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Disease Control and Integrated Management

e-Xtra*

Lack of an Intron in Cytochrome *b* and Overexpression of Sterol 14α-Demethylase Indicate a Potential Risk for QoI and DMI Resistance Development in *Neophysopella* spp. on Grapes

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ABSTRACT

Asian grapevine leaf rust, caused by *Neophysopella meliosmae-myrianthae* and *N. tropicalis*, is often controlled by quinone outside inhibitor (QoI) and demethylation inhibitor (DMI) fungicides in Brazil. Here, we evaluated the sensitivity of 55 *Neophysopella* spp. isolates to pyraclostrobin (QoI) and tebuconazole (DMI). To elucidate the resistance mechanisms, we analyzed the sequences of the cytochrome *b* (CYTB) and cytochrome P450 sterol 14α-demethylase (CYP51) target proteins of QoI and DMI fungicides, respectively. The *CYP51* expression levels were also determined in a selection of isolates. In leaf disc assays, the mean 50% effective concentration (EC_{50}) value for pyraclostrobin was about 0.040 μg/ml for both species. *CYTB* sequences were identical among all 55 isolates, which did not contain an intron immediately after codon 143. No amino acid substitution was identified at codons 129, 137, and 143. The mean EC_{50} value for tebuconazole was 0.62 μg/ml for *N. tropicalis* and 0.46 μg/ml for *N. meliosmae-myrianthae*, and no *CYP51* sequence variation was identified among isolates of the same species. However, five *N. meliosmae-myrianthae* isolates grew on leaf discs treated at 10 μg/ml tebuconazole, and these were further exposed to tebuconazole selection pressure. Tebuconazole-adapted laboratory isolates of *N. meliosmae-myrianthae* showed an eight- to 25-fold increase in resistance after four rounds of selection that was not associated with CYP51 target alterations. In comparison with sensitive isolates, *CYP51* expression was induced in the presence of tebuconazole in three out of four tebuconazole-adapted isolates tested. These results suggest a potential risk for QoI and DMI resistance development in *Neophysopella* spp.

Keywords: antimicrobial or fungicide resistance, Asian grapevine leaf rust, *CYP51* overexpression, ecology and epidemiology, fungicide resistance, intron-less cytochrome *b*, molecular, risk assessment

Asian grapevine leaf rust (AGLR) was reported for the first time in Brazil in 2001 and has become widespread in the main grapevine-growing areas of the country (Primiano et al. 2017; Tessmann et al. 2004). AGLR symptoms occur predominantly on mature leaves, causing reduction in photosynthetic efficiency, leaf necrosis, and premature defoliation, leading to a reduction in carbohydrate accumulation in roots (Nogueira Júnior et al. 2017). These alterations in energy metabolism result in fruit yield and quality losses and reduced vine vigor in the subsequent seasons (Edwards 2015; Nogueira Júnior et al. 2017; Scapin-Buffara et al. 2018). In Brazil, AGLR is caused by two biotrophic basidiomycetes, *N. tropicalis* in the Central-South region and *N. meliosmae-myrianthae* in the Northeast region (Santos et al. 2021), although five *Neophysopella* species have been associated with grapevine leaf rust in other countries (Okane and Ono 2018; Pota et al. 2015).

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*The *e-Xtra* logo stands for "electronic extra" and indicates there are supplementary materials published online.

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The invasion and further spread of the AGLR pathogens have recently led to an increase in the number of late-season fungicide applications in Brazilian viticulture, because most commercial cultivars are susceptible to the disease (Angelotti et al. 2008; Primiano et al. 2017). Fungicides with active ingredients belonging to the quinone outside inhibitor (QoI) and sterol demethylation inhibitor (DMI) groups have provided effective control of AGLR (Angelotti et al. 2014; Naruzawa et al. 2006), which are currently considered a major tool for the disease management. However, only tebuconazole and tetraconazole (both DMIs) and a mixture of pyraclostrobin (QoI) + metiram (dithiocarbamate) are registered and recommended for AGLR control by the Brazilian Ministry of Agriculture, Livestock and Food Supply (AGROFIT 2020). Because of the low diversity of active ingredients registered for AGLR control and because QoIs and DMIs are single-site acting fungicides, resistance to both groups may rapidly evolve, as has been reported for many other plant pathogens (Mair et al. 2016b).

The QoI fungicides inhibit mitochondrial respiration by binding to the Qo site of the cytochrome bc_1 complex, thereby blocking electron transfer and inhibiting ATP synthesis (Bartlett et al. 2002; Gisi et al. 2002). Three amino acid substitutions from phenylalanine to leucine at codon 129 (F129L), glycine to arginine at codon 137 (G137R), and glycine to alanine at codon 143 (G143A) in the cytochrome *b* (*CYTB*) gene have been associated with QoI resistance in plant-pathogenic fungi and oomycetes (Fisher and Meunier 2008; Ma and Michailides 2005). The G143A mutation is associated with high levels of QoI resistance, and F129L and G137R are associated with moderate resistance (Fernández-Ortuño et al. 2008). QoI control failures have been commonly reported in grapevine powdery and downy mildew populations carrying the G143A mutation (Miles et al. 2021; Santos et al. 2020). In rusts, only the F129L mutation has been reported in

Phakopsora pachyrhizi (Klosowski et al. 2016). The G143A mutation has not been reported in rust species belonging to the genera *Hemileia*, *Phakopsora*, *Puccinia*, and *Uromyces* because they harbor a type I intron immediately after codon 143, and a nucleotide substitution at this codon would prevent splicing of the intron, being lethal for the pathogen (Grasso et al. 2006a, b; Oliver 2014). Therefore, the knowledge about the presence or absence of the intron after codon 143 is a crucial information to assess the risk of G143A development in *Neophysopella* spp.

The DMI fungicides affect ergosterol biosynthesis and membrane function in fungi by inhibiting the cytochrome P450 sterol 14α-demethylase (*CYP51*) gene (Lepesheva and Waterman 2007). The increase in genome sequencing data has revealed that some ascomycetes possess up to three *CYP51* paralogs (*CYP51A*, *CYP51B*, and *CYP51C*); however, basidiomycetes have been found to possess a single *CYP51* ortholog (Hawkins and Fraaije 2018; Ziogas and Malandrakis 2015). Resistance against DMIs is conferred by three main mechanisms including nonsynonymous mutations in the *CYP51* gene, increased activity of efflux pumps, and *CYP51* overexpression (Cools and Fraaije 2013; Oliver 2014). In most cases, overexpression is caused by a *CYP51* promoter insert; however, increased number of *CYP51* copies leads to higher gene expression in *Erysiphe necator* (Jones et al. 2014). Despite the economic importance of AGLR and frequent use of QoIs and DMIs in Brazil, there are no fungicide resistance studies in *Neophysopella* spp. Therefore, the objectives of this study were to assess the sensitivity of AGLR pathogens to pyraclostrobin and tebuconazole, characterize nucleotide sequence and intron/exon structure of the *CYTB* and *CYP51* genes, and investigate possible mechanisms of resistance.

MATERIALS AND METHODS

Fungal isolates and fungicide sensitivity testing. *Isolates.* A total of 54 single-uredinium isolates collected from 22 vineyards in six Brazilian states in 2018, soon after harvest, were investigated in this study (Table 1). The *N. tropicalis* AGLR9 isolate collected in 2013, routinely used for AGLR experiments in our laboratory at University of São Paulo, was used as reference isolate. A previous phylogenetic study based on ITS2-D1/D2 sequences revealed that the isolates belong to the species *N. tropicalis* (49 isolates) and *N. meliosmae-myrianthae* (six isolates) (Santos et al. 2021). Isolates were multiplied on detached 'Niagara Rosada' leaves to produce inoculum for the experiments as described previously (Santos et al. 2021).

Fungicides. Pyraclostrobin (Comet, 250 g/liter active ingredient; BASF) was tested at 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml and tebuconazole (Folicur 200 EC, 200 g/liter active ingredient; Bayer) at 0, 0.01, 0.1, 1, and 10 µg/ml. The fungicides were diluted in sterile distilled water immediately before use.

Leaf disc assay. Sensitivity of all 55 isolates to pyraclostrobin and tebuconazole was assessed on leaf discs of 'Niagara Rosada'. Fully expanded healthy leaves were taken from greenhouse-grown grapevines (approximately 1 month after bud break), surface disinfested in 1% sodium hypochlorite solution for 1 min, and rinsed three times with sterile distilled water. After drying, leaves were soaked in the fungicide solution for 1 min and air dried in a laminar flow hood, and then leaf discs were excised with a 24-mm cork borer. Leaf discs were placed with abaxial surface upward on 1% water agar (WA; Difco Laboratories, Franklin Lakes, NJ) amended with streptomycin sulfate (0.1 g/liter) in Petri dishes (90 mm in diameter) and kept at 25°C with a 12-h photoperiod for 24 h before inoculation. Then, urediniospores from 12-day-old cultures were scraped with a sterile brush and suspended in sterile distilled water containing 0.01% Tween 20 (vol/vol), and the suspension was adjusted to 5×10^4 urediniospores/ml. A total of 100 µl of the suspension was sprayed on the abaxial surface of each leaf disc. After the inoculation, Petri dishes were incubated in the dark at 25°C for 24 h and then opened in a laminar flow hood for 2 h to allow any excess moisture to evaporate. Subsequently, plates were incubated for 9 days at 25°C with a 12-h photoperiod. Ten days after inoculation, disease severity on each leaf disc was estimated as the percentage of leaf area covered by rust pustules with a standard area diagram, with a range of disease severity from 0.5 to 48.5% (Supplementary Fig. S1). The standard area diagram was constructed from leaf disc images processed by Quant software (Vale et al. 2001). For each isolate–fungicide concentration combination, 10 leaf discs (five per dish) were used, and the experiment was performed twice.

Urediniospore germination assay. The effect of pyraclostrobin on urediniospore germination was evaluated in 17 *N. tropicalis* and two *N. meliosmae-myrianthae* isolates. Three 30 µl-aliquots of a 1×10^4 urediniospores/ml suspension were deposited on 1.5% WA plates amended or not with pyraclostrobin at the concentrations described previously. After incubation at 25°C in the dark for 24 h, we interrupted urediniospore germination by adding 30 µl of lactoglycerol onto each droplet. We determined the germination percentage by counting 100 urediniospores per droplet under a light microscope, and the mean of three droplets was considered a replicate. A urediniospore was considered germinated when the germ tube was at least twice the diameter of the urediniospore. Three plates were used for each isolate–fungicide concentration combination, and the experiment was performed twice.

Data analysis. For each isolate, the percentage inhibition was calculated relative to the disease severity or urediniospore germination of the control. The effective concentration to inhibit disease severity and urediniospore germination by 50% (EC_{50}) was calculated by linear regression between the disease severity or urediniospore germination inhibition percentage, respectively, on the $\log_{10}$-transformed fungicide concentration in Microsoft Excel software. Data from the two repeated experiments were combined after *F* test indicated homogeneous variances.

DNA and RNA extraction and complementary DNA synthesis. Urediniospores (20 to 30 mg) from each isolate were ground in liquid nitrogen, and the resulting powder was used for DNA extraction via a modified cetyl trimethyl-ammonium bromide extraction procedure (Lo Piccolo et al. 2012). Total RNA from frozen urediniospores (50 mg) was extracted with the PureLink RNA Mini Kit (Invitrogen, Waltham, MA) and subsequently treated with deoxyribonuclease (AMPD1-1KT; Sigma Aldrich, St. Louis, MO) for possible DNA contamination in accordance with the manufacturer's protocols. A quantity of 800 ng of RNA from each sample was reverse transcribed to complementary DNA (cDNA) in the GoScript Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. The reverse transcription conditions were as follows: 5 min at 25°C, 60 min at 42°C, and 15 min at 70°C.

TABLE 1. Origin of *Neophysopella* spp. isolates associated with Asian grapevine leaf rust (AGLR) in Brazil used in this study

Species	State	Number of vineyards sampled	Number of isolates	AGLR isolates
Neophysopella meliosmae-myrianthae	Pernambuco	2	6	54–57, 59, 60
Neophysopella tropicalis	Mato Grosso do Sul	1	2	31, 32
	Minas Gerais	4	10	7, 8, 16–21, 48, 49
	Paraná	1	3	51–53
	Santa Catarina	1	3	36–38
	São Paulo	13	31	1–6, 9–15, 22–30, 33–35, 39, 40, 42, 43, 45, 47

Characterization and analysis of the *CYTB* and *CYP51* genes. All primers used in this study were designed based on transcriptome sequences of *N. tropicalis* (provided by Dr. Marco Loehrer and Dr. Ulrich Schaffrath, RWTH Aachen University, Germany) and sequences of *P. pachyrhizi* available in GenBank (Hirschburger et al. 2015; Maciel et al. 2010; Schmitz et al. 2014). The *CYTB* gene of all 55 isolates was partially amplified with the primers NeoCYTB-F and NeoCYTB-R (Table 2). PCRs for amplification of *CYTB* contained 0.5 µM of each primer, 200 µM of each dNTP, 1× of Easy-A reaction buffer, 1.25 U of Easy-A High Fidelity PCR Cloning Enzyme (Agilent Technologies, Santa Clara, CA) and 60 ng of template DNA at a final volume of 25 µl. The amplification program consisted of an initial denaturation at 94°C for 1 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 8 min. Complete *CYP51* gene was amplified from 19 *N. tropicalis* isolates and six *N. meliosmae-myrianthae* isolates with the primers NeoCYP51-F1 and NeoCYP51-R3 (Table 2). For *CYP51* amplification, PCRs were carried out in a volume of 50 µl with the same reagent concentrations as described previously, except Easy-A High Fidelity PCR Cloning Enzyme was 2.5 U and DNA template was 120 ng. Amplification conditions were 94°C for 1 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 8 min. PCRs were carried out in a SureCycler 8800 thermal cycler (Agilent Technologies). The amplified products were purified and sequenced by MWG Eurofins Genomics (Germany), with the external and internal primers listed in Table 2. Sequences were assembled and aligned in Geneious version 10.0.2 (Kearse et al. 2012). Nucleotide and predicted amino acid sequences were compared with those available in the National Center for Biotechnology Information database via BLASTN and BLASTP algorithms, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments of cDNA and genomic DNA sequences revealed the intron/exon structure of the genes.

Phylogenetic analyses were conducted to examine the genetic relationship between CYTB and CYP51 predicted amino acid sequences from *Neophysopella* spp. and other important plant pathogens deposited in GenBank. *Saccharomyces cerevisiae* was used as the outgroup. Sequences from each gene were aligned separately via the Clustal W algorithm implemented in MEGA X (Kumar et al. 2018). Maximum likelihood phylogenetic trees were carried out in MEGA X with LG + G used as the amino acid substitution model for both CYTB and CYP51 datasets based on the Bayesian information criterion. Nearest neighbor interchange was used as the heuristic method for phylogenetic inference, and support for the nodes was estimated with 1,000 bootstrap replicates.

Selection for reduced sensitivity to tebuconazole. Among all isolates of both species analyzed in the leaf disc assay, five out of six *N. meliosmae-myrianthae* isolates were not completely inhibited by tebuconazole at 10 µg/ml. These five isolates (AGLR54, AGLR55, AGLR56, AGLR57, and AGLR60) were subjected to DMI fungicide selection pressure on 'Niagara Rosada' leaves treated with tebuconazole at 10 µg/ml. Leaves were surface disinfested, soaked in the fungicide solution, and placed on 1% WA with streptomycin sulfate (0.1 g/liter) in 150-mm Petri dishes. Each leaf was inoculated by spraying 800 µl of a suspension (5×10^4 urediniospores/ml) on the abaxial surface. Plates were incubated in the dark for 24 h, opened for 2 h to allow any remaining excessive moisture to evaporate, followed by a 12-h photoperiod at 25°C. Twelve days after inoculation, urediniospores were collected and used for the subsequent inoculation on tebuconazole-treated leaves. This procedure was repeated for a total of four serial passages. After four rounds of selection, the EC_{50} was determined for each isolate via a leaf disc assay of eight tebuconazole concentrations (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/ml). Moreover, genomic DNA was extracted from urediniospores, and the *CYP51* gene was amplified with the primer pair NeoCYP51-F1/NeoCYP51-R3 and sequenced to check for the presence of target alterations after four subcultures. The AGLR54* was not used in the subsequent analysis because of low urediniospore production.

Expression analysis of the *CYP51* gene. Five sensitive (*N. tropicalis*: AGLR9, AGLR18, AGLR21, and AGLR53; *N. meliosmae-myrianthae*: AGLR59) and four tebuconazole-adapted laboratory isolates (*N. meliosmae-myrianthae*: AGLR55*, AGLR56*, AGLR57*, and AGLR60*) obtained after four rounds of selection were used for *CYP51* expression analysis. Potted 'Niagara Rosada' grapevines with five to seven fully expanded leaves were treated with tebuconazole at 3 µg/ml (Schmitz et al. 2014) or sterile distilled water (control) and placed in a growth room at 25°C overnight. Then, leaves were harvested, placed on 1% WA in Petri dishes, inoculated, and incubated as described previously. After 12 days, urediniospores were harvested and freeze-dried, followed by RNA extraction and cDNA synthesis. For each isolate, three replicate leaves were used per treatment.

The *CYP51* expression level was quantified by real-time PCR with the primer pair qNeoCYP51-F/qNeoCYP51-R and normalized to the expression of *actin* and β-*tubulin* amplified with qNeoACT-F/qNeoACT-R and qNeoTUB-F/qNeoTUB-R, respectively (Table 2). Reactions were carried out in a 20-µl volume containing 10 µl of SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich), 0.2 µl of ROX reference dye (Sigma Aldrich), 0.4 µM of each primer, and 2 µl of cDNA (1:10 dilution) on the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA). All samples were analyzed in triplicate with the following program: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s. The relative expression of *CYP51* was calculated via the $2^{-\Delta\Delta C\tau}$ method (Livak and Schmittgen 2001), with *actin* and β-*tubulin* as endogenous controls and AGLR9 as the calibrator sample. These two genes have been widely used as reference genes to normalize *CYP51* expression data (Fan et al. 2013; Schmitz et al. 2014). The data were log-transformed for analysis of variance and means

TABLE 2. Nucleotide sequence and characteristics of primers used in this study

Primer[a]	Sequence 5′–3′	Application	Amplicon size (bp)
NeoCYTB-F	CTAGGATGTTGTCTAATCATTC	Amplification and sequencing of *CYTB*	692
NeoCYTB-R	GGTGTCTGTATYGGATTCGC	Amplification and sequencing of *CYTB*	
NeoCYP51-F1	CATGTCTTCGTCCTCAGAGT	Amplification and sequencing of *CYP51*	2,059
NeoCYP51-R3	GCATCCAGCATCTATTTCCG	Amplification and sequencing of *CYP51*	
NeoCYP51-R1	TGTGATGTTCCATRTGCTCA	Sequencing of *CYP51*	
NeoCYP51-F2	AGAACTTCAAGAGGTATGTG	Sequencing of *CYP51*	
NeoCYP51-R2	TTCTTCAACTAYGTCAGGGC	Sequencing of *CYP51*	
NeoCYP51-F3	TGATGGCTGGTCAACATACTA	Sequencing of *CYP51*	
qNeoCYP51-F	TGGCTCTTATGTCACCTTGCC	Expression analysis of *CYP51*	170
qNeoCYP51-R	GGATGAAGCCTCAAAACCTCCT	Expression analysis of *CYP51*	
qNeoACT-F	GACTGCCGAGCGTGAAATTGT	Expression analysis of *actin*	179
qNeoACT-R	ACAAAGCCTCAGGACAACGGA	Expression analysis of *actin*	
qNeoTUB-F	CGAATGATGGCTACCTTCTCGG	Expression analysis of β-*tubulin*	160
qNeoTUB-R	GGGTACGGAAACAGATGTCGT	Expression analysis of β-*tubulin*	

[a] NeoCYP51-R1, F2, R2, and F3 are internal primers, and all others are external primers.

compared by Scott–Knott test at $P = 0.05$. The correlation between *CPY51* expression and tebuconazole EC_{50} values was evaluated via Pearson's correlation coefficient (r). Statistical analyses were performed in R version 4.0.2 (R Core Team, 2020).

RESULTS

Sensitivity of *Neophysopella* spp. to pyraclostrobin and tebuconazole. All *N. tropicalis* isolates were sensitive to pyraclostrobin. The reference isolate AGLR9 showed EC_{50} values of 0.012 and 0.031 µg/ml based on the leaf disc and urediniospore germination inhibition assays, respectively. EC_{50} values of all isolates ranged from 0.006 to 0.086 µg/ml, with a mean ± SD of 0.040 ± 0.023 µg/ml in the leaf disc assay (Supplementary Table S1). The majority of the isolates (67%) had EC_{50} values <0.050 µg/ml (Fig. 1A). The EC_{50} value range was similar for *N. meliosmae-myrianthae*, between 0.018 and 0.083 µg/ml, with a mean EC_{50} value of 0.043 ± 0.025 µg/ml. In urediniospore germination assays, EC_{50} values of *N. tropicalis* isolates varied from 0.010 to 0.064 µg/ml, and the average value was 0.029 ± 0.019 µg/ml, whereas the mean EC_{50} value of the two *N. meliosmae-myrianthae* isolates was 0.043 ± 0.011 µg/ml (Fig. 1B). All isolates did not germinate and grow at 1 µg/ml of pyraclostrobin in both assays.

The leaf disc EC_{50} values for tebuconazole of *N. tropicalis* and *N. meliosmae-myrianthae* isolates ranged from 0.15 to 1.26 and 0.32 to 0.73 µg/ml, with mean EC_{50} values of 0.62 ± 0.29 and 0.47 ± 0.18 µg/ml, respectively. Most isolates (73%), regardless of the *Neophysopella* species, had EC_{50} values ranging from 0.11 to 0.70 µg/ml (Fig. 2). The reference *N. tropicalis* isolate AGLR9 showed an EC_{50} value of 0.28 µg/ml. All *N. tropicalis* isolates did not grow at 10 µg/ml tebuconazole, and five out of six isolates of *N. meliosmae-myrianthae* grew slightly.

Characterization and analysis of the *CYTB* and *CYP51* genes. Nucleotide sequencing of the *CYTB* gene yielded sequences of 663 bp, spanning all positions that have been associated with QoI resistance in other fungi (Mair et al. 2016b). *CYTB* sequences were identical for all 55 isolates, regardless of species, with wild-type amino acids at codons 129 (TTT, phenylalanine), 137 (GGT, glycine), and 143 (GGT, glycine). A representative sequence of each species was deposited in GenBank under the accession numbers MW024822 and MW024823. The alignment of cDNA and genomic DNA sequences indicated the absence of an intron located directly downstream of codon 143 (Fig. 3A). The BLASTN analysis revealed that both *Neophysopella* spp. share 87 and 89% messenger RNA sequence identity with *P. pachyrhizi* (GQ332420) and *Puccinia triticina* (DQ009925), respectively. Meanwhile, a BLASTP analysis indicated that the deduced amino acid sequences shared 94% identity with *P. pachyrhizi* (YP003795382). The phylogenetic analysis confirmed the similarity of predicted CYTB amino acid sequences between *Neophysopella* spp. and other basidiomycetes; however, they were clustered separately from the others (Supplementary Fig. S2).

A 2,047-bp fragment of the *CYP51* gene was amplified and sequenced for 25 isolates selected based on differences in tebuconazole sensitivity (Fig. 3B). No nucleotide sequence variation was detected among isolates within the same species regardless of the tebuconazole sensitivity, and 122 polymorphic sites were observed between species (GenBank accession numbers MW024820 and MW024821). Therefore, no nucleotide substitution was linked to differences in tebuconazole sensitivity. The coding region was interrupted by five introns from 142 to 219, 464 to 542, 708 to 823, 1,435 to 1,498, and 1,895 to 1,990 bp in both species. A total of 16 amino acid changes were observed between *N. meliosmae-myrianthae* and *N. tropicalis* across the 537-amino-acid protein

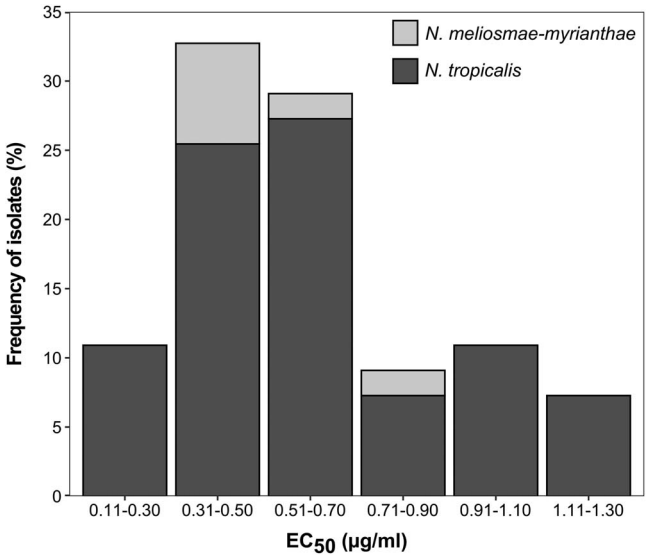

Fig. 2. Frequency distribution of *Neophysopella meliosmae-myrianthae* and *N. tropicalis* isolates according to tebuconazole mean 50% effective concentration (EC_{50}) intervals from leaf disc assays.

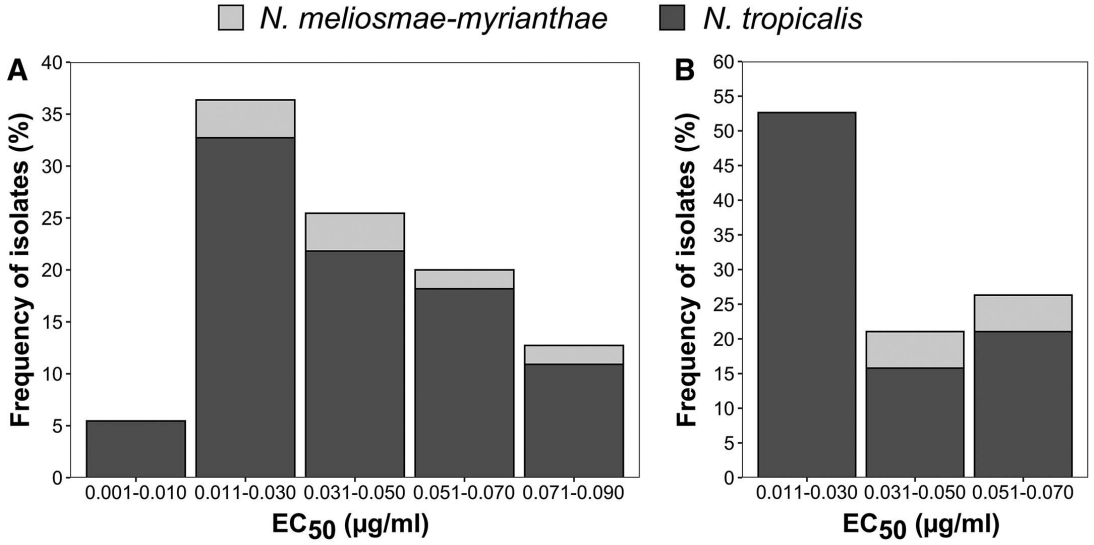

Fig. 1. Frequency distribution of *Neophysopella meliosmae-myrianthae* and *N. tropicalis* isolates according to pyraclostrobin mean 50% effective concentration (EC_{50}) intervals based on **A,** leaf disc assay and **B,** urediniospore germination.

encoded. Both protein sequences showed approximately 70% sequence homology with related protein sequences from *P. pachyrhizi* (AGJ89733) and *P. triticina* (ACS37521). The gene phylogeny revealed that *Neophysopella* spp. were clustered in a well-supported clade with other basidiomycetes, which diverged from the three *CYP51* paralogs (*CYP51A*, *CYP51B*, and *CYP51C*) present in ascomycetes (Fig. 4).

Selection for reduced sensitivity to tebuconazole. After four rounds of tebuconazole selection of five *N. meliosmae-myrianthae* isolates, the EC_{50} values for tebuconazole ranged from 5.19 to 8.83 µg/ml in the leaf disc, representing an eight- to 25-fold increase in resistance compared with the values obtained before the selection (Supplementary Fig. S3, Supplementary Table S2). All five isolates did not grow at 50 µg/ml. *CYP51* sequencing of

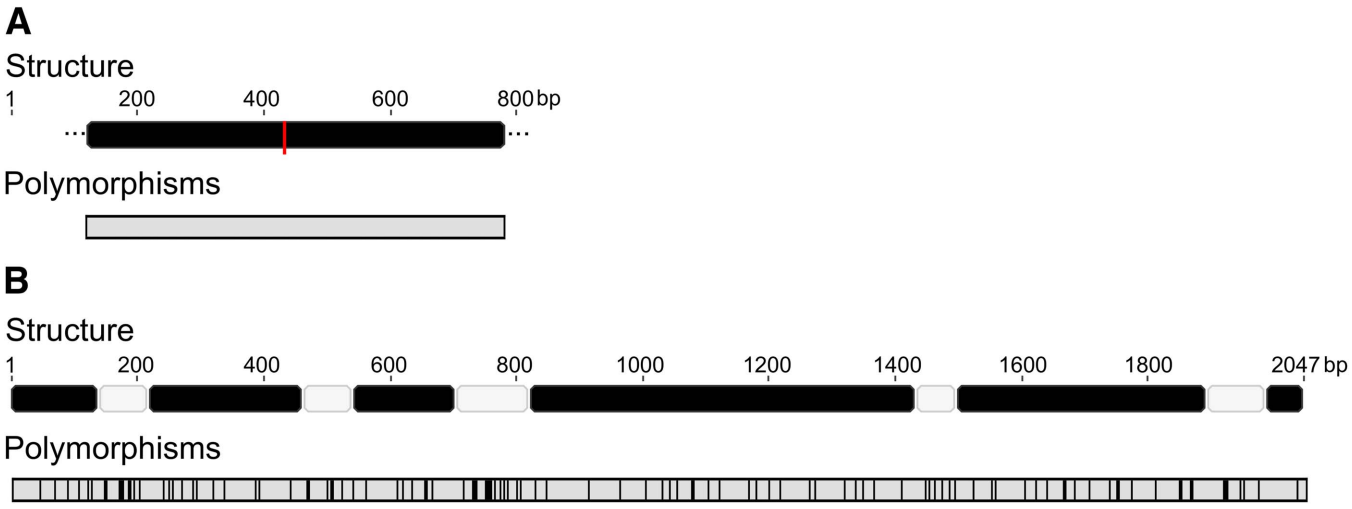

Fig. 3. Schematic structure of the **A,** *CYTB* and **B,** *CYP51* genes and polymorphisms between *Neophysopella meliosmae-myrianthae* and *N. tropicalis*. Exons and introns are represented by black and white boxes, respectively. Polymorphisms are represented by vertical bars. Red or gray line in **A** indicates the position of codon 143.

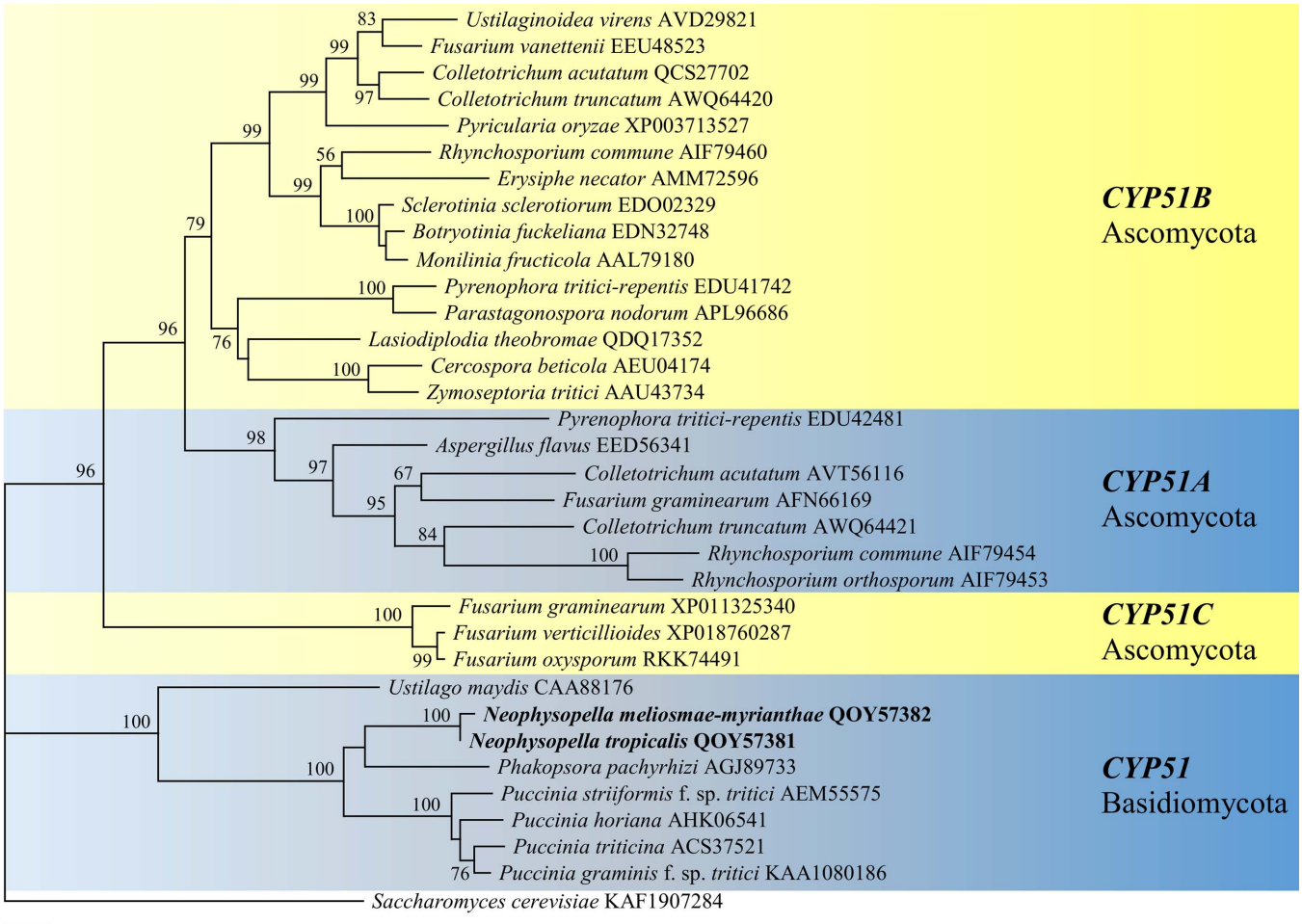

Fig. 4. Maximum likelihood phylogenetic tree based on amino acid sequences of fungal *CYP51* genes. The numbers at the nodes indicate bootstrap support inferred from 1,000 bootstrap replicates; only bootstrap values >50% are shown. *Neophysopella meliosmae-myrianthae* and *N. tropicalis* are highlighted in bold.

these isolates did not identify any nucleotide differences from the sequences obtained before the tebuconazole selection pressure, indicating that target-site alterations are not associated with the shift in tebuconazole sensitivity.

Expression analysis of the *CYP51* gene. *CYP51* gene expression was evaluated in five tebuconazole-sensitive isolates of *N. tropicalis* (n = 4) and *N. meliosmae-myrianthae* (n = 1) (EC_{50} values from 0.16 to 0.32 µg/ml) and four tebuconazole-adapted isolates of *N. meliosmae-myrianthae* (EC_{50} values from 5.19 to 8.83 µg/ml) (Fig. 5). All sensitive *N. tropicalis* isolates were not able to grow on leaves treated with 3 µg/ml of tebuconazole, and a few uredinia showing low sporulation were produced by the sensitive *N. meliosmae-myrianthae* AGLR59, making it difficult to collect enough material for RNA extraction. The *CYP51* expression of AGLR59 was approximately four to eight times higher than that of the sensitive isolates of the other species (Fig. 5). Tebuconazole-adapted isolates showed significantly higher constitutive *CYP51* expression (mean = 17.03) than AGLR59 (mean = 8.46) and the remaining *N. tropicalis* sensitive isolates (mean = 1.61). *CYP51* expression was positively correlated with tebuconazole EC_{50} values (r = 0.93; P = 0.0003). In the presence of tebuconazole, three out of four adapted isolates overexpressed *CYP51* when compared with the untreated control, with a level up to two times higher for AGLR60*.

DISCUSSION

AGLR incidence and severity have increased considerably in recent years in Brazilian viticulture, usually requiring up to four preventive fungicide applications after fruit set (Scapin-Buffara et al. 2018), based almost exclusively on pyraclostrobin and tebuconazole. In the present study, we investigated for the first time the QoI and DMI sensitivity and possible mechanisms of resistance in *N. meliosmae-myrianthae* and *N. tropicalis* by using both phenotypic and molecular approaches. QoI fungicides disrupt mitochondrial respiration and therefore are highly effective in inhibiting spore germination and host penetration, which require large amounts of energy but also have activity against mycelial growth and sporulation (Fernández-Ortuño et al. 2008). Our results demonstrated that all *Neophysopella* spp. isolates tested were highly sensitive to pyraclostrobin, as shown by the low EC_{50} values obtained in the leaf disc (overall mean = 0.040 µg/ml) and urediniospore germination assays (overall mean = 0.030 µg/ml) (Fig. 1). Similarly, high control efficacy of QoIs has also been reported for *P. pachyrhizi* and *Puccinia* spp. (Arduim et al. 2012; Emmitt et al. 2018; Twizeyimana and Hartman 2017).

Since the launch of QoIs into the market in 1996, several ascomycetes and oomycetes have developed resistance to this fungicide group that is, in most cases, associated with the CYTB G143A target alteration (Ishii and Hollomon 2015; Lucas et al. 2015). Although QoIs have been extensively used in the management of grapevine diseases including AGLR, anthracnose, downy mildew, and powdery mildew, there are a limited number of studies on QoI fungicide resistance in Brazil (Santos et al. 2020). In our study, no nucleotide sequence variation in the *CYTB* gene was found among the 55 *Neophysopella* isolates that carried wild-type alleles at codons 129, 137, and 143. In rusts, loss of sensitivity to QoIs has been reported only in *P. pachyrhizi* and *Puccinia horiana*. The F129L mutation was detected in *P. pachyrhizi* in the 2012 to 2013 season after many years of QoI use in soybean in Brazil (Klosowski et al. 2016). Later on, azoxystrobin sensitivity assays revealed that F129L mutant isolates displayed higher EC_{50} values, ranging from 2.10 to >10 µg/ml, compared with EC_{50} values of wild-type isolates that ranged from 0.14 to 1.46 µg/ml (Klosowski et al. 2018). Resistance of *P. horiana* to azoxystrobin has been reported in the United Kingdom (Cook 2001); however, no variation was found between *CYTB* sequences from sensitive and resistant isolates, suggesting that other resistance mechanisms may be involved (Grasso et al. 2006b).

A well-known explanation for the absence of the G143A mutation in rust fungi is the presence of an intron immediately after codon 143 (Grasso et al. 2006a, b). Consequently, a nucleotide change at this codon would affect the intron splicing process and therefore lead to a nonfunctional protein (Grasso et al. 2006a; Vallières et al. 2011).

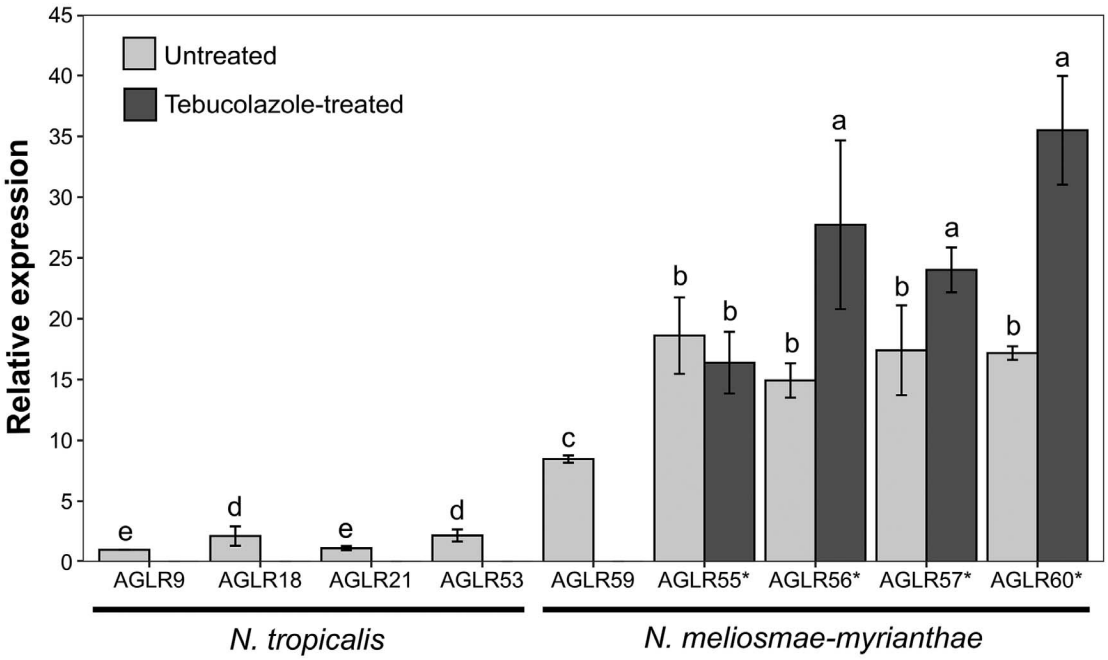

Fig. 5. Relative expression of the *CYP51* gene in *Neophysopella meliosmae-myrianthae* and *N. tropicalis* isolates inoculated on grapevine leaves treated with water or tebuconazole at 3 µg/ml. Sensitive isolates are represented by AGLR9, AGLR18, AGLR21, AGLR53, and AGLR59, and tebuconazole-adapted isolates are represented by AGLR55*, AGLR56*, AGLR57*, and AGLR60*. The expression of *CYP51* was analyzed with *actin* and β-*tubulin* as references genes and AGLR9 as the calibrator sample. Error bars indicate standard error of three biological replicates. Bars with the same letter do not differ significantly according to the Scott–Knott test (P = 0.05).

However, intron-free isolates of rust species may exist in nature (Oliver 2014), because few species have been studied in detail. In this study, no intron was detected between codons 143 and 144 in the *CYTB* gene, suggesting that resistance based on the G143A mutation may occur in both *N. meliosmae-myrianthae* and *N. tropicalis* because that mutation would not be lethal.

As far as we know, this is the first report describing the lack of an intron after codon 143 in a rust fungus. An evolutionary study assessing 465 fungal *CYTB* genes revealed that some introns are present at the same position in distinct species but absent in closely related species (Yin et al. 2012). In ascomycetes, heterogeneity in intron presence or absence between codons 143 and 144 has been reported between *Monilinia* species and even between isolates of the same species, such as *Botrytis cinerea* (Miessner and Stammler 2010; Samuel et al. 2011). The frequent intron loss–gain events in fungal *CYTB* genes described by Yin et al. (2012) may explain the different *CYTB* structure of *Neophysopella* spp. compared with other rust species. Moreover, a recent phylogenetic analysis based on the large subunit region demonstrated that *Neophysopella* belongs to a phylogenetic group distinct from *Phakopsora*, *Puccinia*, and *Uromyces* known to possess the intron after codon 143, indicating that *Neophysopella* is a distinct lineage (Ji et al. 2019).

Analysis of the *CYP51* gene showed that *N. meliosmae-myrianthae* and *N. tropicalis* share 94% nucleotide identity. However, no nucleotide variation was identified among *N. tropicalis* isolates, with EC_{50} values for tebuconazole ranging from 0.15 to 1.26 µg/ml (Fig. 2). Likewise, *N. meliosmae-myrianthae* isolates showed identical *CYP51* sequences among them and similar tebuconazole EC_{50} values (mean EC_{50} = 0.47 µg/ml), even though five out of six isolates were able to grow slightly at 10 µg/ml. The capacity of most *N. meliosmae-myrianthae* isolates to grow on leaf discs treated with 10 µg/ml tebuconazole may be the result of higher selection pressure caused by eight DMI applications performed during the season in the two vineyards sampled, compared with vineyards where *N. tropicalis* was collected that received up to two DMI treatments. Both *Neophysopella* spp. share a similar exon–intron *CYP51* structure with *P. pachyrhizi* and *P. triticina* (Schmitz et al. 2014; Stammler et al. 2009). For rust, CYP51 alteration I145F and the combinations Y131H+F120L, Y131F+K142R, and Y131F+I475T have previously been related to DMI sensitivity shifts in *P. pachyrhizi* (Schmitz et al. 2014), and Y134F had a limited impact on DMI sensitivity in *P. triticina* (Stammler et al. 2009). CYP51 F120L alone was detected in all 25 *Neophysopella* spp. isolates analyzed, including reference isolate AGLR9. Similarly to our results, all *P. triticina* isolates carried L120 alleles regardless of the DMI sensitivity profile (Stammler et al. 2009). The Y131F reported in *P. pachyrhizi* and Y134F in *P. triticina* corresponds to the archetype Y137F in *Zymoseptoria tritici* (Mair et al. 2016b), which is the most widely reported CYP51 alteration associated with a decrease in sensitivity to DMIs (Hawkins and Fraaije 2018).

The eight- to 25-fold reduction in sensitivity of *N. meliosmae-myrianthae* isolates to tebuconazole after four rounds of selection (Supplementary Table S2) suggests that practical resistance to DMIs may occur in vineyards with excessive numbers of applications. This shift in tebuconazole sensitivity was not associated with point mutations in *CYP51*, suggesting that other mechanisms are involved. Besides amino acid changes, overexpression of the *CYP51* gene has also been linked to reduced DMI sensitivity in a number of plant pathogens, including rusts (Mair et al. 2016a; Schmitz et al. 2014; Stammler et al. 2009; Wei et al. 2020). *CYP51* overexpression can occur in either a constitutive or inducible manner, although constitutive expression has been most implicated in DMI resistance (Carter et al. 2014). In this study, we found that constitutive *CYP51* expression in tebuconazole-adapted laboratory isolates of *N. meliosmae-myrianthae* was about two times higher than the sensitive *N. meliosmae-myrianthae* AGLR59, whereas a seven- to 19-fold increase was observed compared with sensitive *N. tropicalis* isolates in the absence of tebuconazole (Fig. 5). Among the sensitive isolates, only AGLR59 produced a few pustules on fungicide-treated leaves, which may be explained by the higher constitutive *CYP51* expression levels on untreated leaves. Another hypothesis is that constitutive *CYP51* expression variation between *N. tropicalis* and *N. meliosmae-myrianthae* may be an intrinsic feature of the species, as reported for *CYP51B* in *Colletotrichum fioriniae* and *C. nymphaeae* (Chen et al. 2020). More *CYP51* expression studies assessing isolates of both species never exposed to DMI fungicides are needed to address this point. After fungicide exposure, induced expression of *CYP51* was found in three tebuconazole-adapted isolates, whereas only constitutive expression has been identified in *P. pachyrhizi* and *P. triticina* (Schmitz et al. 2014; Stammler et al. 2009). Both constitutive and induced *CYP51* expression has been reported as mechanism of DMI resistance in *Cercospora beticola* (Bolton et al. 2012).

Overexpression has been frequently linked to inserts upstream of the *CYP51* gene in hemibiotrophic and necrotrophic pathogens, such as *Monilinia fructicola*, *Venturia inaequalis*, and *Z. tritici* (Cools et al. 2012, 2013; Luo and Schnabel 2008; Schnabel and Jones 2001). Meanwhile, the mechanisms underlying *CYP51* overexpression are unknown in biotrophic pathogens, such as *E. necator*, *P. pachyrhizi*, and *P. triticina* (Frenkel et al. 2015; Schmitz et al. 2014; Stammler et al. 2009). Despite numerous attempts, we could not amplify the upstream fragment of *CYP51*, and further investigation is needed. Overall, our analysis suggests that *N. meliosmae-myrianthae* has higher constitutive *CYP51* expression levels compared with *N. tropicalis* that were associated with reduced tebuconazole sensitivity. However, other resistance mechanisms such as efflux pumps may also be involved.

In conclusion, this work provides data on the sensitivity of *N. tropicalis* and *N. meliosmae-myrianthae* to pyraclostrobin and tebuconazole that can be used as baseline data for future monitoring studies investigating sensitivity shifts. The lack of an intron immediately downstream of codon 143 in *CYTB* indicates that the G143A mutation will not be lethal if it emerges. Overexpression of *CYP51* appears to affect tebuconazole sensitivity, and further target site alterations may develop. Although we did not detect isolates exhibiting practical field resistance, our results indicate a potential risk of *Neophysopella* spp. developing resistance to QoI and DMI fungicides, especially in vineyards where there is a long and frequent exposure to these groups of fungicides.

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