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Sheila F. Almeida[1,2], Marcilene F.A. Santos[1,2], Daniela Stefanelo[1,2,3], Vanessa S. Mattos[2], Paolo L. Rodrigues-Silva[2], Gleiciane P. Sousa[2], Sônia M.L. Salgado[4], Gustavo H. Sera[5], Philippe Castagnone-Sereno[6], Juvenil E. Cares[1], Regina M.D.G. Carneiro[2]

[1]Departamento de Fitopatologia, Universidade de Brasília (UnB), 70910-900, Brasília, DF, Brazil.

[2]EMBRAPA-Recursos Genéticos e Biotecnologia, 70849-979, C.P. 02372, Brasília, DF, Brazil.

[3]Departamento de Ciências Humanas, Universidade do Estado da Bahia (UNEB), 47802-660, Barreiras, BA, Brazil.

[4]EPAMIG-Empresa de Pesquisa Agropecuária de Minas Gerais, 37200-900, C.P. 176, Lavras, MG, Brazil.

[5]IDR-Paraná-Instituto de Desenvolvimento Rural do Paraná – IAPAR-EMATER, 86047-902, C.P. 10030, Paraná, Londrina, PR.

[6]INRAE, Université Côte d'Azur, CNRS, ISA, Sophia Antipolis, France.

Correspondence

Sheila F. Almeida. Departamento de Fitopatologia, Universidade de Brasília (UnB), 70910-900, Brasília, DF, Brazil. Email: sheilafreitas92@hotmail.com

ORCID

Sheila F. Almeida https://orcid.org/0000-0003-3815-7452

Abstract: A new root-knot nematode (RKN) *Meloidogyne izalcoensis* was detected on coffee in Brazil. This species was first described on coffee from El Salvador and later detected in Africa. There are no reports on the genetic diversity of this species. The genetic resistance of coffee to RKN is considered one of the main nematode management strategies and the resistance to *M. izalcoensis* has not yet been studied in coffee cultivars. The objectives of this study were to assess the genetic variability of five *M. izalcoensis* populations from different geographic locations and their phylogenetic relationships based on distinct regions of ribosomal DNA (rDNA), mitochondrial gene cytochrome *c* oxidase II (COII) and nuclear protein coding gene HSP90, and to evaluate the reaction of different coffee genotypes resistant to other RKN species to the Brazilian population of *M. izalcoensis*. All populations were identified by esterase phenotype and SCAR specific markers. Based on RAPD and AFLP markers, a low intraspecific variability was detected among *M. izalcoensis* populations from Africa, Vietnam and Brazil, except for the population from El Salvador that showed fewer genetic differences from the other populations. Phylogenetically, all populations of *M. izalcoensis* from different locations (El Salvador, Kenya, Tanzania, Vietnam and Brazil) grouped with 90% and 69% bootstrap support for COII and HSP90 regions, respectively, indicating that these markers are highly conserved for the species. In addition, both markers allowed the separation between *M. izalcoensis* populations and other important coffee *Meloidogyne* species, including *M. exigua*, *M. paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*. Considering the resistance studies, four separated assays were performed with the objective of evaluating the reaction of fifteen coffee genotypes to *M. izalcoensis*, using the same inoculum concentration and arranged in a completely randomized design with eight replications. Overall, it was observed susceptibility of the genotypes tested with resistance to other *Meloidogyne* spp. (Amphillo x Catuaí, Hybrid of Timor, IAPAR 59,

IPR 99, IPR 100, IPR 102, IPR 103, IPR 105, IPR 106, IPR 107 and IPR 108), except for the rootstock cv. Apoatã IAC 2258 which proved to be moderately resistant, with a genetic segregation for this character of 43.8%.

K E Y W O R D S

AFLP, COII, *Coffea*, genetic variability, RAPD, resistance, root-knot nematode.

INTRODUCTION

Root-knot nematodes (RKN) *Meloidogyne* spp., are amongst the most economically important plant parasitic nematodes infecting coffee (*Coffea* spp.) in several countries in the Americas, including Brazil, El Salvador, Guatemala, Costa Rica and Hawaii, in Africa and in other continents (Campos and Villain, 2005; Villain et al., 2018). Recently, a new species *Meloidogyne izalcoensis* Carneiro, Almeida, Gomes and Hernandez, 2005 was detected on coffee in Brazil (Stefanelo et al., 2018). This species was first described from a population collected in El Salvador where it causes substantial plant damage (Carneiro et al., 2005a; Villain et al., 2018) and detected in Africa in Kenya (Kabete) and Tanzania (Mufindi) without damage information (Jorge Júnior et al., 2016). *Meloidogyne izalcoensis* causes small galls with external egg masses and necrosis at root ends (Figure 1), showing different root symptoms when compared with other *Meloidogyne* spp. from coffee in Brazil (Carneiro et al., 2005a; Stefanelo et al., 2018).

The morphological diagnosis of *M. izalcoensis* is difficult due to the similarity of its perennial pattern to that of *M. incognita* and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos and Almeida, 1996 (Carneiro et al., 2005a). Biochemically, the esterase (Est I4) phenotype is unique and can be used to differentiate *M. izalcoensis* from other RKN species of coffee (Carneiro and Cofcewicz, 2008).

Application of molecular tools, particularly neutral molecular markers, e.g., random amplified polymorphic DNA – RAPD, amplified fragment length polymorphisms – AFLP have been extensively used to analyze the genetic diversity within *Meloidogyne* spp. from coffee, *i.e.*, *M. exigua* Göldi, 1887, *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 and *M. paranaensis* (Carneiro et al., 2004, 2008; Muniz et al., 2008; Randig et al., 2002; Santos et al., 2012, 2018). Phylogenetic analyzes of coffee RKN using molecular data such as rDNA and mt-DNA sequences have been used as

characterization methods for different species, and allowed solving evolutionary processes at deep levels of divergence (Humphrey-Pereira et al., 2014; Janssen et al., 2017; Santos et al., 2019). However, until now there are practically no reports on the genetic variability and phylogenetic diversity of *M. izalcoensis*. Among genomic sequences of interest, species-specific SCAR markers were developed for the identification of Brazilian *Meloidogyne* spp. from coffee: *M. exigua*, *M. incognita* and *M. paranaensis* (Randig et al., 2002) and more recently for *M. izalcoensis* and *M. arabicida* Lopez and Salazar, 1989 (Correa et al., 2013), extending the molecular markers for all RKN species from the Americas, except for *M. lopezi* Humphreys-Pereira, Flores-Chavez, Gomez, Salazar, Gomez-Alpizar and Elling, 2014 described later. Also, the gene encoding heat shock protein 90 (Hsp90) has been increasingly used in phylogenetic studies of plant-parasitic nematodes (Mundo-Ocampo et al., 2008; Skantar and Carta, 2004; Skantar et al., 2008) or for species identification (Skantar et al., 2008), although sequence divergence among nematode species is generally lower than mitochondrial gene differences reported for the same taxa (Skantar and Carta, 2004).

The genetic resistance of coffee is considered one of the main nematode management strategies, since it enables the reduction of nematode population density in the soil and acceptable economic maintenance of crop yield in infested areas (Villain et al., 2018). The main focus to produce nematode resistant rootstocks was in *C. canephora* Pierre ex A. Froehner, *C. liberica* Hiern, *C. dewevrei* De Wild. and T. Durand, and *C. congensis* A. Froehner, since these species present abundant root systems and resistance to pathogens as well. However, resistance genes found in wild or semi-wild lines of *C. arabica* L. from Ethiopia (Fatobene et al., 2017; Holderbaum et al., 2020), as well as accessions of Amphillo could be used in intraspecific hybridization with other *C. arabica* with good agronomic characteristics (Peres et al., 2017).

Most sources of resistance to RKN have been identified in *C. canephora* (Bertrand et al., 2000; Sera et al., 2006). Three cultivars of *C. canephora* are commercially available to be used as rootstocks for resistance to *M. exigua*, *M. incognita* and *M. paranaensis*: 'Apoatã' IAC 2258, 'Nemaya' and 'Goytacá' (Clone 14) and can receive grafts of *C. arabica* and *C. canephora*. These robusta cultivars are highly recommended for planting in extensive *Meloidogyne* spp. infested areas (Ferraz 2008; Fonseca et al., 2008; Gonçalvez et al., 2021; Lima et al., 2015,). Timor hybrid, which is phenotypically an arabica coffee is probably a natural hybrid between *C. arabica* and *C. canephora* that has frequently been used in the Brazilian genetic breeding programs as a source of resistance to *Meloidogyne* spp. Among its derivates are the cultivars Obatã IAC 1669-20, 'Tupi' IAC 1669-33, IAC 125 RN and IAPAR -59, which are resistant to *M. exigua* and to coffee leaf rust caused by the fungus *Hemileia vastatrix* Berk. and Broome (Fazuoli et al., 2018; Ferraz, 2008; Muniz et al., 2009; Salgado et al., 2005). Progenies of 'Icatu Vermelho IAC 4160'resulted from crosses between *C. arabica* and *C. canephora* have been rated as resistant to *M. paranaensis* under greenhouse and field conditions (Ferraz, 2008). An important source of resistance to *M. paranaensis* used by some breeding programs in Brazil was Icatu IAC 925 (Shigueoka et al., 2016), which was used to develop the cultivar (IPR 106) (Sera et al., 2020). For *C. arabica*, most commercial cultivars are highly susceptible to coffee RKN, with the exception of cultivars selected by coffee breeders Acauã, Catucaí 785-15, IAPAR 59, IAC 125 RN, IPR 100, IPR 106, MGS Catiguá, MGS Guaiçara and MGS Vereda, which are available in Brazil to be recommended for areas infested with RKN, depending on the identified nematode species in the planting area. These genotypes have different sources of resistance (Salgado et al., 2021; Villain et al., 2018).

The first objective of this study was to assess the genetic variability of populations of *M. izalcoensis* originating from different geographic locations in the world (El Salvador, Kenya, Tanzania, Vietnam and Brazil) using neutral DNA markers, and their phylogenetic relationships using distinct regions of ribosomal DNA (rDNA), the mitochondrial cytochrome *c* oxidase II (COII) gene and the nuclear protein coding gene HSP90. The second aim of this study was to evaluate the reaction to the Brazilian population of *M. izalcoensis* of different coffee cultivars harbouring resistance genes to other *Meloidogyne* spp.

MATERIALS AND METHODS

Nematode populations

Five *M. izalcoensis* populations from different geographic locations in the world (El Salvador, Kenya, Tanzania, Vietnam and Brazil) were maintained by periodic subculturing on tomato plants (*Solanum lycopersicum* L. cv. Santa Clara) under greenhouse conditions (Table 1). All genetic and phylogenetic studies were performed using these populations. They were identified using esterase (Est) phenotyping, according to Carneiro and Almeida (2001) and confirmed with SCAR-PCR (Correa et al., 2013). One population of *M. enterolobii* Yang and Eisenback, 1983 was included in the study as an outgroup.

Genomic DNA preparation

For each population, total genomic DNA was extracted and purified from 100 µl aliquots of eggs following the method described by Randig et al. (2002). Isolated DNA was quantified in a 1% agarose gel.

RAPD analysis

RAPD-PCR reactions were performed in a 13 µl final volume containing 1.3 µl 10× PCR reaction buffer (Invitrogen®), 0.4 µl 10 µM primer, 2 µl 1.25 mM dNTPs (Invitrogen®), 0.2 µl5U µl −1 Taq DNA polymerase (Invitrogen®) and 3 µl total genomic DNA (3 ng µl −1) of each population. The following 30 random 10-mer oligonucleotide primers (Operon Technologies) were used in the analysis: OPA-12 (TCGGCGATAG), OPAB-06 (GTGGCTTGGA), OPAU-13 (CCAAGCACAC), OPC-07 (GTCCCGACGA), OPC-09 (CTCACCGTCC), OPF-06 (GGGAATTCGG), OPG-04 (AGC GTG TCT G), OPG-13 (CTCTCCGCCA), OPJ-19 (GGACACCACT), OPK-01 (CATTCGAGCC), OPK-01 (CATTCGAGCC), OPK-19 (CACAGGCGGA), OPL-19 (GAGTGGTGAC), OPM-20 (AGGTCTTGGG), OPN-07 (CAGCCCAGAG), OPN-10 (ACAACTGGGG), OPP-01 (GTAGCACTCC), OPP-06 (GTGGGCTGAC), OPR-03 (ACACAGAGGG), OPR-07 (ACTGGCCTGA), OPT-06 (CAAGGGCAGA), OPU-05 (TTGGCGGCCT), OPV-07 (GAAGCCAGCC), OPW-05 (GGCGGATAAG), OPW-06 (AGGCCCGATG), OPW-15 (ACACCGGAAC), OPX-20 (CCCAGCTAGA), OPY-05 (GGCTGCGACA), OPY-16 (GGGCCAATGT), OPZ-04 (AGGCTGTGCT) and OPZ-17 (CCTTCCCACT). Amplifications were performed on a PTC-100 thermocycler, using the following settings: 5 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 36°C, 2 min at 70°C; and a final extension of 10 min at 70°C (Randig et al., 2002). PCR products were separated by electrophoresis in a 1.5% (w/v) agarose gel, stained with ethidium bromide (0.3 µg ml−1) and visualized under UV light. All RAPD analyses were repeated at least twice.

AFLP analysis

For each isolate, 1 µg of total genomic DNA was digested overnight at 37°C with EcoRI (15 U μl^{-1}; Invitrogen®) and ligated to the specific adapters following the method of Suazo and Hall (1999). A series of seven random 22-mer primers (Integrated DNA

Technologies) were used, consisting of the EcoRI adapter core sequence 5 -GACTGCGTACCAATTCAGT-3 plus the 3 selective nucleotides (AGT, ACT, ATT, GGC, CAG, CCT, and TCG). PCR reactions were performed in a 25 µl final volume containing 1 µl (50 ng μl^{-1}) digested DNA, 2.5 µl 10× PCR buffer without magnesium chloride (Invitrogen), 1 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 1 µl 10 µM primer and 0.3 µl Taq DNA polymerase (5 U μl^{-1}; Invitrogen). DNA was amplified using the PT-C100 thermocycler (MJ Research) under the following cycling parameters: 1 min at 95°C; 37 cycles of 1 min at 94°C, 1 min at 56°C, 2 min and 30 s at 72°C; and a final extension of 10 min at 72°C (Suazo and Hall, 1999). PCR products were separated by electrophoresis in a 1.5% (w/v) agarose-synergel (0.7% agarose, 0.4% synergel; Diversified Biotech), stained with ethidium bromide (0.3 µg ml^{-1}) and photographed under UV light. The analysis was repeated at least twice.

Genetic diversity of *Meloidogyne izalcoensis*

DNA fingerprints obtained with RAPD and AFLP markers were used to infer the genetic diversity of the five populations of *M. izalcoensis* plus one population of *M. enterolobii* used as an outgroup. For each marker, amplified bands were scored as present or absent from the digitized photographs of the gels and converted into a 0-1 binary matrix. Phylogenetic reconstruction was performed using the Neighbour-Joining (NJ) algorithm (Saitou and Nei, 1987) in PAUP∗ version 4b10 (Swofford, 2002), considering the data as unordered with no weighting. Testing of node support for the resulting trees was performed on 1000 bootstrap replicates with a cut-off value of 50%. Since the two types of markers could be considered independent from one another, the two datasets were analyzed into a global NJ analysis using the total evidence approach proposed by Huelsenbeck et al. (1996) with the same settings as for the individual NJ analyses.

Phylogenetic analysis of DNA regions

For the phylogenetic analyses, DNA of the five previously characterized populations of *M. izalcoensis* was amplified and investigated using the primers for ITS1-5.8S-ITS2 region of rRNA (primer set: forward 5-TTGATTACGTCCCTGCCCTTT-3 and reverse 5-TCCTCCGCTAAATGATATG-3; Schmitz et al., 1998); the D2-D3 fragment of the 28S rRNA gene (primer set: forward 5-ACAAAGTACCGTGAGGGAAAGTTG-3 and reverse 5-TCGGAAGGAACCAGCTACTA-3; De Ley et al., 1999); mtDNA cytochrome *c* oxidase II (COII) gene (forward 5'-GGTCAATGTTCAGAAATTTGTGG-3' and reverse 5'-TACCTTTGACCAATCACGCT-3'; Powers and Harris, 1993) using the PCR conditions described by Subbotin et al. (2000) and HSP90 (primer set: forward 5'-GCYGATCTTGTYAACAACCYTGGAAC-3' and reverse 5'-TCGAACATGTCAAAAGGAGC-3' PCR conditions according to Nischwitz et al. (2013). PCR products were cleaned using the Wizard® SV Gel/PCR Clean Up System (Promega) and cloned into the pGem-T® Easy Vector (Promega), following the manufacturer's instructions. Sequencing of the insert was carried out on two independent clones by Macrogen.

Meloidogyne izalcoensis populations cloned sequences were aligned using ClustalW in Mega 5.0.3 (Tamura et al., 2011) and compared with other retrieved sequences from the NCBI database of important coffee *Meloidogyne* species, *i.e. M. exigua*, *M. paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*. The program MrModeltest (Nylander, 2004) implemented in PAUP* was used to identify the best-fit models of each analysis. The phylogenetic trees were generated based on Maximum Likelihood (ML) analysis in IQtree (Trifinopoulos et al., 2016). The phylogram was bootstrapped 1,000 times, and only support values above 50% were considered.

Resistance assays

The inoculum of *M. izalcoensis* used in the experiments was multiplied in tomato and coffee plants for 3 and 8 months, respectively. The eggs of *M. izalcoensis* were extracted from infected roots according to the protocol described by Hussey and Barker (1973), using a blender for 40 seconds instead of hand shaking. Plants of coffee were sown in trays and later transplanted to polyethylene pots (20x40x0,015 cm and capacity for 5 L), filled with a mixture of autoclaved soil and Bioplant® compost (1:1). When they hit 4 to 6 pairs of leaves they were inoculated with 10,000 eggs of *M. izalcoensis*/plant.

Fifteen coffee genotypes were tested: Catuaí Amarelo IAC 62 and Catuaí Vermelho IAC 99 (susceptible controls), Catuaí Vermelho x Amphillo 2-161 (28-2-II), Catuaí Vermelho x Amphillo 2-161 (16-5-III), Hybrid of Timor UFV 408-01 MG (6-I-III), IAPAR 59, IPR 99, IPR100, IPR 102, IPR 103, IPR 105, IPR 106, IPR 107, IPR 108 and Apoatã IAC 2258 (*C. canephora*) (Table 2). The plants were arranged in a completely randomized design with eight replications for each experiment. Four experiments were conducted under greenhouse conditions at Embrapa Genetic Resources and Biotechnology (Cenargen, Brasilia, Brazil): the first one was implanted on 3/27/2018, the second on 4/11/2019 (repetition), the third and fourth on 3/18/2020 and 6/12/2020, respectively.

The evaluation of the four experiments was performed at 240-350 days after inoculation (DAI). The following variables were determined: fresh root weight, gall index (GI), total number of eggs per gram of root, total number of eggs, reproduction factor (RF) and global reaction. Before evaluating the trials, some supplementary control plants were always analyzed to determine whether the reproduction factor was high enough to evaluate the bioassays (Hussey and Jansen, 2002).

To determine fresh root weight, roots were carefully washed to remove adhered soil and, after excess water was removed, they were weighed. The root systems were

stained with Phloxine B at 0.0015 % (15 mg/L) for 15-20 minutes and evaluated for gall index. The gall index (GI) was determined based on a 0 to 5 scale proposed by de Hartman and Sasser (1985), as follows: 0 = absence; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100, and 5 = more than 100 galls per plant. The extraction of eggs was performed according to the methods mentioned above, using 1% NaOCl. The total number of eggs per plant was determined under a light microscope using Peters' slides in triple count.

The reproduction factor (RF) was calculated as RF = FP/IP, where FP corresponds to the final nematode population and IP refers to the initial nematode population (IP = 10,000), according to Oostenbrink (1966). Genotypes for which RF < 1 were considered resistant (R), while those for which RF > 1 were considered susceptible (S), according to Sasser et al. (1984). Genotypes were also scored using intermediate criteria as: low susceptibility (LS), susceptibility (S), high susceptibility (HS) and moderate resistance (MR), using statistical analysis. Data were transformed in log (x+1) prior to analysis of variance, and treatments were compared using Scott-Knott test (P<0,05), using the software SISVAR (Ferreira, 2011).

RESULTS

Characterization of nematode populations

The esterase phenotype (Est I4, Rm: 0.86, 0.96, 1.24, 1.30) allowed the identification for all *M. izalcoensis* populations (Figure 2), as reported in the description of Carneiro et al. (2005a). To confirm the identification, the specific SCAR marker developed for *M. izalcoensis*, i.e., a single amplicon of 670 bp, was obtained for all the *M. izalcoensis* populations (Iza 1-5) used in this study (Figure 3).

Genetic diversity of *Meloidogyne izalcoensis*

For the study on genetic diversity, a total of 7 AFLP and 30 RAPD primers were used. The sizes of amplified fragments ranged from 200 to 4500 bp and the cumulative number of reproducible amplified fragments was 306 for the two markers. Overall, there was a low level of polymorphism in the *M. izalcoensis* populations (22%) (Figure 4). The 0-1 binary matrix (absence/presence of fragments) obtained from the entire set of markers was used to infer the genetic relationships among the isolates. The results from the NJ dendrogram confirmed a low intraspecific variability among *M. izalcoensis* populations from Africa (Kenya and Tanzania), Vietnam and Brazil, with 91% bootstrap, while the population from El Salvador exhibited a few genetic differences from the other populations (Figure 4).

Phylogenetic analysis of DNA regions

In the phylogenetic analysis obtained from the intergenic ITS1-5.8S-ITS2 gene and the D2D3 region of the 28S gene, respectively, *M. izalcoensis* populations did not form cohesive clusters (Figure 5a and 5b), suggesting a poor phylogenetic resolution of these sequences.

However, for the two other sequences, all populations of *M. izalcoensis* from different origins grouped together with 90% and 69% bootstrap support for COII and Hsp90 regions, respectively (Figures 6 and 7), indicating that these markers are highly conserved for this species. In addition, both markers allowed the separation between *M. izalcoensis* populations and other important RKN coffee species, including *M. exigua*, *M. paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*, showing to be excellent specific markers for these species.

Resistance and susceptibility assays

In the four assays (Tables 3, 4, 5 and 6), the differences of fresh root weight between all cultivars, especially the Apoatã rootstock and cv IPR 103 were evidenced by

304 statistical analysis, these differences are not very important since plants genetically very
305 different were compared.
306 In the first experiment (Table 3), regarding the number of eggs per gram of root,
307 only the genotype Catuaí Vermelho x Amphillo 2-161 (16-5-III) and the rootstock Apoatã
308 IAC 2258 differ statistically from the control. When considering the reproduction factor
309 (RF), the genotypes IPR 100, Hybrid of Timor and Catuaí Vermelho x Amphillo 2-161
310 (28-2-II) did not differ statistically from the control and were considered highly
311 susceptible (HS) to *M. izalcoensis*. In contrast, the genotype Catuaí Vermelho x Amphillo
312 2-161 (16-5-III) and Cv. Apoatã IAC 2258 differed statistically from the control and are
313 considered susceptible and moderately resistant with a RF reduction of 83 %,
314 respectively.
315 In the repetition of the first experiment (Table 4), the results were similar to those
316 of the first trial, with no differences in the reaction between cultivars. Again, the 'Apoatã
317 rootstock' was the only one that proved to be moderately resistant. Analyzing the 16
318 individual replications of cv Apoatã, a genetic segregation of 43.8% occurred, with RFs
319 values ranging from 9.8 to 0, showing susceptible and resistant plants in the same
320 treatments (data not shown).
321 In the third and fourth experiments (Tables 5 and 6), the genotypes IAPAR 59,
322 IPR 102, IPR 105, IPR 106, IPR 107, IPR 108 and IPR 99 showed a higher number of
323 eggs per gram of root when compared to the control. In the third experiment, there was
324 no statistical difference between the cultivars IPR 100, IPR 103 and the control treatment
325 regarding the number of eggs per gram of root (Table5). As the reproduction factor (RF)
326 is concerned, the genotypes IAPAR 59, IPR 100, IPR 102, IPR 105, IPR 106, IPR 108
327 and IPR 99 did not differ statistically from the control and were considered susceptible to
328 *M. izalcoensis*. In both experiments, the IPR 103 genotype had the lower RF when

329 compared to all other treatments and was considered with low susceptibility (LS).
330 Probably due to the smaller size of the roots (1/5 of the control), the reproduction factor
331 was smaller, since this parameter depends on the total number of eggs/plant, which
332 depends on the root size.

333 The gall index was the same (GI = 5) for all genotypes in the four experiments,
334 except for the rootstock cv. Apoatã IAC 2258, which presented GI=4 in experiment 1
335 (Table 3) and GI=3 in experiment 2 (Table 4). All coffee cultivars and genotypes tested
336 showed characteristic symptoms of *M. izalcoensis*: presence of small rounded galls,
337 usually in greater numbers on younger roots, necrosis at the root tip and external egg
338 masses. Nevertheless, Apoatã IAC 2258 showed typical symptoms of *M. izalcoensis* in
339 some plants evaluated, whereas in other plants these symptoms were not observed (data
340 not shown). This behavior is probably related to the segregation present in this cultivar as
341 commented above.

342 Most genotypes evaluated in the four experiments (Catuaí Amarelo 62, Catuaí
343 Vermelho IAC 99, Hybrid of Timor HT UFV 408-01 MG 0294 pl.1 R1 (6-I-III), Catuaí
344 Vermelho x Amphillo 2-161 MG 0179 pl3 R1 (28-2-II), Catuaí Vermelho x Amphillo 2-
345 161 MG 0179 pl1 R1 (16-5-III), IAPAR 59, IPR 99, IPR 100, IPR 102, IPR 105, IPR
346 106, IPR 107 and IPR 108) showed susceptibility to *M. izalcoensis*. Only the rootstock
347 cv. Apoatã IAC 2258 can be considered as moderately resistant with some plants
348 segregating for this character (data not shown).

349

350 **DISCUSSION**

351 Intraspecific variation and genetic structure are not well characterized for *M.*
352 *izalcoensis* populations, but such information is crucial to avoid misidentification and to
353 optimize effective control strategies (Monteiro et al., 2016). Genetic studies along with

354 traditional taxonomic methods integrated with enzymatic and molecular approaches
355 proved to be useful in solving problems of nematode identification (Santos et al., 2012;
356 2018). This is the first study on genetic and phylogenetic characterization of different
357 populations of *M. izalcoensis,* a species from coffee recently detected in Brazil (Stefanelo
358 et al., 2018).

359 The development of molecular techniques has opened new prospects for
360 identification of *Meloidogyne* species and for studies on intraspecific variability and
361 phylogenetic relationships in distinct regions of ribosomal DNA (rDNA), mitochondrial
362 gene cytochrome *c* oxidase II (COII) and nuclear protein coding gene (Hsp90) for RKNs
363 (Carneiro et al., 2004; Leite et al., 2020; Randig et al., 2002; Santos et al., 2012; 2018).
364 SCAR markers have been developed for RKNs often associated with coffee in Brazil and
365 the Americas (Correa et al., 2013; Randig et al., 2002), *i.e. M. exigua, M. paranaensis*
366 and *M. incognita* (Randig et al., 2002) and for the new species *M. izalcoensis* and *M.*
367 *arabicida* (Correa et al., 2013). In the present study, this approach was validated by
368 analyzing five *M. izalcoensis* populations from different geographic locations in the world
369 and exhibiting the same esterase phenotype (Est I4). While esterase isozyme phenotypes
370 are restricted to the characterization of RKN females (Carneiro and Cofcewicz, 2008),
371 PCR-based methods are more suitable for routine diagnosis, especially, in coffee roots
372 infected with *Meloidogyne* spp., where females collected from fields are frequently in bad
373 conditions of preservation, making the identification difficult when using esterase
374 isozyme only (Muniz et al., 2008). PCR is fast, can be used on a large number of samples
375 and can be applied to eggs and J2 from field soil samples and roots. It also does not require
376 nematode multiplication on the host plant until they reach female adult stages (Carneiro
377 et al., 2000).

Neutral molecular markers, such as RAPD and AFLP, have been used to analyze the genetic diversity of *Meloidogyne* species from coffee (Carneiro et al., 2004; Muniz et al., 2008; Randig et al., 2002; Santos et al., 2012; 2018). Here, using these markers, a low genetic variability was found among five *M. izalcoensis* populations from different geographic locations in the world. This low genetic variability was also observed for other *Meloidogyne* spp. related to coffee, *i.e*., *M. incognita* (Santos et al., 2012) and *M. paranaensis* (Santos et al., 2018). However, the opposite was observed among populations of *M. exigua*, among which a high level of genetic variability was observed (Muniz et al., 2008). This result is to be linked to the mode of reproduction of the nematodes, i.e., mitotic pathenogenesis for *M. incognita*, *M. paranaensis* and M. izalcoensis, and meiotic parthenogenesis for *M. exigua.*

Sequences of ITS1-5.8S-ITS2, D2-D3 (28S) rRNA, mitochondrial COII and HSP90 genes have been used to infer phylogenetic relationships in RKN species and may allow evolutionary processes to be solved at deep levels of divergence (Humphreys-Pereira et al., 2014; Janssen et al., 2017; Santos et al., 2019). However, in the present study, an overall lack of polymorphic sequences was noticed for *M. izalcoensis* populations.

The ribosomal DNA is relatively conserved with high similarity among the three most common tropical root-knot nematode species, *M. incognita*, *M. arenaria* (Neal, 1889) Chitwood, 1949 and *M. javanica* (Treub, 1885) Chitwood, 1949, and is therefore not useful as a barcode region for identifying these species (Janssen et al., 2017; Ye et al., 2015). Despite this limitation, it can be an useful tool for identification of other species such as *M. chitwoodi* Golden, O'Bannon, Santo and Finley, 1980 and *M. fallax* Karssen, 1996 (Onkendi and Moleleki, 2013; Ye et al., 2015). The combined analyses of 18S and 28S nuclear rDNA sequences also distinguished haplotypes of *Meloidogyne* species on

403 coffee (Herrera et al., 2011). The multi-copy basis of rDNA provides a sample target for
404 PCR amplification, and it allows the intraspecific variation to be detected in *Meloidogyne*
405 spp. populations (Adam et al., 2007; Zijlstra et al., 1995). However, in the analyzes
406 obtained from the D2D3 regions of the 28S and intergenic ITS1-5.8S-ITS2 gene from the
407 rDNA, *M. izalcoensis* populations did not form cohesive clusters, demonstrating a
408 phylogenetic limitation of these markers.

409 In complement, the region encoding the HSP90 gene has also been used to
410 distinguish different *Meloidogyne* species (Blouin, 2002; Onkendi and Moleleki, 2013;
411 Skantar et al., 2008; Tigano et al., 2005). Here, a low diversity was reported for HSP90
412 sequences among *M. izalcoensis* populations.

413 From the perspective of identification, the mitochondrial genome provides a rich
414 source of genetic markers for identification (Hu and Gasser, 2006). This region has a
415 faster rate of evolution than the corresponding nuclear genes (Blok and Powers, 2009). In
416 fact, the mitochondrial coding region is already considered a better potential barcode
417 region for some *Meloidogyne* spp. groups, since intergenic regions have been shown to
418 contain AT repeats that appear not to correlate with speciation events (Pagan et al., 2015).
419 This region has been used for diagnosis of *Meloidogyne* spp. (Blok et al., 2002; Brito et
420 al., 2004; Powers and Harris, 1993). In the present study, *M. izalcoensis* COII sequences
421 proved to be useful for both species identification and phylogenetic studies, as observed
422 for some other RKN coffee species, *i.e.*, *M. paranaensis* (Santos et al., 2019). Indeed, this
423 marker allowed the separation between *M. izalcoensis* populations and other important
424 *Meloidogyne* species of coffee, including *M. exigua*, *M. paranaensis*, *M. incognita*, *M.*
425 *arabicida* and *M. lopezi*.

426 Considering coffee resistance to *M. izalcoensis*, our study both confirmed
427 previous results and provided new information. Similar results to those obtained here for

428 the Timor Hybrid UFV 408-01 have been reported by Carneiro et al. (2008), testing the
429 H419-5-4-5 progeny obtained from artificial crossing between the cultivar Catuaí
430 Amarelo IAC 30 X Timor UV 445-46, which was susceptible to *M. izalcoensis* but
431 resistant to *M. exigua*. Hernandez *et al.* (2004) did not observe resistance to *M. izalcoensis*
432 in cultivars of *C. arabica* from Ethiopia, but resistance was observed to *M. paranaensis*
433 (Boisseau et al., 2009). The same occurred with the genotypes whose source of resistance
434 are 'Amphillo' (*C. arabica* from Ethiopia) (Peres et al., 2017). Probably, resistance from
435 'Amphillo' is not efficient in controlling *M. izalcoensis*, altough it controlled *M.*
436 *paranaensis* in greenhouse (Peres et al., 2017) and under field conditions (Salgado et al.,
437 2014).

438 Some of the genotypes or crosses evaluated in this study with the Hybrid of Timor
439 as resistance source, like IAPAR 59, have already been tested for other *Meloidogyne*
440 species, in greenhouse conditions and were shown resistant to *M. exigua* and susceptible
441 to *M. incognita* and *M. paranaensis* (Muniz et al., 2009). Unfortunately, in this study the
442 five genetic materials tested with this resistance source mentioned in Table 2 were
443 susceptible to *M. izalcoensis.*

444 Other studies had shown that the rootstock cultivar Apoatã IAC 2258 is resistant
445 to *M. paranaensis* (Fonseca et al., 2008; Santos et al., 2018), *M. exigua* (Salgado et al.,
446 2005) and *M. incognita* (Fonseca et al., 2008; Sera et al., 2006). Since 1987, the adoption
447 of such multi-resistant rootstock cultivars has been recommended for nematode
448 management in infested coffee-growing areas, being justified in field conditions because
449 more than one species of *Meloidogyne* may occur simultaneously (Carneiro et al., 2005b;
450 Sera et al., 2017). The resistance of this rootstock originates from *C. canephora* (clone
451 T3561) belonging to the CATIE germplasm collection from Costa Rica (Bertrand and
452 Anthony, 2008). In this study, we observed only moderate resistance of the cultivar

Apoatã IAC 2258 to *M. izalcoensis*, with confirmed segregation. The same was observed for different populations of *M. paranaensis* in greenhouse conditions (12% segregation, Santos et al., 2018). Although the use of resistant cultivars or rootstocks is an environmentally correct and efficient control method, it shows limitations, such as segregation for susceptibility to nematodes (10 to 15%), breakage in the grafting region and need of replanting (about 10 to 15%) (Gonçalves and Silvarolla, 2007).

All the cultivars developed by IDR-Paraná evaluated in this study are considered susceptible to *M. izalcoensis*, although, in other studies, some of these cultivars showed resistance to other *Meloidogyne* species, and represent an important tool, as they enable the reduction of nematode population density in an economically acceptable way (Barrantes et al., 2020). The Arabica cultivar IPR 100 carrying genes from *C. liberica* (Sera et al., 2017) is resistant to *M. exigua* (Rezende et al., 2017), *M. paranaensis* (Andreazi et al., 2015; Santos et al., 2018; Sera et al., 2017) and *M. incognita* populations (Sera et al., 2017). The resistance of cultivar IAPAR 59 to different populations of *M. exigua* was observed by Muniz et al. (2009) and Salgado et al. (2005), but this cultivar is susceptible to *M. paranaensis*, *M. incognita* (Muniz et al., 2009) and *M. izalcoensis* (this study). There is evidence that IAPAR 59 resistance to *M. exigua* originates from the Timor Hybrid, since the resistance to this nematode has been shown to be controlled by one major gene in this genotype (Noir et al., 2003) with incomplete dominance (Alpizar et al., 2007). Barrantes et al. (2020) showed that the cultivars IPR 99, IPR 100, IPR 102, IPR 103 and IPR 107 also showed resistance to *M. exigua* from Costa Rica. Despite the cultivar IPR 106 has not shown resistance to *M. izalcoensis*, the resistance of this cultivar has already been demonstrated to *M. paranaensis* and *M. incognita* (Sera et al., 2020). It is believed that the cultivars IPR 98, IPR 99 and IPR 107 inherited the *Mex-1* gene from the Timor Hybrid CIFC 832/2, as demonstrated for the ‘IAPAR 59’, while cultivars IPR 102 and

IPR 103 would have inherited the resistance of *C. canephora* from Icatu (Barrantes et al., 2020).

Sera et al. (2020) evaluated the performance of Arabica coffee cultivars in area infested by the nematode *M. paranaensis* and *M. incognita* and confirmed that the cultivar IPR 100 and IPR 106 showed the best performance with a high vegetative vigor and yield, while the cultivars IPR 103, IAPAR 59, and Catuaí Vermelho IAC 81 showed reduction in yield production and poor vegetative development of plants with up to 45% of dead plants after seven years of planting.

In studies of genetic diversity of coffee root-knot nematodes, *M. izalcoensis* was separated phylogenetically from all other species (*M. exigua*, *M. paranaensis*, *M. incognita*, *M. arenaria, M. enterolobii* and *M. lopezi*). In this way, it is expected that the sources of genetic resistance to this species are also different. The present study has demonstrated that the sources of resistance of the coffee plants to *M. izalcoensis* are not the same as those previously reported for *M. paranaensis*, *M. incognita* and *M. exigua*. Although some available sources of resistance as the Hybrid of Timor, Amphillo, Icatu IAC 925 and IPR 100 (*C. liberica*) showed susceptibility to *M. izalcoensis*, cultivars as Apoatã IAC 2258, whose source of resistance originates from a pure *C. canephora*, are promising for studying resistance to *M. izalcoensis.* Other pure *C. canephora* genotypes should be studied, such as the cultivar 'Goytacá' (=Clone 14) recently registered by INCAPER, and the polyclonal cultivars from IAC, composed of clones CcK1, CcR2, CcR8 and CcR10, which according to Gonçalves et al. (2021) are resistant to *M. exigua*, *M. paranaensis* and *M. incognita*. Seed fields for commercial production of IAC clones will be established after registration and protection of the clones.

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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 are available from the corresponding author upon reasonable request.

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FIGURES LEGENDS

FIGURE 1. Symptoms caused by the root-knot nematode *Meloidogye izalcoensis* on coffee (*Coffea arabica* cv. Mundo Novo) roots stained with Phoxina B . A) Small round

galls induced by the nematode, egg-mass outside the gall tissue and necrosis at the end of roots. B) Whole root view. Photos: Cláudio Bezerra.

FIGURE 2. Esterase phenotypes of *Meloidogyne izalcoensis* (Est I4). *M. javanica* (Est J3) is included as a reference.

FIGURE 3. PCR amplification patterns for five populations of *Meloidogyne izalcoensis* generated with specific SCAR primer izAB02F/R (Correa et al., 2013). (1-5): *M. izalcoensis,* (-) DNA: negative control. M: 1kb Plus DNA ladder (Invitrogen).

FIGURE 4. Concatenated Neighbor-Joining (NJ) tree showing the analysis of genetic variability (by RAPD and AFLP) of *Meloidogyne izalcoensis* populations. Numbers to the left of the arms are the bootstrap values of 1000 replicates. Population codes detailed in Table 1.

FIGURE 5. Maximum Likehood analysis showing the phylogenetic relationships of *Meloidogyne izalcoensis* populations with related species based on the ITS1-5.8S-ITS2 rRNA sequences (a), and on the D2-D3 fragment of 28S rRNA sequences (b), respectively. Numbers to the left of the branches are bootstrap values for 1000 replications. Populations codes and accession numbers for gene sequences retrieved from Genbank are listed following species names.

FIGURE 6. Maximum Likehood analysis showing the phylogenetic relationships of *Meloidogyne izalcoensis* populations with related species based on the COII-mtDNA sequences. Numbers to the left of the branches are bootstrap values for 1000 replications. Populations codes and accession numbers for gene sequences retrieved from Genbank are listed following species names.

FIGURE 7. Maximum Likehood analysis showing the phylogenetic relationships of *Meloidogyne izalcoensis* populations with related species based on the Hsp90 gene sequences. Numbers to the left of the branches are bootstrap values for 1000 replications.

Populations codes and accession numbers for gene sequences retrieved from Genbank are listed following species names.