performed for each locus using MUSCLE (Edgar, 2004) and including the complete sequence from the genome of isolate ARG\_18\_001 as a reference. Conserved loci were detected in the reference genome sequence using *C. beticola* sequences of each locus recovered from the NCBI against a local BLAST database. Coding regions and introns for each locus were identified by comparing with the mentioned *C. beticola* sequences (Sautua *et al.*, 2019b).

## 2.9 | 3-D modelling of the sterol 14α-demethylase protein

In order to locate the positions of mutations within the 3-D structure of the *C. kikuchii* sterol 14α-demethylase cytochrome P450 protein (CYP51), the enzyme structure was modelled by comparative protein modelling methods using the SWISS-MODEL server (<http://swissmodel.expasy.org>). The modelling structure of the *C. kikuchii* CYP51 was obtained with the 5eah.1 template (*Saccharomyces cerevisiae* CYP51 complexed with difenoconazole) by the On-line Swiss-model software.

## 2.10 | Data analysis

Dose–response curves and EC<sub>50</sub> values were generated for each isolate × fungicide combination (isolates 1–35). Function `drc()` from `drc` package v. 2.5-12 (Ritz *et al.*, 2015) was used in the R environment v. 3.6.3 (R Core Team, 2020) for nonlinear regression analysis. A log-logistic model, specifically a Weibull type I four-parameter model, was fitted and estimation of parameters was based on the maximum-likelihood estimate as described in Carmona *et al.* (2017b). Mean EC<sub>50</sub> values and their 95% confidence intervals were estimated by combining all the data from the two (or three) experiments and using the `ED()` function of the `drc` package. A normality test for EC<sub>50</sub> values was performed using the Shapiro–Wilk test of normality with the `stats` package of R. In all cases, data were non-normally distributed. Therefore, a Kruskal–Wallis one-way analysis of variance was used with the `stats` package in R to assess for significant differences among distributions of EC<sub>50</sub> values for the different fungicides. Dunn's multiple comparison test was used post hoc to compare distributions among fungicide a.i. ( $p = .05$ ) with the `dunnTest()` function of the `FSA` package in R (Ogle *et al.*, 2019). For AOX analysis, mean EC<sub>50</sub> values (or growth inhibition values at 100 µg/ml if EC<sub>50</sub> values could not be estimated due to resistance) for SHAM-amended PDA and nonamended PDA were compared using a two-sample *t* test with the `stats` package in R.

## 3 | RESULTS

### 3.1 | Fungal isolates and pathogenicity tests

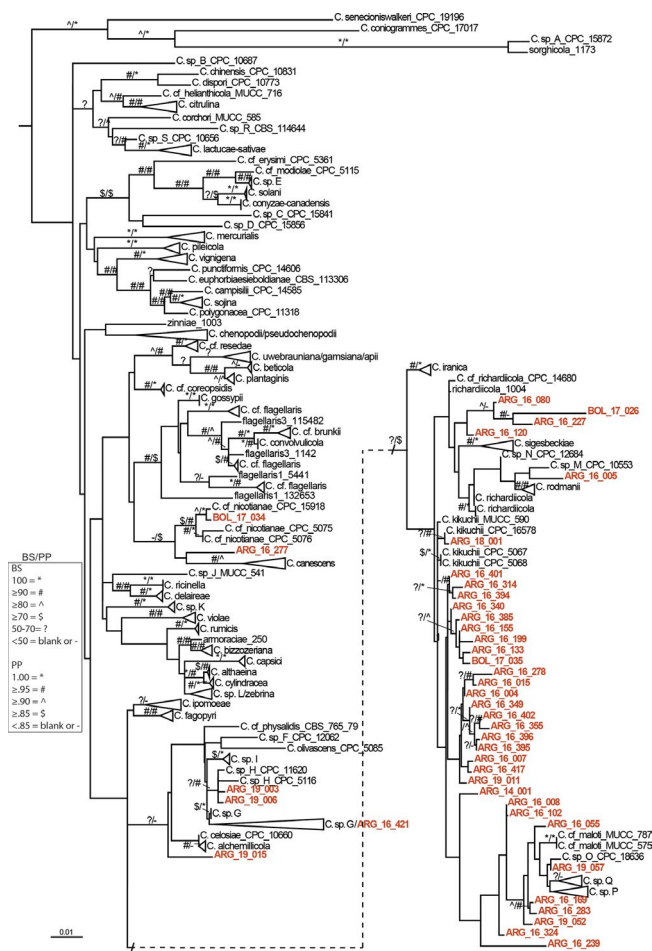
All the fields sampled in all locations and in all years were infected with CLB/PSS with 100% prevalence, confirming that these diseases are fully distributed throughout Argentina and Santa Cruz, Bolivia. In all, 554 single-conidium isolates of *Cercospora* spp. were collected from different fields as follows: a total of 516 isolates were obtained from 67 locations in nine different provinces of Argentina in 2012 (1), 2014 (1), 2015 (20), 2016 (364), 2017 (67), 2018 (6), and 2019 (57); and 38 isolates were obtained from Santa Cruz, Bolivia in 2017. In Argentina, 196 isolates were collected from the north-west region, 241 isolates from the centre, and 74 isolates from the

south-east (green, yellow, and orange GPS points in Figure S1, respectively). The north-east (two isolates) and west (three isolates) regions were not included as they were not represented in the same proportion as the rest. Within each region, the isolates were chosen randomly. In Argentina, 336 isolates were obtained from seeds, 177 from leaves, two from peduncles, and one from a pod, whereas the 38 Bolivian isolates were obtained from leaves. Of the 516 isolates from Argentina, 492 were collected from soybean, 10 from *A. hybridus*, and 14 from *A. hortorum*. All isolates were deposited in the fungal culture collection of the Plant Pathology Department of the University of Buenos Aires (FAUBA, Argentina).

All isolates were pathogenic to soybean leaves, causing blight symptoms typical of CLB within 21 days of inoculation. The results were confirmed in repeated experiments. All the inoculated varieties were infected and showed CLB symptoms. Disease incidence on inoculated plants was 100%. There was no differential response among cultivars. Negative controls were symptomless. Leaves with symptoms were incubated in Petri dishes and isolates were recovered from all soybean varieties tested. Recovered isolates and monoconidial cultures resembled original inoculum on PDA, completing Koch's postulates.

### 3.2 | Species identification

The 42 isolates from Argentina and Bolivia were distributed among eight lineages. The majority of previously circumscribed species represented by multiple isolates in the concatenated multilocus phylogeny were moderately to strongly supported as monophyletic in both the Bayesian and maximum-likelihood analyses (Figure 1). Those species that are paraphyletic or polyphyletic include *C. chenopodii*, *C. apii*, *C. cf. flagellaris*, *C. richardiicola*, and *C. kikuchii*. In each of these cases, other species are nested within the clade that otherwise includes conspecific isolates. *C. pseudochenopodii* is nested within the clade that includes isolates of *C. chenopodii*. Isolates of *C. uwebrauniana* and *C. gamsiana* are nested within the clade that includes all isolates of *C. apii*. *C. cf. flagellaris* isolates are partitioned into multiple subclades that include isolates of *C. gossypii*, *C. cf. brunckii*, and *C. convolvulicola*. *C. richardiicola* is polyphyletic with *C. sigesbeckiae*, *C. sp. N*, *C. sp. M*, and *C. rodmanii*, and is referred to as the *C. richardiicola* complex (CRC) below. Finally, *C. kikuchii* is paraphyletic with *C. cf. maloti*, *C. sp. O*, *C. sp. Q*, and *C. sp. P* nested within the clade that includes all isolates of *C. kikuchii*. This clade that includes isolates of *C. kikuchii*, which we refer to as the *C. kikuchii* complex (CKC), contains the majority of the isolates collected for this study, including 30 isolates from Argentina (one collected in 2014, 25 in 2016, one in 2018, and three in 2019) and one isolate from Bolivia. Of these isolates, 30 are assigned to *C. kikuchii* based on the multilocus concatenated analysis and the remaining isolate, 19\_057 from Argentina, is assigned to *C. sp. O* despite it being weakly supported as sister to a single isolate of *C. sp. O*. Five of the remaining 11 isolates were nested within the CRC, and four of these (ARG\_16\_080, ARG\_16\_120, ARG\_16\_227, and BOL\_17\_026) are denoted as such



**FIGURE 1** Maximum-likelihood phylogeny of the genus *Cercospora* inferred from a concatenated alignment of nrITS, *actA*, *cmdA*, *gapdh*, *tef1*, and *his3*. Isolates collected and sequenced for this study are highlighted in bold red font. The tree is rooted with *Septoria provencialis*. Some clades that are not the focus of this study have been collapsed for visualization purposes. Maximum-likelihood bootstrap support (BS) values greater than 50% and Bayesian posterior probabilities (PP) greater than 0.85 are shown on the branches (BS/PP). Symbols represent the range of values indicated in the legend. Unlabelled branches were found in the maximum-likelihood tree search, but were not found at a frequency greater than or equal to 50% among the bootstrap replicate searches and were not present or received low support (<0.85) in the Bayesian analysis. The scale bar represents the estimated number of substitutions per site [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

in Table S1 because their affinity with any previously described species is unclear based on the phylogenetic results. A single isolate within the CRC was assigned to *C. sp. M* based on a weakly supported sister relationship to a single isolate of *C. sp. M* (*C. sp. M\_CPC\_10553*). Four of the six remaining isolates were assigned to *C. sp. H* (ARG\_19\_003, ARG\_19\_006), *C. sp. G* (ARG\_16\_421), and *C. nicotianae* (BOL\_17\_034). The last two isolates could not be definitively assigned to species based on their uncertain placement in the concatenated phylogeny. ARG\_16\_277 was weakly supported as sister to *C. canescens* in the multilocus concatenated phylogeny, but



in the *gapdh*, *tef1*, and *his3* phylogenies this isolate was strongly supported as sister to BOL\_17\_034, thus it was assigned to *C. nicotianae* in Table S1. The last remaining isolate, ARG\_19\_015, was not assigned to a species (species indet.) in Table S1 because its placement varied considerably among individual locus phylogenies. This isolate was weakly supported as sister to *C. sp. H* in the *actA* phylogeny, uncertain in *his3*, weakly supported as sister to *C. sp. G* in *tef1*, strongly supported as nested within *C. sp. G* in *gapdh*, and closely related to isolates of *C. kikuchii* and *C. fagopyri*, albeit with weak support, in the *cmdA* phylogeny. This is the first report of *C. kikuchii* and *C. sp. O* isolated from *A. hortorum*, and *C. sp. H* from *A. hybridus*.

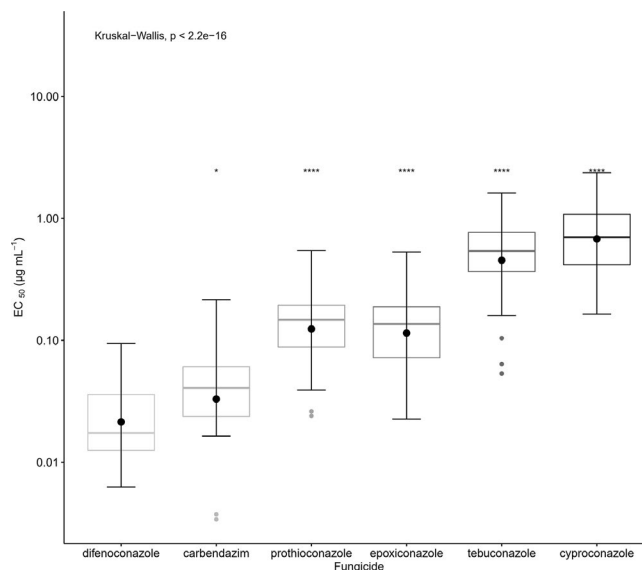
### 3.3 | Determination of alternative respiration

While all isolates were extremely resistant to the QoI fungicides tested and the  $EC_{50}$  values could not be estimated, there were no significant differences when comparing between mycelial growth inhibition values with and without the addition of SHAM at a given fungicide concentration. This was observed in 35 isolates of *Cercospora* (data not shown). These results are in agreement with those of Price *et al.* (2015). Therefore, it was assumed that for the isolates tested here, AOX did not occur, and SHAM was not used in subsequent experiments. Nevertheless, future experiments including sensitive isolates should elucidate whether or not the AOX pathway is present in *Cercospora* isolates from Argentina.

### 3.4 | Activity of fungicides against mycelial growth and nucleotide and amino acid sequences of *cytb*, *tub2*, *cyp51*, and *sdh* genes

#### 3.4.1 | DMI

A total of 62 Argentinian isolates were sensitive to all DMIs tested.  $EC_{50}$  values of DMIs estimated in 32 Argentinian isolates ranged from 0.006 to 0.094  $\mu\text{g/ml}$  for difenoconazole, from 0.024 to 0.545  $\mu\text{g/ml}$  for prothioconazole, from 0.022 to 0.577  $\mu\text{g/ml}$  for epoxiconazole, from 0.055 to 1.762  $\mu\text{g/ml}$  for tebuconazole, and from 0.164 to 2.371  $\mu\text{g/ml}$  for cyproconazole (isolates 1–32 in Tables S1 and S4). Among the five DMI fungicides tested, difenoconazole exhibited the highest fungitoxicity (Figure 2; Table 2). Prothioconazole and epoxiconazole did not differ from each other ( $p = 1$ ), being less fungitoxic than difenoconazole but more fungitoxic than tebuconazole and cyproconazole, which also did not differ from each other ( $p = 1$ ). Two Bolivian isolates (numbers 33 and 34) were sensitive to all DMI fungicides and one isolate (number 35, *C. kikuchii*) was the only strain considered resistant to cyproconazole ( $EC_{50} = 45.8 \mu\text{g/ml}$ ) and tebuconazole ( $EC_{50} = 10 \mu\text{g/ml}$ ) and less sensitive to prothioconazole ( $EC_{50} = 6 \mu\text{g/ml}$ ). Cyproconazole was the least fungitoxic, with a 14.5- and 107.4-fold range of  $EC_{50}$  values for the Argentinian and Bolivian isolates, respectively (max/min values in Table S4). Based on these results, two discriminatory doses were chosen to test new



**FIGURE 2** Sensitivity distribution ( $EC_{50}$ ) of 32 and 19 Argentinian *Cercospora* isolates causing cercospora leaf blight/ purple seed stain to five demethylation inhibitors (DMIs) and to carbendazim (MBC), respectively. Pairwise comparisons using the Wilcoxon rank sum test (Mann–Whitney  $U$  test) are shown against difenoconazole, the most fungitoxic active ingredient against all *Cercospora* isolates tested in the present study

**TABLE 2** Average fungicide effective concentration required to inhibit mycelial growth by 50% ( $EC_{50}$ ) in Argentinian isolates of *Cercospora* sensitive to DMI and carbendazim (MBC), resistant to QoI, and insensitive to SDHI and mancozeb

Active ingredient	Mode of action	No. isolates tested	$EC_{50}$ ( $\mu\text{g/ml}$ )
Difenoconazole	DMI	32	0.027 a
Carbendazim	MBC	19	0.048 ab
Prothioconazole	DMI	32	0.157 b
Epoxiconazole	DMI	32	0.154 b
Tebuconazole	DMI	32	0.602 c
Cyproconazole	DMI	32	0.889 c
Azoxystrobin	QoI	62	>100
Pyraclostrobin	QoI	62	>100
Trifloxystrobin	QoI	62	>100
Boscalid	SDHI	62	>100
Pydiflumetofen	SDHI	62	>100
Fluxapyroxad	SDHI	62	>100
Mancozeb	Multisite	62	>10

Note: Means followed by the same letter do not significantly differ as determined by Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparison test. All 62 Argentinian isolates were completely resistant or insensitive to QoI and SDHI fungicides, respectively.

isolates in a new round of experiments: 10  $\mu\text{g/ml}$  allows for the detection of decreased sensitivity and 100  $\mu\text{g/ml}$  allows for the detection and confirmation of extremely resistant isolates. The other 30

Argentinian isolates (nos. 36–65, Table S1) exposed to discriminatory doses of each DMI fungicide were sensitive to all fungicides tested, confirming the lack of variation in sensitivity with respect to the oldest isolates (Table 3).

Despite having found 103 single nucleotide polymorphisms (SNPs) along the *cyp51* partial sequence in 16 DMI-sensitive isolates, no apparent mutations known to be associated with DMI resistance in *Cercospora* or other fungi were found in the 32 Argentinian and 2 Bolivian sensitive isolates sequenced. Most of the SNPs found (84%, 87/103) were synonymous substitutions and thus did not alter the amino acid sequence. There is no clear correlation or association between SNPs and *Cercospora* species at this locus. The only observed pattern corresponded to 19 SNPs shared by 14 isolates of different species (*C. kikuchii*, *C. nicotianae*, CRC, *C. sp. M*, *C. sp. G*). Nevertheless,

two new single-nucleotide mutations detected in the *cyp51* gene of the DMI-resistant Bolivian isolate BOL\_2017\_035 resulted in the amino acid substitutions I387M and D440N. The complete amino acid sequence for the CYP51 enzyme and the modelling structure of the CYP51 of isolate *C. kikuchii* BOL\_17\_035 are shown in Figures S2 and S3, respectively. According to the modelling structure of the CYP51 of DMI-resistant isolate *C. kikuchii* BOL\_17\_035, the mutation I387M is present in a residue next to the prosthetic heme group (Figure S3).

### 3.4.2 | Carbendazim (MBC)

In total, 33 isolates were sensitive and 32 were resistant to carbendazim, the only MBC fungicide tested. Carbendazim had a high

**TABLE 3** Inhibition of mycelial growth of sensitive or insensitive/resistant *Cercospora* spp. isolates exposed to discriminatory doses of different fungicides

Fungicide	Sensitive			Resistant or insensitive		
	No. isolates	Inhibition (%)		No. isolates	Inhibition (%)	
		10 µg a.i./ml	100 µg a.i./ml		10 µg a.i./ml	100 µg a.i./ml
<b>Argentina</b>						
Difenoconazole	62	98 (92–100)	100 (100–100)	0	–	–
Prothioconazole	62	87 (73–98)	100 (100–100)	0	–	–
Epoxiconazole	62	96 (84–100)	100 (100–100)	0	–	–
Tebuconazole	62	88 (72–100)	100 (100–100)	0	–	–
Cyproconazole	62	82 (71–99)	100 (100–100)	0	–	–
Carbendazim	33	100 (100–100)	100 (100–100)	29	0 (0–0)	6 (0–19)
Azoxystrobin	0	–	–	62	25 (0–49)	33 (21–48)
Trifloxystrobin	0	–	–	62	31 (18–39)	38 (24–48)
Pyraclostrobin	0	–	–	62	33 (29–53)	48 (37–59)
Boscalid	0	–	–	62	9 (0–36)	19 (0–40)
Fluxapyroxad	0	–	–	62	11 (3–20)	12 (3–52)
Pydiflumetofen	0	–	–	62	20 (2–41)	23 (3–58)
Mancozeb	0	–	–	62	37 (19–40)	100 (100–100)
<b>Bolivia</b>						
Difenoconazole	3	94 (93–96)	100 (100–100)	0	–	–
Prothioconazole	3	72 (62–85)	100 (100–100)	0	–	–
Epoxiconazole	3	85 (82–90)	100 (100–100)	0	–	–
Tebuconazole	2	63–70	100–100	1 <sup>a</sup>	51	85
Cyproconazole	2	61–67	100–100	1 <sup>a</sup>	38	76
Carbendazim	0	–	–	3	0 (0–0)	0 (0–0)
Azoxystrobin	0	–	–	3	25 (16–34)	39 (21–44)
Trifloxystrobin	0	–	–	3	32 (22–39)	44 (35–49)
Pyraclostrobin	0	–	–	3	36 (25–37)	49 (39–52)
Boscalid	0	–	–	3	5 (3–7)	16 (9–21)
Fluxapyroxad	0	–	–	3	13 (4–19)	11 (2–36)
Pydiflumetofen	0	–	–	3	19 (14–25)	21 (3–44)
Mancozeb	0	–	–	3	31 (24–42)	100 (100–100)

<sup>a</sup>Isolate BOL\_2017\_035.

fungitoxicity on 19 Argentinian sensitive isolates with  $EC_{50}$  values ranging from 0.003 to 0.2  $\mu\text{g/ml}$  (Table S4). These  $EC_{50}$  values did not significantly differ from difenoconazole ( $p = 1$ ), epoxiconazole ( $p = .1$ ) or prothioconazole ( $p = .06$ ; Table 2; Figure 2). In the 33 sensitive isolates (19 from isolates 1 to 35 and 14 from isolates 36 to 65), the mycelial growth was inhibited 100% at 10  $\mu\text{g/ml}$ . However, in the other 32 resistant isolates tested (16 from isolates 1 to 35 and 16 from isolates 36 to 65) carbendazim at 10  $\mu\text{g/ml}$  did not inhibit any isolate (0% inhibition of the mycelial growth) and at 100  $\mu\text{g/ml}$  inhibited between 0% and 19%, but 78% of these resistant isolates (25/32) had a percentage inhibition of 10% or less (Table 3).

The E198A mutation was the only mutation detected in the partial sequence of the *tub2* gene in 31 of 32 isolates found resistant to carbendazim. One resistant isolate (ARG\_19\_016) does not have this mutation or any other mutation in the entire *tub2* gene. The percentage of mycelial growth inhibition for this isolate was 0% and 19% at 10  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$ , respectively.

The *C. kikuchii*  $\beta$ -tubulin protein is coded by a 1,611 bp gene with four introns (Table 1). This gene is highly conserved; no differences were observed in the partial or complete exon sequences between species or between any isolate. Although a few intronic SNPs were found in the entire *tub2* sequence for eight isolates, including the *C. kikuchii*, *C. nicotianae*, and CRC species (nine, nine, eight, and four SNPs in introns 1, 2, 3, and 4, respectively), no clear association was observed between intron sequences and assigned species. The complete amino acid sequence for the  $\beta$ -tubulin protein is shown in Figure S4.

### 3.4.3 | QoI

Because no differences in mycelial growth inhibition were observed in any isolate along the concentration gradient used in the in vitro sensitivity tests of azoxystrobin, trifloxystrobin, and pyraclostrobin,  $EC_{50}$  values could not be estimated ( $EC_{50} > 100 \mu\text{g/ml}$ ; Table 2). Mycelial growth of all 65 isolates was highly insensitive to these fungicides as none of them were 100% inhibited when exposed at the highest concentration tested (100  $\mu\text{g/ml}$ ). The 62 Argentinian isolates had a mycelial inhibition range of 0%–49%, 18%–39%, and 29%–53% for azoxystrobin, trifloxystrobin, and pyraclostrobin, respectively, at 10  $\mu\text{g/ml}$ ; and a range of 21%–48%, 24%–48%, and 37%–59%, respectively, at 100  $\mu\text{g/ml}$  (Table 3). Because none of the QoI tested suppressed mycelial growth at 100  $\mu\text{g/ml}$  for any of the isolates tested, we consider these isolates strobilurin-resistant.

Although 65 isolates tested in this study were found to be resistant to QoI fungicides, the G143A mutation was detected in the *cytb* gene of only 53 isolates (82%; Table 4). In order to rule out sequencing errors, the 12 isolates that do not possess the G143A mutation were sequenced separately and independently, and the complete *cytb* gene was amplified and sequenced. No mutations associated with QoI resistance, F129L and G137R, nor any other mutation was found in the partial or complete sequence of this gene in any tested isolate. The *C. kikuchii* cytochrome b protein is coded by a 1,161 bp

intron-free gene (Table 1). This gene is highly conserved; no differences were observed in the partial or complete sequence among isolates. The complete amino acid sequence for the cytochrome b protein is shown in Figure S5.

### 3.4.4 | SDHI

All isolates in this study were insensitive to boscalid, fluxapyroxad, and pydiflumetofen. Mycelial growth of all 65 isolates was highly insensitive to SDHI fungicides as none of them were 100% inhibited when exposed to the highest concentration tested (100  $\mu\text{g/ml}$ ). The 62 Argentinian isolates had a mycelial inhibition range of 0%–36%, 3%–20%, and 2%–41% for boscalid, fluxapyroxad, and pydiflumetofen, respectively, at 10  $\mu\text{g/ml}$ ; and a range of 0%–40%, 3%–52%, and 3%–58%, respectively, at 100  $\mu\text{g/ml}$  (Table 3).

There were no mutations present in the *sdhB*, *sdhC*, and *sdhD* genes sequenced in a subset of 11 isolates for *sdhB* and *sdhC* and eight isolates for *sdhD*. The *C. kikuchii* SDHB, SDHC, and SDHD proteins are coded by 1,015, 881, and 775 bp genes, respectively, with two introns each (Table 1). The three loci are highly conserved; no differences were observed in the complete sequence between species or between any isolate. A few intronic SNPs were found in the entire *sdhB*, *sdhC*, and *sdhD* sequences for 11 isolates, including the *C. kikuchii*, *C. nicotianae*, *C. sp. G*, and CRC species (11 and 8 SNPs in introns 1 and 2, respectively, for *sdhB*; zero and one SNP in introns 1 and 2, respectively, for *sdhC*; one and zero SNP in introns 1 and 2, respectively, for *sdhD*). No clear association was observed between intron sequences and assigned species. The complete amino acid sequences for SDHB, SDHC, and SDHD proteins for these isolates are shown in Figures S6–S8.

### 3.4.5 | Mancozeb (multisite)

All isolates tested were insensitive to mancozeb at 10  $\mu\text{g/ml}$ , with a mycelial growth inhibition range of 19%–40% for the Argentinian isolates. However, all isolates were completely inhibited (100%) at 100  $\mu\text{g/ml}$  (Table 3).

Lastly, the results were consistent between the first and second round of tests (the first to estimate  $EC_{50}$  values and the second to use discriminatory doses to detect loss of sensitivity or resistance) for all fungicide modes of action and a.i. here evaluated. We did not find any impact of species in sensitivity to different a.i. The mycelial growth inhibition versus concentration of each a.i. are shown in Figures S9–S30.

## 4 | DISCUSSION

To our knowledge, this is the first study in Argentina investigating sensitivity of *Cercospora* species causing CLB/PSS to technical and commercial grade fungicides from different MoA groups. In total, 65

TABLE 4 Phenotype (P) and genotype (G) of each *Cercospora* spp. isolate tested in this study against DMI, MBC, Qol, and SDHI fungicides

No.	Isolate	Species	Host plant	MBC		tub2		Qol		cytb		DMI		cyp51		SDHI		sdhB		sdhC		sdhD	
				P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G
1	ARG_2014_001	<i>Cercospora kikuchii</i>	Glycine max	R	E198A	R	—	R	—	R	—	S	—	R	—	R	—	—	—	—	—	—	—
2	ARG_2016_004	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
3	ARG_2016_005	<i>C. sp. M</i>	G. max	S	— <sup>c</sup>	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
4	ARG_2016_007	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
5	ARG_2016_008	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
6	ARG_2016_015	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
7	ARG_2016_055	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
8	ARG_2016_080	CRC <sup>a</sup>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	—	—	—	—	—	—
9	ARG_2016_102	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
10	ARG_2016_120	CRC	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
11	ARG_2016_133	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
12	ARG_2016_155	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
13	ARG_2016_169	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
14	ARG_2016_199	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
15	ARG_2016_227	CRC	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
16	ARG_2016_239	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
17	ARG_2016_277	<i>Cercospora nicotianae</i>	G. max	R	E198A	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
18	ARG_2016_278	<i>C. kikuchii</i>	G. max	S	—	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
19	ARG_2016_283	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
20	ARG_2016_314	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
21	ARG_2016_324	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
22	ARG_2016_340	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
23	ARG_2016_349	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
24	ARG_2016_355	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
25	ARG_2016_385	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
26	ARG_2016_394	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
27	ARG_2016_395	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
28	ARG_2016_396	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
29	ARG_2016_401	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	—	—	—	—	—	—
30	ARG_2016_402	<i>C. kikuchii</i>	G. max	S	—	R	—	R	—	R	—	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns
31	ARG_2016_417	<i>C. kikuchii</i>	G. max	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	ns	ns	ns	ns	ns	ns

(Continues)

TABLE 4 (Continued)

No.	Isolate	Species	Host plant	MBC		tub2		Qol		cytb		DMI		cyp51		SDHI		sdhB		sdhC		sdhD	
				P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G	P	G
32	ARG_2016_421	<i>C. sp. G</i>	<i>G. max</i>	S	—	R	—	R	—	R	—	S	—	R	—	R	—	R	—	R	—	R	—
33	BOL_2017_026	CRC	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	R	—	R	—	R	—
34	BOL_2017_034	<i>C. nicotianae</i>	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	R	—	R	—	R	—
35	BOL_2017_035	<i>C. kikuchii</i>	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	R	I387M?, D440N?	R	—	R	—	R	—	R	—	R	—
36	ARG_2018_001	<i>C. kikuchii</i>	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	—	R	—	R	—	R	—	R	—	R	—
37	ARG_2019_003	<i>C. sp. H</i>	<i>Amaranthus hybridus</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns <sup>d</sup>	R	—	R	—	R	—	R	—	R	—
38	ARG_2019_006	<i>C. sp. H</i>	<i>A. hybridus</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
39	ARG_2019_011	<i>C. kikuchii</i>	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
40	ARG_2019_012	nd <sup>b</sup>	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
41	ARG_2019_013	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
42	ARG_2019_014	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
43	ARG_2019_015	species indet.	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
44	ARG_2019_016	nd	<i>G. max</i>	R	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
45	ARG_2019_017	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
46	ARG_2019_018	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
47	ARG_2019_019	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
48	ARG_2019_020	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
49	ARG_2019_029	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
50	ARG_2019_030	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
51	ARG_2019_031	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
52	ARG_2019_032	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
53	ARG_2019_033	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
54	ARG_2019_034	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
55	ARG_2019_035	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
56	ARG_2019_036	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
57	ARG_2019_037	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
58	ARG_2019_038	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
59	ARG_2019_039	nd	<i>G. max</i>	S	—	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
60	ARG_2019_040	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
61	ARG_2019_041	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—
62	ARG_2019_042	nd	<i>G. max</i>	R	E198A	R	G143A	R	G143A	R	G143A	S	ns	R	—	R	—	R	—	R	—	R	—

(Continues)



TABLE 4 (Continued)

No.	Isolate	Species	Host plant	MBC		tub2		Qol		cytb		DMI		cyp51		SDHI		sdhB		sdhC		sdhD	
				P	S	G	—	P	R	G	G	P	S	G	G	P	R	G	G	G	G	G	G
63	ARG_2019_043	nd	<i>G. max</i>	S	S	—	—	R	R	G143A	S	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
64	ARG_2019_52	<i>C. kikuchii</i>	<i>Araujia hortorum</i>	S	S	—	—	R	R	G143A	S	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
65	ARG_2019_57	<i>C. sp. O</i>	<i>A. hortorum</i>	S	S	—	—	R	R	G143A	S	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

Abbreviations: DMI, cyproconazole, difenoconazole, epoxiconazole, prothioconazole, tebuconazole; MBC, carbendazim; Qol, azoxystrobin, trifloxystrobin, pyraclostrobin; R, resistant; S, sensitive; SDHI, boscalid, fluxapyroxad, pydiflumetofen.

<sup>a</sup>CRC, *C. richardiicola* complex.

<sup>b</sup>nd, not determined in the present study.

<sup>c</sup>—, no mutation detected.

<sup>d</sup>ns, not sequenced.

*Cercospora* isolates were tested, and were highly resistant to Qol, and half of these isolates were highly resistant to carbendazim. Similarly, *Cercospora* isolates were reported resistant to Qol and MBC fungicides in Louisiana, USA (Price *et al.*, 2015). All isolates tested here were insensitive to SDHI fungicides. In contrast, all tested isolates were sensitive to the DMI fungicides, except for one *C. kikuchii* isolate from Bolivia (BOL\_17\_035).

All Qol molecules tested (azoxystrobin, trifloxystrobin, and pyraclostrobin) were ineffective against all isolates, indicating that these isolates possess cross-resistance to multiple Qol fungicides. It was not possible to test picoxystrobin in this study, but we speculate that it would most likely exhibit a similar response against the *Cercospora* isolates tested here, as cross-resistance was reported in the in vitro tests, and field observations from location Alejo Ledesma, where isolates 49–56 were recovered, showed that plots were heavily infected with CLB/PSS after being treated twice with a picoxystrobin + cyproconazole mixture (300 ml/ha).

In general, Qol-resistance and MBC-resistance in ascomycetes are usually caused by mutations in the *cytb* and *tub2* genes, respectively (Imazaki *et al.*, 2006a). In the present study, we identified the most frequently found mutations that are known to confer resistance to Qols and MBCs in the majority of tested isolates.

In Argentina, the G143A mutation in the *cytb* gene was the only one found in most of the isolates tested that contributed to the field control failures of CLB treated with Qols, and a similar situation in USA and Bolivia has been observed (Price *et al.*, 2015; Sautua *et al.*, 2019a). No other known mutations, such as the F129L and G137R (Sierotzki, 2015), were identified in this gene among the 65 sequenced isolates. The G143A mutation is the most frequent mutation associated with resistance to Qol fungicides in plant pathogens (Sierotzki, 2015). However, although all isolates tested were resistant to all Qols, we only detected the G143A mutation in the *cytb* gene in 82% of these isolates. Thus, it might be possible that factor(s) other than target-site modification are involved in the resistance of *Cercospora* species to Qol fungicides. For example, Vielba-Fernández *et al.* (2018) recently reported on the role of mitochondrial heteroplasmy for the *cytb* gene in *Podosphaera xanthii* as a mechanism explaining resistance to Qols. Their findings suggest that the proportion of G143 and A143 alleles in an isolate may determine the Qol resistance level.

Similarly, in this study the E198A mutation in the *tub2* gene was present in 31 of 32 (97%) isolates resistant to carbendazim. No other mutation was found in the full-length sequence of *tub2*, suggesting that mechanism(s) other than fungicide target-site modification may confer resistance to carbendazim. In Japan, MBC-resistance was attributed to the E198A mutation only, as 154 of 247 (62.3%) isolates of *C. kikuchii* were highly resistant to thiophanate-methyl (MBC) and the 12 resistant isolates that were sequenced possessed the E198A mutation (Imazaki *et al.*, 2006b).

On the other hand, boscalid, fluxapyroxad, and pydiflumetofen were ineffective against all isolates tested here. For instance, at 10 µg/ml boscalid and fluxapyroxad/pydiflumetofen had an average inhibition percentage of 9%, 11%, and 20% in the 62 Argentinian

*Cercospora* isolates, respectively, whereas *Corynespora cassiicola* isolates from cucumber in Japan highly resistant to boscalid had  $EC_{50}$  values from 8.9 to 10.7  $\mu\text{g/ml}$  (Miyamoto *et al.*, 2010). The H278Y and H278R mutations in the *sdhB* subunit were responsible for the resistance (Miyamoto *et al.*, 2010). However, in the present study, no mutations were found in the complete sequence of the *sdhB*, *sdhC*, and *sdhD* genes of the sequenced isolates. Moreover, in Argentina SDHI fungicides have only recently begun to be applied on soybean crops. These fungicides are used with low frequency and always mixed with QoI and/or DMI fungicides. Although the SDHI began to be used in soybean fields in Argentina in 2015, currently, the application of these a.i. represent approximately 3%–4% of the total area planted to soybean or 7%–8% of the total area applied with fungicides. Therefore, the *Cercospora* isolates found insensitive were only exposed in the field to SDHI for a couple of years. It would be unlikely that a resistance mechanism developed after being exposed briefly to these fungicides. Therefore, we hypothesize that *Cercospora* species are insensitive (naturally resistant) to SDHI fungicides *in vitro* because of unknown factors. Our results are in line with those reported by Nikou *et al.* (2009), who tested different fungicides on the *in vitro* mycelial growth of *C. beticola* and found that boscalid had the lowest fungitoxicity, with an average inhibition percentage of 22% (1.7%–39%) at 5  $\mu\text{g/ml}$ . Recently, Ishii *et al.* (2016) reported that isolates of *Colletotrichum gloeosporioides*, *C. acutatum*, *C. cereale*, and *C. orbiculare* tested insensitive to SDHI fungicides (boscalid, fluxapyroxad, and fluopyram). In that study, authors sequenced the *sdhB*, *sdhC*, and *sdhD* genes but did not find mutations associated with SDHI resistance. Consequently, these authors proposed that *Colletotrichum* species are insensitive or naturally resistant to SDHI. These results are similar to those presented here for *Cercospora*. Future studies such as *in vitro* and *in vivo* inhibition tests involving more *Cercospora* isolates and different SDHI a.i. should seek to test this hypothesis. In order to fully confirm the insensitivity of *Cercospora* to SDHI, it is important to establish the relationship between *in vitro* inhibition assays and CLB/PSS control efficacy in field trials. Secondly, to rule out that mechanisms other than point mutations could confer resistance to SDHI, genome-wide association approaches could help identify different mechanisms that could generate resistance to certain fungicides.

The *C. kikuchii* CYP51 protein is coded by a 1,632 bp intron-free gene with homology to other fungal *cyp51* genes and is present as a single copy in the *C. kikuchii* genome (Sautua *et al.*, 2019b). Polymorphisms were detected in the partial sequences of the *cyp51* gene sequenced from the 36 isolates tested here, and none of the SNPs corresponded to any mutation known in *Cercospora* or other genera of phytopathogenic fungi that confers resistance to DMI. In the present study, the lowest average  $EC_{50}$  value corresponded to difenoconazole, which only began to increase in use in recent growing seasons and in mixtures with SDHI or QoI fungicides. In contrast, we have detected higher  $EC_{50}$  values for cyproconazole, the most commonly used DMI fungicide in the history of soybean production in Argentina, Bolivia, and Brazil (Carmona *et al.*, 2017a). This indicates a loss of sensitivity of the *Cercospora* populations in Argentina towards

this particular DMI molecule. Nevertheless, all isolates were sensitive to all DMI molecules tested, except for a single *C. kikuchii* isolate from Bolivia, which was resistant to some DMI molecules. Two new mutations were identified in the *cyp51* gene for this Bolivian isolate, conferring nonsynonymous amino acid substitutions I387M and D440N. Neither of these match the mutations that correspond to previously reported amino acid polymorphisms associated with DMI resistance in *C. beticola* (E169, I330T, P384S, E297K; Nikou *et al.*, 2009). However, because these mutations are solely present in the only DMI-resistant isolate, we speculate that they may be involved in the loss of sensitivity to cyproconazole, tebuconazole, and prothioconazole ( $EC_{50}$  values equal to 45.8, 10, and 6  $\mu\text{g/ml}$ , respectively). Resistance could be attributed to this mutation, but future experiments and analysis should confirm this hypothesis as mechanisms other than single-site mutations in the *cyp51* gene could also be involved in the DMI resistance observed in the Bolivian isolate.

Multisite fungicides have been widely used in horticulture for many years, and during the last 10 years their use has increased significantly in Brazil to control soybean rust (*Phakopsora pachyrhizi*; Carmona *et al.*, 2017a). Even though the risk of developing resistance to mancozeb is considered low (Carmona *et al.*, 2018), the appearance of several tolerant or resistant fungal species on various crops has been reported (Yang *et al.*, 2019). We cannot confirm that the *Cercospora* species evaluated here should be considered resistant or naturally insensitive to mancozeb, because mancozeb has never been used on soybean for the control of any disease in Argentina.

Our findings suggest that QoI-resistance is widespread in Argentinian *Cercospora* populations causing CLB/PSS, as all of the populations tested appear to be composed of strobilurin-resistant individuals and the G143A mutation is present in the majority of the isolates collected from soybean and from alternative hosts such as *A. hybridus* and *A. hortorum*. Likewise, half of the tested isolates were highly resistant to carbendazim, suggesting that resistance to this fungicide is spreading in *Cercospora* populations as well. Moreover, this confirms that isolates of the Argentinian population of *Cercospora* are harbouring multiple amino acid substitutions that confer resistance to fungicides with different modes of action, which may have implications when mixes of fungicide a.i. are applied. Secondly, SDHI fungicides are a special case for which we propose that the *Cercospora* species evaluated here are insensitive (naturally resistant) *in vitro*. Similarly, mancozeb, an example of a multisite fungicide, only exhibited complete inhibition of mycelial growth at 100  $\mu\text{g/ml}$ . Consequently, it was only possible to establish the *in vitro* baseline sensitivity of *Cercospora* isolates causing CLB/PSS to five DMIs and carbendazim. This is the first fungicide sensitivity study to be carried out for *Cercospora* species causing CLB/PSS of soybean in Argentina. Therefore, we tested several isolates and technical- and commercial-grade fungicides. The  $EC_{50}$  values estimated in this work represent the baseline fungicide sensitivity to DMI and carbendazim, as there are no isolates available from previous years. The results presented herein will be useful to guide detection and evaluation of sensitivity shifts in *Cercospora* populations towards these fungicides during subsequent years. Finally, further

studies are needed to (a) fully elucidate the basis of different molecular mechanisms that may be involved in the resistance to QoI, MBC, and DMI fungicides; (b) confirm insensitivity or natural resistance to SDHI; and (c) confirm the lack of fungitoxicity of mancozeb in *Cercospora*.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study (all alignments and phylogenetic trees) are openly available in Github at <https://github.com/vinsondoyle/SautuaPMS2020>. Accession numbers of the 438 nucleotide sequences for *Cercospora* spp. isolates generated in this study are available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/> with accession numbers shown in Table S2.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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