The Multi-Resistant Reaction of Drought-Tolerant Coffee ‘Conilon Clone 14’ to *Meloidogyne* spp. and Late Hypersensitive-Like Response in *Coffea canephora*

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ABSTRACT

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Root-knot nematodes (RKN), *Meloidogyne* spp., have major economic impact on coffee production in Central and South America. Genetic control of RKN constitutes an essential part for integrated pest management strategy. The objective of this study was to evaluate the resistance of *Coffea canephora* genotypes (clones) to *Meloidogyne* spp. Sensitive and drought-tolerant coffee genotypes were used to infer their resistance using nematode reproduction factor and histopathology. Eight clonal genotypes were highly resistant to *M. paranaensis*. ‘Clone 14’ (drought-tolerant) and ‘ESN2010-04’ were the only genotypes highly resistant and moderately resistant, respectively, to both *M. incognita* races

3 and 1. Several clones were highly resistant to both avirulent and virulent *M. exigua*. Clone 14 and ESN2010-04 showed multiple resistance to major RKNs tested. Roots of ‘clone 14’ (resistant) and ‘clone 22’ (susceptible) were histologically studied against infection by *M. incognita* race 3 and *M. paranaensis*. Reduction of juvenile (J2) penetration in clone 14 was first seen at 2 to 6 days after inoculation (DAI). Apparent early hypersensitive reaction (HR) was seen in root cortex between 4 and 6 DAI, which led to cell death and prevention of some nematode development. At 12 to 20 DAI, giant cells formed in the vascular cylinder, besides normal development into J3/J4. From 32 to 45 DAI, giant cells were completely degenerated. Late, intense HR and cell death were frequently observed around young females and giant cells reported for the first time in coffee pathosystem. These results provide rational bases for future studies, including prospection, characterization, and expression profiling of genomic loci involved in both drought tolerance and resistance to multiple RKN species.

Root-knot nematodes (RKN), *Meloidogyne* spp., represent major threat to most coffee-growing areas worldwide. Currently, 17 RKN species have been reported as pathogenic to coffee (19,42). Among the most damaging species, *M. exigua* Göldi 1887, *M. incognita* (Kofoid & White), Chitwood 1949, and *M. paranaensis* Carneiro et al. 1996 constitute serious agronomic constraint to coffee due to their wide distribution and damage in Central and South America (17).

Genetic control of RKN is an essential part for integrated pest management strategy since the use of resistant cultivars or rootstocks constitutes an effective, inexpensive, nonpolluting method of control (11). Resistance to *M. exigua* has been sought in coffee germplasm of several countries (Brazil, Colombia, and Central America). To date, no resistance has been found in several *Coffea arabica* cultivars (Caterra, Catuaí, Mundo Novo, among others) or in wild coffee trees collected in Ethiopia (9,13,20,25,26,36). In contrast, several resistant accessions have been identified in *C. canephora* and in some progenies of interspecific hybrids—i.e., *C. arabica* × *C. canephora* (2,10,13,34,53). In *C. arabica*, all cultivars have been considered susceptible to *M. incognita* (33) and *M. paranaensis* (7,8), while numerous wild coffee trees from Ethiopia were considered resistant to *M. paranaensis* (7,8,16).

The resistant rootstocks ‘Nemaya’ (cross between *C. canephora* clones T3561 and T3751) and Apoatã IAC 2258 (*C. canephora* ‘2258’ from the Catie germplasm collection, Turrialba, Costa Rica) were developed to collectively overcome major problems associated with RKN in Central America and Brazil (6,26). *C. arabica* grafted on ‘Nemaya’ or ‘Apoatã’ is the only feasible control measure that allows economic return of coffee planted in areas infested with *M. incognita*, *M. paranaensis*, and *M. arabicida* López & Salazar 1989. However, ‘Apoatã’ and ‘Nemaya’ rootstocks showed intolerance to drought during dry season under field conditions (W. Gonçalves, IAC, São Paulo, Brazil, *personal communication*).

Coffee breeding for durable resistance to RKN is now a major goal in coffee-producing countries. A few years ago, a simply inherited major gene (*Mex-1*) from the related coffee ‘IAPAR 59’ was found to confer resistance to *M. exigua* (46). Upon avirulent RKN infection, *Mex-1*-carrying coffee plants show hypersensitive-like symptoms around 4 to 6 days after inoculation (DAI), preventing formation of most giant cells (3).

The objective of this work was to investigate novel sources of resistance in Conilon coffee to major *Meloidogyne* spp. In this study, using standard pathogenicity assays we showed that Conilon coffee genotype ‘clone 14’, which is drought-tolerant (22,29,48), showed resistance to three highly aggressive RKN species (e.g., *M. exigua*, *M. incognita*, and *M. paranaensis*). We then examined histological alterations in susceptible and resistant coffee genotypes following infection with *M. incognita* and *M. paranaensis* in order

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to assess new defense mechanisms that may operate to resist nematode infection in roots of *C. canephora*.

MATERIALS AND METHODS

Clonal varieties of *C. canephora*. In the first inoculation test we used the clonal variety Vitória-Incaper 8142 released in 2004, including 13 superior clones selected among genetic materials considered elite by Incaper's breeding program (31). The other materials used in this study are also genotypes that showed superior agronomic traits in Incaper's breeding program. Most genotypes are part of one of the eight clonal varieties released and recommended by Incaper. Clones 14 and 120 are drought-tolerant (22,29,48), while clones 109A and 22 are sensitive to drought (29). The 13 clones, ESN2010-01 to ESN2010-13, are promising varieties for breeding programs, including rust resistance (M. A. Ferrão, Incaper, Vitória, ES, Brazil, *personal communication*). The cultivar Catuaí Vermelho IAC 81 was used as susceptible control. In the first pathogenicity assay, we tested clones Vitória-Incaper 8142 (V1 to V13), in addition to clones 14 and 22. The second assay included clones 109A, 120, and ESN2010-01 to ESN2010-13. In a third assay, clones 14 (resistant) and 22 (susceptible) used in the histopathological study were retested in the pathogenicity test.

Meloidogyne spp. populations. Five RKN populations from Brazil were used in this study. Species were identified using esterase phenotypes (Est) (18) and confirmed with sequence characterized amplified region-polymerase chain reaction (50). Races of *M. incognita* were characterized according to Hartman and Sasser (37). *Meloidogyne incognita* (Est I1) race 1 was obtained from Avilândia-SP, *M. incognita* (Est I2) race 3 from Londrina-PR, avirulent *M. exigua* (Est E2) from Lavras-MG, virulent *M. exigua* (Est E1) from Bom Jesus de Itabapoana-RJ, and *M. paranaensis* (Est P1) from Rolândia-PR. *Meloidogyne* populations were maintained on coffee plants 'IAC 15'.

Nematode inoculation. Prior to inoculation, RKN populations were multiplied in tomato (*Solanum lycopersicum* L. 'Santa Clara') for 3 months under greenhouse conditions. Eggs were extracted from infected roots using 0.5% NaOCl, according to Hussey and Barker (39), using a blender instead of manual agitation. For histopathological studies, freshly hatched second-stage juveniles (J2) were collected using a modified Baermann funnel. In both cases, counting was done under a light microscope using Peters' slides.

Experimental design. Inoculated plants were arranged in a completely randomized design with 80 treatments (16 coffee genotypes $\times$ 5 *Meloidogyne* spp. populations) and eight replications. The inoculation assay was carried out in three separate experiments. The cultivar Catuaí Vermelho IAC 81 was used as susceptible check in all assays. In the first assay, clones Incaper 8142 (V1 to V13) and clones 14 (drought-tolerant) and 22 (drought-sensitive) were phenotyped. The second assay included genotype clones 109A, 120, and ESN2010-01 to ESN2010-13. In a third assay, clones 14 (resistant) and 22 (susceptible) used in the histopathological study were reinoculated to ensure their response against challenged nematodes.

Evaluation of nematode resistance in controlled conditions. Plants of each accession were grown in 3 liter pots filled with a mixture (1:1) of autoclaved soil and Plantimax compost under greenhouse conditions. Seedlings of about 15 to 20 cm height were inoculated with 10,000 eggs of *Meloidogyne* spp. by pipetting the nematode suspension around the stem base. Plants were arranged in the design mentioned above and maintained under greenhouse conditions at $\sim$ 25 to 30°C, with watering and fertilization as needed. Eight months after inoculation, the root system was rinsed with tap water and weighed. Roots were stained with phloxine B and evaluated for gall and egg mass numbers (galling index [GI] or egg mass index [EMI]), using a 0 to 5 scale, where 0 = no galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3

to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = >100 galls or egg masses per root system (35).

Eggs were extracted as mentioned above using 1% NaOCl and quantified under a light microscope using Peters' slides. The reproduction factor (RF) was calculated as $RF = FP/IP$, where FP = final nematode population and IP = initial nematode population ($IP = 10,000$). The average RF was transformed as $\log(x + 1)$, submitted to analysis of variance and the means separated using Scott-Knot's test ($P < 0.05$). Accessions for which $RF < 1$ were considered highly resistant (HR), while those for which $RF \geq 1$ were considered moderately resistant (MR) or susceptible (S) (52). The highly resistant cultivar clone 14 and the susceptible clone 22 were retested to corroborate the resistance observed against *M. incognita* race 3 and *M. paranaensis*.

Histopathological observations. Plantlets of the resistant *C. canephora* clone 14 and the susceptible check clone 22 were grown in pots (3 liters) containing washed, sterilized, and weekly fertilized sand. Plantlets were inoculated with 20,000 J2 of *M. incognita* or *M. paranaensis* per plant. Three plantlets each of the resistant and susceptible, checked per time point, were carefully removed from cups at 2, 4, 8, 12, 16, 20, 32, 38, 45, and 50 DAI, and their roots were rinsed with water. A portion of roots from susceptible and resistant plants were stained with acid fuchsin to observe J2 penetration, localization, and subsequent development within roots. After staining, root segments were observed under a stereo microscope and those parts that showed nematode infection were mounted on a glass slide for observation under light microscope (Axiophoto, Zeiss).

Subsamples of roots were embedded in resin to produce thin sections. Root fragments showing galls/swellings or without symptoms were excised under a stereo microscope, fixed, and embedded in Technovit 7100 epoxy resin (Kulzer Friedrichsdorf) as described by Pegard et al. (47) and according to the manufacturer's recommendations. Unstained root sections were mounted on glass slides and fluorescence was observed after UV excitation (UV filter set A2 Zeiss 02; 488002-0000). The same sections were subsequently stained (1 min at 60°C) with 0.5% toluidine-blue in 0.1 M sodium phosphate buffer, pH 5.5, and observed using a light microscope.

RESULTS

Reaction of coffee genotypes to *Meloidogyne* spp. There were no differences among nematode populations and vegetative growth of coffee genotypes: plants showed no obvious above ground symptoms caused by *Meloidogyne* spp. This is probably due to frequent fertilization of coffee plants during the experiments.

Coffee resistance was evaluated based on three criteria: galling index (GI), egg mass index (EMI), and reproduction factor (RF). As expected, the susceptible check Catuaí 'IAC 81' exhibited high number of galls and egg masses, and high rate of nematode reproduction. The GI and EMI (Tables 1 to 7) were sometimes a subjective parameter because some species such as *M. exigua* forms typical galls whereas others do not (e.g., *M. paranaensis* and *M. incognita*). In some cases, egg masses of coffee roots infected with *Meloidogyne* spp., do not extend beyond root tissues and thus are not stained with phloxine B, underestimating the real number of galls. Therefore, these two parameters are useful to describe symptoms but not to evaluate rates of nematode infection. Statistical analysis showed that GI and EMI (*M. exigua*) were correlated with coffee infection (RF) ($r = 0.58$ to 0.71 ; 0.35 to 0.78 , respectively), however, sometimes with low Pearson correlation coefficient. For *M. incognita* and *M. paranaensis*, GI and EMI were not significantly correlated with RF, showing that these pathosystems do not allow evaluation of nematode infection using GI and EMI.

In the first assay, Incaper-Vitória clones 2V, 3V, 6V, 7V, 13V, and clone 14 were highly resistant to *M. paranaensis* ($RF < 1$), while clones 1V, 4V, 5V, 10V, 12V, and clone 22 were susceptible ($RF \geq 1$)

(Table 1). Considering the statistical analysis, clones 8V, 9V, and 11V were considered moderately resistant (MR) to this nematode.

Considering the reaction of coffee genotypes to *M. incognita*, clone 14 was the only one resistant to both races tested (races 1 and 3), whereas clones 2V, 3V, 6V, 8V, and 13V were highly resistant to *M. incognita* race 1 (Table 2). Our results demonstrated that *M. incognita* race 3 was more pathogenic to coffee genotypes than *M. incognita* race 1. In addition, clones 6V and 8V can be considered moderately resistant (MR) to *M. incognita* race 3 and highly resistant (HR) to *M. incognita* race 1, respectively (Table 2).

The avirulent population of *M. exigua* had a low reproduction rate on most clones (RF < 1) (Table 3). Clones 10V and 13V can be

considered MR. Regarding the virulent population of *M. exigua*, genotypes 1V, 2V, 4V, 5V, 7V, 9V, 11V, 12V, and clone 14 were highly resistant (RF < 1) (Table 3). The other clones 3V, 6V, 8V, 10V, 13V, and 22 showed RF ≥ 1 and were considered susceptible or MR.

In the second assay, with cultivar Catuaí Vermelho, the genotype clones 109A and 120, and ES clones N2010-06, ES-07, ES-08, ES-09, ES-10, and ES-12 were susceptible to *M. paranaensis* (Table 4). ES clones N2010-01, 02, 03, 05, and 11 were classified as MR, while genotypes ESN2010-04 and ESN2010-13 were highly resistant (HR) to *M. paranaensis* (Table 4). Regarding *M. incognita* race 3, most genotypes were classified as susceptible, except ES N2010-04 was classified as MR (Table 5). For *M. incognita* race 1, clones ESN2010-04, ESN2010-03, and ESN2010-13 were highly resistant and moderately resistant, respectively, while genotypes 109A, 120, and ESN2010-01, ES-02, ES-05, ES-06, ES-07, ES-08, ES-09, ES-10, ES-11, and ES-12 were all susceptible (Table 5). For the avirulent *M. exigua*, most genotypes were highly resistant (clones 109A, 120, ESN2010-01, 02, 03, 04, 05, 06, 07, 08, 10, 11, 12, and 13) (Table 6). Only 'Catuaí Vermelho' and genotype ESN2010-09 allowed considerable reproduction of the avirulent *M. exigua* population (Table 6). Genotypes inoculated with virulent *M. exigua* population showed similar results. The cultivar Catuaí Vermelho was susceptible, while genotype ESN2010-09 behaved as MR. All other genotypes (109A and 120 and ES clones N2010-01, 02, 03, 04, 05, 06, 07, 08, 10, 11, and 12) behaved as highly resistant (Table 6).

The multi-resistant genotype Conilon clone 14 and clone 22, susceptible to *M. paranaensis* and *M. incognita* race 3, were confirmed with inoculation in the third assay (Table 7).

Histopathology of *M. paranaensis* and *M. incognita* in coffee genotypes. Histological features from ca. 10,000 root sections were observed in both susceptible (Fig. 1) and resistant (Fig. 2) *C. canephora* genotypes inoculated with *M. incognita* race 3 and *M. paranaensis*. Nematode-infected roots were compared with noninoculated controls (data not shown).

The susceptible *C. canephora* clone 22 and resistant clone 14 were chosen for the histopathological observations of resistance in compatible and incompatible interactions, respectively. Because reaction of these clones to both *M. incognita* race 3 and *M. paranaensis* was very similar, only results for the later species are shown.

Examination of acid fuchsin-stained roots and toluidine blue-stained sections showed that J2 were able to penetrate root tips,

TABLE 1. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with *Meloidogyne paranaensis* under controlled conditions

Genotypes ^w	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuaí 'IAC 81'*	23.00	4.50	4.43	29.33 a	S
1V	32.43	4.25	5.00	15.45 a	S
2V	22.37	2.50	0.00	0.25 d	HR
3V	21.69	2.75	0.125	0.60 d	HR
4V	28.00	3.75	4.00	7.96 b	S
5V	41.75	3.75	3.25	8.23 b	S
6V	18.68	2.62	0.125	0.20 d	HR
7V	20.81	2.87	2.25	0.61 d	HR
8V	48.87	3.37	0.125	2.30 c	MR
9V	30.12	3.12	2.125	1.35 c	MR
10V	30.25	4.37	4.00	8.41 b	S
11V	29.44	3.50	3.00	1.53 c	MR
12V	32.93	3.50	4.625	7.38 b	S
13V	18.09	2.37	1.875	0.35 d	HR
Clone 14	16.25	3.00	0.00	0.51 d	HR
Clone 22	32.50	3.50	3.50	15.06 a	S

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. paranaensis*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficient of variation = 22%.

^z Reaction of inoculated coffee genotypes: S = susceptible; HR = highly resistant; and MR = moderately resistant.

TABLE 2. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with *Meloidogyne incognita* races 1 and 3 under controlled conditions

Genotypes ^w	<i>M. incognita</i> race 3					<i>M. incognita</i> race 1				
	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuaí 'IAC 81'*	42.43	4.88	4.87	111.8 a	S	45.75	4.87	5.0	30.83 a	S
1V	52.56	3.13	5.0	34.0 a	S	23.44	4.0	4.25	13.8 b	S
2V	27.87	2.75	4.25	4.08 b	S	38.81	1.75	2.62	0.68 d	HR
3V	42.93	2.5	2.62	6.35 b	S	54.50	2.75	0.37	0.78 d	HR
4V	36.06	2.0	4.37	10.01b	S	20.94	2.87	3.87	11.18 b	S
5V	14.31	3.0	4.87	6.31 b	S	55.56	2.62	4.0	2.38 c	S
6V	24.12	2.75	1.75	2.0 c	MR	14.12	1.87	0.5	0.01 d	HR
7V	45.43	3.5	5.0	10.61 b	S	24.12	2.75	3.12	1.01 c	MR
8V	71.37	3.13	1.5	2.48 c	MR	33.12	2.62	0.0	0.28 d	HR
9V	51.75	3.25	3.75	5.76 b	S	46.12	3.75	3.5	2.35 c	S
10V	43.56	3.63	4.12	9.95 b	S	14.37	3.12	3.75	4.88 b	S
11V	66.00	3.38	3.87	7.73 b	S	59.64	3.12	4.25	5.1 b	S
12V	72.18	4.63	5.0	16.28 a	S	39.12	2.87	4.0	1.53 c	S
13V	45.57	2.0	4.0	6.20 b	S	16.69	2.5	2.25	0.71 d	HR
Clone 14	20.26	2.2	0.5	0.26 d	HR	43.00	2.16	0.0	0.04 d	HR
Clone 22	58.81	3.38	3.87	5.10 b	S	23.64	3.29	4.71	12.05 b	S

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. incognita* races 1 and 3) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficients of variation = 28 and 31%.

^z Reaction of inoculated coffee genotypes: S = susceptible; HR = highly resistant; and MR = moderately resistant.

migrate along with sieve elements, and develop normally after having initiated differentiation into feeding sites (Fig. 1A to F). At 2 DAI, numerous J2 were observed localized in the subapical meristem of roots (Fig. 1A). At 4 DAI, J2 were present in the root cortex (Fig. 1B), probably migrating toward the vascular cylinder, thus causing cell wall damage. At 4 to 8 DAI, numerous J2 were observed within the vascular cylinder (Fig. 1B to C), and feeding J2 were visible inside the vascular cylinder at 8 DAI, close to giant cells (Fig. 1C). At 16 to 32 DAI, numerous J3/J4 had established feeding sites with 10 to 12 well-defined giant cells associated with each nematode (Fig. 1F). Giant cells were hypertrophied, oval-shaped, vacuolated and with dense cytoplasm containing some nuclei (Fig. 1D to F). At this time, enlargement of the vascular cylinder region took place, and small galls were apparent. Giant cells displayed thickened cell walls (Fig. 1D and E). At 32 DAI, adult females were observed associated with severe injuries to the surrounding cells and disruption of root cortex. At 45 DAI, egg masses were observed inside and at root surface.

For the histopathological observations in the resistant *C. canephora* clone 14–*M. paranaensis* interactions, at 2 to 6 DAI, macroscopic observations of cleared roots stained with acid fuchsin revealed a reduced number of J2 in the subapical region compared with the distribution of nematodes in infected susceptible roots. At 4 DAI, infected samples of clone 14 showed that nematodes reached the vascular cylinder; however, no gall was observed at this time. Moreover, only J2 were detected until 6 DAI, indicating that a great majority of invading nematodes were not capable of proceeding to other stages in their life cycle within resistant roots. Fluorescence microscopy using UV excitation of root sections harvested at 8 DAI showed a strong yellowish autofluorescence around nematodes in every infection site examined (Fig. 2B). The same section visualized under bright field microscopy after toluidine-blue staining showed a bluish staining, indicating degradation of cells surrounding nematodes (Fig. 2A), which can be associated with an HR-like response. These HR-like lesions were found around nematodes when they were probably migrating through the cortex at early infection stage (Fig. 2A and B). At 8 DAI, some nematodes were seen close to feeding sites in which the giant cells displayed similar features to those in susceptible roots (Fig. 2C).

The main difference between the susceptible coffee clone 22 and the resistant clone 14 was detected at 12 to 20 DAI, when

giant cells adjacent to nematodes in resistant roots displayed small to large vacuoles that sometimes occupied almost the entire volume of the cells, whereas giant cells in susceptible roots had uniformly dense cytoplasm with less vacuolization. At this time, J2/J4 nematodes were present in the vascular cylinder (Fig. 2C to F). At 32 to 45 DAI, a complete degeneration of giant cells was observed (Fig. 2G to H). Fluorescence microscopy at 20 to 45 DAI, using UV excitation of root sections, showed a strong blue or gray autofluorescence around nematodes and giant cells in all infection sites examined. Observed under bright field after toluidine blue staining, the same sections revealed dark blue regions indicating major alterations of cells (cell death) in contact

TABLE 4. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with *Meloidogyne paranaensis* under controlled conditions

Genotypes ^w	FRM(g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuaf 'IAC 81'*	29.58	4.17	4.33	11.66 a	S
Clone 109A	136.83	5.0	5.0	16.64 a	S
Clone 120	100.0	4.8	3.8	4.13 b	S
ES N2010-01	158.83	3.33	3.0	2.19 c	MR
ES N2010-02	120.67	4.83	4.5	1.91 c	MR
ES N2010-03	134.08	3.83	2.67	2.19 c	MR
ES N2010-04	64.08	2.0	0.17	0.00 d	I
ES N2010-05	70.67	3.83	3.0	2.32 c	MR
ES N2010-06	230.33	3.67	3.5	3.85 b	S
ES N2010-07	145.83	4.83	4.5	5.88 b	S
ES N2010-08	174.25	5.0	4.33	11.88 a	S
ES N2010-09	119.5	4.83	4.67	11.75 a	S
ES N2010-10	99.67	5.0	4.83	8.47 a	S
ES N2010-11	123.5	3.83	3.83	2.04 c	MR
ES N2010-12	120.92	4.67	4.67	13.96 a	S
ES N2010-13	108.83	3.0	2.67	0.39 d	HR

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. exigua*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficient of variation = 29%.

^z Reaction of inoculated coffee genotypes: S = susceptible; HR = highly resistant; MR = moderately resistant; and I = immune.

TABLE 3. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with avirulent and virulent *Meloidogyne exigua* populations under controlled conditions

Genotypes ^w	Virulent <i>M. exigua</i>					Avirulent <i>M. exigua</i>				
	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuaf 'IAC 81'*	47.44	4.88	3.88	78.92 a	S	59.37	5.0	4.5	53.4 a	S
1V	34.71	4.29	3.29	0.84 c	HR	48.07	0.29	0.57	0.82 c	HR
2V	20.75	2.13	0.0	0.02 d	HR	46.94	0.0	0.0	0.0 c	I
3V	42.61	4.78	2.78	20.93 b	S	62.94	1.88	0.38	0.08 c	HR
4V	43.0	3.5	0.33	0.02 d	HR	38.44	0.25	0.13	0.07 c	HR
5V	32.21	3.86	0.57	0.02 d	HR	37.62	0.0	1.0	0.05 c	HR
6V	25.69	2.88	1.0	10.7 b	S	37.69	0.0	0.0	0.0 c	I
7V	51.56	3.63	0.0	0.0 d	HR	35.25	1.88	0.0	0.0 c	I
8V	60.94	3.88	1.5	1.07 c	MR	62.0	0.0	0.25	0.03 c	HR
9V	59.31	2.75	0.38	0.02 d	HR	56.0	0.0	0.0	0.06 c	HR
10V	45.0	3.86	1.71	7.05 b	S	41.18	0.38	3.38	3.82 b	MR
11V	48.94	3.88	0.13	0.05 d	HR	33.25	1.63	0.75	0.18 c	HR
12V	45.75	1.83	0.0	0.84 d	HR	62.87	0.0	1.38	0.4 c	HR
13V	25.5	2.25	2.20	2.45 c	MR	39.72	0.78	1.56	1.44 b	MR
Clone 14	38.92	2.83	0.0	0.04 d	HR	47.33	0.0	0.0	0.0 c	I
Clone 22	21.69	4.25	3.38	7.9 b	S	40.18	0.5	1.5	0.08 c	HR

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. exigua*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficients of variation = 32 and 34%.

^z Reaction of inoculated coffee genotypes: S = susceptible; I = immune; HR = highly resistant; and MR = moderately resistant.

TABLE 5. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with *Meloidogyne incognita* races 1 and 3 under controlled conditions

Genotypes ^w	<i>M. incognita</i> race 3					<i>M. incognita</i> race 1				
	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuai 'IAC 81'*	98.0	5.0	5.0	190.3 a	S	64.83	4.0	4.67	44.62 a	S
Clone 109A	140.92	5.0	5.0	35.76 b	S	178.17	5.0	5.0	39.66 a	S
Clone 120	79.7	5.0	5.0	16.32 c	S	99.75	5.0	5.0	16.0 b	S
ES N2010-01	111.25	5.0	5.0	21.35 c	S	168.8	5.0	5.0	9.81 b	S
ES N2010-02	104.75	5.0	5.0	13.5 c	S	120.58	5.0	5.0	13.19 b	S
ES N2010-03	19.06	5.0	5.0	37.16 b	S	159.0	4.83	4.5	4.60 c	MR
ES N2010-04	150.08	4.83	3.83	2.29 d	MR	123.0	1.17	0.0	0.0 d	I
ES N2010-05	27.6	4.4	3.8	6.91 c	S	76.9	3.67	3.67	11.29 b	S
ES N2010-06	141.92	5.0	5.0	12.65 c	S	201.83	5.0	5.0	38.82 a	S
ES N2010-07	191.17	5.0	5.0	49.96 b	S	122.92	5.0	5.0	16.51 b	S
ES N2010-08	214.2	5.0	5.0	50.52 b	S	201.33	5.0	5.0	37.16 a	S
ES N2010-09	126.67	5.0	5.0	27.36 b	S	112.5	5.0	5.0	31.1 a	S
ES N2010-10	64.33	5.0	5.0	10.84 c	S	103.83	5.0	5.0	47.83 a	S
ES N2010-11	151.42	5.0	5.0	50.83 b	S	170.42	5.0	5.0	59.24 a	S
ES N2010-12	105.67	5.0	5.0	38.71 b	S	83.83	5.0	5.0	22.67 a	S
ES N2010-13	105.4	4.83	4.67	17.38 c	S	105.42	4.83	4.83	4.84 c	MR

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. exigua*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficients of variation = 33 and 35%.

^z Reaction of inoculated coffee genotypes: S = susceptible; I = immune; and MR = moderately resistant.

TABLE 6. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with virulent and avirulent *Meloidogyne exigua* under controlled conditions

Genotypes ^w	Virulent <i>M. exigua</i>					Avirulent <i>M. exigua</i>				
	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuai 'IAC 81'*	155.92	5.0	4.67	125.6 a	S	118.92	3.83	1.17	17.89 a	S
Clone 109A	179.78	0.18	0.0	0.02 c	HR	211.17	0.0	0.0	0.01 b	HR
Clone 120	106.83	0.0	0.0	0.04 c	HR	77.67	0.0	0.0	0.01 b	HR
ES N2010-01	160.33	1.33	0.33	0.02 c	HR	147.25	0.17	0.0	0.0 b	I
ES N2010-02	92.42	1.33	0.0	0.04 c	HR	114.25	0.0	0.0	0.01 b	HR
ES N2010-03	108.5	0.0	0.0	0.02 c	HR	187.9	0.0	0.0	0.0 b	I
ES N2010-04	125.6	0.2	0.0	0.02 c	HR	109.4	0.0	0.0	0.0 b	I
ES N2010-05	88.9	0.2	0.0	0.27 c	HR	45.5	0.0	0.0	0.0 b	I
ES N2010-06	254.08	0.0	0.0	0.0 c	HR	187.17	0.0	0.0	0.03 b	HR
ES N2010-07	158.83	0.0	0.0	0.0 c	HR	117.42	0.0	0.0	0.01 b	HR
ES N2010-08	183.08	2.33	1.83	0.08 c	HR	272.42	0.0	0.0	0.03 b	HR
ES N2010-09	200.92	4.17	3.0	5.13 b	MR	194.8	4.33	3.83	23.73 a	S
ES N2010-10	130.5	1.33	0.66	0.26 c	HR	223.5	0.0	0.0	0.11 b	HR
ES N2010-11	86.67	0.67	0.33	0.01 c	HR	125.67	0.0	0.0	0.03 b	HR
ES N2010-12	63.42	1.0	0.5	0.15 c	HR	66.5	0.0	0.0	0.01 b	HR
ES N2010-13	119.5	0.33	0.17	0.0 c	HR	151.0	0.0	0.0	0.0 b	HR

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. exigua*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficients of variation = 27 and 28%.

^z Reaction of inoculated coffee genotypes: S = susceptible; I = immune; HR = highly resistant; and MR = moderately resistant.

TABLE 7. Mean values of fresh root mass (FRM), gall index (GI), egg mass index (EMI), and reproduction factor (RF) of coffee genotypes 8 months after inoculation with *Meloidogyne* spp. under controlled conditions

Genotypes ^w	<i>M. incognita</i> race 3					<i>M. paranaensis</i>				
	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z	FRM (g)	GI ^x	EMI ^x	RF ^y	R ^z
Catuai 'IAC 81'*	44.3	4.9	4.8	36.2 a	S	46.2	4.5	5.0	32.5	S
Clone 14	40.6	2.2	0.5	0.28 c	R	43.0	2.2	0.0	0.35	R
Clone 22	56.1	3.4	4.7	8.4 b	S	38.4	3.28	4.71	18.05	S

^w * indicates susceptible check.

^x Mean values ($n = 8$) of gall and egg mass index rated on a 0 to 5 scale: 0 = absence of galls or egg masses; 1 = 1 to 2 galls or egg masses; 2 = 3 to 10 galls or egg masses; 3 = 11 to 30 galls or egg masses; 4 = 31 to 100 galls or egg masses; and 5 = over 100 galls or egg masses (54).

^y Values ($n = 8$) of reproduction factor (RF = final population/10,000 eggs of *M. exigua*) were transformed as $\log(x + 1)$. Means followed by different letters in the column are significantly different according to Scott-Knot's test ($P < 0.05$). Coefficients of variation = 21 and 23%.

^z Reaction of inoculated coffee genotypes: S = susceptible; and R = resistant.

with nematodes and surrounding giant cells (Fig. 2E to H). Although at 32 to 45 DAI young females with developed early stages of ovaries were observed; no egg masses were seen (Fig. 2G to H).

External observations of roots at 45 to 50 DAI revealed large and well-developed swelling or small galls in susceptible roots, whereas in resistant roots nothing was observed. Acid fuchsin-stained roots at 45 to 50 DAI revealed that in susceptible clone 22, females had reached reproductive maturity and begun to lay eggs, whereas in resistant roots the underdeveloped females showed no signs of egg production. These results agree with those obtained from coffee genotype inoculations (Tables 1 to 7).

DISCUSSION

The identification and selection of *C. canephora* clones combining drought tolerance with other agronomic traits (e.g., efficient flowering, productivity, and vigor) and multiple-resistance to RKN is of particular interest in generating new varieties better adapted to climate changes and biotic stress (27,29,31). All 30 *C. canephora* genotypes screened in this work had never been tested against *Meloidogyne* spp. populations.

Plant genotypes can be evaluated for RKN resistance based on levels of galling, egg mass number, and total number of eggs collected from infected roots. However, for some crops root galling is not a completely satisfactory indicator of RKN resistance (suppressed reproduction) (41). The galling and egg mass indexes were not a reliable indicator of nematode multiplication rates because of damage symptoms caused by different *Meloidogyne* spp., on coffee were variable and difficult to quantify. Based on these findings, the most pertinent parameter for assessing *Meloidogyne* spp. reproduction on coffee germplasms and the resistance status of these coffee genotypes to several *Meloidogyne* spp. populations are the number of eggs per gram of roots or the nematode reproduction factor. Our findings agree with results reported by Muniz et al. (45) for *C. arabica* and disagree with those reported by Hernandez et al. (38), who considered galling index as a relatively good indicator of *Meloidogyne* multiplication rate on coffee. Considering these contradictory results, these indexes are also included in this work. Hussey and Boerma (40) recommended a preliminary test to determine whether there is a strong correlation among galling, egg masses, and nematode reproduction rate.

Our results demonstrate that only clone 14 showed high multiple resistance to *M. exigua*, *M. incognita* races 1 and 3, and

Fig. 1. Root sections of *Coffea canephora* clonal variety clone 22 (susceptible control) infested with *Meloidogyne paranaensis*. **A**, Fuchsin acid staining; second-stage juvenile (J2) nematodes located in the root cortex 2 days after inoculation (DAI). **B to F**, Toluidine blue staining; **B**, intercellular localization of a J2 nematode in the central cylinder; **C**, J2/J3 nematodes close to initial giant cells inside the central cylinder; **D**, **E**, and **F**, J4 or female nematodes close to giant cells that display dense cytoplasm, vacuoles, and thickened blue-stained cell walls. N = nematode, gc = giant cells, co = cortex, vc = vascular cylinder. Sections visualized after staining with acid fuchsin (**A**) and toluidine-blue (**B to F**).

M. paranaensis, with HR-like lesions as suggested by histological observations. In contrast, clone 22 was susceptible to these RKN species. Specific resistance to either *M. exigua*, *M. incognita*, *M. paranaensis* or *Meloidogyne* spp. was also found in coffee germplasm lines (11,17), however, multiple resistance was usually not found —i.e., it was restricted to one or two *Meloidogyne* species. This was the case in our study in which some genotypes were resistant or moderately resistant to one or two *Meloidogyne*

spp., except for clone 14, which showed high levels of resistance to five RKN populations. Similarly, cultivar IAPAR 59 harboring the *Mex-1* gene is only resistant to *M. exigua* (11).

The widespread distribution of *Meloidogyne* spp. in Brazil and Central America, along with its high aggressiveness led nematologists to look for alternatives to chemical control (28,55,57). For example, an accession of *C. canephora* ‘2258’ from Catie’s germplasm collection in Costa Rica showed high resistance to

Fig. 2. Root sections of *Coffea canephora* clonal variety clone 14 (resistant) infested with *Meloidogyne paranaensis*. **A, C, D, E, and G**, Toluidine blue staining; **B, F, and H**, UV fluorescence observation. **A and B**, Second-stage juvenile (J2) nematode located in the root cortex and surrounded by blue-stained degraded cells (A) which display a yellow fluorescence under UV illumination (B); **C, D, E, F, G, and H**, nematodes present in the vascular cylinder. Giant cells can be observed 8 days after inoculation associated with the presence of nematodes (C). A blue-gray fluorescence is seen in cells near the nematode and in walls of vessels (F and H); **D, E, and G**, parenchyma cells close to J2s exhibiting a dark-stained material, suggesting severe alteration of the cytoplasm; **E and G**, giant cells showing retracted cytoplasm containing numerous vacuoles. N = nematode, HR = hypersensitive reaction indicating cell death (cd), co = cortex, gc = giant cells, vc = vascular cylinder, va = vacuole. Sections visualized under UV light (B, F, and H) and after staining with toluidine blue (A, C, D, E, and G).

M. exigua and resistance and/or tolerance to *M. incognita* populations (24). The same accession was later reported as resistant to *M. incognita* races 1, 2, and 3 (32, 35) and to *M. paranaensis* (26). Initially, the '2258' accession had a resistance level around 70%; however, this level has been raised considerably by subsequent selection in fields infested by *M. incognita*. This improved line was later released as 'Apoatã', a rootstock resistant to *Meloidogyne* spp. (26). 'Nemaya', another multi-resistant rootstock (cross between the *C. canephora* clones 'T3561' and 'T3751'), was developed to overcome major problems associated with RKNs in Central America (7,14) and can be reproduced through seeds. Somatic embryogenesis had to be used to speed up propagation of mother plants (15). Unfortunately, in Central America and Brazil *C. canephora* rootstocks 'Nemaya' and 'Apoatã' encountered serious reduction in growth and yield related to sensitivity to low temperatures and drought, respectively (12; W. Gonçalves, IAC, São Paulo, Brazil, *personal communication*). As a consequence of global warming, extended periods of drought and an increase in nematode populations are now key factors affecting coffee plant development and production (21). The identification of RKN multi-resistant coffee rootstock with drought tolerance, like Conilon clone 14, has now turned into one of the priorities for many coffee research institutes.

In the present study, resistance of *C. canephora* clone 14 to *Meloidogyne* spp. was confirmed and characterized at the histological level. Defense mechanisms associated with resistance to *M. incognita* race 3 and *M. paranaensis* resulted in (i) reduced penetration of roots by few J2, (ii) early appearance of HR-like symptoms during invasion of cortex cells by the nematodes, and (iii) altered feeding sites localized next to strong HR-like areas.

The failure of J2 to penetrate the roots of clone 14 indicates the occurrence of physical and/or chemical barriers. Such barriers were suggested previously for resistant grape rootstock (4,30), cotton (5), soybean 256 (23), pepper (47), coffee IAPAR 59 (3), and peanut (49). Besides these barriers, at least two additional mechanisms could be involved in the expression of resistance. One occurs around 8 DAI, which blocks development of J2 that have penetrated the roots, as observed for other RKN-*C. arabica* interactions (1,3). Microscopic investigation showed that degradation of plant cells in contact with penetrating or migrating J2s at 6 to 8 DAI is closely concomitant to the arrest of nematode development. This observation is in accordance with previous reports on HR-like response involved in resistance against nematodes in other plant species, including cotton (44), peanut (49), and pepper (47). These HR-like areas in infected cortical cells displayed a gray-blue autofluorescence under UV light, indicating a possible presence of phenols that could play a role in coffee defense strategy (1,3). Other research (47) has identified chlorogenic acid as a major phenolic compound present in root extract of inoculated RKN-resistant pepper. Likewise, (3) demonstrated that resistance conferred by the *Mex-1* gene introgressed from *C. canephora* into the coffee Iapar 59 genome is strongly associated with a HR phenotype. Correlatively, differential staining with toluidine-blue also revealed the same early HR staining observed in clone 14. This interesting feature was also seen lately in infected resistant roots, with the occurrence of feeding sites and nematodes close to dead cells or HR-like areas. Until 20 DAI, giant cells in this cultivar were easily recognized by their increased size, presence of some small nuclei and vacuole-like structure and small cell death. After this time, 32 to 45 DAI, the cytoplasm degradation of giant cells, the dark blue (cell death) and blue-green coloration (HR) next to nematodes and feeding sites were strongly visible, suggesting accumulation of phenolic compounds. These degraded giant cells were frequently associated with collapsed young females. These results suggest that the defense response of *C. canephora* roots was activated later and are related to inhibition and degradation of nematodes feeding sites, rather than obstruction of nematode infection and migration

through the roots. This late mechanism seems to be the most important in the Conilon clone 14 pathosystem.

In coffee, the HR in root cells of clone 14 triggered by *M. paranaensis* and *M. incognita* infection may be indicative of a specific gene-for-gene interaction. This would suggest that resistance to *Meloidogyne* spp. in this clone is probably controlled by more than one resistance gene, which could be used to produce new coffee varieties resistant to RKNs. Currently, a segregating population has been built up for mapping and further investigation of the resistance gene(s) that could control clone 14-RKN interactions. Improvement of coffee plants to achieve RKN resistance is crucial for most coffee-producing countries and could be even more urgent in the near future, given the negative impact that climate change will have on coffee crops (32).

Traditional breeding programs usually rely on related diploid species *C. canephora* or *C. racemosa* as sources of resistance to RKN (43). However, the crossing limitations of this strategy incur high costs and long-term investments to develop new coffee cultivars that combine good cup quality and resistance to parasites. Another strategy consists in using selected resistant *C. canephora* as rootstocks. In Costa Rica, Guatemala, and Brazil, grafting onto robusta rootstocks is effective in the field, with productivity of the grafted plants being four times higher than ungrafted plants (56). The use of resistant rootstocks constitutes an inexpensive, non-polluting, and efficient control method. Compared with viruses, bacteria, or fungi, RKNs are characterized by low natural dispersal, gene flow, and genetic diversity between populations (51). This leads to a durable resistance in clone 14 that combines multiple resistance to RKN and drought tolerance in a unique clonal variety. These results provide rational bases for future studies involving gene expression analysis, which will reveal the resistance of clone 14 to RKNs at a molecular level.

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