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Morphological, enzymatic and molecular characterization of root-knot nematodes parasitizing vegetable crops

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ABSTRACT

Species of the genus *Meloidogyne* are limiting factors in vegetable crop production. Studies in Brazil about the occurrence of root-knot nematodes in areas of vegetable crop growth have been conducted without using advanced techniques. Using modern techniques, such as biochemical and molecular methods, improves the accuracy of *Meloidogyne* species identification. The present study characterized species of *Meloidogyne* in 36 samples associated with vegetable crops using isoenzyme electrophoresis, SCAR markers, and morphological markers, in addition to validating SCAR markers for accurate species identification. The species *M. incognita*, *M. javanica*, *M. hapla*, *M. morocciensis*, and *M. arenaria* were identified, with the first two being the most frequent. Here, the species *M. arenaria* parasitizing scarlet eggplant and *M. morocciensis* parasitizing pumpkin and cabbage are reported in Brazil for the first time. Esterase electrophoresis efficiently separated the species of *Meloidogyne* found in vegetable crops; however, SCAR markers were only effective for the identification of *M. incognita*, *M. javanica*, and *M. hapla*, since the primer pair Far/Rar yielded no amplification product to confirm the identity of *M. arenaria*. The species *M.arenaria* and *M. morocciensis* could not be distinguished by the female perineal patterns. Based on the present results, new primers should be designed for the identification of *M. arenaria* and *M. morocciensis*.

Keywords: *Meloidogyne*, diagnosis, molecular biology, vegetable crops.

RESUMO

Caracterização morfológica, enzimática e molecular de nematoide das galhas, parasitas de hortaliças

Espécies do gênero *Meloidogyne* constituem um dos fatores limitantes à produção de olerícolas. Estudos têm sido realizados no Brasil a respeito da ocorrência de espécies de nematoide de galhas em áreas de cultivo de olerícolas sem o uso de técnica avançada. O uso de técnicas modernas, tais como, os métodos bioquímicos e moleculares aumentam a segurança na identificação das espécies de *Meloidogyne*. Neste trabalho foram caracterizadas espécies de *Meloidogyne* associadas a olerícolas em um total de 36 amostras por meio de eletroforese de isoenzimas, marcadores SCAR e morfológico. Além disso, foram validados marcadores SCAR para identificação acurada dessas espécies. Desta forma, foram identificadas as espécies *M. incognita*, *M. javanica*, *M. hapla*, *M. morocciensis* e *M. arenaria*, sendo as duas primeiras espécies mais frequentes. Foram ainda relatadas, pela primeira vez no Brasil, as espécies: *M. arenaria* parasitando plantas de jiló e *M. morocciensis* parasitando plantas de abóbora e repolho. A eletroforese de esterase foi eficiente em separar todas as espécies de *Meloidogyne* encontradas em olerícolas. Entretanto, os marcadores SCAR foram eficientes, somente, na identificação de *M. incognita*, *M. javanica* e *M. hapla*, no entanto, os primers Far/Rar não promoveram amplificação para confirmação da identidade de *M. arenaria*. Na avaliação da configuração perineal de fêmeas, não foi possível distinguir as espécies *M. arenaria* e *M. morocciensis*. Com base nos resultados, novos primers devem ser desenvolvidos para identificação de *M. arenaria* e *M. morocciensis*.

Palavras chave: *Meloidogyne*, diagnose, biologia molecular, hortaliças.

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Plant parasitic nematodes are the major pest problem in vegetable crops in the tropics (Carneiro *et al*., 2008b). In certain production areas, root-knot nematodes are responsible for an approximate 30% yield reduction in vegetable crops (Anwar *et al*., 2009). Vegetable productivity reductions are directly related to preplant infestation levels in the soil; as soil infestation increases, the amount of damage and yield loss also increases (Noling, 2012). *Meloidogyne* is the most important genus in Brazil due to its wide distribution throughout different vegetable production regions, polyphagia, and physiological variability among populations of the same species (Moens *et al*., 2009).

There exist three techniques for *Meloidogyne* identification, representing the evolution of knowledge regarding this nematode species over time: perineal pattern, electrophoresis of isoenzymes, and sequence-characterized amplified region (SCAR). Firstly, in 1949, Chitwood described the *Meloidogyne* genus and subsequently developed a perineal pattern method that was able to distinguish between five species of *Meloidogyne*. During 1950−70, the descriptions were improved (Taylor & Sasser, 1978). Secondly, application of electrophoresis to *Meloidogyne* species identification was well established by Esbenshade & Triantaphyllou (1990).

Thirdly, studies by Zijlstra *et al*. (2000); Randig *et al*. (2002); Meng *et al*. (2004) and Tigano *et al*. (2010) using SCAR markers were widely accepted by plant-parasitic nematode researchers.

Identifying *Meloidogyne* species based only on morphological characteristics such as the perineal pattern in females is not reliable, since the analysis may not reflect the species diversity. For instance, *Meloidogyne incognita* in coffee was characterized for 22 years as *M. incognita* race 5 or biotype IAPAR, which only occurred in Brazil; however, it was defined as a novel species by electrophoresis (Carneiro *et al*., 1996). The electrophoresis of isoenzymes is more precise and permits the identification of species in a mixture and characterization of atypical populations (Carneiro *et al*., 2016). Nevertheless, identification using this technique is limited to the stage of adult females, but females are not always suitable for analysis due to the state of root degradation (Salgado *et al.*, 2015). SCAR markers, however, represent a fast and accurate tool with which to determine the identity of *Meloidogyne* species, in addition to allowing the detection of species in a mixture and making it possible to identify species based on eggs and juveniles, which eliminates the problem of inadequate females (Salgado *et al.*, 2015). Yet, only nine *Meloidogyne* species detected in Brazil can be identified by SCAR markers (Carneiro *et al*., 2016).

The main *Meloidogyne* species parasitizing Brazilian vegetables are *Meloidogyne javanica*, *M. incognita*, *M. arenaria*, and *M. hapla*. Another species of great importance, *M. enterolobii*, has also been detected in vegetables (Carneiro *et al*., 2006; Almeida *et al*., 2008). Despite the importance of the correct identification of *Meloidogyne* species, few studies in Brazil have characterized these species based on esterase and SCAR markers. Using electrophoresis analysis, *M. enterolobii* was found in the states of Mato Grosso, Ceará, São Paulo, and eastern Minas Gerais (Carneiro *et al*., 2006; Oliveira *et al*., 2007; Almeida *et al*., 2008; Silva *et al*., 2016); *M. incognita*, *M. javanica*, and *M. arenaria* were found in eastern Minas Gerais (Oliveira *et al*., 2007); and *M. ethiopia* was reported in the Federal District (Carneiro *et al*., 2005). Based on electrophoresis and SCAR markers, different species were identified in Brazil in association with potatoes, such as *M. javanica*, *M. incognita*, *M. arenaria*, and *M. ethiopica* (Medina *et al.*, 2017). Using only the perineal pattern technique, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla* were detected in several potato-growing states in Brazil (Charchar, 1997), and *M. incognita* and *M. javanica* were found in vegetable crops in Maranhão (Silva, 1991).

The combined use of enzymatic, molecular, and morphological methods better characterize the diversity of *Meloidogyne* species in vegetables with higher identification reliability. Breeders and agribusiness consultants benefit from these studies with respect to the selection of genotypes for research and the recommendation of cultivars specific to the species of nematode. Familiarization and validation of biomolecular protocols for *Meloidogyne* tropic species are also important.

Thus, the objectives of the present study were to: 1) evaluate the diversity of *Meloidogyne* species associated with vegetables in various states in Brazil using morphological, enzymatic, and molecular characterization; and 2) validate the SCAR markers for the accurate identification of these species, with a view to such markers being used in diagnostic laboratories in the future.

MATERIAL AND METHODS

Collecting and obtaining females of *Meloidogyne* spp.

Thirty-six populations of *Meloidogyne* were obtained from vegetable-producing areas in seven different states in Brazil (Table 1). Five sub-samples were collected composing one sample of soil and roots removed from the rhizosphere of plants with root-knot symptoms at approximately 20-cm depth. The samples were placed in plastic bags, stored in styrofoam boxes with identification labels, and subsequently processed.

Nematodes were extracted from the roots containing galls. Roots were rinsed in standing water and dissected with tweezers and fine scalpels under a stereoscopic microscope to release the milky-white females. The females were used for subsequent evaluation of the perineal pattern and isoenzyme electrophoresis.

Multiplication of *Meloidogyne* spp. populations

Samples with roots in an advanced rotting state and not containing appropriate females for morphological and electrophoretic studies had the roots washed in standing water. Subsequently, the samples were cut into 0.5-cm pieces, and the eggs extracted using the process described by Hussey & Barker (1973). Tomato seedlings (cv. Santa Clara) were inoculated with the eggs in a greenhouse for nematode multiplication for approximately two months, when it was possible to obtain appropriate females for the study.

Morphological studies of *Meloidogyne* spp. populations

Ten milky-white females removed from infected roots were immediately transferred to one drop of 45% lactic acid. The perineal area of each female was cut, cleaned, and mounted on slides with glycerol for identification using a light microscope.

Isozyme characterization of *Meloidogyne* spp. populations

Adult females of milky-white color in early oviposition were taken either from newly collected field samples or tomato roots resulting from greenhouse multiplication for 60 days. The females were transferred to 0.2-mL microfuge tubes containing 10 µL protein extraction buffer, according to Davis (1964), and were subsequently smashed with a rounded-end glass rod in an ice block to prevent protein denaturation. Vertical polyacrylamide gel electrophoresis was used with a bis-acrylamide concentration of 8% for the stacking gel and 4% for the running gel. When electrophoresis was not performed on the same day, the females were kept in the freezer at a temperature of approximately-10°C.

For electrophoresis, nine females

were used for each population, e.g., one female per well. The protein extract from *M. javanica* was placed in the first, sixth, and final well of each gel as a standard for comparison with the phenotypes found. Electrophoresis was performed in a refrigerator at approximately 4°C, with a voltage of 80 V through the stacking gel for 15 minutes and 200 V through the running gel for 30 minutes. Migration was monitored by means of displacement of the frontline of bromophenol blue, and electrophoresis was stopped when this line was 1 cm from the bottom of the gel. After stopping the electrophoretic run, the gel was removed from the plate. The running gel was immersed in a developing solution prepared immediately prior to use, to study esterase isozyme (EST) pattern. The protein bands were compared with the positions presented by the migration of the *M. javanica* esterase pattern, as reported in the literature (Esbenshade & Triantaphyllou 1990; Carneiro *et al*., 2008a).

DNA extraction

The extraction of genomic DNA was performed using the protocol described by Holterman *et al*. (2006), with slight modifications. To obtain nematodes, the roots were cut into 0.5-cm pieces, from which eggs were extracted using the procedure described by Hussey & Barker (1973). The eggs were placed in a hatching chamber, and 10 second-stage juveniles (J2) were transferred to PCR microtubes containing 50 µL HLB buffer [0.2 M NaCl, 0.2 M Tris-HCl (pH= 8.0), 1%(v/v) β-mercaptoethanol, and 800 µL/mL proteinase K]. The samples were centrifuged at 14,000 rpm for one minute, incubated at 65°C for 2 hours and 99°C for 5 minutes, and subsequently stored at -20°C for later use in PCR reactions.

Identification of *Meloidogyne* species by SCAR markers

Based on studies of perineal pattern and esterase phenotypes, a prior identification was carried out and confirmed using SCAR markers. The SCAR markers used to identify the species of *Meloidogyne* are listed in Table 2.

Amplification reactions using the SCAR markers were performed in a final volume of 25 µL, containing 1 µL DNA, 0.7 µL each primer (10 µM), 0.5 µL acetylated BSA (10 mg/mL), 12.5 µL GoTaq Colorless PCR Master Mix (Promega, Madison, USA), and 9.6 µL nuclease-free water. PCR reactions were performed using a My Cycler ™thermal cycler (BIO-RAD). The amplification conditions for each primer set are described in Table 3. All primers were synthesized by Sigma-Aldrich (Madrid, Spain). The PCR products were subjected to agarose gel electrophoresis at 0.7% and stained with GelRed (Biotium®) to visualize the bands. The length of each amplified fragment was compared with the Axygen 1 kb DNA ladder.

RESULTS AND DISCUSSION

In the present study, analysis of the diversity of *Meloidogyne* species in 36 soil samples and vegetable roots with galls collected from seven different states in Brazil yielded six different species: *M. incognita*, *M. javanica*, *M. hapla*, *M. arenaria*, and *M. morocciensis* (Table 1). The most frequent species were *M. incognita* (55.5%) and *M. javanica* (44.4%), and the least frequent were *M. arenaria* (11.1%), *M. hapla* (8.3%), and *M. morocciensis* (5.5%). Mixed populations occurred in 22% samples. The predominance of the *M. incognita* and *M. javanica* species in vegetable crops and the lower frequency of *M. arenaria* and *M. hapla* found in the present study are in accordance with reports by Charchar (1997), Silva (1991), Oliveira *et al*. (2007), and Rosa *et al*. (2013); however, only the latter two references used electrophoretic pattern of esterase for species identification. Nevertheless, to date, there have been no reports of *M. arenaria* parasitizing

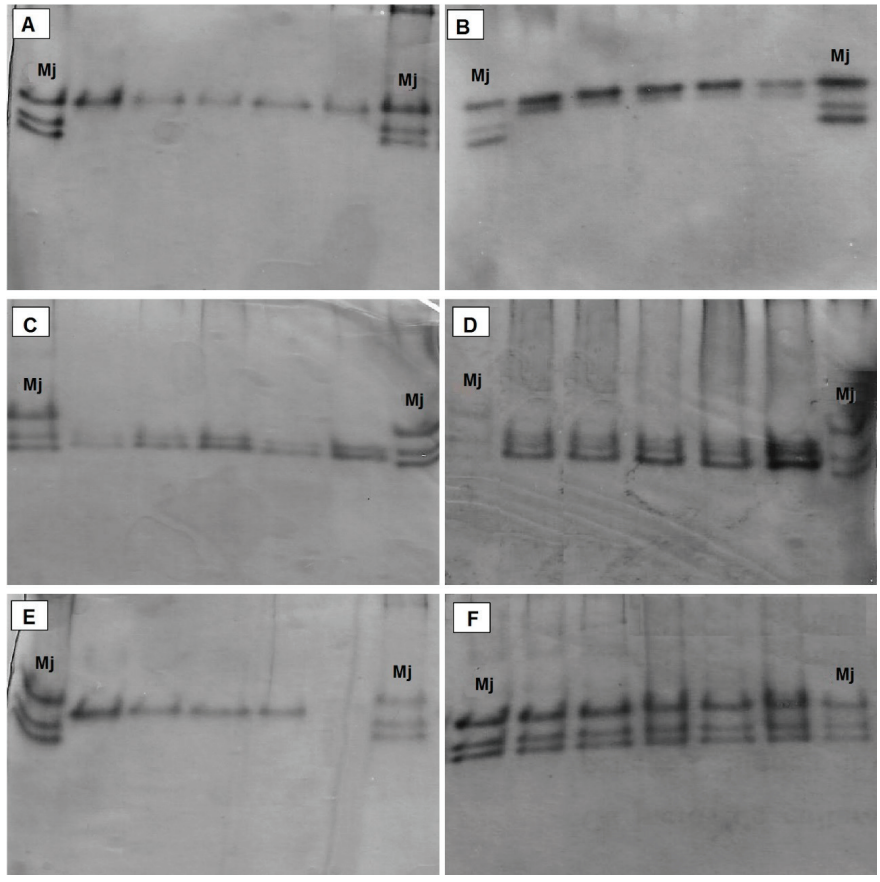

Figure 1. Phenotypes of esterase of *Meloidogyne* spp. associated with vegetable crops. **A:** phenotype I1 of *M. incognita*; **B:** phenotype I2 of *M. incognita;* **C:** phenotype A2 of *M. arenaria*; **D:** phenotype A3 of *M. morocciensis*; **E:** phenotype H1 of *M. hapla*; **F:** phenotype J3 of *M. javanica*. **Mj:** phenotype of *M. javanica* used as comparing patterns. Lavras, UFLA, 2015.

scarlet eggplant (*Solanum aethiopicum*) or *M. morocciensis* parasitizing pumpkin (*Cucurbita pepo*) and cabbage (*Brassica oleracea*) in Brazil.

The species found in the present study showed the following characteristics: *M. incognita* had a high trapezoidal dorsal arch and striae varying from fine to coarse; *M. javanica* had a distinct perineal pattern with incisures on the lateral lines, interrupting the streaks of ventral and dorsal fields, where few or no striae crossed the lateral lines of the perineal pattern; *M. hapla* had smooth striae, a rounded dorsal arch,

Table 1. Phenotype of esterase and species names of *Meloidogyne* associated to vegetable with galls in several states, Brazil. Lavras, UFLA, 2015.

Population Code	Nematode species	Geographical origin	Host plant	Phenotype of esterase
1	*M. incognita*	Viçosa-MG	*Cucurbita pepo*	I1
2	*M. arenaria*	Cajuri-MG	*Solanum aethiopicum*	A2
3	*M. morocciensis*	Cajuri-MG	*Cucurbita pepo*	A3
4	*M. javanica* and *M. incognita*	Cajuri-MG	*Solanum lycopersicum*	J3 and I1
5	*M. javanica*	Cajuri-MG	*Solanum aethiopicum*	J3
6	*M. incognita*	Cajuri-MG	*Cucurbita pepo*	I2
7	*M. incognita*	Santana da Vargem-MG	*Lactuca sativa*	I2
8	*M. arenaria* and *M. incognita*	Santana da Vargem-MG	*Lactuca sativa*	A2 and I1
9	*M. incognita*	Santana da Vargem-MG	*Abelmoschus esculentus*	I1
10	*M. hapla*	Pouso Alegre-MG	*Fragaria vesca*	H1
11	*M. javanica*	Lavras-MG	*Solanum tuberosum*	J3
12	*M. javanica*	Lavras-MG	*Lactuca sativa*	J3
13	*M. incognita*	Ijaci-MG	*Cucurbita pepo*	I1
14	*M. javanica* and *M. hapla*	Rio Paranaíba-MG	*Daucus carota*	J3 and H1
15	*M. javanica* and *M. incognita*	Rio Paranaíba-MG	*Solanum tuberosum*	J3 and I1
16	*M. incognita*	Rio Paranaíba-MG	*Daucus carota*	I1
17	*M. javanica* and *M. incognita*	Rio Paranaíba-MG	*Daucus carota*	J3 and I2
18	*M. incognita*	Muzambinho-MG	*Cucumis sativus*	I2
19	*M. incognita*	Ibertioga-MG	*Arracacia xanthorrhiza*	I2
20	*M. javanica*	Piedade-SP	*Beta vulgaris*	J3
21	*M. javanica*	Piedade-SP	*Solanum lycopersicum*	J3
22	*M. hapla*	Piedade-SP	*Solanum lycopersicum*	H1
23	*M. incognita*	Piedade-SP	*Lactuca sativa*	I2
24	*M. incognita, M. javanica* and *M. arenaria*	Piedade-SP	*Cichorium endivia*	I1, J3 and A2
25	*M. incognita*	Holambra-SP	*Solanum lycopersicum*	I1
26	*M. javanica* and *M. incognita*	Casa Nova-Ba	*Cucumis melo*	J3 and I1
27	*M. morocciensis*	Mucugê-Ba	*Brassica oleracea*	A3
28	*M. incognita*	Piracanjuba-GO	*Abelmoschus esculentus*	I1
29	*M. incognita*	Burite Alegre-GO	*Solanum lycopersicum*	I2
30	*M. javanica*	Caldas Novas-GO	*Abelmoschus esculentus*	J3
31	*M. incognita*	Bom Jesus-GO	*Solanum aethiopicum*	I1
32	*M. javanica*	Morrinhos-GO	*Abelmoschus esculentus*	J3
33	*M. javanica*	Petrolina-PE	*Solanum lycopersicum*	J3
34	*M. javanica* and *M. incognita*	Petrolina-PE	*Beta vulgaris L.*	J3 and I1
35	*M. javanica*	Venda Nova do Imigrante-ES	*Solanum lycopersicum*	J3
36	*M. arenaria*	Pelotas-RS	*Solanum tuberosum*	A3

Table 2. Sequences of SCAR markers used for identifying species of *Meloidogyne.* Lavras, UFLA, 2015.

SCAR Primers	Sequence (5'→ 3')	Size of the SCAR (bp)	Identified species	References
FhN RhN	GCCTTCTTTGGATTCCTCTCA GGCTCATCCTTGCTGTAAAT	420	*M. hapla*	Zijlstra (2000)
Far Rar	TCGGCGATAGAGGTAAATGAC TCGGCGATAGAGGTAAATGAC	420	*M. arenaria*	Zijlstra *et al.* (2000)
Fjav Rjav	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	670	*M. javanica*	Zijlstra *et al*. (2000)
inc-K14-F inc-K14-R	GGGATGTGTAAATGCTCCTG CCCGCTACACCCTCAACTTC	399	*M. incognita*	Randig *et al*. (2002)

Table 3. Amplification conditions used for different primers in identification of *Meloidogyne* species. Lavras, UFLA, 2015.

Primers	35 cycles					
FhN/RhN	2 min/ 94ºC	30 s/94ºC	30 s/58º C	1 min/72ºC	4ºC /∞	
Far/Rar	2 min/ 94ºC	30 s/94ºC	30 s/61º C	1 min/72ºC	4ºC /∞	
Fjav/Rjav	2 min/ 94ºC	30 s/94ºC	30 s/64° C	1 min/72ºC	4ºC /∞	
inc-K14-F/inc-K14-R	5 min/ 94ºC	30 s/94ºC	45 s/55ºC	1 min/70ºC	8 min/ 70ºC	4ºC /∞

and visible points near the end of the tail; *M. arenaria* and *M. morocciensis* had a similar perineal pattern, becoming impossible to distinguish, with a low rounded dorsal arch, coarse striae, and some striae bent toward the vulva. All perineal patterns were similar to those already described by Jepson (1987) and Carneiro *et al*. (2008a).

Although perineal patterns have long been used for the identification of *Meloidogyne* species, this method has shown inconsistent results, besides being subjective and prone to many mistakes (Carneiro *et al*., 2004). For instance, *M. arenaria* and *M. morocciensis* cannot be distinguished by perineal patterns, which has also been corroborated by Conceição *et al*. (2012). Perineal patterns of *M. incognita* described here were also similar to other species such as *M. paranaensis*, *M. enterolobii* (Syn. *M. mayaguensis*), and *M. izalcoensis*.

In the present study, the electrophoretic pattern of esterase was sufficient to distinguish *Meloidogyne* species, including *M. arenaria* and *M. morocciensis*, leaving no doubt of species identity. Of the 36 *Meloidogyne* populations assessed, we found six phenotypes in electrophoretic analysis using esterase (Table 1; Figure 1). The phenotypes were J3, characteristic of *M. javanica*; I1 and I2, characteristic of *M. incognita*; H1, characteristic of *M. hapla*; A2, characteristic of *M. arenaria;* and A3, characteristic of *M. morocciensis*. The phenotype I1 (66.7%) of *M. incognita* was more frequent than I2 (33.3%) of the same species. Other studies have used isozyme electrophoresis to confirm identities by perineal patterns (Oliveira *et al*., 2007; Carneiro *et al*., 2008a; Barros *et al*., 2011).

The pairs of SCAR markers usually employed in the diagnosis of *Meloidogyne* species were more efficient in distinguishing the species *M. incognita*, *M. javanica*, and *M. hapla* associated with vegetable crops (Figure 2); however, the primer pair (Far/Rar) indicated for the identification of *M. arenaria* was inefficient, since no PCR product was detected even following optimization of the annealing temperature. In accordance, Carneiro *et al.* (2008a) found no amplification of DNA fragments of three populations of the *M. arenaria* phenotype A2 using the same primer pair. Therefore, the development of a novel pair of species-specific primers for *M. arenaria* and *M. morocciensis* is necessary.

Several field samples showed mixed *Meloidogyne* populations (Table 1). Since DNA was extracted from 10 J2 samples, some could have predominated, such as sample 8, in which *M. arenaria* prevailed over *M. incognita*; thus, the sample DNA was not amplified using the inc-K14-F/inc-K14-R primer pair.

Root samples sometimes arrive already degraded to the laboratory, precluding withdrawal of females or even eggs; thus, DNA extraction from J2 may facilitate diagnosis in laboratories, since J2 may be found in the soil.

Isozyme electrophoresis in conjunction with SCAR markers was efficient in diagnosing *Meloidogyne* species that parasitize vegetable crops, expanding the pool of *Meloidogyne* species in different states in Brazil. Despite SCAR markers being efficient, only nine species could be identified in Brazil by this method, whereas there is a pattern of esterase for all species detected in Brazil, which makes isoenzyme electrophoresis more advantageous (Carneiro *et al*., 2016). In the present study, we verified that the primer pair Far/Rar does not

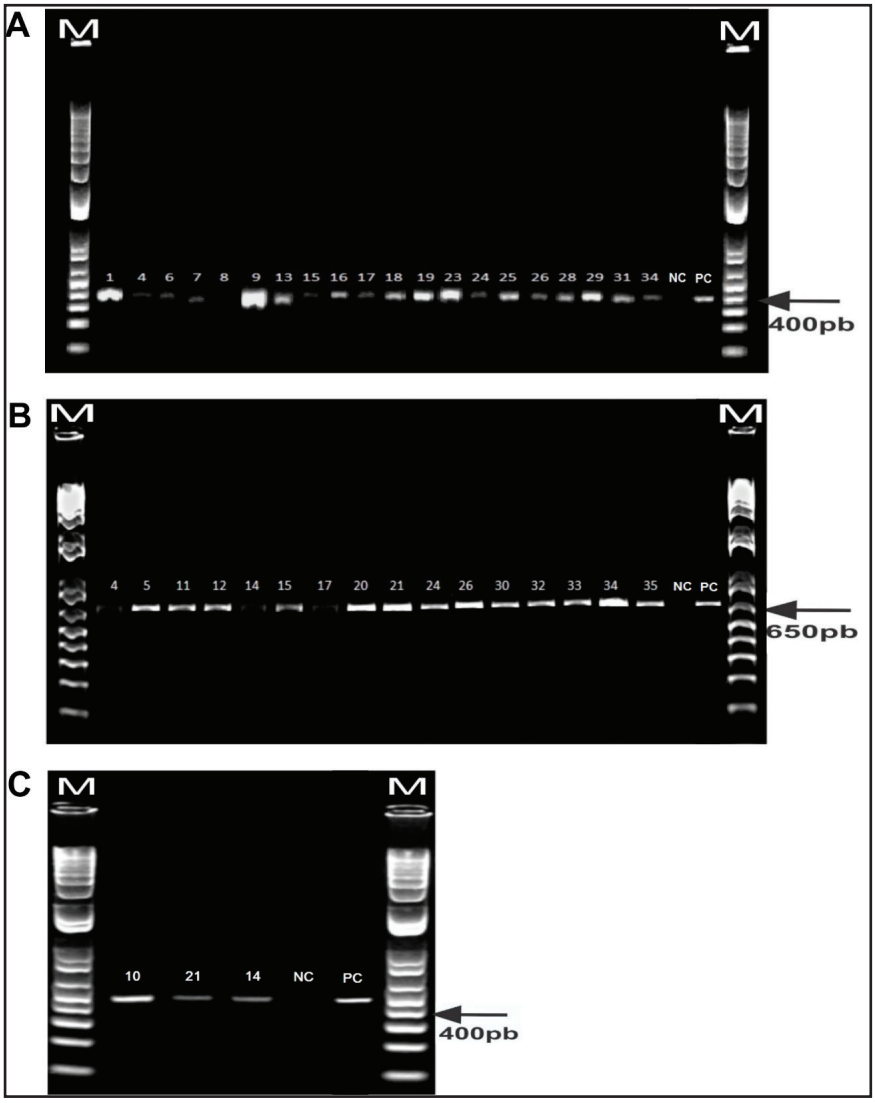

Figure 2. Specific amplification by PCR from DNA of *Meloidogyne* spp. associated with vegetables crops: **(A)** inc-K14-F/inc-K14-R, **(B)** Fjav/Rjav and **(C)** FhN/RhN. **M:** 1 kb plus ladder; **1-36** populations of *Meloidogyne*; **NC:** negative control; **PC:** positive control. Lavras, UFLA, 2015.

identify *M. arenaria*, and there is no primer described for *M. morocciensis*; however, the inc-K14-F/inc-K14-R, Fjav/Fjav, and FhN/RhN primer pairs were validated to characterize *M. incognita*, *M. javanica*, and *M. hapla*, respectively. Molecular, enzymatic, and morphological techniques must be used in combination to provide reliable diagnosis.

Our data allowed us to access the diversity of *Meloidogyne* species associated with vegetables crops, and to verify that a plant may be infected with more than one species simultaneously. Here, we report, for the first time in Brazil, the presence of *M. arenaria* parasitizing scarlet eggplant and *M. morocciensis* parasitizing pumpkin and cabbage. The present findings will contribute to future studies regarding genetic improvement and control methods.

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