Root-Knot Nematodes (*Meloidogyne* spp.) Parasitizing Resistant Tobacco Cultivars in Southern Brazil

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Abstract

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Our study surveyed *Meloidogyne* spp. from tobacco cultivars in Brazil carrying the *Rk* gene, which confers resistance to *Meloidogyne incognita* races 1 and 3. Identification of *Meloidogyne* populations from 39 tobacco fields was based on the analysis of esterase phenotypes, perineal patterns, and 18S-internal transcribed spacer (ITS)1-5.8S ribosomal RNA sequences. Biometric characterization and differential host tests of isolates were determined as well. We detected *M. incognita*, *M. javanica*, *M. enterolobii*, *M. arenaria*, and *M. inornata* in 18 (46.2%), 16 (41%), 10 (25.6%), 2 (5.1%), and 1 (2.6%) samples, respectively. Mixtures of species were found in 25.6% of the samples. This is the first report of *M. inornata*

parasitizing tobacco in Brazil's southern region. Two morphological and biochemically unusual populations had host ranges and ITS1 sequences nearly identical to *M. enterolobii* and *M. incognita*, respectively. Pathogenic and biometric intraspecific variations were observed. Based on our results and considering the limited efficiency of the *Rk* gene, it is indispensable not only to search for resistance sources to *M. incognita* virulent strains but also to consider resistance to *M. enterolobii*, *M. inornata*, *M. arenaria*, and *M. javanica* in tobacco breeding programs. Changes in integrated management procedures should be considered to avoid increased crop damage in the future.

Tobacco (*Nicotiana tabacum* L.) is the main economic nonfood crop worldwide (Sinditabaco 2013). Brazil is the second leading world producer and the greatest exporter of tobacco. Total production during the 2011–12 growing season was approximately 737,000 metric tons; the southern region of Brazil accounted for 96% of this production. In Brazil, 200,000 small farms are involved in the production of tobacco, generating an annual income of U.S.\$2 billion (ABIFUMO 2013; Souza Cruz 2013). Tobacco production in Brazil is threatened by various pathogens due to a narrow genetic base of currently grown cultivars as well as intensive cropping practices. Among production-limiting pathogens, plant-parasitic nematodes are a concern among growers (Ferraz and Brown 2002). Several plant-parasitic nematode species have been reported to be associated with tobacco (Johnson et al. 2005) but *Meloidogyne* spp. are the most economically important, causing extensive yield losses of 15 to 28% (Díaz-Silveira and Herrera 1998; Shew and Lucas 1991).

In recent decades, *Meloidogyne* spp. control has been based on the use of resistant tobacco cultivars carrying a single gene with complete dominance, called *Rk* (Yi et al. 1998). This gene was transferred from *N. tomentosa* and confers resistance to races 1 and 3 of *Meloidogyne incognita* (Kofoed & White) Chitwood and race 1 of *M. arenaria* (Neal) Chitwood (Shew and Lucas 1991). Despite this, *Meloidogyne* spp. are still a serious problem in Brazil. Changes in the composition of *Meloidogyne* spp. and *M. incognita* virulent strains induced by the compulsory and exclusive use of the gene *Rk* could explain this, as in other countries (Rich and Garcia 1985; Stanton et al. 1998).

Surveys in infested Brazilian tobacco-growing areas are scarce and very old. *M. incognita*, *M. javanica* (Treub) Chitwood, and *M. arenaria* have all been reported in Brazil (Johnson et al. 2005; Zem et al. 1985). A recent report noted the occurrence of *M. enterolobii*

Yang & Eisenback (= *M. mayaguensis* Rammah & Hirschmann) (Gomes et al. 2008). Other species were described or revalidated at this time (Moens et al. 2010). These identifications were based only on morphology and included overlapping intraspecific and intrapopulation characteristics commonly observed in *Meloidogyne* spp. (Brito et al. 2004; Carneiro et al. 2000; Cofcewicz et al. 2004), potentially resulting in misidentifications. For this reason, the integration of classic, biochemical, and molecular tools for effective diagnosis has often been recommended (McClure et al. 2012).

The objectives of this research were to (i) document the occurrence of *Meloidogyne* spp. in resistant tobacco cultivars (*Rk*) in the major tobacco-producing regions of Brazil, with identification based on integrated analyses of esterase (Est phenotypes), perineal patterns, and partial sequencing of the 18S-internal transcribed spacer (ITS) 1-5.8S ribosomal RNA (rRNA) region (ITS1-rRNA); (ii) analyze the phylogenetic relationships among collected *Meloidogyne* spp.; (iii) characterize isolates biometrically; and (iv) determine the races of *Meloidogyne* present in Brazil. To our knowledge, this is the first survey in Brazilian tobacco-growing areas based on the integration of biochemical, molecular, and morphological approaches.

Materials and Methods

Collection and maintenance of *Meloidogyne* populations and isolates. Roots samples were collected in 39 naturally infested tobacco fields comprising several resistant cultivars (Table 1). The sampling was made in a targeted way: only symptomatic plants were collected in each field. All samples were placed in plastic bags and transported to the laboratory. The *Meloidogyne* spp. populations were established and maintained (1 year) on susceptible ‘Santa Clara’ tomato (*Solanum lycopersicum*) from specimens obtained by the sodium hypochlorite method (Hussey and Barker 1973). Plants were grown under greenhouse conditions at temperatures of 25 to 30°C.

Monospecific isolates from each population (Laboratório de Genética Molecular [LGM] isolates) were established from a single egg mass, inoculated onto Santa Clara tomato, and maintained as described above. These isolates were subsequently used for the biometric studies and differential host tests. Due to the parthenogenetic reproduction of these *Meloidogyne* spp. (Ferraz and Brown 2002; Trudgill and Blok 2001), each isolate was considered a clonal line.

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Later, the specific identity of each isolate was confirmed again by Est phenotypes. When more than one isolate was obtained from a mixed population, we assigned different numbering to the codes (i.e., LGM 07.1 and LGM 07.2).

Morphological and biochemical analyses of *Meloidogyne* populations. At least 10 perineal patterns from each population cultured in the greenhouse were studied under a light microscope (LM) using standard procedures (Hartman and Sasser 1985). All slides

Table 1. Geographic origin, codes for populations, esterase (Est) phenotypes, and GenBank accession numbers of *Meloidogyne* spp. populations collected from tobacco fields in Brazil planted with cultivars containing *Rk* resistance

<i>Meloidogyne</i> sp., city, state ^a	Cultivar	Codes ^b	Est phenotypes	Accession number ^c
<i>Meloidogyne incognita</i>				
Araranguá, SC	CSC 444	IPR LGM 01	I ₁ and I ₂	KF053005
Içará, SC	CSC 467	IPR LGM 02	I ₁	KF053006
Araranguá, SC	CSC 444	IPR LGM 06	I ₂	KF053009
Sombrio, SC	CSC 467	IPR LGM 07 ^d	I ₁	KF053010
Sombrio, SC	CSC 405	IPR LGM 09	I ₂	KF053011
Sombrio, SC	CSC 444	IPR LGM 10 ^d	I ₁	KF053012
Venâncio Aires, RS	CSC 444	IPR LGM 14	I ₂	KF053016
Venâncio Aires, RS	CSC 401	IPR LGM 16	I ₁	...
Apiúna, SC	CSC 467	IPR LGM 32 ^d	I ₁	...
Ituporanga, SC	CSC 405	IPR LGM 35	I ₂	KF053028
Mercedes, PR	BRAVO 702	IPR LGM 36	I ₁	KF053029
Mercedes, PR	BRAVO 702	IPR LGM 39	I ₂ and I ₁	KF053032
Mercedes, PR	BRAVO 702	IPR LGM 40	I ₁	KF053033
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 41	I ₁ and I ₂	KF053034
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 42	I ₁	KF053035
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 43 ^d	I ₁	KF053036
Missol, PR	BRAVO 702	IPR LGM 44 ^d	I ₁	KF053037
Mercedes, PR	BRAVO 702	IPR LGM 45 ^d	I ₁	...
<i>M. javanica</i>				
Içará, SC	CSC 405	IPR LGM 05	J3	...
Sombrio, SC	CSC 467	IPR LGM 07 ^d	J3	...
Araranguá, SC	CSC 444	IPR LGM 08	J3	...
Sombrio, SC	CSC 444	IPR LGM 10 ^d	J3	...
Vale do Sol, RS	CSC 467	IPR LGM 12	J3	KF053014
Vale do Sol, RS	CSC 439	IPR LGM 13	J3	KF053015
Rio Pardo, RS	CSC 401	IPR LGM 17 ^d	J3	...
Rio Pardo, RS	CSC 416	IPR LGM 18	J3	KF053017
Vera Cruz, RS	CSC 444	IPR LGM 21	J3	KF053018
Presidente Nereu, SC	CSC 405	IPR LGM 23 ^d	J3	KF053019
Agronômica, SC	CSC 444	IPR LGM 29 ^d	J3	...
Agrolândia, SC	CSC 416	IPR LGM 33	J3	KF053026
Agronômica, SC	CSC 405	IPR LGM 34 ^d	J3	KF053027
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 37	J3	KF053030
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 43 ^d	J3	...
Missol, PR	BRAVO 702	IPR LGM 44 ^d	J3	...
<i>M. enterolobii</i>				
Treze de Maio, SC	CSC 404	IPR LGM 03	VS1-S1	KF053007
Pedras Grandes, SC	CSC 438	IPR LGM 04	VS1-S1	KF053008
Treze de Maio, SC	CSC 404	IPR LGM 11	VS1-S1	KF053013
Presidente Nereu, SC	CSC 405	IPR LGM 23 ^d	VS1-S1	...
Ascurra, SC	CSC 439	IPR LGM 26	VS1-S1	KF053020
Ascurra, SC	CSC 439	IPR LGM 28	VS1-S1	KF053022
Agronômica, SC	CSC 444	IPR LGM 29 ^d	VS1-S1	KF053023
Agronômica, SC	CSC 444	IPR LGM 30	VS1-S1	KF053024
Apiúna, SC	CSC 467	IPR LGM 32 ^d	VS1-S1	...
Agronômica, SC	CSC 405	IPR LGM 34 ^d	VS1-S1	...
<i>M. arenaria</i>				
Rio Pardo, RS	CSC 401	IPR LGM 17 ^d	A2	...
Ituporanga, SC	CSC 405	IPR LGM 31	A2	KF053025
<i>M. inornata</i>				
Mercedes, PR	BRAVO 702	IPR LGM 45 ^d	I3	KF053038
<i>Meloidogyne</i> sp. 1				
Vidal Ramos, SC	CSC 405	IPR LGM 27	F2 ^e	KF053021
<i>Meloidogyne</i> sp. 2				
Marechal Cândido Rondon, PR	BRAVO 702	IPR LGM 38	F3 ^e	KF053031

^a Abbreviations for states of the Southern Brazil: PR = Paraná, SC = Santa Catarina, and RS = Rio Grande do Sul.

^b Codes for *Meloidogyne* populations: IPR = Instituto Agronômico do Paraná, where slides were deposited, and LGM = Laboratório de Genética Molecular, the laboratory where identification analyses were performed.

^c Internal transcribed spacer 1 ribosomal RNA.

^d Mixed populations.

^e Unknown Est phenotypes.

were deposited (Table 1) at the Instituto Agronômico do Paraná [IPR], Londrina, Paraná State, Brazil. The Est phenotypes were determined using protein extract from 18 young egg-laying females from each population. Females were placed in a hematocrit containing 5 μ l of extraction solution (Carneiro et al. 2000), macerated, and transferred to wells on polyacrylamide gel with 4% stacking (pH 6.8) and 7.5% separating (pH 8.9) gel using Tris-glycine buffer (pH 8.9). Homogenates of the *M. javanica* isolate IPR 81 (phenotype J3, relative mobility rate [Rm] = 1.0, 1.3, and 1.4) was used as a known reference. Electrophoresis was carried out in a Mini Protean III (Bio-Rad Laboratories) unit at 4°C (Brito et al. 2008). Gels were stained with α -naphthyl acetate (Carneiro et al. 2000) and Rm was calculated for each gel.

DNA extraction, polymerase chain reaction, sequencing, and phylogenetic analysis of *Meloidogyne* populations. DNA was extracted from single females according to Stanton et al. (1998) and stored at -20°C. The polymerase chain reaction (PCR) assays for ITS1-rRNA (50 μ l) were performed using Taq PCR Master Mix Kit (Promega Corp.) and the primers rDNA2 (5'-TTGATTACGTCCCTGC CCTTT-3') and rDNA1.58S (5'-ACGAGCCGAGTGATCCACCG-3') (Cherry et al. 1997). Taq PCR Master Mix Kit (25 μ l), 1.5 μ l (0.3 mM) of each primer, 18 μ l of milli-Q water, and 4 μ l of total DNA were placed in a microfuge tube. The thermal cycler was programmed as follows: 94°C (2 min) followed by 40 cycles of 94°C (1 min), 57°C (1 min), and 72°C (2 min).

DNA sequences were analyzed using the BLASTn megablast program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and deposited in GenBank (Table 1). The ITS1 sequences were aligned and edited using CLUSTAL X 2 (Thompson et al. 1997) and GeneDoc (Nicholas et al. 1997). Descriptive statistics, divergence estimates, and dendrograms were obtained using MEGA 5.1 (Tamura et al. 2011). The phylogenetic tree was constructed using the unweighted pair group method with arithmetic means algorithm with the maximum-likelihood model and complete deletion (1,500 replicates). *Pratylenchus coffeae* (AB053485.1 from Japan) was used as the outgroup taxon. An *M. enterolobii* sequence (JF309158.1 from Costa Rica) was included for comparison purposes.

Due to similarities between LGM 38 and *M. incognita*, we used the sequence-characterized amplified regions (SCAR) primer pair inc-K14 (5'-GGGATGTGTAAATGCTCCTG-3' and 5'-CCCCTACACCCT CAACTTC-3') (Randig et al. 2002) to differentiate these populations. The thermal cycler was programmed as follows: 94°C (5 min), followed by 30 cycles of 94°C (30 s), 64°C (45 s), and 70°C (1 min); and a final incubation of 70°C (8 min).

Scanning electron microscopy. Scanning electron microscopy (SEM) analysis was performed on partially dissected females obtained from the roots of tomato culture plants. After dissection, samples were transferred to glass vials containing 2 ml of Karnovsky fixative solution (2.5% [vol/vol] glutaraldehyde and 2.5% [vol/vol] paraformaldehyde in 0.05 M sodium cacodylate buffer + 0.001 M calcium chloride, pH 7.0) and stored at 4°C. Samples were postfixed in 1% osmium tetroxide for 2 h at 25°C, dehydrated in a graded acetone series (30, 50, 70, 90, and 100%), and dried to the critical point in CO₂ (Bal-tec CPD 030; Balzers). Root tissues containing nematodes were mounted on aluminum stubs, sputter coated with gold (Bal-tec SCD 050; Balzers), and examined with a SEM (LEO-435 VP) operating at 20 kV with a working distance ranging from 10 to 30 mm.

Race determination using North Carolina differential host test. Bioassays were carried out at the IPR. All LGM isolates were subjected to the North Carolina differential host test (NCDHT) (Hartman and Sasser 1985). The host set used in the test was 'Rutgers' tomato, 'NC95' tobacco, 'Deltapine 61' cotton (*Gossypium hirsutum*), 'Early California Wonder' pepper (*Capsicum annuum*), 'Charleston Gray' watermelon (*Citrullus vulgaris*), and 'Florunner' peanut (*Arachis hypogaea*). Seedlings were planted in plastic pots (500 cm³) containing a sterile sandy soil.

Three seedlings of each differential host were inoculated with a suspension containing 2,000 nematodes (Nono-Womdim et al. 2002). After 60 days, roots were removed, washed, and stained with Phloxine

B (15 mg/liter for 20 min), and the number of galls and egg masses were recorded, as follows: 0 = no gall or egg mass and 1 = 1 to 2, 2 = 3 to 10, 3 = 11 to 30, 4 = 31 to 100, and 5 = >100 galls or egg masses. Differential hosts were rated as resistant (≤ 2 egg masses) or susceptible (>2 egg masses) (Hartman and Sasser 1985). The host test was repeated once.

Biometric characterization of *Meloidogyne* isolates. Biometric parameters from 10 second-stage juveniles (J2) and 10 males were measured for each isolate. Specimens were measured live under LM after relaxation at 5°C for 5 min. The following parameters were measured: total body length, maximum body width, body width at anus level (J2), body width at stylet bulb level (males), body width at excretory pore level (males), stylet length, stylet bulb height, stylet bulb width, esophagus length (males), distance from stylet base to anterior region terminus (J2), dorsal pharyngeal gland opening (DGO), distance from medium bulb to anterior region terminus, medium bulb valve length (J2), medium bulb valve width (J2), distance from excretory pore to anterior region terminus, hyaline tail terminus (J2), tail length, spicule length (males), and gubernaculum length (males). The de Man's ratios a, c, and c' along with stylet bulb height/stylet bulb width (males), length/distance from medium bulb to anterior region terminus, and distance from excretory pore to anterior region terminus $\times$ 100/length were calculated as well (Siddiqi 2000).

The biometric values were analyzed between species using parametric one-way analysis of variance (ANOVA) and means were compared by Tukey's honestly significant difference test ($P < 0.05$). This analysis allowed us to view differences between species based on each characteristic and, when present, to indicate which variables are capable of distinguishing one species from another. Additionally, the data set was submitted to principle component analysis (PCA) and later to hierarchical clustering analysis (Ward's method). This multivariate analysis allowed for the determination of the most robust discriminant trait and obtaining an overview of morphometric similarities at inter- and intra-specific levels. Both analyses were performed with the R 2.15 program (R Development Core Team 2012).

Results

Morphological and biochemical analyses of *Meloidogyne* populations. The isozyme electrophoresis revealed eight Est phenotypes, six of them species specific (Fig. 1). There were 18 (46.2%) samples with phenotypes of *M. incognita* (I1 and I2, Rm = 1.0 and 1.1), 16 (41%) of *M. javanica* (J3, Rm = 1.0, 1.3, and 1.4), 10 (25.6%) of *M. enterolobii* (VS1-S1, Rm = 0.75, 0.85, 1.0 and 1.1), 2 (5.1%) of *M. arenaria* (A2, Rm = 1.25 and 1.35), and 1 (2.6%) of *M. inornata* Lordello, 1956 (I3 = Y3, Rm = 0.90, 1.25, and 1.35). Two Est phenotypes were detected in 10 (25.6%) samples. Unusual phenotypes were isolated for LGM 27 (F2, Rm = 0.75, 0.90 and 0.95) and LGM 38 (F3, Rm = 0.90, 1.0 and 1.1).

From the 483 specimens, we found perineal patterns for four species: *M. javanica* ($n = 127$), *M. incognita* ($n = 141$), *M. enterolobii* ($n = 74$), and *M. arenaria* ($n = 37$). Inconsistent patterns were found for *M. incognita* (13.5%), small variations for *M. javanica*, and large variability for *M. enterolobii* (39.3%) and *M. arenaria* (27%). Specimens of *M. javanica* displayed small variations but always with the usual features, such as dorsal arch varying between low and moderately low and two conspicuous lateral incisures (Fig. 1A). For *M. incognita*, lateral lines often marked by bifurcations and high dorsal arch were observed (Fig. 1G). Although the majority of specimens of *M. enterolobii* showed perineal patterns similar to its description or to *M. mayaguensis*, we observed large variability, with *M. arenaria* or *M. incognita*-like types observed. Patterns varied between oval and rounded, with fine and spaced striae and moderately high dorsal arch (Fig. 1D). Some specimens had conspicuous lateral lines and others had one or two lateral wings.

The *M. arenaria* specimens had ovoid shape perineal patterns, with low dorsal arch, often dorsum-laterally compressed, forming small shoulders (Fig. 1C). Some specimens had one or two prominent lateral wings, as previously described (Carneiro et al. 2008b). In relation to LGM 27, patterns varied between round and oval, and there was absence of punctuations or lateral lines, with some

specimens having one or two lateral wings (Fig. 1F). On the other hand, LGM 38 patterns were very close to *M. incognita* (Fig. 1E).

Sequencing of ITS1-rRNA gene. Amplification of the rRNA produced a unique fragment of about 429 bp (354 to 442 bp), with the ITS-1 sequence varying from 202 and 215 bp. In general, the identification based on biochemical and morphological criteria was confirmed by the ITS1 sequences. Sequence variation was in accordance with *Meloidogyne* spp.; that is, high for *M. incognita* (0 to 38.8%) and low for *M. javanica* (0 to 1.6%) and *M. enterolobii* (0 to 2.8%). For *M. incognita*, sequences of LGM 01, 02, 06, 09, and 10 accounted for this heterogeneity. There was low polymorphism between the sequences of *M. javanica*, *M. arenaria*, and most sequences of *M. incognita* (0 to 1.1%). The sequence of *M. inornata* differed from those of *M. incognita* (3.9 to 32.4%), *M. javanica* (3.9 to 5.1%), *M. enterolobii* (9.8 to 11%), LGM 27 (10.4%), and LGM 38 (3.9%). There was low divergence between sequences of *M. enterolobii* and JF309158.1 (1.6 to 2.8%) and LGM 27 (0 to 0.5%). Furthermore, there was a variable 12 nucleotide region found in the *M. enterolobii* sequences (data not shown).

Phylogenetic analysis allowed us to distinguish five clades, which are shown in Figure 2. The A+T content varied among clades, with values of 61.42% (clade i), 60% (clade ii), 61.9% (clade iii), 63.27% (clade iv), and 56.9% (clade v). Low divergence was observed in clades i (0.2%) and iv (1.3%) but high divergence was observed in clade v (10.9%). Divergences varied considerably among clades (3.70 to 28.80%).

Race determination using NCDHT. The NCDHT allowed us to categorize the 40 isolates as five *Meloidogyne* spp. (Table 2). We did not obtain an isolate from population LGM 05. Most of the results are in agreement with the specific delimitations obtained with other methods used in this study. Only five isolates (12.8%) displayed unexpected host range: LGM 23, LGM 26, LGM 30, LGM 34, and LGM 10.1. Almost all *M. incognita* isolates were classified as race 2 (79%) but LGM 36, LGM 39, and LGM 42 were classified as race 1, because they parasitized watermelon, tomato and pepper, but not cotton, peanut or tobacco. About 60% of the *M. enterolobii* isolates were similar to *M. incognita* race 2, reproducing on tomato, tobacco, watermelon and pepper, as noted in previous works (Brito et al. 2004; Rammah and Hirschmann 1988; Yang and Eisenback 1983). Some variants (LGM 23, LGM 26, LGM 30, and LGM 34) were unable to reproduce on pepper. Almost all *M. javanica* isolates showed a usual host range. Tomato, tobacco and watermelon were good hosts for them. Only LGM 10.1 parasitized pepper. This variant was also detected in another study (Ogbuji 1981).

Identification of the LGM 45 (*M. inornata*) isolate was confirmed, because its host range was identical to *M. javanica*, as in the *M. inornata* description (Cameiro et al. 2008a). The LGM 31 isolate was classified as *M. arenaria* race 2. Interestingly, LGM 27 had a host range similar to *M. incognita* race 2 and *M. enterolobii*. LGM 38 was similar to *M. incognita* race 1, not parasitizing tobacco, cotton, or peanut.

Biometric characterization of *Meloidogyne* isolates. Variations were observed for all species but most remained within the

Fig. 1. Esterase phenotypes (right) and perineal patterns (left) for *Meloidogyne* spp. collected from tobacco fields in southern Brazil. Isolate of *Meloidogyne javanica* (J3) was used as reference. **A**, *M. javanica* (J3); **B**, *M. inornata* (I3); **C**, *M. arenaria* (A2); **D**, *M. enterolobii* (VS1-S1); **E**, LGM 38 (F3); **F**, LGM 27 (F2); and **G** and **G'**, *M. incognita* (I1 and I2).

limits reported in the original descriptions (data not shown). Significant differences were observed only on *M. inornata* males, a finding that could be attributed to the host plant, number of specimens, and measurement method (Brown and Tophan 1984).

For LGM 27, the obtained measurements were (i) for J2 specimens, length = 455.7 μm (± 15.5), maximum body width = 16.7 μm (± 1.0), body width at anus level = 11.4 μm (± 0.5), stylet length = 10.5 μm (± 0.5), distance from stylet base to anterior region terminus = 14.6 μm (± 0.5), stylet bulb height = 1.6 μm (± 0.2), diameter of the bulb of the stylet = 2.5 μm (± 0.0), DGO = 3.3 μm (± 0.3), distance from medium bulb to anterior region terminus = 50.2 μm (± 2.0), medium bulb valve length = 4.5 μm (± 0.0), medium bulb valve width = 3.0 μm (± 0.0), distance from excretory pore to anterior region terminus = 90.2 μm (± 2.1), tail length = 51.5 μm (± 2.8), hyaline tail terminus = 12.2 μm (± 2.1), a =

27.5 (± 2.1), c = 8.9 (± 0.4), c' = 4.5 (± 0.2), length/distance from medium bulb to anterior region terminus = 9.1 (± 0.5), and distance from excretory pore to anterior region terminus $\times 100/\text{length}$ = 19.8 (± 0.8); and (ii) for males, length = 2,052.1 μm (± 124.6), maximum body width = 52.9 μm (± 4.5), stylet bulb width = 20.5 μm (± 0.7), diameter at the level of the excretory pore = 25.9 μm (± 0.7), esophagus length = 99.8 μm (± 4.9), stylet length = 23.1 μm (± 0.7), stylet bulb height = 3.5 μm (± 0.4), stylet bulb width = 5.6 μm (± 0.4), DGO = 4.1 μm (± 0.4), distance from medium bulb to anterior region terminus = 96.5 μm (± 6.1), distance from excretory pore to anterior region terminus = 153.2 μm (± 7.0), tail length = 16.3 μm (± 1.4), spicule length = 25.3 μm (± 0.7), gubernaculum length = 8.6 μm (± 1.1), a = 38.9 (± 2.5), c = 126.4 (± 8.1), length/distance from medium bulb to anterior region terminus = 21.3 μm (± 1.9), stylet bulb height/stylet bulb width = 1.6 (± 0.1), and distance from excretory pore to anterior region terminus $\times 100/\text{length}$ = 7.5 (± 0.5).

Fig. 2. Phylogenetic tree (unweighted pair group method with arithmetic means) resulting from the alignment of the internal transcribed spacer 1 sequences of several populations of *Meloidogyne* spp. from tobacco fields in Brazil. Bootstrap support more than 70% for appropriate clade. Phylogenetic analysis distinguished five clades: (i) *Meloidogyne incognita*, *M. javanica*, *M. arenaria*, and LGM 38 populations; (ii) LGM 45; (iii) LGM10; (iv) *M. enterolobii* and LGM 27 populations; and (v) *M. incognita* populations.

For LGM 38, the measurements were (i) for J2 specimens, length = 416.0 μm (± 27.4), maximum body width = 15.9 μm (± 1.7), body width at anus level = 10.5 μm (± 0.7), stylet length = 10.7 μm (± 0.7), distance from stylet base to anterior region terminus = 15.1 μm (± 0.3), stylet bulb height = 1.5 μm (± 0.0), diameter of the bulb of the stylet = 2.5 μm (± 0.0), DGO = 3.1 μm (± 0.2), distance from medium bulb to anterior region terminus = 52.1 μm (± 3.0), medium bulb valve length = 4.1 μm (± 0.3), medium bulb valve width = 3.2 μm (± 0.3), distance from excretory pore to anterior region terminus = 86.7 μm (± 7.0), tail length = 51.5 μm (± 3.4), hyaline tail terminus = 11.4 μm (± 1.3), a = 26.2 (± 1.4), c = 8.1 (± 0.4), c' = 4.9 (± 0.3), length/distance from medium bulb to anterior region terminus = 8.0 (± 0.7), and distance from excretory pore to anterior region terminus $\times 100/\text{length}$ = 20.9 (± 2.3); and (ii) for males, length = 1,519.5 μm (± 50.0), maximum body width = 42.1 μm (± 2.5), stylet bulb width = 18.8 μm (± 0.8), diameter at the level of the excretory pore = 29.0 μm (± 0.8), esophagus length = 101.5 μm (± 3.0), stylet length = 24.7 μm (± 0.7), stylet bulb height = 4.4 μm (± 0.4), stylet bulb width = 6.3 μm (± 0.4), DGO = 4.0 μm (± 0.4), distance from medium bulb to anterior region terminus = 115.5 μm (± 7.2), distance from excretory pore to anterior region terminus = 153.3 μm (± 7.2), tail length = 24.8 μm (± 0.8), spicule

length = 29.0 μm (± 0.8), gubernaculum length = 9.3 μm (± 0.8), a = 37.9 (± 1.7), c = 64.2 (± 2.9), length/distance from medium bulb to anterior region terminus = 13.8 (± 0.9), stylet bulb height/stylet bulb width = 1.4 (± 0.1), and distance from excretory pore to anterior region terminus $\times 100/\text{length}$ = 9.6 (± 0.5).

The ANOVA revealed significant differences between species for all variables ($P < 0.05$). Nevertheless, a small number of variables were useful to differentiate *Meloidogyne* spp., such as body width at anus level and hyaline tail terminus (J2) for *M. enterolobii*; tail length and distance from excretory pore to anterior region terminus $\times 100/\text{length}$ (J2) for *M. arenaria*; c (J2) for *M. inornata*; and DGO, a, distance from excretory pore to anterior region terminus $\times 100/\text{length}$, and length/distance from medium bulb to anterior region terminus (males) for *M. javanica*. The LGM 27 differed from *M. enterolobii* in only one variable (stylet bulb height/stylet bulb width) for males.

Low intraspecific variability was observed by PCA analysis from J2 measurements, with high correspondence (computable general equilibrium, CGE) between phenetic clusters and species characterized using other methods in this study but with slight interspecific overlap. The percentage of variance explained (inertia) by the first two principle components (PC) was 52.6%. PC1 was positive for

Table 2. Codes of isolates and host range results for *Meloidogyne* spp. collected from tobacco fields in Brazil planted with cultivars containing *Rk* resistance

Species	Codes	Differential hosts ^a					
		Tobacco	Tomato	Watermelon	Peanut	Pepper	Cotton
<i>Meloidogyne incognita</i>	LGM 01	4.67 $\pm$ 0.52	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	3.83 $\pm$ 0.41	0 $\pm$ 0
	LGM 02	4.5 $\pm$ 0.55	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	3.83 $\pm$ 0.41	0 $\pm$ 0
	LGM 06	4.5 $\pm$ 0.55	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	2.17 $\pm$ 0.75	0 $\pm$ 0
	LGM 07.2	4.0 $\pm$ 0.63	3.83 $\pm$ 0.41	4.8 $\pm$ 0.45	0 $\pm$ 0	2.83 $\pm$ 0.41	0 $\pm$ 0
	LGM 09	4.67 $\pm$ 0.52	4.83 $\pm$ 0.41	4.4 $\pm$ 0.89	0 $\pm$ 0	4.33 $\pm$ 0.52	0 $\pm$ 0
	LGM 10.2	4.83 $\pm$ 0.41	4.5 $\pm$ 0.55	4.4 $\pm$ 0.55	0 $\pm$ 0	2.83 $\pm$ 0.75	0 $\pm$ 0
	LGM 14	4.83 $\pm$ 0.41	4.0 $\pm$ 0.63	4.0 $\pm$ 0	0 $\pm$ 0	2.5 $\pm$ 0.84	0 $\pm$ 0
	LGM 16	3.67 $\pm$ 1.37	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	3.67 $\pm$ 0.81	0 $\pm$ 0
	LGM 35	4.6 $\pm$ 0.55	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	3.4 $\pm$ 0.55	0 $\pm$ 0
	LGM 36	0.17 $\pm$ 0.41	5.0 $\pm$ 0	4.4 $\pm$ 0.55	0 $\pm$ 0	2.5 $\pm$ 0.55	0 $\pm$ 0
	LGM 39	0 $\pm$ 0	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	4.0 $\pm$ 0	0 $\pm$ 0
	LGM 40	3.17 $\pm$ 0.75	5.0 $\pm$ 0	4.5 $\pm$ 0.55	0 $\pm$ 0	3.33 $\pm$ 0.52	0 $\pm$ 0
	LGM 41	4.33 $\pm$ 0.82	4.83 $\pm$ 0.41	5.0 $\pm$ 0	0 $\pm$ 0	4.17 $\pm$ 0.41	0 $\pm$ 0
	LGM 42	0.33 $\pm$ 0.82	5 $\pm$ 0	4.67 $\pm$ 0.52	0 $\pm$ 0	1.8 $\pm$ 1.79	0 $\pm$ 0
	LGM 07.1	4.5 $\pm$ 0.55	5.0 $\pm$ 0	3.4 $\pm$ 0.55	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 08	4.67 $\pm$ 0.52	4.4 $\pm$ 0.55	4.6 $\pm$ 0.55	0 $\pm$ 0	0.33 $\pm$ 0.82	0 $\pm$ 0
	LGM 10.1	5 $\pm$ 0	5 $\pm$ 0	5 $\pm$ 0	0 $\pm$ 0	4.2 $\pm$ 0.84	0 $\pm$ 0
	LGM 12	3.67 $\pm$ 0.52	3.33 $\pm$ 0.52	4.6 $\pm$ 0.55	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 13	5.0 $\pm$ 0	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 17	4.33 $\pm$ 0.52	4.17 $\pm$ 0.75	5.0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
<i>M. javanica</i>	LGM 18	4.5 $\pm$ 0.55	4.83 $\pm$ 0.41	5.0 $\pm$ 0	0 $\pm$ 0	0.4 $\pm$ 0.89	0 $\pm$ 0
	LGM 21	4.67 $\pm$ 0.52	5.0 $\pm$ 0	4.6 $\pm$ 0.55	0 $\pm$ 0	0.17 $\pm$ 0.41	0 $\pm$ 0
	LGM 33	4.67 $\pm$ 0.52	4.83 $\pm$ 0.41	4.83 $\pm$ 0.41	0 $\pm$ 0	0.5 $\pm$ 0.84	0 $\pm$ 0
	LGM 37	4.33 $\pm$ 0.52	5 $\pm$ 0	5 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 43	5.0 $\pm$ 0	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 44	4.67 $\pm$ 0.52	4.67 $\pm$ 0.52	4.2 $\pm$ 0.84	0 $\pm$ 0	0.33 $\pm$ 0.51	0 $\pm$ 0
	LGM 03	5.0 $\pm$ 0	5.0 $\pm$ 0	4.0 $\pm$ 0.89	0 $\pm$ 0	3.67 $\pm$ 0.52	0.33 $\pm$ 0.52
	LGM 04	4.83 $\pm$ 0.41	4.83 $\pm$ 0.41	4.67 $\pm$ 0.52	0 $\pm$ 0	3.5 $\pm$ 0.55	0 $\pm$ 0
	LGM 11	4.2 $\pm$ 0.40	4.8 $\pm$ 0.40	5.0 $\pm$ 0	0 $\pm$ 0	3.2 $\pm$ 0.40	0 $\pm$ 0
	LGM 23	3.0 $\pm$ 0	3.5 $\pm$ 0.55	5.0 $\pm$ 0	0 $\pm$ 0	0.17 $\pm$ 0.41	0 $\pm$ 0
<i>M. enterolobii</i>	LGM 26	3.17 $\pm$ 0.41	3.5 $\pm$ 0.55	4.4 $\pm$ 0.55	0 $\pm$ 0	0.17 $\pm$ 0.41	0 $\pm$ 0
	LGM 28	3.83 $\pm$ 0.75	5.0 $\pm$ 0	4.83 $\pm$ 0.41	0 $\pm$ 0	3.4 $\pm$ 0.55	0 $\pm$ 0
	LGM 29.1	4.83 $\pm$ 0.41	5.0 $\pm$ 0	4.0 $\pm$ 1.22	0 $\pm$ 0	3.83 $\pm$ 0.41	0.17 $\pm$ 0.41
	LGM 30	4.67 $\pm$ 0.52	3.67 $\pm$ 0.52	4.4 $\pm$ 0.55	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 32	4.5 $\pm$ 0.83	4.7 $\pm$ 0.52	3.6 $\pm$ 0.55	0 $\pm$ 0	4.2 $\pm$ 0.75	0 $\pm$ 0
	LGM 34	4.67 $\pm$ 0.52	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	1.5 $\pm$ 0.54	0 $\pm$ 0
	LGM 31	4.8 $\pm$ 0.40	4.7 $\pm$ 0.52	4.4 $\pm$ 0.55	0 $\pm$ 0	0.83 $\pm$ 0.98	0 $\pm$ 0
	LGM 45	3.5 $\pm$ 0.55	5.0 $\pm$ 0	4.67 $\pm$ 0.52	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	LGM 27	4.17 $\pm$ 0.41	5.0 $\pm$ 0	4.0 $\pm$ 0	0 $\pm$ 0	2.5 $\pm$ 1.38	0 $\pm$ 0
	LGM 38	1 $\pm$ 0.60	5.0 $\pm$ 0	5.0 $\pm$ 0	0 $\pm$ 0	3.8 $\pm$ 0.40	0 $\pm$ 0
<i>M. arenaria</i>							
<i>M. inornata</i>							
<i>Meloidogyne</i> sp. 1							
<i>Meloidogyne</i> sp. 2							

^a Gall rating index according to the scale 0 = no galls and 1 = 1 to 2, 2 = 3 to 10, 3 = 11 to 30, 4 = 31 to 100, and 5 = >100 galls. Differential hosts were rated as resistant (≤ 2) or susceptible (> 2) (Hartman and Sasser 1985).

10 traits and closely correlated with maximum body width (0.92) and body width at anus level (0.95); PC2 was positive for 15 traits and closely correlated with length (0.93). From this analysis, we could observe three clusters (Fig. 3): (i) all *M. incognita* isolates (100% of CGE), LGM 27, LGM 38, LGM 32 (*M. enterolobii*), and *M. javanica* (LGM 43 and LGM 44); (ii) the majority (70% of

CGE) of *M. enterolobii* isolates (LGM 34, LGM 04, LGM 28, LGM 29.1, LGM 30, LGM 23, and LGM 26); and (iii) the majority (85% of CGE) of *M. javanica* isolates (LGM 07.1, LGM 08, LGM 10.1, LGM 12, LGM 13, LGM 17, LGM 18, LGM 21, LGM 29.2, LGM 33, and LGM 37), *M. enterolobii* (LGM 03 and LGM 11), *M. arenaria*, and *M. inornata*.

Fig. 3. Dendrograms obtained from morphometric data of **A**, second-stage juveniles and **B**, males of *Meloidogyne* spp. from tobacco fields in Brazil subjected to principle components analysis.

The findings for the adult male specimens were consistent with those for J2 but with higher CGE indices. The inertia explained by the two first PC was 76.7%. PC1 was positive for 10 traits and closely correlated with length (0.97), maximum body width (0.97), and c (0.98); PC2 was positive for 9 traits and closely correlated with diameter of the bulb of the stylet (0.93). We could observe three clusters (Fig. 3): (i) all *M. enterolobii* isolates (100% of CGE) and LGM 27; (ii) the majority (92% of CGE) of *M. javanica* isolates (LGM 07.1, LGM 08, LGM 10.1, LGM 12, LGM 17, LGM 18, LGM 21, LGM 29.2, LGM 33, and LGM 37); and (iii) all *M. incognita* isolates (100% of CGE), *M. javanica* (LGM 43 and LGM 44), *M. arenaria*, *M. inornata*, and LGM 38.

Discussion

Our results demonstrated that disease caused by *Meloidogyne* spp. on *Rk* tobacco varieties occurs due to virulent *M. incognita* populations and the presence of other *Meloidogyne* spp., mainly *M. javanica* and *M. enterolobii*. Because there is no information about the national scenario before the introduction of *Rk* tobacco varieties, the hypothesis of directional selection favoring virulent populations is probable but not conclusive.

Perineal patterns showed limited power for identification of *Meloidogyne* spp., because unusual specimens occur commonly in monospecific populations and sibling species can coexist in the same sample. These situations are difficult even for expert taxonomists, resulting in misdiagnosis (Cofcewicz et al. 2004). High intraspecific variability in perineal patterns from populations of *M. enterolobii* was found in this study as well as in other studies (Almeida et al. 2008; Brito et al. 2008). Moreover, a considerable number of

species exhibited perineal patterns similar to those of *M. incognita* (Handoo et al. 2005; Moens et al. 2010). Therefore, the adoption of less-subjective approaches to identify *Meloidogyne* spp. such as Est electrophoresis is indispensable (Brito et al. 2004; Carneiro et al. 2000).

The presence of *M. incognita* populations in two distinct clades corroborates previous studies (Hugall et al. 1999), in which the complexity of ribosomal genes in apomictic tropical species was documented. Disparities between morphological data and ITS-1-RNA sequence data have been documented not only for *Meloidogyne* spp. but also for all nematodes (Mes and Cornelissen 2004). This incongruence restricts the diagnostic value of these sequences (Handoo et al. 2005). There are likely morphological or biochemical species indistinguishable from *M. incognita* sensu stricto, which could have evolved in a convergent manner. For instance, various *Meloidogyne* spp. have been described from *M. incognita* similar isolates (Carneiro et al. 2008a; Handoo et al. 2005). In relation to *M. enterolobii*, the polymorphism between sequences from *M. enterolobii* and others is a promising finding because it will enable the development of specific primers. The observation of a cluster composed only of LGM 45 supports its unique taxonomic status.

Biometrically, high CGE ratifies the taxonomic value of quantitative variables, as long as these variables are submitted to the proper multivariate statistical analyses. Analyses of these combinations allowed a better understanding of the morphometric relationships between *Meloidogyne* populations. On the other hand, univariate analyses demonstrated a low capacity to discriminate *Meloidogyne* spp.

Fig. 4. Distribution of *Meloidogyne* spp. identified in tobacco fields in southern Brazil. Symbols represent the species.

Unfortunately, the taxonomic status of LGM 27 and LGM 38 could not be resolved here. LGM 38 could be a unique biochemical variant (new phenotype) from *M. incognita*, because there was amplification of species-specific fragments by SCAR markers. However, this hypothesis should be taken with caution due to the limitations of these markers. LGM 38 is perhaps only a closely related taxon. The proximity between LGM 27 and *M. enterolobii* was very evident. Morphological and additional molecular studies will be necessary to solve the taxonomic status of both isolates.

The predominance of virulent genotypes under high-pressure selection has already been observed in the United States, Australia, and Brazil (Rich and Garcia 1985; Stanton et al. 1992; Zem et al. 1985). In the United States and Australia, this predominance was attributed to directional selection. The unexpected occurrence of avirulent populations (LGM 36, LGM 38, LGM 39, and LGM 42) to tobacco NC95 (*Rk*), as observed with the NCHT, parasitizing and causing disease on *Rk* cultivars, is probably explained by the occurrence of high temperatures (30 to 35°C) at which the *Rk* gene are rendered ineffective (Ogbuji 1981). In relation to *M. enterolobii*, variations in virulence have been observed worldwide, and our data further support the existence of host races of this parasite (Brito et al. 2004).

The present results showed that the range of *Meloidogyne* spp. that occur in tobacco crops in Brazil was higher than expected and included *M. incognita*, *M. javanica*, *M. enterolobii*, *M. arenaria*, and *M. inornata*. This diversity of species could be due to the more judicious identification but also to alterations in species composition as a result of intensive and prolonged use of the *Rk* gene. Indeed, the second hypothesis is corroborated by the NCHT results, in which prevalence of *M. incognita* race 2 was observed. Despite the predominance of *M. incognita*, its distribution was lower than that of *M. javanica* and *M. enterolobii*.

Contrary to a previous report (Zem et al. 1985), in our study, *M. arenaria* had a lower percentage of occurrence (5.1%). *M. enterolobii*, in turn, was the third most prevalent species (25.6%). This finding, combined with previous reports (Gomes et al. 2008), shows the importance of *M. enterolobii* as a tobacco pathogen due to not only its high incidence but also its distribution, especially in Santa Catarina (Fig. 4). However, questions about its recent detection need to be answered. Was *M. enterolobii* introduced and spread during this lapse of time? What is the cause of its rising incidence? Is *M. enterolobii* an indigenous species? Was *M. enterolobii* wrongly identified, probably due to the inherent difficulty of the methods used at that time? Based on the premise that *M. enterolobii* exhibits high morphological variability and that, in the past, the *Meloidogyne* spp. identification was performed almost exclusively by observation of morphology, the hypothesis that *M. enterolobii* has been misidentified as *M. arenaria* or *M. incognita* is plausible. Indeed, due to the adoption of nonsubjective criteria such as biochemical markers, the number of reports of *M. enterolobii* has increased worldwide (Almeida et al. 2008; Brito et al. 2008). In addition, *M. enterolobii* was detected parasitizing *Senefeldera multiflora* in an Atlantic Forest area in Rio de Janeiro state, demonstrating the indigenous presence of this nematode in Brazil and refuting the hypothesis of introduction (Lima et al. 2005). Moreover, the distribution of *M. enterolobii* overlaps the areas of the original Atlantic Forest biome. Corroborating this theory is the fact that the E180 population, from which *M. mayaguensis* (= *M. enterolobii*) was described, was first identified as *M. arenaria* (Rammah and Hirschmann 1988).

To our knowledge, this is the first report of *M. inornata* parasitizing tobacco in the southern region of Brazil. Previously, this species, first described in the 1950s, was observed on tobacco in the state of São Paulo (Figueiredo 1958). We believe that this nematode has often been identified as *M. incognita* due to the low use of biochemical markers and to the failure to observe morphological parameters of males and juveniles. The identification of *M. inornata* in our study was possible only by Est phenotypes, because its perineal pattern is very similar to *M. incognita* (Carneiro et al. 2008a).

Our findings have great practical importance, because they imply a considerable alteration in *Meloidogyne* management. Plant species

used for rotation or succession schemes are limited for *M. enterolobii* when compared with the options available for *M. javanica* and *M. incognita*. There are few recognized nonhosts of *M. enterolobii* (Brito et al. 2007; Moens et al. 2010). This line of reasoning is also applied to management with plant resistance. Although there are no cultivars with high resistance to virulent biotypes of *M. incognita* (races 2 and 4), *M. arenaria* (race 2), and *M. javanica*, resistance sources are known in wild *Nicotiana* spp. (Davis et al. 1988; Di Vito et al. 1998; Johnson et al. 2005). However, there is no information on plant resistance for *M. enterolobii*. This is important because *M. enterolobii* can parasitize even cultivars carrying resistance genes, which generally confer resistance to *M. incognita*, *M. javanica*, and *M. arenaria* (Brito et al. 2007; Cetintas et al. 2007). Another question is the nonexistence of economic threshold levels for *M. enterolobii* on tobacco plants. It is known that, on some crops, *M. enterolobii* exhibits higher multiplication rates and pathogenicity levels than *M. incognita* (Cetintas et al. 2007); the implications of this on tobacco are unknown. In summary, it is necessary to conduct studies in the future that will support the integration of several management strategies targeting *Meloidogyne* spp. on tobacco crops.

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Literature Cited

- ABIFUMO. 2013. Associação Brasileira da Indústria do Tabaco. Online publication. <http://www.abifumo.org.br/produ.htm>
- Almeida, E. J., Soares, P. L. M., Silva, A. R., and Santos, J. M. 2008. Novos registros de *Meloidogyne mayaguensis* no Brasil e estudo morfológico comparativo com *M. incognita*. Nematol. Bras. 32:236-241.
- Brito, J. A., Kaur, R., Cetintas, R., Stanley, J. D., Mendes, M. L., McAvoy, E. J., Powers, T. O., and Dickson, D. W. 2008. Identification and isozymes characterization of *Meloidogyne* spp. infecting horticultural and agronomic crops, and weed plant in Florida. Nematology 10:757-766.
- Brito, J. A., Powers, T. O., Mullin, P. G., Inseña, R. N., and Dickson, D. W. 2004. Morphological and molecular characterization of *Meloidogyne mayaguensis* isolates from Florida. J. Nematol. 36:232-240.
- Brito, J. A., Stanley, J. D., Kaur, R., Cetintas, R., Di Vito, M., Thies, J. A., and Dickson, D. W. 2007. Effects of the *Mi-1*, *N*, *Tabasco* genes on infection and reproduction of *M. mayaguensis* on tomato and pepper genotypes. J. Nematol. 39:327-332.
- Brown, D. J. F., and Tophan, P. B. 1984. A comparison of the reported variation in the morphometrics of *Xiphinema diversicaudatum* (Nematoda: Dorylaimida) and the effect of preparing specimens for examination by optical microscopy. Nematol. Mediterr. 12:169-186.
- Carneiro, R. M. D. G., Almeida, M. R. A., and Quénéhervé, P. 2000. Enzyme phenotypes of *Meloidogyne* spp. populations. Nematology 2:645-654.
- Carneiro, R. M. D. G., Mendes, L. M., Almeida, M. R. A., Santos, M. F. A., Gomes, A. C. M. M., and Karssen, G. 2008a. Additional Information on *Meloidogyne inornata* Lordello, 1956 (Tylenchida: Meloidogynidae) and its characterization as valid species. Nematology 10:123-136.
- Carneiro, R. M. D. G., Santos, M. F. A., Almeida, M. R. A., Mota, F. C., Gomes, A. C. M. M., and Tigano, M. S. 2008b. Diversity of *Meloidogyne arenaria* using morphological, cytological and molecular approaches. Nematology 10:819-834.
- Cetintas, R., Kaur, R., Brito, J. A., Mendes, M. L., Nyczepir, A. P., and Dickson, D. W. 2007. Pathogenicity and reproductive potential of *Meloidogyne mayaguensis* and *M. floridensis* compared with three common *Meloidogyne* spp. Nematologica 37: 21-31.
- Cherry, T., Szalanski, A. L., Todd, T. C., and Powers, T. O. 1997. The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaimidae). J. Nematol. 29:23-29.
- Cofcewicz, E. T., Carneiro, R. M. D. G., Castagnone-Sereno, P., and Quénéhervé, P. 2004. Enzyme phenotypes and genetic diversity of root knot nematodes parasitizing *Musa* in Brazil. Nematology 6:85-95.
- Davis, E. L., Rich, J. R., Gwynn, G. R., and Sisson, V. 1988. Greenhouse evaluation of *Nicotiana* spp. for resistance to root knot nematodes. Nematologica 18:99-107.
- Díaz-Silveira, M. F., and Herrera, J. O. 1998. An overview of nematological problems in Cuba. Nematologica 28:151-164.
- Di Vito, M., Lombardi, D. A., Zaccaro, G., and Catalano, F. 1998. Response of lines and cultivars of tobacco and *Nicotiana* species to Italian populations of *Meloidogyne* species. Nematol. Mediterr. 26:271-274.
- Ferraz, L. C. C. B., and Brown, D. J. F. 2002. An Introduction to Nematodes: Plant Nematology. Pensoft Publishers, Sofia, Bulgaria.

- Figueiredo, M. B. 1958. Algumas observações sobre os nematóides que atacam o fumo no estado de São Paulo. *Rev. Agric.* 33:69-73.
- Gomes, C. B., Couto, M. E. O., and Carneiro, R. M. D. G. 2008. Registro de ocorrência de *Meloidogyne mayaguensis* em goiabeira e fumo no sul do Brasil. *Nematol. Bras.* 32:244-247.
- Handoo, Z. A., Skantar, A. M., Carta, L. K., and Erbe, E. F. 2005. Morphological and molecular characterization of a new root-knot nematode, *Meloidogyne thailandica* n. sp. (Nematoda: Meloidogynidae), parasitizing ginger (*Zingiber* sp.). *J. Nematol.* 37:342-353.
- Hartman, K. M., and Sasser, J. N. 1985. Identification of *Meloidogyne* species on the basis of differential host and perineal pattern morphology. Pages 68-69 in: *An Advanced Treatise on Meloidogyne*. Vol. II: Methodology. K. R. Barker, C. C. Carter, and J. N. Sasser, eds. North Carolina State University, Raleigh.
- Hugall, A., Stanton, J., and Moritz, C. 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomitic *Meloidogyne*. *Mol. Biol. Evol.* 16:157-164.
- Hussey, R. S., and Barker, K. R. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. *Plant Dis. Rep.* 57: 1025-1028.
- Johnson, C. S., Way, J., and Barker, K. R. 2005. Nematode parasites of tobacco. Pages 675-708 in: *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*. M. Luc, R. A. Sikora, and J. Bridge, eds. CABI International, St. Albans, UK.
- Lima, I. M., Souza, R. M., Silva, C. P., and Carneiro, R. M. G. 2005. *Meloidogyne* spp. from preserved areas of Atlantic Forest in the State of Rio de Janeiro, Brazil. *Nematol. Bras.* 29:31-38.
- McClure, M. A., Nischwitz, C., Skantar, A. M., Schmitt, M. E., and Subbotin, S. A. 2012. Root-knot nematodes in golf course greens of the western United States. *Plant Dis.* 96:635-647.
- Mes, T. H. M., and Cornelissen, A. W. C. A. 2004. Non-homogenized ITS regions in the parasitic nematode *Cooperia oncophora*. *Parasitology* 129:213-222.
- Moens, M., Perry, R. N., and Star, J. L. 2010. *Meloidogyne* species—A diverse group of novel and important plant parasites. Pages 1-13 in: *Root-Knot Nematodes*. M. Moens, R. N. Perry, and J. L. Star, eds. CABI International, Wallingford, UK.
- Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield, D. W., II. 1997. GeneDoc, analysis and visualization of genetic variation. *EMBL NEWS* 4:14.
- Nono-Womdim, R., Swai, I. S., Mrosso, L. K., Chadha, M. L., and Opeña, R. T. 2002. Identification of root-knot nematode species occurring on tomatoes in Tanzania and resistant lines for their control. *Plant Dis.* 86:127-130.
- Ogbuji, O. R. 1981. Variations in infectivity among populations of *Meloidogyne javanica* on tobacco and pepper. *Plant Dis.* 65:65-66.
- Rammah, A., and Hirschmann, H. 1988. *Meloidogyne mayaguensis* n. sp. (Meloidogynidae), a root knot nematode from Puerto Rico. *J. Nematol.* 20:58-69.
- Randig, O., Bongiovanni, M., Carneiro, R. M. D. G., and Castagnone-Sereno, P. 2002. Genetic diversity of root-knot nematodes from Brazil and development of Scar markers specific for the coffee-damaging species. *Genome* 45:862-870.
- R Development Core Team. 2012. R: A Language and Environment for Statistical Computing. Online publication. R Foundation for Statistical Computing, Vienna. <http://www.R-project.org>
- Rich, J. R., and Garcia, M. R. 1985. Nature of the root knot disease in Florida tobacco. *Plant Dis.* 69:972-974.
- Shew, H. D., and Lucas, G. B. 1991. Compendium of Tobacco Diseases. American Phytopathological Society, St. Paul, MN.
- Siddiqi, M. R., ed. 2000. *Tylenchida Parasites of Plants and Insects*, 2nd ed. CABI Publishing, Wallingford, UK.
- Sinditabaco. 2013. Online publication. Sindicato Interestadual da Indústria do Tabaco. <http://www.sindifumo.com.br>
- Souza Cruz. 2013. Online publication. <http://www.souzacruz.com.br>
- Stanton, J. M., McNicol, C. D., and Steele, V. 1998. Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction. *Australas. Plant Pathol.* 27:112-115.
- Stanton, J. M., O'Brien, P. C., Schipke, L. G., Hugall, A., and Moritz, C. 1992. Species of root-knot nematode (*Meloidogyne* spp.) affecting tobacco in north Queensland, including two new host races of *M. arenaria*. *Australas. Plant Pathol.* 21:150-157.
- Tamura, K., Peterson, D., Perterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. Mega 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731-2739.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The Clustal X windows interface, flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876-4882.
- Trudgill, D. L., and Blok, V. C. 2001. Apomitic, polyphagous root knot nematodes: Exceptionally Successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* 39:53-77.
- Yang, B., and Eisenback, J. D. 1983. *Meloidogyne enterolobii* n. sp. (Meloidogynidae), a root knot nematode parasitizing Pacara earpod tree in China. *J. Nematol.* 15: 381-391.
- Yi, H. Y., Ruft, R. C., Wernsman, E. A., and Conkling, M. C. 1998. Mapping the root knot nematode resistance gene (*Rk*) in Tobacco with RAPD markers. *Plant Dis.* 82:1319-1322.
- Zem, A. C., Zanon, J. I., and Monteiro, A. R. 1985. Nota sobre a ocorrência de nematóides associados à cultura do fumo no Estado de Santa Catarina e Rio Grande do Sul. Page 62 in: *Reunião Brasileira de Nematologia*, 9. Resumos Sociedade Brasileira de Nematologia/Copersucar, Piracicaba, SP, Brazil.