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ANALYSIS OF RESISTANCE TO FUNGAL PATHOGEN *HEMILEIA VASTATRIX* OF LIBERICA COFFEE BASED ON FUNCTIONAL MARKER

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ABSTRACT

Coffee Leaf Rust (CLR) disease caused by fungal pathogen *Hemileia vastatrix* is one of devastated diseases in coffee plants. Disease RGA (resistance gene analog) primer pair CARF 005 has been reported for leaf rust-resistant screening in Arabica coffee and has never been reported in Liberica coffee. Previously, Liberoid Meranti 1 and 2 (Lim 1 and Lim 2) from Meranti Islands Indonesia were officially published by the government as CLR resistant cultivars and adaptive to peat soil. Our study aimed to analyze the resistance of Liberica coffee plants based on functional primer CARF 005. We sampled healthy plants of three Liberica genotypes (Lim 1, Lim 2, Bengkalis) in commercial farmer fields. DNA was extracted from young leaves, amplified and sequenced using CARF 005 primers. All samples generated DNA band about 400 bp. In addition, nucleotide sequences are similar to Arabica putative disease resistance gene. All the three sequences contain NB-ARC conserved domain which contribute to pathogenic-resistant trait. The regions also contain one motif sequence of P-loop/Walker-A domain. Our result confirmed that DNA fragments amplified by CARF 005 are linked to RGA region and eventually we suggested that CARF 005 can be used to identify resistance to CLR in Liberica. It will particularly contribute for supporting Liberica breeding program and conservation of Liberica germplasm.

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INTRODUCTION

Coffee is one of important agricultural commodities in Indonesia particularly, Robusta (*Coffea canephora* Pierre ex A. Froehner) and Arabica (*Coffea arabica* L.). Another coffee species, Liberica (*Coffea liberica* W. Bull ex Hiern), was quite unpopular especially in Indonesia probably due to relatively lower yield compared to other species (Harni *et al.*, 2015). However, Liberica coffee plants are able to grow and develop in peat land area which is predominantly found in Riau Province, Indonesia (Prasetyo *et al.*, 2019; Maryani and Novriadhy, 2022).

Reportedly, Liberica varieties from Meranti Island Indonesia namely Liberoid Meranti 1 or Lim 1 (Litbang, 2015) and Liberoid Meranti 2 or Lim 2 (Litbang, 2019) showed phenotypically resistance to Coffee Leaf Rust (CLR) disease caused by the obligate parasitic fungal pathogen *Hemileia vastatrix*. Thus, the Liberica cultivars can be source of resistant germplasm. Coffee Leaf Rust (CLR) disease is one of main devastated diseases in coffee plants. The disease may lead to significant reduction of coffee berry yields up to 35% (Silva *et al.*, 2022; McCook and Vandermeer, 2015; Talhinhas *et al.*, 2017). Symptoms of the disease can be recognized by orange spore mass on the lower surface of leaf resulting in premature leaf fall (Talhinhas *et al.*, 2017). It has been considered that the most effective strategy to maintain coffee productivity and sustain their existence is by providing resistant cultivars. There are several

approaches to identify resistance against plant pathogen such as morphology, biochemistry and molecular methods. Molecular markers are believed as valuable tools to assess the plant trait since they can detect at any stage of plant development including for determining CLR disease resistance such as SCAR (sequence characterized amplified region) markers (Alkimim *et al.*, 2017), receptor-like kinase marker (Almeida *et al.*, 2021) and RGA-based primer CARF 005 (Alvarenga *et al.*, 2011; Barka *et al.*, 2020).

CARF 005 marker has been developed to distinguish resistant and susceptible Arabica populations (Alvarenga *et al.*, 2011; Barka *et al.*, 2020). The primer pair anneal to R gene region encoding NBS-LRR protein. NBS-LRR protein is the largest class of R protein which are found in various plant species. Plants have two immune systems called plant triggered immunity (PTI) and effector triggered immunity (ETI) (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The first layer of plant defence, PTI, require PRR (pattern-recognition receptors) protein whereas ETI system involve R gene encoding resistance protein including NBS-LRR protein (Lee and Yeom, 2015). Hence, objective of our study was to analyze resistance to CLR disease of Liberica coffee grown under peatland area in Meranti Islands Indonesia based on molecular marker CARF 005.

MATERIALS AND METHODS

Plant material

Two Liberica coffee varieties (Lim 1 and Lim 2) officially released by Agricultural Ministry of Republic Indonesia and one Liberica local genotype (Bengkalis/B1) were used in this study. We collected leaves from three healthy plants for each variety or genotype from commercial fields on peatland area in Meranti Islands, Indonesia. Leaf samples were wrapped in aluminium foil, kept in cooling box and brought to the laboratory. Eventually the samples were stored in the deep freezer before DNA isolation.

DNA extraction

Total genomic DNA was extracted from a young and fresh leaf according to the manufacturer protocol of Genomic DNA Mini Kit (Plant) Geneaid (Geneaid Biotech Ltd., New Taipei City, Taiwan). Around 100 mg of leaf tissue per sample was ground to fine powder using mortar and pestle with liquid nitrogen. RNase was used to remove RNA contamination. Integrity of extracted DNA was checked under 1% agarose gel with additional of FluoroVue™ Nucleic Acid Gel Stain (SMOBIO Technology Inc., Taiwan).

PCR and sequencing

DNA amplification used CARF 005 primer pair (F: 5'-GGACATCAACACCAACCTC-3' and R: 5'-ATCCCTACCATCCACTTCAAC-3') (Integrated DNA Technologies, Singapore) based on Barka *et al.* (2020). A volume of 50 µl PCR mixture consisted of 5 µl of 10x DreamTaq buffer, 5 µl of 2 mM dNTPs, 5 µl of 10 µM each for forward and reverse primer, 0.4 µl 5 U/ µl Taq DNA Polymerase (Thermo Fisher Scientific Inc., USA), nuclease free water and genomic DNA solution up to 50 µl total volume. Amplification cycles as Barka *et al.* (2020) described was denaturation at 95 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 60 s and post-PCR at 72 ℃ for 10 min. A volume of 5 µl PCR product was visualized by electrophoresis under 1% agarose gel. Remain PCR product about 45 µl was subsequently sent to First Base Laboratories, Malaysia through PT. Genetika Science Jakarta Indonesia, a company partner of First Base Laboratories, for sequencing. The laboratory performed purification treatment our PCR product followed by cycle sequencing with bidirectional sequencing method.

Sequence analysis

The nucleotide sequence data was subjected to BioEdit ver 7 prior to analyze using BLAST for similarity alignment with NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) and identification of conserved domain. Nucleotide sequence was converted into amino acid sequence by Augustus program (http://bioinf.uni-greifswald.de/augustus/) and for checking the particular amino acid motif. Alignment of predicted protein sequence was performed by Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo).

Phylogenetic relationships based on predicted protein sequence was constructed using Molecular Evolutionary Genetics Analysis (MEGA X) program (Kumar *et al.*, 2016).

RESULTS AND DISCUSSION

Total genomic DNA was successfully extracted from the three samples of Liberica coffee and amplified using primer pair CARF 005. The primer pair was able to generate a well-defined DNA band with the size around 400 bp (Figure 1). This size of PCR product is similar to result of study on *C. arabica* genome using the same primer (Barka *et al.*, 2020). The functional

primer CARF 005 was previously reported to amplify DNA segment which is related to a partial open reading frame of *C. arabica* genome encoding a disease resistance protein (Alvarenga *et al.*, 2011). The marker CARF 005 was developed from Resistance Gene Analog(s) (RGA) sequences to distinguish between resistant and susceptible Arabica coffee plants to *H. vastatrix* based on presence or absence of PCR product (Alvarenga *et al.*, 2011).

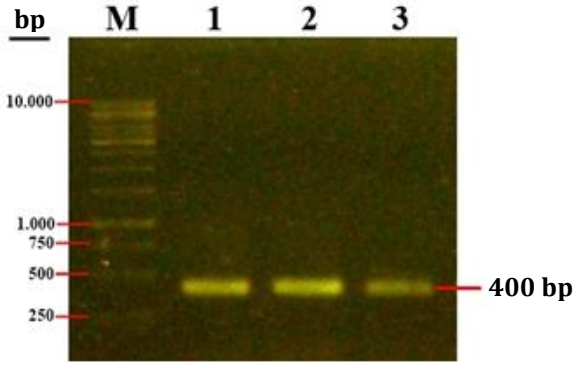

Figure 1. DNA amplification result. PCR using CARF005 primer generated a clear band of about 400kb. M = 1 kb DNA ladder, Coffee varieties: 1 = Lim 1, 2 = Lim 2, 3 = Bengkalis.

A number of studies on mechanism of coffee plants resistance against CLR disease primarily have focused on Arabica and Robusta (Cao *et al.*, 2013; Pestana *et al.*, 2015; Jibat, 2020; Santos *et al.*, 2022; Silva *et al.*, 2022). As mentioned above, functional marker CARF 005 was also initially for identifying resistance on Arabica against CLR disease. Therefore, this study is the first experiment using CARF 005 for Liberica coffee resistant to CLR.

Our PCR result indicated that Lim 1, Lim 2 and Bengkalis may genetically contain NBS-LRR sequence related to resistance against CLR disease. To confirm, the PCR product subsequently was subjected to sequencing reaction. Result of sequence cycles was analyzed to check similarity of nucleotide sequence to NCBI database. The result showed that all Liberica DNA fragments were similar to nucleotide sequence encoding *C. arabica* putative disease resistance protein RGA4 (Table 1).

Table 1. Sequence similarity between the three liberica coffee (Lim 1, Lim 2, B1) and disease resistance protein that have been deposited in Genebank.

Samples	Genebank accession	Query coverage (%)	e-value	Identity (%)
Lim_1	XM_027246611 Coffea arabica putative disease resistance protein RGA4	91	4e-156	96.48
Lim_2	XM_027246611 Coffea arabica putative disease resistance protein RGA4	92	8e-153	95.89
B1	XM_027246611 Coffea arabica putative disease resistance protein RGA4	92	2e-173	98.31

To confirm that our DNA fragments contained any conserved domain related to disease resistance gene, we checked the sequence by CDD (conserved domain database)-NCBI. We found that our plants have conserved domain expressing nucleotide binding site-ARC/APAF-1, R protein and CED-4) (NB-ARC) protein (Figure 2). The NB-ARC region has been reported as functional ATPase domain and its nucleotide-binding state is considered to regulate activity of the R protein (Van Ooijen *et al.*, 2008). NB-ARC domain also act as a molecular switch, converting between ADP (repressed) and ATP (active) bound forms (Steele *et al.*, 2019).

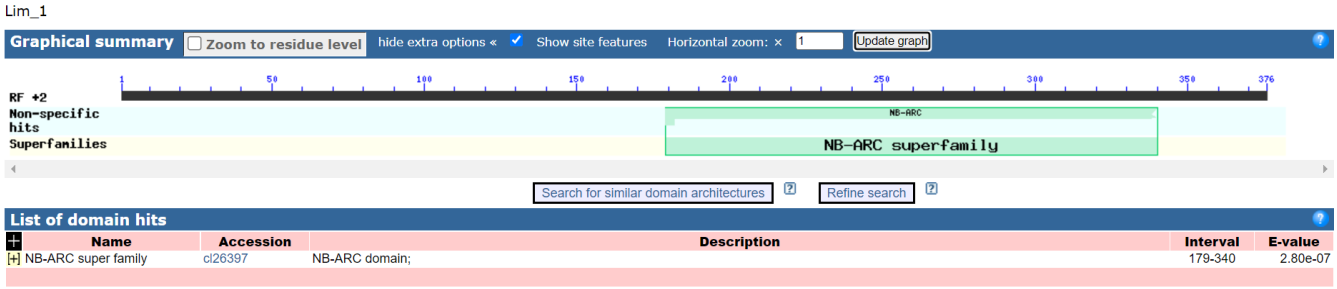

Figure 2. Conserved domain NB-ARC was found in Liberica coffee varieties Lim 1, Lim 2 and B1.

The DNA sequence of our samples were converted into amino acid sequence using Augustus program prior to be subjected into ClustalOmega to align the similarity of the deduced amino acid sequences. Result of amino acids alignment showed that there is variation of amino acid residue amongst the three Liberica coffee genotypes (Figure 3). There are three amino acids with strongly similar substitution (colon), two amino acids with weakly similar substitution (period), eleven unrelated amino acid substitution (space). In addition, there is one conserved motif P-loop/kinase-1a/Walker a (GGLGKTT) that is one of characteristics of NB-ARC. P-loop motif is essential in nucleotide binding and the alterations of this motif may lead to a loss of function of the NBS-LRR proteins. GGLGKTT motif is also found in Arabica and Robusta coffee genome. Conserved motif of P-loop can be GGL/V/M/IGKTT (Zimmer and Wen, 2015), for example the motif is GGVGKTT in banana (Martanti *et al.*, 2015) whereas P-loop motif of this study was GGLGKTT.

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Lim_1   ---DINTNLERINRKHQKEADATGATTSRPTDSIVVPNVVGRAGDESKIVEMLLTPSERV     57
Lim_2   HQHQPRED-----RKHQKEADATGATTSRPTDSIVVPNVVGRAGDESKILEMLLTPSERV     55
B1    HQHQPREDHWGLVRKHQKEADATGATTSRPTDSIVVPNVVGRAGDESKIVEMLLTPSERV     60
      :.:  ************************************.**********

Lim_1   VSVIPITGMGGLGKTTLAKSVYNNTKIDENFGIKSWACVAREIKIVELFKLILESLTRTK     117
Lim_2   VSVIPITGMGGLGKTTLAKSVYNNTKIDENFGKKSWACVAREIKIVELFKLILESLTRTK     115
B1    VSVIPITGMGGLGKTTLAKSVYNNTKIDENFGIKSWVCVAREIKIVELFKLILESLTRTK     120
      ************************************ *** ********************

Lim_1   VEVDGRD  124
Lim_2   VEVDGRD  122
B1    VEVDGRD  127
      *******
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Figure 3. Alignment of predicted amino acid sequence using Clustal Omega.

The majority of plant R (resistance) genes belong to the NB-LRR gene. The NB-LRR class genes can commonly be categorized into two groups: Toll/interleukin-1 receptor (TIR)–NB-LRRs (TNLs) and coiled-coil (CC)-NB-LRRs (CNLs) (Kim *et al.*, 2012).The NB-ARC domain functions as nucleotide-binding site and hydrolysing ATP to trigger changes of R protein conformation (Van Ghelder *et al.*, 2019). Conserved motifs in NB-ARC domain, including the P-loop, eventually contribute in influencing activation of R gene (Steele *et al.*, 2019). Proteins expressed R genes are involved in recognition of effector produced by plant pathogen. Binding between R protein and pathogenic effector molecule induce second layer of plant defence, the effector triggered immunity (ETI), which can hamper growth of pathogen (de Araújo *et al.*, 2019). Thus, RGA fragment of Liberica coffee samples in this study may correspondent to phenotype of resistance to fungal pathogen *H. vastatrix* as previously reported that the CARF 005 marker was derived from RGA sequence (Alvarenga *et al.*, 2010; Barka *et al.*, 2020). Our results ascertain resistant characteristic of Lim 1 and Lim 2 that have been described by Litbang (2015) and Litbang (2019).

Phylogenetic analysis of the deduced amino acid of our

samples and putative disease resistance protein RGA of *Coffea arabica* (Figure 4) showed that Lim 1 and Lim 2 are grouped and separated from Bengkalis coffee. The result supports that Lim 1 and Lim 2 are from same region in Meranti Island, whereas Bengkalis coffee is from another island, Bengkalis Island, in Indonesia. In addition, our samples revealed high similarity to putative amino acid sequences for disease resistance in Arabica coffee. According to (Noir *et al.*, 2001), evolution of NBS-encoding genes experience accumulation of mutation gradually with low of divergence rate instead of a rapid mechanism.

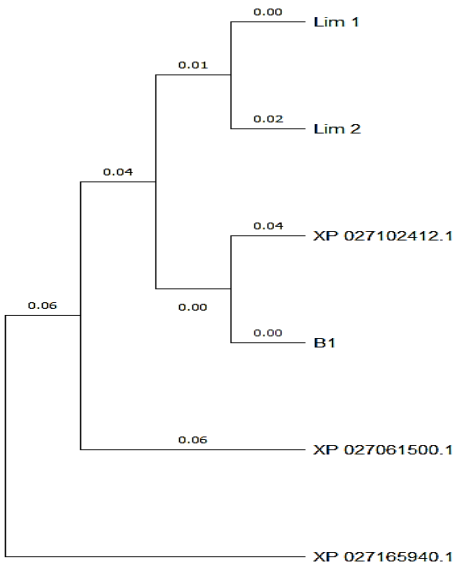

Figure 4. Phylogenetic tree of sequence of predicted amino acid between Liberica coffee varieties and putative disease resistance protein RGA *Coffea arabica* based on Neighbour Joining method.

Another study using CARF 005 marker (Pestana *et al.*, 2015) showed that the marker produced polymorphic bands amongst parents and segregant F2 Arabica populations. Barka *et al.* (2020) used CARF 005 marker to reveal candidate RGA by in silico technique. Other marker, SCAR, was used by Alkimim *et al.* (2017) to assess resistant and susceptible coffee population. All above studies have focused on Arabica and Robusta coffee populations. Thus, studying Liberica coffee is essential work. Therefore, we supposed that our result contributes to provide a new insight into molecular-based data in assessment resistance trait of Liberica coffee. We also suggested that the results of the study be used as basic data for further analysis in coffee breeding program particularly for Liberica coffee (selection or identification) and conservation. The marker may also be used for evaluation in the fields for the release of new resistant Liberica coffee variety.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

REFERENCES

Alkimim, E. R., E. T. Caixeta, T. V. Sousa, A. A. Pereira, A. C. B. de Oliveira, L. Zambolim and N. S. Sakiyama. 2017. Marker-assisted selection provides arabica coffee with genes from other *Coffea* species targeting on multiple resistance to rust and coffee berry disease. Molecular Breeding, 37: 1-10.

Almeida, D. P. d., I. S. L. Castro, T. A. d. O. Mendes, D. R. Alves, G. D. Barka, P. R. R. M. Barreiros, L. Zambolim, N. S. Sakiyama and E. T. Caixeta. 2021. Receptor-Like Kinase (RLK) as a candidate gene conferring resistance to *Hemileia vastatrix* in

coffee. Scientia Agricola, 78: e20200023.

Alvarenga, S. M., E. T. Caixeta, B. Hufnagel, F. Thiebaut, E. Maciel-Zambolim, L. Zambolim and N. S. Sakiyama. 2011. Molecular markers from coffee genome expressed sequences potentially involved in resistance to rust. Pesquisa agropecuária brasileira, 46: 890-98.

Alvarenga, S. M., E. T. Caixeta, B. Hufnagel, F. Thiebaut, E. Maciel-Zambolim, L. Zambolimand and N. S. Sakiyama. 2010. In silico identification of coffee genome expressed sequences potentially associated with resistance to diseases. Genetics and Molecular Biology, 33: 795-806.

Barka, G. D., E. T. Caixeta, S. S. Ferreira and L. Zambolim. 2020. In silico guided structural and functional analysis of genes with potential involvement in resistance to coffee leaf rust: A functional marker based approach. PLoS One, 15: e0222747.

Cao, H., Y. Yang, Z. Pei, W. Zhang, D. Ge, Y. Sha, W. Zhang, K. Fu, Y. Liu and Y. Chen. 2013. Intellectualized identifying and precision controlsystem for horticultural crop diseases based on small unmanned aerial vehicle. Computer and Computing Technologies in Agriculture VI: 6th IFIP WG 5.14 International Conference, CCTA 2012, Zhangjiajie, China.

de Araújo, A. C., F. C. D. A. Fonseca, M. G. Cotta, G. S. C. Alves and R. N. G. Miller. 2019. Plant NLR receptor proteins and their potential in the development of durable genetic resistance to biotic stresses. Biotechnology Research and Innovation, 3: 80-94.

Dodds, P. N. and J. P. Rathjen. 2010. Plant immunity: Towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics, 11: 539-48.

Harni, R., E. Taufiq and B. Martono. 2015. Ketahanan pohon induk kopi liberika terhadap penyakit karat daun (*Hemileia vastatrix* B. et Br.) di Kepulauan Meranti. Jurnal tanaman Industri dan penyegar, 2: 35-42.

Jibat, M. 2020. Review on resistance breeding methods of coffee leaf rust in Ethiopia. International Journal of Research in Agriculture and Forestry, 7: 32-41.

Jones, J. D. and J. L. Dangl. 2006. The plant immune system. Nature, 444: 323-29.

Kim, J., C. J. Lim, B.-W. Lee, J.-P. Choi, S.-K. Oh, R. Ahmad, S.-Y. Kwon, J. Ahn and C.-G. Hur. 2012. A genome-wide comparison of NB-LRR type of resistance gene analogs (RGA) in the plant kingdom. Molecules and cells, 33: 385-92.

Kumar, S., G. Stecher and K. Tamura. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution, 33: 1870-74.

Lee, H.-A. and S.-I. Yeom. 2015. Plant NB-LRR proteins: Tightly regulated sensors in a complex manner. Briefings in functional genomics, 14: 233-42.

Litbang. 2015. Kopi Liberoid Meranti 1 (Lim 1), Accessed 2 October 2022. http://www.litbang.pertanian.go.id/varietas.php/1136/.

Litbang. 2019. Liberoid Meranti 2, Varietas Unggul Kopi Spesifik Lahan Gambut Bercitarasa Tinggi, Accessed 2 October 2022. http://www.litbang.pertanian.go.id/info-teknologi/3513/.

Martanti, D., U. Widyastuti, Y. S. Poerba and R. Megia. 2015. Identification of gene candidate of nucleotide binding site (NBS) from banana *Musa acuminata* Colla var Malaccensis (Riddl.) Nasution and Musa, AAA, Cavendish sub-group. Pakistan Journal of Biological Sciences, 18: 99-106.

Maryani, S. and D. Novriadhy. 2022. The potency of *Coffea liberica* to remediate peat soil after fires in the musi-belida peat hydrological unity, Indonesia. IOP Conference Series: Earth and Environmental Science.

McCook, S. and J. Vandermeer. 2015. The big rust and the red queen: Long-term perspectives on coffee rust research. Phytopathology, 105: 1164-73.

Noir, S., M.-C. Combes, F. Anthony and P. Lashermes. 2001. Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees (*Coffea* L.). Molecular Genetics and Genomics, 265: 654-62.

Pestana, K. N., A. S. Capucho, E. T. Caixeta, D. P. de Almeida, E. M. Zambolim, C. D. Cruz, L. Zambolim, A. A. Pereira, A. C. B. de Oliveira and N. S. Sakiyama. 2015. Inheritance study and linkage mapping of resistance loci to *Hemileia vastatrix* in Híbrido de Timor UFV 443-03. Tree Genetics and Genomes, 11: 1-13.

Prasetyo, P., R. Hidayat and H. Purnomo. 2019. Budidaya Kopi Liberika di lahan gambutCenter for International Forestry Research. Bogor, Indonesia. pp. 3-6.

Santos, M. d. L., M. L. V. de Resende, B. A. dos Santos Ciscon, N. C. Freitas, M. H. d. B. Pereira, T. Reichel and S. M. Mathioni. 2022. LysM receptors in *Coffea arabica*: Identification, characterization, and gene expression in response to *Hemileia vastatrix*. PLoS One, 17: e0258838.

Silva, M. d. C., L. Guerra-Guimarães, I. Diniz, A. Loureiro, H. Azinheira, A. P. Pereira, S. Tavares, D. Batista and V. Várzea. 2022. An overview of the mechanisms involved in Coffee-*Hemileia vastatrix* interactions: Plant and pathogen perspectives. Agronomy, 12: 326.

Steele, J. F., R. K. Hughes and M. J. Banfield. 2019. Structural and biochemical studies of an NB-ARC domain from a plant NLR immune receptor. PLoS One, 14: e0221226.

Talhinhas, P., D. Batista, I. Diniz, A. Vieira, D. N. Silva, A. Loureiro, S. Tavares, A. P. Pereira, H. G. Azinheira and L. Guerra-Guimarães. 2017. The coffee leaf rust pathogen *Hemileia vastatrix*: one and a half centuries around the tropics. Molecular plant pathology, 18: 1039.

Van Ghelder, C., G. J. Parent, P. Rigault, J. Prunier, I. Giguère, S. Caron, J. Stival Sena, A. Deslauriers, J. Bousquet and D. Esmenjaud. 2019. The large repertoire of conifer NLR resistance genes includes drought responsive and highly diversified RNLs. Scientific Reports, 9: 1-13.

Van Ooijen, G., G. Mayr, M. M. Kasiem, M. Albrecht, B. J. Cornelissen and F. L. Takken. 2008. Structure–function analysis of the NB-ARC domain of plant disease resistance proteins. Journal of experimental botany, 59: 1383-97.

Zimmer, E. A. and J. Wen. 2015. Using nuclear gene data for plant phylogenetics: Progress and prospects II. Next-gen approaches. Journal of Systematics and Evolution, 53: 371-79.