Distribution of *Meloidogyne* species in carrot in Brazil

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ABSTRACT: *Root-knot nematodes (RKN – Meloidogyne spp.) are one of the most serious threats to carrot production worldwide. In Brazil, carrots are grown throughout the year, and economic losses due to RKN are reported. Since little is known on the distribution of RKN species in carrot fields in Brazil, we collected plant and soil samples from 35 fields across six states. Based on the morphology of perineal patterns, esterase phenotypes and species-specific PCR, three Meloidogyne species were identified: 60% of the fields were infested with Meloidogyne incognita, M. javanica was reported in 42.9% of the areas, whereas M. hapla was detected in 17.1% of carrot fields. Mixed populations were reported in 20% of the areas with a predominance of M. incognita + M. javanica. The combination of morphological, biochemical, and molecular techniques is a useful approach to identify RKN species.*
Key words: *Daucus carota, integrative taxonomy, isozyme phenotypes, species-specific PCR.*

Distribuição de espécies de *Meloidogyne* em cenoura no Brasil

RESUMO: *Os nematoides-das-galhas (RKN - Meloidogyne spp.) são uma das mais sérias ameaças à produção de cenoura no mundo. No Brasil, as cenouras são cultivadas ao longo do ano, e as perdas econômicas devido à RKN são frequentemente relatadas. Como pouco se sabe sobre a distribuição de espécies RKN em campos de cenoura no Brasil, coletamos amostras de plantas e solo de 35 campos em seis estados. Baseado na morfologia do padrão perineal, fenótipos de esterase e/ou PCR espécie-específica, três espécies de Meloidogyne foram identificadas: 60% dos campos estavam infestados por Meloidogyne incognita, M. javanica foi encontrada em 42,9% das áreas, enquanto M. hapla foi detectada em 17,1% dos campos de cenoura. Populações mistas foram encontradas em 20% das áreas, com predominância de M. incognita + M. javanica. A combinação de técnicas morfológicas, bioquímicas e moleculares é uma abordagem útil para identificar espécies de RKN.*
Palavras-chave: *Daucus carota, taxonomia integrativa, fenótipos de isoenzimas, PCR espécie-específica.*

INTRODUCTION

Plant-parasitic nematodes are one of the main biotic causes of carrot (*Daucus carota* L.) crop losses worldwide (WALKER, 2004). This crop can be parasitized by more than ninety species of nematodes, belonging to the genera *Pratylenchus* Filipjev, *Longidorus* (Micoletzky) Filipjev, *Paratylenchus* Micoletzky, *Belonolaimus* Steiner, *Paratrichodorus* Siddiqi, *Rotylenchus* Filipjev, *Ditylenchus* Filipjev, *Hemicycliophora* de Man and *Meloidogyne* Goeldi (WALKER, 2004; TEKLU et al., 2016).

Root-knot nematodes (*Meloidogyne* spp.) can induce galling, forking, stunting, and fasciculation of carrot roots. Ultimately, attack by root-knot nematodes can lead to quality losses of the taproot (WALKER, 2004; WESEMAEL et al., 2011; HEVE et al., 2015). Severity of symptoms depends on a combination of factors, such as soil texture, temperature, and water content, carrot cultivar, nematode species and its population density in soil (HEVE et al., 2015).

In Brazil, carrots are grown throughout the year under irrigation, in deep and medium-textured soils. Thus, soil conditions in the main areas of carrot cultivation in the country are conducive to root-knot nematodes (BONTEMPO et al., 2014). Furthermore, most of the carrot genotypes used in
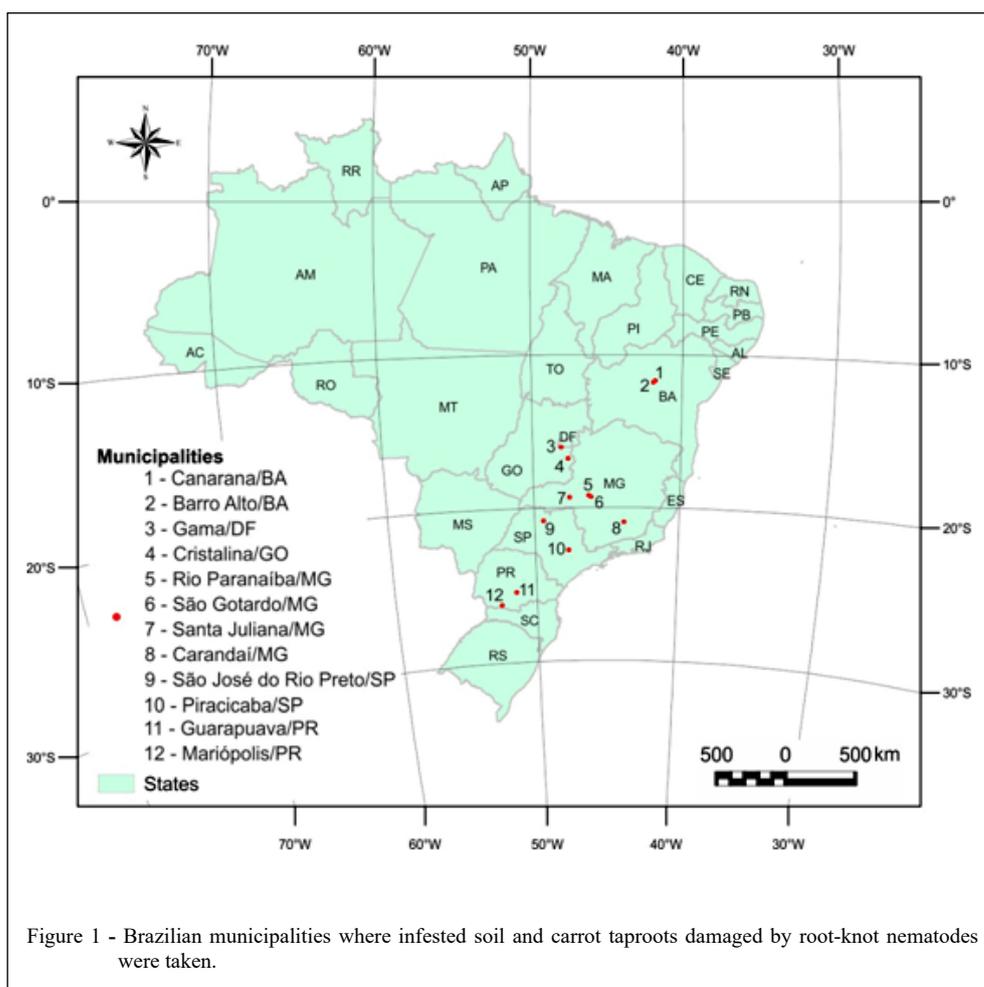
the country are susceptible to root-knot nematodes. The municipalities of Rio Paranaíba, São Gotardo, Carandaí, and Santa Juliana, Minas Gerais State and Cristalina, Goiás State are among the major producers of carrots in Brazil. In these places, carrots are eventually grown in nematode-infested fields, due to the limited availability of pathogen-free areas. Thus, information on the geographical distribution of *Meloidogyne* species can be critical for designing efficient control strategies, including crop rotation with non-host plants, the development of resistant cultivars and the application of chemical and biological nematicides (CUNHA et al., 2018; LOPES et al., 2018).

The major root-knot nematode species that cause losses in carrot worldwide are *M. javanica* (Treub) Chitwood, *M. incognita* (Kofoid & White) Chitwood, *M. hapla* Chitwood, *M. arenaria* (Neal) Chiwood, *M. fallax* Karssen, *M. chitwoodi* Golden et al. (WESEMAEL et al., 2011; ONKENDI et al., 2014). In Brazil, little is known about the distribution of *Meloidogyne* species in carrot fields. It is assumed that *M. fallax* and *M. chitwoodi* are not present in Brazil (MAPA, 2018) and *M. incognita* and *M. javanica* are the most prevalent species in the country. To study the distribution of *Meloidogyne* species on the carrot in Brazil, we collected plant and soil samples from 35 fields across six states and identified the species using morphological, biochemical, and molecular techniques.

MATERIALS AND METHODS

The root-knot nematode populations were collected from various carrot fields across Brazil (Figure 1). Sampled sites were georeferenced using a GPS receiver (Garmin®, Model Etrex H). In each field, we collected a composite sample with 1,500 cm^3 of

Figure 1 - Brazilian municipalities where infested soil and carrot taproots damaged by root-knot nematodes were taken.

soil and 1 to 2 kg of roots with galls. Styrofoam boxes were used to provide temperature-buffering capacity during sample transportation to the laboratory.

Soil samples were homogenized, and we used 100 cm^3 for nematode extraction by the centrifugal-flotation method (JENKINS, 1964). RKN females were extracted from the root samples for species identification. The remaining of the soil was stored in a refrigerator at 8 °C or used for growing tomato cv. Santa Cruz Kada in greenhouse (26 ± 2 °C) to maintain the population for further use.

Identification of Meloidogyne species

We identified *Meloidogyne* species by the integration of the morphological (perineal pattern), biochemical (esterase phenotypes) and molecular (species-specific PCR) techniques (CUNHA et al., 2018). For identification based on morphology, we randomly picked 10–20 mature RKN females from roots from each carrot field for analysis of perineal patterns under light microscopy (HARTMAN & SASSER, 1985). Esterase activity of ten mature females from each field was analyzed according to the method of ESBENSHADE & TRIANTAPHYLLOU (1990). For DNA extraction, we used the protocol reported by LOPES et al. (2018), with modifications. Fifteen second-stage juveniles or females were individually transferred to a microtube with 15 µl of nuclease-free water, 3 µl of 5X GoTaq® Flexi PCR buffer (Promega, USA), and five 1-mm glass beads (Thistle Scientific, UK). Nematodes were disrupted in a dental amalgamator (Dentomat®, Model 600 BR, Degussa, Germany) for 30 sec at 50/60 Hz. Two microliters of proteinase K (100-mg ml^{-1}) were added to each microtube, followed by incubation for 60 min at 60 °C, 15 min at 95 °C and 2 min at 15 °C. DNA was stored at −20 °C until use.

RKN species were identified using the molecular diagnostic key of ADAM et al. (2007) with modifications. For *M. incognita*, we used the primers MI2F4/MIR1 (KIEWNICK et al., 2013) due to the absence of amplification products using the primers MI-F/MI-R (MENG et al., 2004). We used the primers Fjav/Rjav (ZIJLSTRA et al., 2000) for *M. javanica*, JMV primers for *M. hapla* (WISHART et al., 2002), Far/Rar set for *M. arenaria* (ZIJLSTRA et al., 2000), and the universal primers 194/195 for the region 5S-18S rDNA (BLOK et al., 1997).

The amplification of DNA fragments was performed in a Veriti® Thermal Cycler (Applied Biosystems, USA) in a final volume of 12.5 µl and using 2.5 µl a 5X GoTaq® Flexi PCR buffer (Promega), 1.25 µl of each dNTP at 2 mM (Promega), 0.75 µl of 25 mM MgCl$_2$ (Promega), 0.5 µl of each primer at 10 µM, 0.0625 µl of GoTaq® Flexi (Promega), 1 µl of DNA, and 6.43 µl of nuclease-free water. All amplifications included positive controls for *M. javanica* or *M. incognita*, and a control without template DNA.

Except for *M. incognita*, amplifications were conducted using cycling conditions of 94 °C for 2 min, followed by 45 cycles of 94 °C for 30 sec, with specific annealing conditions for each primer set (50 °C/30 sec for 194/195 and JMV primers; 61 °C/30 sec for Far/Rar; 64 °C/30 sec for Fjav/Rjav), 72 °C for 90 sec (194/195 and JMV primers) or 60 sec (Fjav/Rjav and Far/Rar), with a final cycle of 72 °C for 7 min. For *M. incognita* using MI2F4/MIR1 primers, thermal cycling conditions were of initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 40 sec, annealing at 64 °C for 60 sec and extension at 72 °C for 60 sec, with a final extension of 72 °C for 7 min PCR products were separated by 1.14% agarose gel electrophoresis at 45 V for 90 min using 1X TBE buffer. Gels were stained with 0.7 µl of SYBR Safe (10.000 x, Invitrogen, Carlsbad, USA) and visualized under UV illumination and photographed.

RESULTS AND DISCUSSION

Meloidogyne incognita, *M. javanica*, and *M. hapla* were the species reported in 35 carrot fields in six Brazilian states (Table 1, Figure 2). Sixty percent of the carrot fields were infested with *M. incognita*. The second most prevalent species was *M. javanica*, which was found in 42.9% of the areas, alone or in a mixed population with *M. hapla* or *M. incognita*. *Meloidogyne hapla* was detected in 17.1% of the areas, all located in São Gotardo – MG. Mixed populations of *Meloidogyne* were detected in 20% of the fields, with a predominance of *M. incognita* + *M. javanica*. We found the mixture of *M. incognita* and *M. javanica* in all fields from Gama – DF and Carandaí – MG (Table 1).

The perineal patterns of the females were consistent with that expected for *M. incognita*, *M. javanica* and *M. hapla*. The esterase (EST) phenotype J2 (Rm = 1.0; 1.25) was observed in the populations RIO 04 and RIO 11 of *M. javanica* (Table 1). All other populations of this species showed the EST-phenotype J3 (Rm = 1.0; 1.25; 1.4). EST-phenotype H1 (Rm = 1.1) was detected in all *M. hapla* populations. The phenotype I1 (Rm = 1.0) was detected in all *M. incognita* populations, except for RIO 16, which showed two isoforms: one strong band at Rm = 1.0 and a weak band at Rm = 1.05 (Figure 3).

Table 1 - Distribution of *Meloidogyne* species in 35 carrot production fields in Brazil.
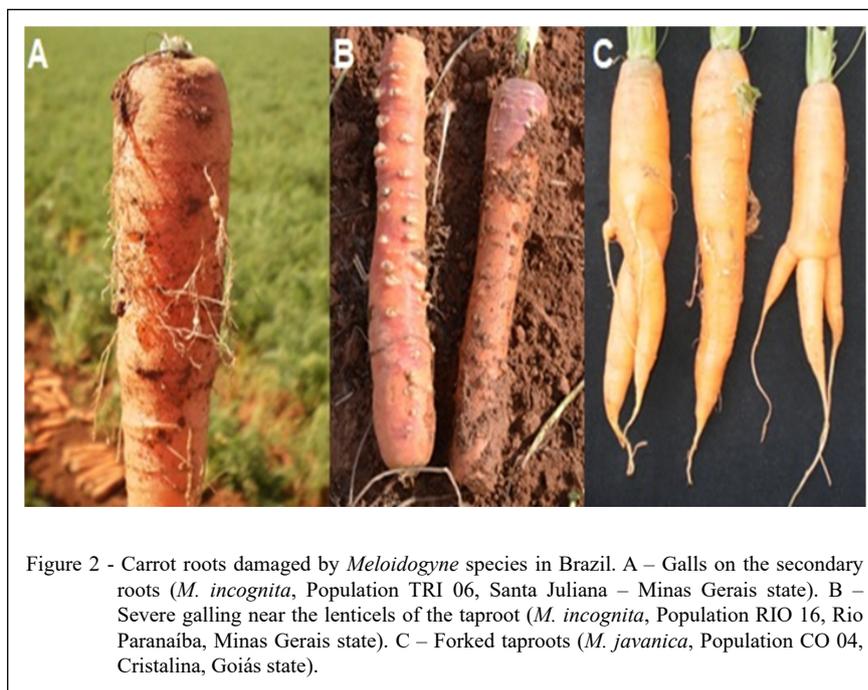
Municipality (State)	Field	Species
Barro Alto (BA)	N 02	*M. incognita*[1,3]
Canarana (BA)	N 01	*M. incognita*[1,3]
Carandaí (MG)	CAD 01	*M. incognita*[1,2] + *M. javanica*[3]
Carandaí (MG)	CAD 02	*M. incognita*[1,2] + *M. javanica*[1,3]
Carandaí (MG)	CAD 09	*M. incognita*[1,2] + *M. javanica*[2,3]
Carandaí (MG)	CAD 10	*M. incognita*[1,2,3] + *M. javanica*[2]
Cristalina (GO)	CO 03	*M. javanica*[1,3]
Cristalina (GO)	CO 04	*M. javanica*[1,3]
Gama (DF)	CO 01	*M. incognita*[1,3] + *M. javanica*[1,3]
Gama (DF)	CO 02	*M. incognita*[1,3] + *M. javanica*[1,3]
Guarapuava (PR)	S 02	*M. javanica*[1,3]
Mariópolis (PR)	S 01	*M. incognita*[1,3]
Piracicaba (SP)	SP 02	*M. incognita*[1,2]
Rio Paranaíba (MG)	RIO 03	*M. incognita*[1,2,3]
Rio Paranaíba (MG)	RIO 04	*M. javanica*[1,2,3]
Rio Paranaíba (MG)	RIO 05	*M. incognita*[1,2,3]
Rio Paranaíba (MG)	RIO 06	*M. javanica*[1,2,3]
Rio Paranaíba (MG)	RIO 09	*M. incognita*[1,2,3]
Rio Paranaíba (MG)	RIO 10	*M. javanica*[1,2,3]
Rio Paranaíba (MG)	RIO 11	*M. javanica*[1,2,3]
Rio Paranaíba (MG)	RIO 16	*M. incognita*[1,2,3]
Santa Juliana (MG)	TRI 06	*M. incognita*[1,2,3]
Santa Juliana (MG)	TRI 07	*M. incognita*[1,2,3]
Santa Juliana (MG)	TRI 08	*M. incognita*[1,2,3]
São Gotardo (MG)	SG 01	*M. incognita*[1,2]
São Gotardo (MG)	SG 02	*M. incognita*[1,2]
São Gotardo (MG)	SG 03	*M. javanica*[1,2,3] + *M. hapla*[1,2]
São Gotardo (MG)	SG 04	*M. javanica*[1,2,3]
São Gotardo (MG)	SG 05	*M. hapla*[1,2]
São Gotardo (MG)	SG 06	*M. hapla*[1,2]
São Gotardo (MG)	SG 07	*M. hapla*[1,2]
São Gotardo (MG)	SG 08	*M. hapla*[1,2]
São Gotardo (MG)	SG 09	*M. hapla*[1,2]
São Gotardo (MG)	SG 10	*M. incognita*[1,2,3]
São José do Rio Pardo (SP)	SP 01	*M. incognita*[1,2]

Brazilian states: MG – Minas Gerais; SP – São Paulo; GO – Goiás; PR – Paraná; BA – Bahia; DF – Distrito Federal. [1]Identification based on perineal pattern of females; [2]Identification based on esterase phenotypes; [3]Identification based on polymerase chain reaction.

Meloidogyne javanica and *M. hapla* were identified by PCR using the primers Fjav/Rjav and JMV, with amplicons of 720 bp and 440 bp, respectively (Figure 3). The use of the primers MIF/MIR, recommended on the molecular key proposed by ADAM *et al.* (2007), did not result in the amplification of 999 bp fragments in populations previously identified as *M. incognita* by esterase phenotypes and perineal patterns. Amplicons were not observed even when pure populations of *M. incognita* were used as positive controls. However, typical amplicons of 300 bp were observed for *M. incognita* when the primers MI2F4/MIR1 were used.

In our survey, we reported *M. incognita*, *M. javanica,* and *M. hapla* in carrot crops in Brazil, with the occurrence of *M. incognita* and *M. javanica* in more than 85% of the production fields. These species have been reported to cause damage to vegetable crops in Brazil, including carrots (CARNEIRO et al., 2008; ROSA et al., 2013; SILVA et al., 2016). *Meloidogyne incognita* and *M. javanica* have various hosts, especially among vegetable crops, which

Figure 2 - Carrot roots damaged by *Meloidogyne* species in Brazil. A – Galls on the secondary roots (*M. incognita*, Population TRI 06, Santa Juliana – Minas Gerais state). B – Severe galling near the lenticels of the taproot (*M. incognita*, Population RIO 16, Rio Paranaíba, Minas Gerais state). C – Forked taproots (*M. javanica*, Population CO 04, Cristalina, Goiás state).
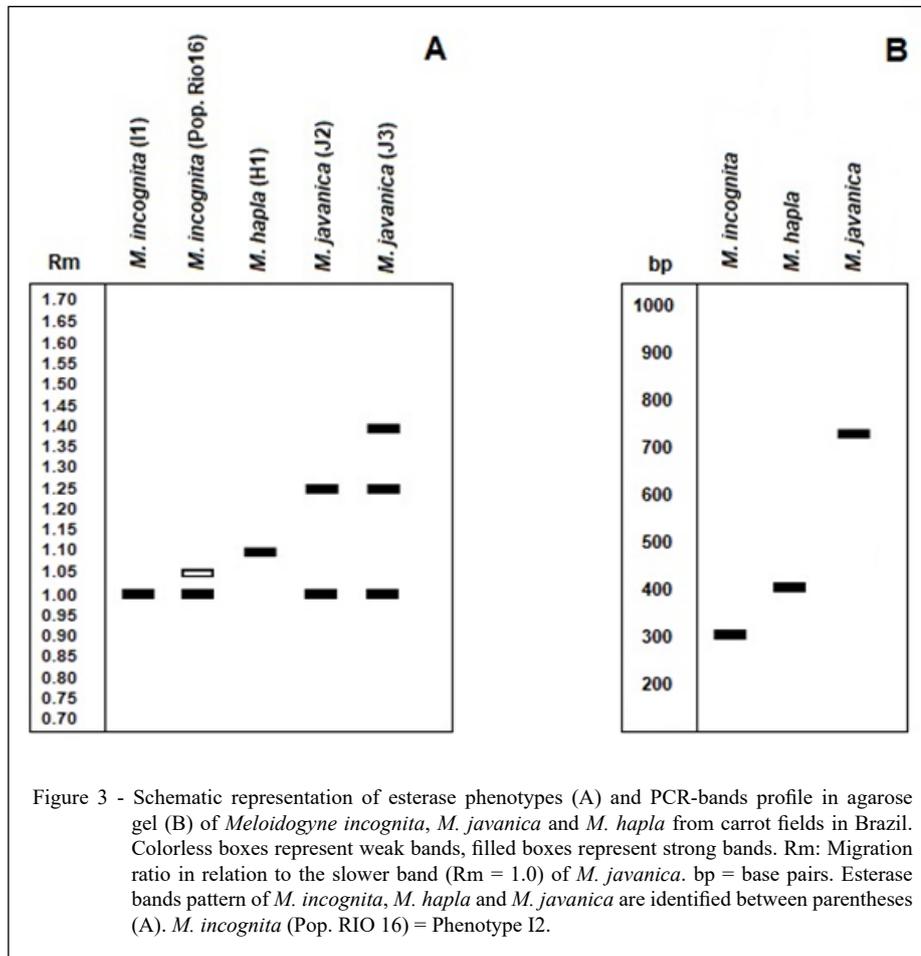
facilitate their establishment and survival in the field. As a result, crop rotation including commercial crops is limited, with few options remaining, which include cover crops and green manures, including *Brachiaria decumbens* Stapf and *B. ruziziensis* Germain et Evrard, pearl millet [*Pennisetum glaucum* (L). R. Br], crotalaria (*Crotalaria spectabilis* Roth), sunn hemp (*Crotalaria juncea* L.), and velvet bean [*Mucuna pruriens* (L). DC.] (LOPES et al., 2019). The occurrence of mixed populations of *M. incognita* and *M. javanica*, as we reported here makes management even more difficult. In this study, other tropical root-knot nematodes, such as *M. arenaria* and *M. enterolobii* Yang & Eisenback, were not detected in carrot fields; although, they have been reported in areas of vegetable production in Brazil (ROSA et al., 2013; SILVA et al., 2016).

In the cooler regions, *M. hapla* causes economic losses in carrot (GUGINO et al., 2006). The neighboring municipalities of São Gotardo and Rio Paranaíba stand out as one of the largest areas of carrot production in Brazil with productivity above the national average (HORTIFRUTI, 2019). As they are located above the 1000-m altitude, the temperature in these places is mild, which favored the establishment of *M. hapla*, which may cause losses mainly in the cooler periods of the year.

In 2009, the species *M. polycephannulata* CHARCHAR et al. (2009), the carrot root-knot nematode, was described from a population reported in a carrot field in Rio Paranaíba (CHARCHAR et al., 2009). We collected samples in the same farm where the type population was reported and concluded that the population RIO 16 had similarities to those described for *M. polycephannulata*, especially the esterase phenotype I2. However, recently MONTEIRO et al. (2019) have shown that *M. polycephannulata* is t a junior synonym of *M. incognita*, based on morphological and morphometric characters, and biochemical, molecular, and phylogenetic studies. For this reason, in agreement with MONTEIRO et al. (2019), we classified the population RIO 16 as *M. incognita*.

The use of integrative taxonomy is an approach that should be used for accurate identification of root-knot nematode populations (OLIVEIRA et al., 2011; CUNHA et al., 2018). The observation of female perineal patterns is simple, it is relatively reliable for the identification of species such as *M. javanica*, but it may not be accurate enough to separate several species of *Meloidogyne*, including *M. incognita*, *M. enterolobii,* and *M. inornata* Lordello (CUNHA et al., 2018). The analysis of esterase phenotypes is commonly used in routine laboratories in Brazil as a diagnosis technique for root-knot nematodes. However, molecular-based diagnostics have become more popular recently, due to the number of protocols available in the literature and reduction in the cost of

Figure 3 - Schematic representation of esterase phenotypes (A) and PCR-bands profile in agarose gel (B) of *Meloidogyne incognita*, *M. javanica* and *M. hapla* from carrot fields in Brazil. Colorless boxes represent weak bands, filled boxes represent strong bands. Rm: Migration ratio in relation to the slower band (Rm = 1.0) of *M. javanica*. bp = base pairs. Esterase bands pattern of *M. incognita*, *M. hapla* and *M. javanica* are identified between parentheses (A). *M. incognita* (Pop. RIO 16) = Phenotype I2.

equipment and consumables. Eggs, juveniles, and adults can be used as the DNA source, which is an advantage of the molecular methods compared to perineal patterns and isoenzyme phenotypes that require mature females (OLIVEIRA et al., 2011).

The use of various diagnostic techniques may improve the accuracy of diagnostics and increase the chance of detecting mixed populations, since more nematodes will be used. Considering the 01-population with CAD as an example, a maximum of 30 females were used for identification based on perineal pattern of females and esterase phenotypes. If only these individuals were evaluated, we would conclude that only *M. incognita* was infecting carrots in that specific field. When 15 additional specimens were used in the molecular assay, *M. javanica* was detected. This was not due to a previous failure of diagnosis since the perineal and esterase patterns of *M. incognita* and *M. javanica* are distinct, but simply because the number of nematodes assessed

was higher and by chance, we used individuals of *M. javanica* for molecular analysis.

The DNA extraction method used in this research is simple and produces enough material for identification by species-specific PCR. The molecular identification protocol proposed by ADAM et al. (2007) proved to be efficient, except for failing to identify *M. incognita* populations using MIF/MIR primers (MENG et al., 2004). KIEWNICK et al. (2013) also reported a failure in the molecular diagnosis of *M. incognita* using this same set of primers. Based on the *M. incognita*-specific amplicons of 399 bp using primers inc-K14-F / inc-K14-R (RANDIG et al., 2002), KIEWNICK et al. (2013) designed primers MI2F4/MIR1, which amplify a 300 bp fragment and can be used in a multiplex reaction to identify *M. incognita*, *M. javanica*, and *M. arenaria*. The populations of *M. incognita* in carrots from this study may be genetically similar to the Brazilian populations of *M. incognita* that were used to design

the SCAR primers inc-K14-F / inc-K14-R, and the starting point for designing the primers MI2F4/MIR1.

Information on the occurrence and distribution of root-knot nematodes in carrots can be used for designing integrated management systems adapted to local root-knot nematode problems (WESEMAEL et al., 2011). Carrot breeding programs and producers of chemical and biological nematicides may benefit from our findings by targeting the most prevalent RKN species.

CONCLUSION

Meloidogyne incognita, *M. javanica,* and *M. hapla* infect carrots in six Brazilian states, with a prevalence of *M. incognita* and *M. javanica*, and the occurrence of mixed populations in some fields. *Meloidogyne hapla* is reported in São Gotardo – MG, where temperatures are mild year-round. The combination of morphological, biochemical, and molecular techniques is a useful approach to identify RKN species.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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