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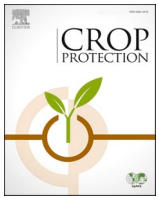

First report of *Meloidogyne javanica* infecting *Thymus vulgaris* in the state of São Paulo, Brazil

Edicleide Macedo da Silva [a,*], Ricardo Koroiva [b], Rivanildo Júnior Ferreira [c], Daniel Dalvan do Nascimento [c], João Pedro Peixoto Fernandes [c], Benedito Charlles Damasceno Neves [a], Antonio Cesar de Araujo Filho [a], Rafaelle Fazzi Gomes [d], Nynyve Thaynar Brito de Almeida [a], Andréia Mitsa Paiva Negreiros [a], Pablo Forlan Vargas [c], Laura Raissa Fagundes Costa Bezerra [a], Lindomar Maria da Silveira [a], Glauber Henrique de Sousa Nunes [a], Pedro Luiz Martins Soares [c]

[a] Federal Rural University of the Semi-Arid (UFERSA), Department of Agronomic and Forestry Sciences, Mossoró, Rio Grande do Norte, Brazil
[b] Federal University of Pará (UFPA), Institute of Biological Sciences, Belém, Pará, Brazil
[c] Paulista State University (UNESP), Faculty of Agricultural and Veterinary Sciences (FCAV), Jaboticabal Campus, São Paulo, Brazil
[d] Federal Rural University of the Amazon (UFRA), Capanema Campus, Pará, Brazil

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ABSTRACT

Thymus vulgaris L. is found all over the world and is cultivated in several countries. It is considered an important medicinal plant with anti-inflammatory and aromatic properties. Its cultivation can suffer from infestation by numerous pathogens, which contributes to lower production. Thus, in 2020, a nematological survey in a vegetable growing area in Jaboticabal, São Paulo, Brazil, detected the presence of galls on the root, indicating that it was *Meloidogyne* spp. To identify the species, a sample with soil and roots was sent to the laboratory. After the analyzes performed, the species was identified as *Meloidogyne javanica*. This result was based on the morphological characteristics of the adults and the genetic identification. In the morphological part, the following characteristics were found: Perineal region of females low trapezoidal dorsal arch with two lines in laterals, while males have broader basal nodules with a non-raised labial disk, with the head region not separated from the body. Molecular confirmation was performed by genetic sequencing and sequence characterized amplified regions technique (SCAR). This is the first report of *T. vulgaris* as a host for *M. javanica* confirmed by Koch's postulate and several lines of evidence. Based on this report, farmers wishing to grow this vegetable should be aware of plants that are also hosts for this species.

1. Introduction

Aromatic and medicinal plants have been used since ancient times, especially in traditional medicine. Over time and with advances in various fields, these plants have been widely researched and their uses have become more widespread and well-known. Among the many medicinal plants found worldwide, we can mention *Thymus vulgaris* L., (Lamiaceae), commonly known as "thyme" an herb that is often used to add flavor and aroma to foods and is also often used for its expectorant, antitussive, antibroncholytic, antispasmodic, vermifuge, carminative, and diuretic properties (Marinelli et al., 2016; Miraj and Kiani, 2016; Nabissi et al., 2018). It is also rich in essential oils, which are often used in folk medicine and in the development of medicines (Galavicová et al., 2021; Lorenzo et al., 2019; Patil et al., 2021).

Global production of *T. vulgaris* is estimated at 14,000 tons/year, with Turkey being the largest producer with more than 12,000 ha under cultivation (Lorenzo et al., 2019; Stahl-Biskup and Venskutonis 2012). In Brazil, production is mainly through extractivism and it is mainly known as part of the so-called "unconventional food plants" (UFP) or "unconventional vegetables" (see more details on UFP in Barbosa et al., 2021). The areas where this species is cultivated are exposed to the occurrence of numerous pathogens that can directly affect plant productivity. Among the most important pathogens is the presence of nematodes, mainly species of *Meloidogyne* spp.

* Corresponding author.
E-mail address: edicleide.c.c@gmail.com (E.M. Silva).

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Meloidogyne spp., known as root-knot nematodes (RKNs), are considered the most agriculturally important nematode genus with a wide distribution around the world due to their wide range of hosts (Pan et al., 2023). In Brazil, the species with wide occurrence are *M. incognita* and *M. javanica* (Charchar, 1999). Depending on the species, host crop and population level in the region, they can cause crop losses of up to 100 % in a carrot crop (*Daucus carota* L.) (Pinheiro, 2017; Vrain et al., 1981).

Therefore, the identification of nematode species in new hosts in cultivated areas is important, especially root knot nematodes, considering that they have a wide range of hosts (Pan et al., 2023) and reproduce under different environmental conditions (Rusinque et al., 2023). Correct identification of species is also essential to ensure proper management. Identification using different methods provides more certainty, and morphological characterization, which is considered cost-effective, must be carried out in addition to identification using molecular methods (Rusinque et al., 2023). Therefore, this research is necessary and essential and serves as a warning to producers and researchers of the incidence of nematodes in new hosts.

Considering this situation, the aim of this study was to confirm the presence of root knot nematodes for *T. vulgaris* by morphological characteristics, pathogenicity tests and molecular analyzes.

2. Material and methods

2.1. Sample collection and processing

In March 2020, in the municipality of Jaboticabal, in the state of São Paulo, Brazil (21°14′38.7″ S, 48°17′10.6″ W), a nematological survey found that thyme plants (*Thymus vulgaris*) showed characteristic symptoms of the presence of nematodes in their roots, with reduced growth, chlorotic leaves and the presence of galls on the roots (Fig. 1). Therefore, soil and roots of thyme plants were carefully collected, packed in plastic bags, identified and sent to the Nematology Laboratory (LabNema) of the FCVA/UNESP to determine the species.

First, a thyme plant was analyzed in the laboratory. This sample came from the area where the nematological study was carried out. When the sample was analyzed, 18,000 eggs and second-stage juveniles (J2s) of *Meloidogyne* spp. were estimated in 10 g of plant roots after extractions according to the method proposed by Coolen and D'Herde (1972) (n = 1). Morphological and molecular analyzes were performed to identify the nematode. For the morphological analysis, 15 females extracted directly from the root were used. The perineal pattern of these females was then cut out and placed on microscope slides for later identification. To identify the males, 15 individuals caught in the suspension obtained from the sample were also used. The procedure was repeated twice. The first sample was taken directly on the production field, the second was carried out when the postulate was completed.

2.2. Pathogenicity test, Koch's postulate and morphological characterization

A pathogenicity test was carried out using Koch's postulate (1890) to confirm the species' ability to host. This purpose, the test was carried out in a greenhouse where four plants (n = 4) were inoculated with the original thyme population. Thyme plants were also used as a control where the nematode population was not inoculated. The inoculation suspension was prepared, extraction was performed and then the egg population and J2 were estimated. At the time of transplanting, inoculation was performed using a graduated pipette, applying 10 ml of the suspension, the concentration of which was adjusted to 300 eggs and J2 per ml per seedling, for a total of 3000 eggs and J2 per plant. The plants were kept in plastic pots with a capacity of 2 L, filled with a mixture of soil-sand-manure in the proportion of. 3:1:1, respectively, previously autoclaved (120 °C and 1 ATM for 1 h).When extracting the nematodes from the thyme plants, the aerial part of all plants was removed. The roots were then collected and carefully washed to obtain the galls and egg masses. The nematodes were extracted from the roots of the inoculated plants after 90 days. Initially, the root system was cut and processed in a blender for 20s with hypochlorite (0.5%). Subsequently, the sample was poured into 60 over 500 mesh sieves, obtaining the suspension. Next, the samples were process according to the method proposed by Hussay and Baker (1973). To centrifuged the samples, the methodology of Coolen and D'Herde (1972), which consists of the addition of 1 g of kaolin in each sample and then centrifuged for 5 min at 1750 rpm. In the second stage of processing, sucrose (45%) was added, and the sample was centrifuged again for 1 min at 1750 rpm.

Quantification was then carried out in a Peters chamber. The method of Oostenbrink (1966) was used to calculate the reproductive factor (RF), which is the ratio between the total number of eggs and J2s in the roots (NTOJ in the roots), i.e. the final population (FP), and the initial inoculated population (IP).

After extraction, sections were also taken from the perineal region of females (n = 10) (Netscher and Taylor, 1974) and the labial region of males (n = 10) (Eisenback and Hirschmann, 1981) to allow comparison with the original description of the species by Chitwood (1949).

Fig. 1. A. Plant and roots of thyme, *Thymus vulgaris* L. with galls formed by *Meloidogyne javanica* (Treub, 1885; Chitwood, 1949); B. Thyme root system with symptoms characteristic of the presence of *M. javanica* in the root; C. Apparent roots with nodules characterizing the galls and females removed from the gall; D. Males and females of *M. javanica* observed on thyme root.

2.3. Molecular analyzes

To confirm the identification of our specimens, total genomic DNA of one specimen was extracted using the DNA Blood & Tissue Kit (Qiagen, Germany). PCR amplifications were performed for the 18srRNA gene using 988F (CTC AAA GAT TAA GCC ATG C) and 1912R (TTT ACG GTC AGA ACT AGG G) from Holterman et al. (2006). All PCRs consisted of 25 μl reagent mixture containing: 10 × reaction buffer (Promega); 25 mM MgCl2; 2 mM dNTPs; 2 mM primers and 2 μl GoTaq® G2 Hot Start Polymerase (Promega); 2 μl DNA template. The PCR cycling program was used for both primer sets: 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and finally a 10-min extension to 72 °C. The PCR products were sequenced bidirectionally in the ABI 3130 Genetic Analyzer (Applied Biosystems).

We used GENEIOUS PRIME v 2023.2.1. (Kearse et al., 2012) to check the sequence quality of the strands by comparison with the respective chromatograms and to assemble and edit them if necessary. The sequences of *Meloidogyne* available in GenBank were used to analyze the genetic regions (AB905324, AB905325, AY268121, JX100419, JX100422, KJ130033, KJ636261, KP901057, KP901058, KP901059, KP901062, KP901063, KP901064, MF157427, MF157428, MF157429, MF157430, MF157431, MF157432, MG273438 and MG273439). *Pratylenchus thornei* (AJ966499) was considered as outgroup. The phylogenetic analysis, we aligned the sequences using MAFFT v. 7.017 (Katoh et al., 2002), a module implemented in GENEIOUS PRIME v 2023.2.1. (Algorithm:Auto). We constructed maximum likelihood (ML) tree with RAxML (v.8.2.12) (Stamatakis, 2014) using the GTR GAMMA I model and 1000 bootstrap (BP) replicates. Our sequence was deposited in GenBank (NCBI) under accession number OP422209. Finally, species-specific primers (Fjav/Rjav, henceforth "Mj"; Me-F/Me-R, henceforth "Me"; and Finc-F/Finc-R, henceforth "Mi") developed by Zijlstra (2000), Zijlstra et al. (2000), Randig et al. (2002) and Long et al. (2006) were used to identify *Meloidogyne* spp by the sequence characterized amplified regions technique (SCAR). The PCR parameters followed the description of Zijlstra et al. (2000).

3. Results

After 90 days, the inoculated plants showed symptoms similar to those originally observed in plants with galls on the roots, with the leaves yellow and reduced in size, with chlorotic roots and visible gall formation (Fig. 1). No symptoms were observed in plants that were not inoculated.

When Koch's postulate was analyzed, the following results were obtained for the thyme samples: (n = 1) eggs and juveniles 11,600 (RF = 3.87); (n = 2) eggs and juveniles 48,700 (RF = 16.23); (n = 3) eggs and juveniles 4100 (RF = 1.37) and (n = 4) eggs and juveniles 16,300 (RF = 5.43), so that the media of eggs and juveniles was 20,175 (RF = 6.73). The high multiplication observed in all plant samples indicates that there was a constant pathogen-host association, with the nematode

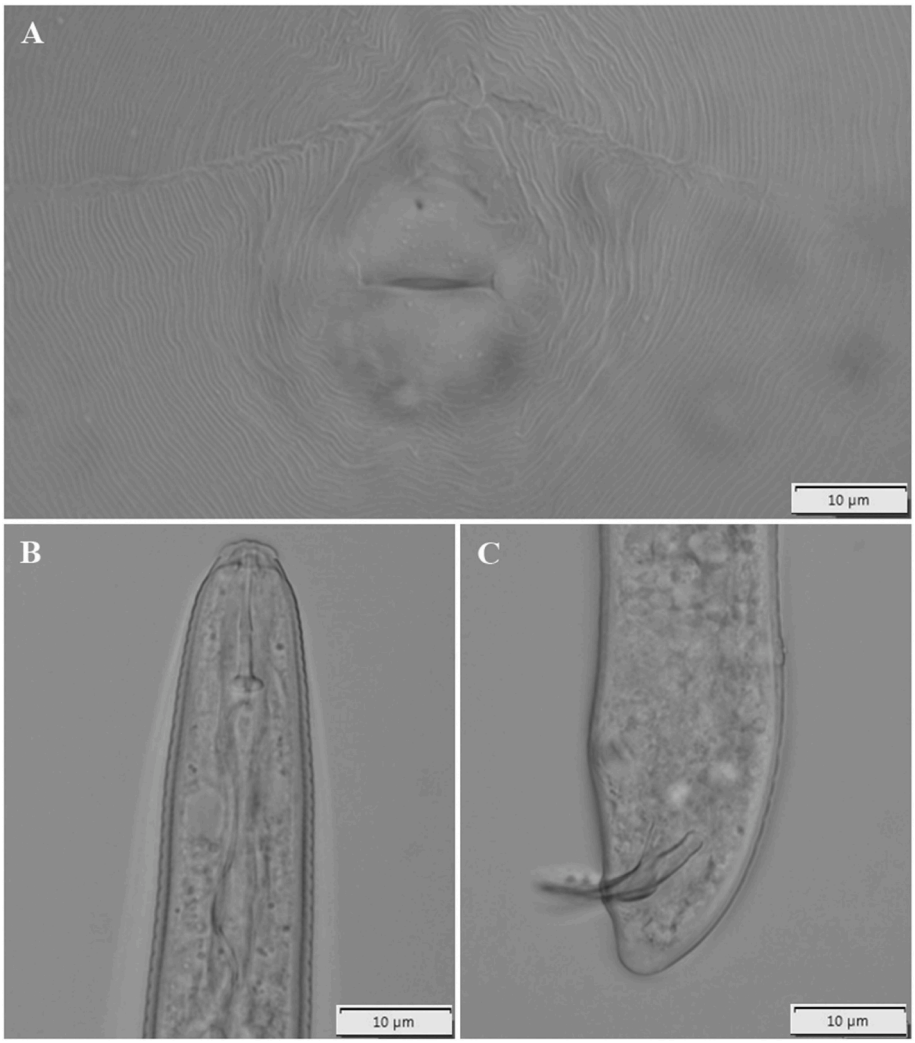

Fig. 2. A. Perineal pattern of a mature female of *M. javanica*; B. Intersex male; C. Labial region of the male.

present in all symptomatic plants and absent in healthy, non-inoculated plants.

The following features were observed in the perineal region of females: a low trapezoidal dorsal arch with two lines in the lateral field, in addition to a rounded perineal pattern with distinct lateral lines, confirming the original description of the species by Chitwood (1949), while males show broader basal nodules with a non-raised labial disk, the head region not being separated from the body and the male being intersexed (Eisenback and Hirschmann, 1981) (Fig. 2).

The ML is shown in Fig. 3 - A. The 18srRNA sequences grouped *Meloidogyne* sp. with species such as *Meloidogyne enterolobii*, *Meloidogyne incognita* and *M. javanica*. A fragment of approximately 700bp DNA size was amplified with *Mj* primers, but not with *Me* and *Mi* primers, confirming the identification of *M. javanica* (Fig. 3 - B).

4. Discussion

Meloidogyne javanica is widespread and parasitizes several agriculturally important crops worldwide, including *Glycine max* (Adamu et al., 2024; Ballardin et al., 2022), *Triticum* spp. (Carraro-Lemes et al., 2022), *Solanum lycopersicum* (Iberkleid et al., 2014) and others.

Thymus vulgaris, as reported in our study, is host to *M. javanica*, and although this plant does not have the acreage of the previously mentioned crops, it can help to increase the populations of *M. javanica* in the region as it adapts well to different soil types. It is also important to know which plants are potential nematode hosts so that preventive management can be done. However, *Meloidogyne* spp. has also been reported to cause problems on other Lamiaceae such as basil, perilla, mint and lion's ear (Bui et al., 2022; Quenéhervé et al., 2011; Zhanar et al., 2021). Root-knot nematodes are considered one of the most damaging plant parasites (Almohithef et al., 2020). These nematodes have been detected in association with more than 770 plant species, including vegetables, medicinal herbs, weeds, legumes and fruit trees (Cabi, 2022).

Based on all results, this is the first report of infection of thyme plants with the root-knot nematode *M. javanica* in Brazil. Thyme is not only a perennial plant, but can also be propagated vegetatively, which favour the spread of pathogens, including nematodes. This report urges caution to prevent the spread of this nematode in areas not yet infested and the search for new management strategies.

It should be noted that there are several management strategies that can be used for *M. javanica*. Among the available management methods, several studies have reported the importance of identifying genotypes with genetic resistance to this nematode (Bellé et al., 2024; Bhuiyan and Garlick, 2020; Gontijo et al., 2024) For example, Soares and Nascimento (2021) found that in an area with heavy infestations of root-knot nematodes (*Meloidogyne incognita* and *M. javanica*), a resistant soybean variety yielded 70 % more than the susceptible variety.

In addition to genotypes with genetic resistance, crop rotation can be used and the farmers should pay attention to the type of crop in succession/rotation. Recommendations may include *Crotalaria spectabilis* Roth. (Miamoto et al., 2021; Scupinari et al., 2024). Besides these strategies, chemical and biological control is still one of the most commonly used strategies by farmers. Available chemical nematicides include those based on abamectin, fluopyram, cadusaphos, etc. The main biological nematicides are based on *Pochonia chlamydosporia*, *Bacillus amyloliquefaciens*, *B. subtilis* and others (see AGROFIT, 2024).

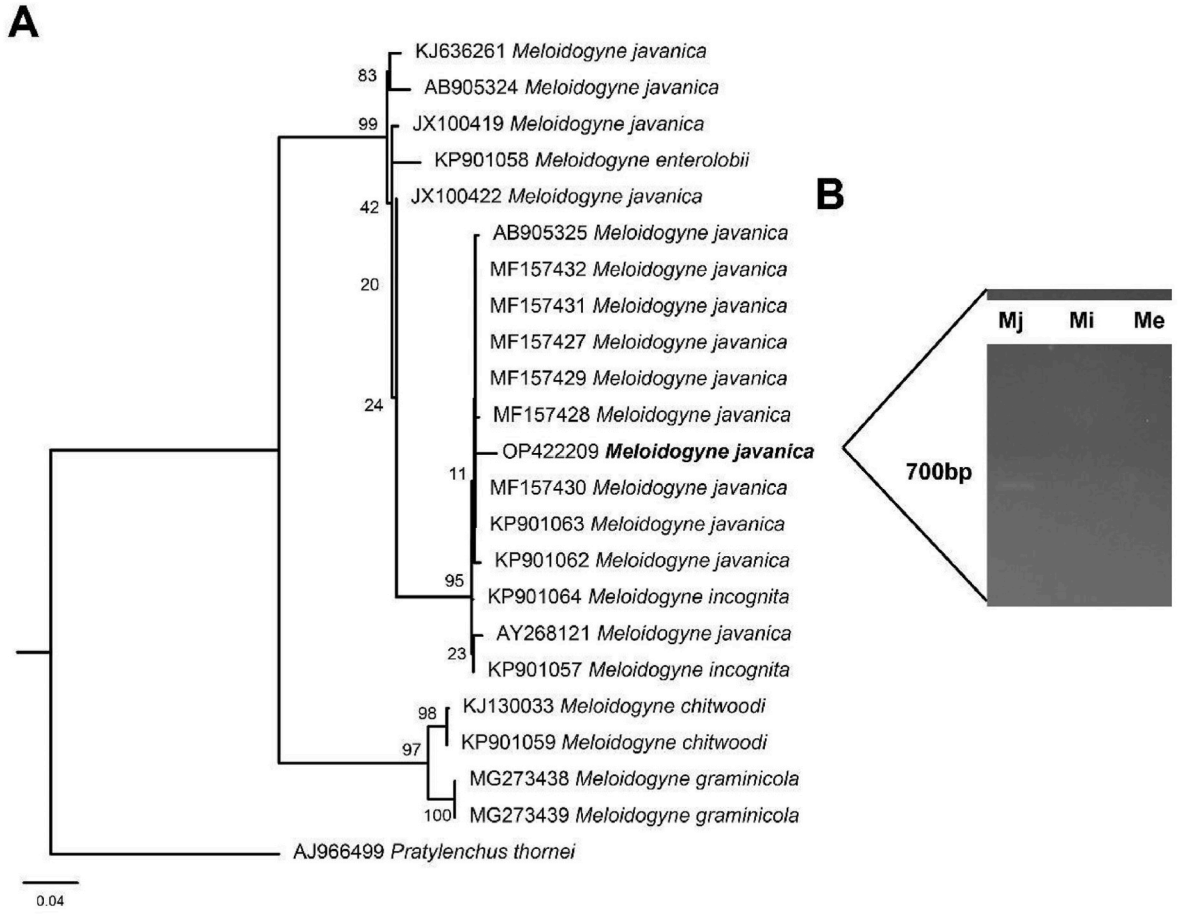

Fig. 3. A. Maximum likelihood phylogenetic relationships of *Meloidogyne* based on 18s rRNA; B. PCR amplification of our specimen with *Mj* (Fjav/Rjav), *Me* (*Me*-F/*Me*-R) and *Mi* (Finc-F/Finc-R) primers. *Pratylenchus thornei* (AJ966499) was considered as outgroup.

5. Conclusion

This is the first report of infection of thyme plants with the root-knot nematode *M. javanica* in Brazil, and this report is important to warn farmers of other crops that are also hosts of *M. javanica*. In addition, known control strategies can be used to minimize the impact caused by the nematode.

CRediT authorship contribution statement

Edicleide Macedo da Silva: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ricardo Koroiva:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rivanildo Júnior Ferreira:** Writing – review & editing, Methodology, Formal analysis. **Daniel Dalvan do Nascimento:** Writing – original draft, Methodology, Data curation. **João Pedro Peixoto Fernandes:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Benedito Charlles Damasceno Neves:** Writing – review & editing, Writing – original draft, Conceptualization. **Antonio Cesar de Araujo Filho:** Writing – review & editing, Writing – original draft. **Rafaelle Fazzi Gomes:** Writing – review & editing, Writing – original draft, Visualization. **Nynyve Thaynar Brito de Almeida:** Writing – review & editing. **Andréia Mitsa Paiva Negreiros:** Writing – original draft. **Pablo Forlan Vargas:** Writing – review & editing, Writing – original draft. **Laura Raissa Fagundes Costa Bezerra:** Writing – review & editing. **Lindomar Maria da Silveira:** Writing – review & editing. **Glauber Henrique de Sousa Nunes:** Writing – review & editing, Writing – original draft. **Pedro Luiz Martins Soares:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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