Tropical Plant Pathology
https://doi.org/10.1007/s40858-023-00556-x

SHORT COMMUNICATION

Sequence analysis of cytochrome *b* gene in Vietnamese isolates of *Hemileia vastatrix*, the causal agent of coffee leaf rust, in relation to potential QoI fungicide resistance

Cham Thi Mai Le[1,2] · Hideo Ishii[3] · Izumi Okane[3] · Yuichi Yamaoka[3]

Received: 13 November 2022 / Accepted: 17 January 2023

Abstract
Coffee leaf rust, caused by *Hemileia vastatrix*, is a devastating fungal disease threat to coffee production and the economy globally. Spraying fungicides including quinone outside inhibitors (QoIs) can effectively extinguish a disease outbreak quickly. However, mutations in the cytochrome *b* (*CYTB*) gene associated with QoI resistance have been reported in rust fungi and many other pathogens. In the Asian soybean rust fungus, *Phakopsora pachyrhizi*, a nucleotide mutation in the first exon of the *CYTB* gene, leading to the amino acid substitution F129L, is responsible for QoI resistance. Together with the F129L, amino acid substitutions at positions 137 and 143 are linked with moderate or high resistance to QoIs, respectively, in other pathogens. As an important obligate parasitic fungus causing disease to major and valuable tropical plant, therefore, it is important to develop molecular methods to detect resistance development in this pathogen *H. vastatrix*. Difference from the soybean rust fungus, codon 129 is located in exon 2 of the *CYTB* gene of this fungus, while exon 3 harbors codons 137 and 143. In this study, three PCR primer pairs were designed to amplify exons 2, 3, and 4 of the *H. vastatrix CYTB* gene and used for 40 isolates from Vietnam, the second-largest coffee producer worldwide. The following sequencing results showed that Vietnamese isolates did not harbor F129L and G143A mutations in exons 2 and 3 of the *CYTB* gene. The three primer pairs used in this study can be applied for live or dried mycelia to detect potential resistance to QoI fungicides in *H. vastatrix* in the future.

Keywords Coffee leaf rust · Cytochrome *b* · Disease control · Fungicide resistance · QoIs sensitivity

Coffee leaf rust (CLR) caused by *Hemileia vastatrix* Berk. & Broome (*Pucciniales*, *Basidiomycota*) is a major threat to global coffee production. This disease causes early defoliation, making coffee plants more vulnerable to being attacked by other fungal pathogens (Talhinhas et al. 2017). Urediniospores of *H. vastatrix* can survive for several weeks in the wild (McCook 2006) and are easily spread by the wind, rain, insects, animals, and humans (Avelino et al. 2015). Therefore, global CLR epidemics have occurred and caused severe yield loss affecting the livelihoods of more than 100 million people worldwide (Talhinhas et al. 2017).

An effective strategy for controlling CLR is applying chemical fungicides. Three fungicide classes are among the most effective for controlling rust fungi: quinone outside inhibitors (QoIs), sterol demethylation inhibitors (DMIs), and succinate dehydrogenase inhibitors (SDHIs) (Oliver 2014). Of these, QoIs (strobilurins) are especially important because of their effectiveness at controlling a broad range of fungal and oomycete pathogens (Bartlett et al. 2002). QoI fungicides inhibit mitochondrial respiration by binding the Qo site of the cytochrome bc1 enzyme complex, which leads to energy deficiency (Bartlett et al. 2002). To control CLR and increase yield, QoI fungicides are applied alone or in mixtures with other systemic fungicides, like triazoles (DMI group) (Souza et al. 2011; Honorato et al. 2015) or copper-based fungicides (copper oxychloride and hydroxide, cuprous oxide, and Bordeaux mixture) (Zambolim 2016; Costa et al. 2019). In

✉ Cham Thi Mai Le
ltmcham.snn@tphcm.gov.vn; ltmaicham.bio@gmail.com

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

[2] Division of Microbial Technology, Biotechnology Center of Ho Chi Minh City, Trung My Tay Ward, District 12, 71507 Ho Chi Minh, Vietnam

[3] Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

Published online: 16 February 2023

Vietnam, coffee farmers have been using the QoI fungicides azoxystrobin, pyraclostrobin, picoxystrobin, and trifloxystrobin to control CLR and other fungal diseases (Circular no. 19/2021/TT-BNNPTNT dated 28/12/2021 2021).

Field resistance to QoIs has been reported in many pathogenic agents (FRAC 2020). Resistance to QoI fungicides is usually related to a single-point mutation in the *CYTB* gene that changes the encoded amino acid (Sierotzki et al. 2000). Three amino acid substitutions are associated with QoI resistance in fungi: from phenylalanine to leucine at position 129 (F129L), from glycine to arginine at position 137 (G137R), and from glycine to alanine at position 143 (G143A) (Fernández-Ortuño et al. 2008). In rust fungi, the first report on a change in QoI fungicide sensitivity in *Puccinia horiana*, the causative agent of chrysanthemum white rust, was from England (Cook 2001), although the *CYTB* gene of *P. horiana* was characterized, no mutations were found (Grasso et al. 2006). Several years later, in Japan, curative spraying of azoxystrobin against *P. horiana* isolates which were subjected to QoI selection pressure in the field was ineffective. Sequencing the *CYTB* gene of these isolates revealed that they did not contain the F129L mutation (Matsuura 2019). Nonetheless, the F129L mutation in the first exon of the *CYTB* gene of the Asian soybean rust fungus, *P. pachyrhizi*, can lead to QoI resistance (Klosowski et al. 2016; Muller et al. 2021). Likewise, QoI resistance associated with G143A has never been reported in rust fungi previously because the presence of intron type I directly after this codon leads to the substitution at this position being lethal (Grasso et al. 2006). QoI fungicides are being applied in coffee cultivation (Souza et al. 2011; Honorato et al. 2015; Zambolim 2016; Costa et al. 2019), thus it is crucial to monitor QoI resistance of CLR to devise disease management strategies.

The *CYTB* gene structure is different among rust fungal genera. *Phakopsora pachyrhizi CYTB* gene includes two exons and an intron, while *P. horiana* harbors three exons and two introns in its *CYTB* gene (Grasso et al. 2006). However, the *H. vastatrix CYTB* gene contains four exons and three introns (Grasso et al. 2006). Until now, although there have been no reports on QoI resistance in *H. vastatrix*, it is epidemiologically important to detect such QoI resistance relating gene mutation rapidly. Therefore, in this study, we designed specific PCR primers to amplify fragments of exons 2 to 4 in the *H. vastatrix CYTB* gene and tested them using CLR specimens collected in Vietnam. The purpose of this work was to examine mutations in the *CYTB* gene to propose an appropriate control strategy for CLR disease.

Vietnam has a long history of coffee cultivation. In southern Vietnam, farmers usually intercrop coffee with black pepper, while coffee is frequently cultivated as a mono-crop in large areas of northern Vietnam. Besides managing CLR and other diseases on coffee, southern farmers sometimes apply fungicides including biological and chemical compounds to control root rot, charcoal rot, anthracnose, and so forth on black pepper, while coffee is grown naturally in high mountains in the northwest and some areas of the Central Highlands (data not shown). In this study, we examined 40 CLR specimens (Table S1) identified based on rDNA-ITS sequences (Le et al. 2022). These specimens were collected in three main coffee planting regions in Vietnam including Northwest, Southeast, and Central Highlands. Genomic DNA was isolated from the CLR specimens using the modified thermal-shock method (Le et al. 2022). Briefly, *H. vastatrix* urediniospores were collected from rust-infected leaves and immersed in a tube containing 30 µL Buffer 1. After a 15 min incubation at 95 °C, the tube was placed in a deep freezer at − 80 °C for 10 min. Finally, it was centrifuged and the supernatant was transferred to a new tube. A NanoDrop DS-11 spectrophotometer (DeNovix, Delaware, USA) was used to evaluate the quality and quantity of the extracted DNA.

The *H. vastatrix CYTB* gene is large, with four exons and three introns (Grasso et al. 2006). Therefore, to survey potential nucleotide substitutions in the *CYTB* gene of *H. vastatrix* isolates, the three PCR primer pairs CLRE2-FW and CLRE2-RV, CLRE3-FW and CLRE3-RV, CLRE4-FW and CLRE4-RV were designed (Fig. 1 and Table 1) to amplify fragments containing exons 2 to 4 based on a whole reference sequence of the *CYTB* gene using the Primer-BLAST tool at NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The reference sequence of *H. vastatrix CYTB* gene fragment, including full sequence of exons 2, 3 and fragments of exons 1, 4 (Grasso et al. 2006), was obtained from NCBI (https://www.ncbi.nlm.nih.gov/nuccore) with GenBank accession number DQ209282. The primer melting temperature (Tm) ranged from 57 to 63 °C; the maximum Tm difference between two primers in a pair was 3 °C and the GC content was 40 to 60%. The primer pairs with the smallest Tm difference were chosen.

Gradient PCR was conducted to determine the optimal annealing temperature for each primer pair. A 25 µL reaction mixture was prepared that contained 1–2 µL DNA template (30–50 ng/µL), 0.2 µM each primer (2.5 µL), 12.5 µL Gene RED PCR Mix Plus (Nippon Gene, Tokyo, Japan), and 5.5–6.5 µL autoclaved distilled water. The PCR process

Fig. 1 The positions of the exons of the *Hemileia vastatrix CYTB* gene and designed primers

Table 1 Information on the primer pairs designed to amplify exon fragments of the *Hemileia vastatrix* cytochrome *b* gene

Primer name		Sequence (5' →3')	Tm (°C)	GC percentage (%)	Optimal annealing temperature (°C)	Product length (bp)
CLRE2-FW	Forward	TGTTGAGTATGGATGACTTATCAGG	58.18	40	57	280
CLRE2-RV	Reverse	GTAGCTTTCGGCTGGCTCTA	59.54	55		
CLRE3-FW	Forward	TCGGTTCTATGCAGGATCGC	59.97	55	53	475
CLRE3-RV	Reverse	TGATCATCGTCGTTCAGCGT	59.83	50		
CLRE4-FW	Forward	ACAGTCGATGTTCCCGTAGC	59.83	55	57	691
CLRE4-RV	Reverse	TTCAGGAACGATCGACGGTG	60.11	55		

consisted of an initial 5 min denaturation at 95 °C, 30 cycles of 95 °C for 30 s, annealing from 48 to 58 °C for 45 s, and 72 °C for 1 min, followed by a final 8 min extension at 72 °C. The amplicon was confirmed by electrophoresis in 1.2% agarose gels. After being purified using the FastGene™ Gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan), the PCR products were consigned to Eurofins Genomics (Tokyo, Japan) for sequencing. ATGC software (GENETYX, Tokyo, Japan) was used to assemble the obtained sequences. Finally, ClustalX ver. 2.0 (Larkin et al. 2007) was used to identify the nucleotide substitutions in exons 2 to 4.

The primers CLRE2-FW and CLRE2-RV amplified a 280 bp fragment that contains site F129 (exon 2). The primers CLRE3-FW and CLRE3-RV amplified a 475 bp fragment containing sites G137 and G143 (exon 3). The primers CLRE4-FW and CLRE4-RV amplified a 691 bp fragment including a part of exon 4 (Table 1, Fig. 1). After gradient PCR, the optimal annealing temperature for the primer pairs CLRE2-FW and CLRE2-RV, and CLRE4-FW and CLRE4-RV was determined to be 57 °C, while that for the primer pair CLRE3-FW and CLRE3-RV was 53 °C (Table 1). After aligning the nucleotide sequences, amino acids were deduced and compared to those of *H. vastatrix* CYTB in GenBank (ABB54711). No mutations at positions F129, G137, and G143 were detected in any isolates analyzed (Fig. 2A, B). Nonetheless, all isolates had third-base nucleotide substitutions in codons T145 and T148 compared with reference sequence DQ209282 (Fig. 2C). The thymine (T) at both codons in the DQ209282 sequence was replaced with adenine (A) in all Vietnamese isolates, although the deduced amino acid remained unchanged (threonine).

The *CYTB* gene structure of *H. vastatrix* was revealed with three sandwich introns (Grasso et al. 2006). In this study, the primer pairs CLRE3-FW, CLRE3-RV, CLRE4-FW, and CLRE4-RV were designed based on sequences of introns 2 and 3 and exon 4 (Fig. 1). An intron between exons 1 and 2 (intron 1) of the *CYTB* gene of *H. vastatrix* (Fig. 1) was considered for the designed forward primer CLRE2-FW. However, we could not design a forward primer in the target intron 1 because no sequence data were available. No forward primer located in exon 1 amplified a product. Finally, the primer CLRE2-FW was designed which included 10 nucleotides in exon 1 and 15 nucleotides in exon 2, while CLRE2-RV covered part of intron 2 (Fig. 1). The success of amplifying the exon 2 fragment using these primers questions the existence of intron 1 in the *H. vastatrix CYTB* gene. The presence of this intron needs to be confirmed because the frequent loss of introns was reported in the fungal *CYTB* gene (Yin et al. 2012).

The main mechanism of QoI resistance in fungal pathogens is the nucleotide substitutions in some positions including 129, 137, and 143 in their *CYTB* gene (Fernández-Ortuño et al. 2008). Howbeit, in this study, no mutations were detected at these positions in 40 *H. vastatrix* isolates in Vietnam although some host plants were exposed to QoI fungicides several times (data not shown). The G143A mutation has not been detected in the rust fungus and the presence of type I intron immediately after this codon might be one of the reasons (Grasso et al. 2006; Fernández-Ortuño et al. 2008). Similar to *H. vastatrix*, F129L mutation was not observed in *P. horiana* isolates in Japan (Matsuura 2019). Nonetheless, the F129L mutation associated with QoI resistance was detected in 51% of the *P. pachyrhizi* populations in Brazil in 2013–2014 (Klosowski et al. 2016). Subsequently, these F129L mutant isolates increased rapidly in Brazil (Müller et al. 2021). In soybean cultivation, rust-resistant cultivar is yet planted, thus, chemical sprays including triazoles and strobilurins have been applied two or three times per season as an effective method to control rust disease in Brazil (Zambolim et al. 2022). The periodic QoI applications could be a reason for developing resistance in soybean rust fungal populations in Brazil. As QoIs are being applied to control CLR disease, it is important to monitor fungicide resistance in the rust fungal populations subjected to fungicide selection pressure in the field to propose rational and timely management strategy for disease control. Meanwhile, to avoid QoI resistance, farmers need to be guided to save QoI fungicide applications.

Even though F129L, G137R, and G143A mutations were not detected in 40 *H. vastatrix* isolates in Vietnam, it cannot

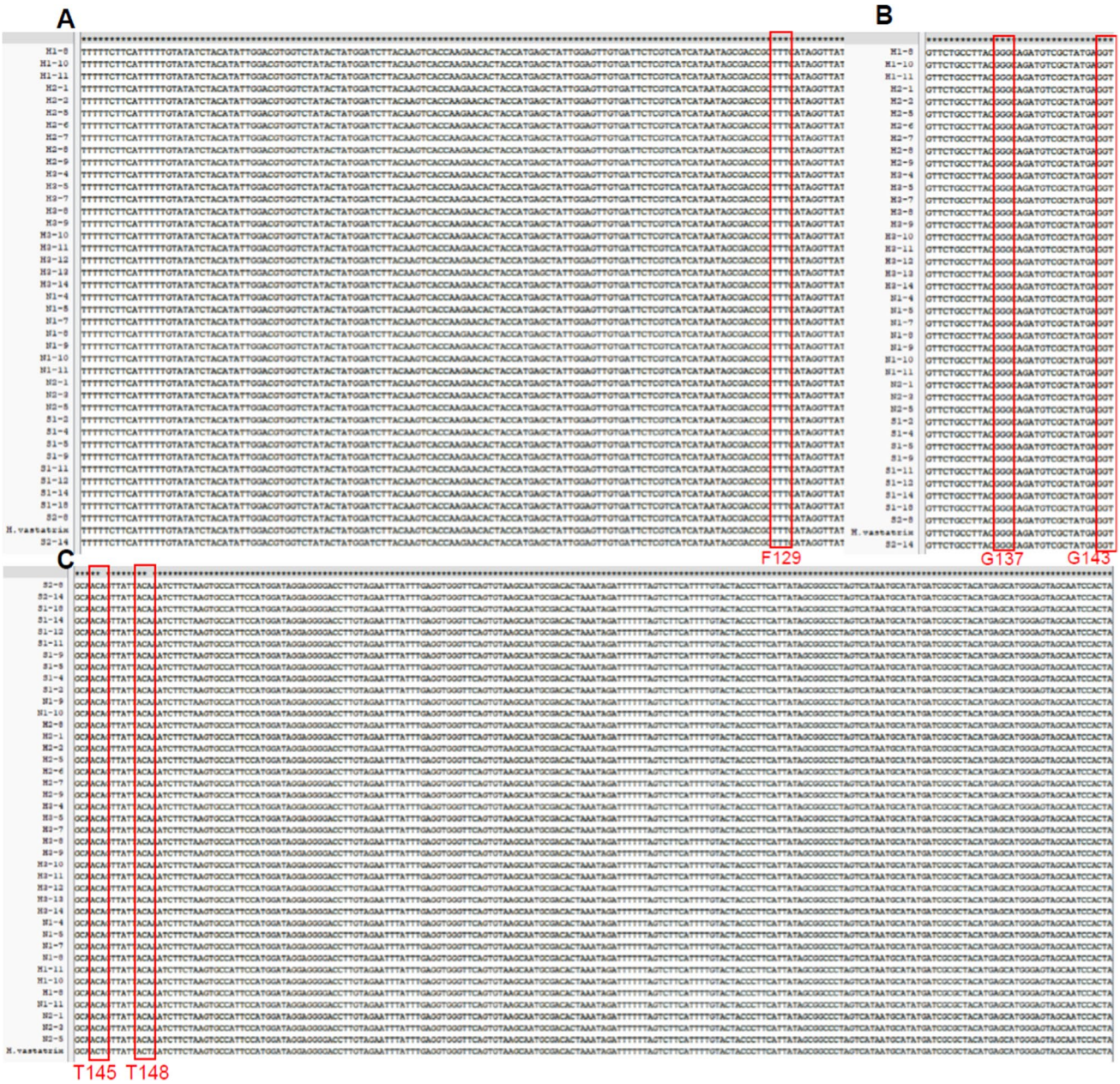

Fig. 2 Sequence alignment of *CYTB* gene fragments of *Hemileia vastatrix* collected in Vietnam and the reference GenBank accession DQ209282. Alignments of the exon **A** 2, **B** 3, and **C** 4 fragments

be excluded that other mechanisms are involved in resistance in this fungus. Indeed, two *Colletotrichum nymphaeae* isolates causing strawberry anthracnose fruit rot showed resistance to azoxystrobin without mutations in the *CTYB* gene (Chechi et al. 2020). In the case of *P. horiana*, F129L mutation was not detected in some isolates collected in 2017 (Matsuura 2019). However, several years later, the substitutions L299F, L275F+L299F, and N256S+L299F in the third exon were identified in some isolates possessing azoxystrobin resistance (Matsuzaki et al. 2020; 2021). In this study, the amplicon using primers CLRE4-FW and CLRE4-RV did not cover positions L275F, L299F, and N256S. We need to analyze these positions and others in exon 4 of *H. vastatrix* in the future. In the meantime, field survey and bioassay will be necessary to be conducted in order to know whether performance of QoI fungicides is still maintained or not.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40858-023-00556-x.

Acknowledgements We would like to thank the Board of Directors of the Biotechnology Center of Ho Chi Minh City, Dinh A. H., Nguyen T. T. D., Tran C. H., and Nguyen T. N. B., for their assistance in collecting samples in Vietnam.

Author contribution CTML collected samples. HI, IO, YY, and CTML conceived and directed this study. CTML designed and performed experiments. CTML, HI, and IO analyzed and biologically interpreted the data. CTML wrote the draft manuscript. HI, IO, and YY revised and approved the final version of the manuscript.

Funding This research was financially supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 20H03006 (IO) and the fund for student research from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.

Data availability This study has no data sharing as no datasets were generated or analyzed during the current research.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All the specimens were collected under the permission of the Vietnamese Government and experiments were performed with the current laws of Japan.

References

Avelino J, Cristancho M, Georgiou S, Georgio S, Imbach P, Aguilar L, Bornemann G, Laderach P, Anzueto F, Hruska AJ, Morales C (2015) The coffee rust crises in Colombia and Central America (2008–2013): impacts, plausible causes and proposed solutions. Food Security 7:303–321

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B (2002) The strobilurin fungicides. Pest Management Science 58:649–662

Chechi A, Ivey MLL, Kaufman RR, Bryson KP, Schnabel G (2020) Quinone outside inhibitor-resistant Colletotrichum nymphaeae isolates from strawberry lack mutations in cytb gene. Journal of Plant Pathology 102:681–683

Circular No. 19/2021/TT-BNNPTNT dated 28/12/2021 (2021). Danh mục thuốc bảo vệ thực vật được phép sử dụng tại Việt Nam. Available at: https://snnptnt.thuathienhue.gov.vn/UploadFiles/TinTuc/2022/3/18/phuluc1(2).pdf. Accessed 8 Oct 2022

Cook RTA (2001) First report in England of changes in the susceptibility of *Puccinia horiana*, the cause of chrysanthemum white rust, to triazole and strobilurin fungicides. Plant Pathology 50:792

Costa GA, Lira JB, Lopes RLF, Lopes UP (2019) Tank mix application of copper hydroxide either with cyproconazole or pyraclostrobin fungicides reduced the control of coffee leaf rust. Crop Protection 124:104856

Fernández-Ortuño D, Torés JA, de Vicente A, Pérez-García A (2008) Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology 11:1–9

Fungicide Resistance Action Committee (2020) List of first confirmed cases of plant pathogenic organisms resistant to disease control agents. CropLife Int., Brussels, Belgium. Available at: https://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-first-confirmed-cases-of-plant-pathogenic-organisms-resistant-to-disease-control-agents_05_2020.pdf?sfvrsn=7073499a_2. Accessed 22 Sept 2022

Grasso V, Palermo S, Sierotzki H, Garibaldi A, Gisi U (2006) CYTB gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. Pest Management Science 62:465–472

Honorato J, Zambolim L, do Nascimento Lopes U, Pedro Lopes U, de Silva Silveira Duarte H (2015) DMI and QoI fungicides for the control of coffee leaf rust. Australasian Plant Pathology 44:575–581

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

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–8

Matsuura S (2019) Does QoI (strobilurin) resistance in isolates of *Puccinia horiana*, the causal agent of chrysanthemum white rust, occur in western Japan? Journal of Plant Diseases and Protection 126:469–473

Le CTM, Okane I, Ono Y, Tsuda Y, Yamaoka Y (2022) Incidence of coffee leaf rust in Vietnam, possible original sources and subsequent pathways of migration. Frontiers in Plant Science 13:872877

Matsuzaki Y, Harada T, Iwahashi F (2020) Amino acid substitutions responsible for different QoI and SDHI sensitivity patterns in *Puccinia horiana*, the causal agent of chrysanthemum white rust. Plant Pathol 70:377–386. https://doi.org/10.1111/ppa.13298

Matsuzaki Y, Harada T, Iwahashi F (2021) New cytochrome *b* haplotypes, harboring L299F or N256S + L299F substitutions, were found in azoxystrobin-resistant *Puccinia horiana*, the causal agent of chrysanthemum white rust. European Journal of Plant Pathology 160:963–972

McCook S (2006) Global rust belt: *Hemileia vastatrix* and the ecological integration of world coffee production since 1850. Journal of Global History 1:177–195

Müller MA, Müller MA, Stammler G, Stammler G, Larissa May De Mio L (2021) Multiple resistance to DMI, QoI and SDHI fungicides in field isolates of *Phakopsora pachyrhizi*. Crop Protection 145:105618

Oliver RP (2014) A reassessment of the risk of rust fungi developing resistance to fungicides. Pest Management Science 70:1641–1645

Sierotzki H, Wullschleger J, Gisi U (2000) Point mutation in CYTB gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f sp *tritici* field isolates. Pesticide Biochemistry and Physiology 68:107–112

Souza AF, Zambolim L, Jesus VC Jr, Cecon PR (2011) Chemical approaches to manage coffee leaf rust in drip irrigated trees. Australasian Plant Pathology 40:293

Talhinhas P, Batista D, Diniz I, Vieira A, Silva DN, Loureiro A, Tavares S, Pereira AP, Azinheira HG, Guerra-Guimarães L, Várzea V, Silva MDC (2017) The coffee leaf rust pathogen *Hemileia vastatrix*: one and a half centuries around the tropics. Molecular Plant Pathology 18:1039–1051

Yin LF, Hu MJ, Wang F, Kuang H, Zhang Y, Schnabel G, Li GQ, Luo CX (2012) Frequent gain and loss of introns in fungal CYTB genes. PLoS One 7:e49096

Zambolim L (2016) Current status and management of coffee leaf rust in Brazil. Tropical Plant Pathology 41:1–8

Zambolim L, Reis EM, Guerra WD, Juliatti FC, Menten JOM (2022) Integrated management of Asian soybean rust. European Journal of Applied Sciences 10:602–633