SCAP
SOCIEDADE DE CIÊNCIAS AGRÁRIAS DE PORTUGAL

Morphological, biochemical and molecular characterisation of *Meloidogyne javanica*, from North Portugal, in tomato

Caracterização morfológica, bioquímica e molecular de *Meloidogyne javanica*, do Norte de Portugal, em tomateiro

Leidy Rusinque[1,2*], M. Lurdes Inácio[1], Mariana Mota[2] and Filomena Nóbrega[1]

[1] *Instituto Nacional de Investigação Agrária e Veterinária, I.P., Unidade Estratégica de Sistemas Agrários e Florestais e Sanidade Vegetal, Quinta do Marquês, 2780-159 Oeiras, Portugal*
[2] *LEAF, Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal*
*(*E-mail: Leidycrmora@gmail.com)*
http://dx.doi.org/10.19084/RCA17078

Received/recebido: 2017.03.29
Received in revised form/recebido em versão revista: 2017.09.09
Accepted/aceite: 2017.09.18

ABSTRACT

Plant-parasitic nematodes are highly damaging pests in many crops of great economic importance. A substantial part of this damage is caused by root-knot nematodes (RKN), *Meloidogyne* spp. represents losses of millions of euros, due to their wide geographical distribution and range of host plants. Therefore, an accurate and reliable identification is needed to establish effective, sustainable and environmentally safe control measures. The main goal of this study was to characterise morphological, biochemical and molecularly one Portuguese isolate of *Meloidogyne* found associated with tomato crop. Morphometric studies were based on second-stage juveniles (body length, stylet length, tail length, hyaline terminus length and DGO) and female perineal patterns. Biochemical assays using esterases (EST isozymes) were performed on females and the PAGE enzymatic patterns compared to previous descriptions. Molecular analysis was based on PCR using the species-specific SCAR primers Fjav/Rjav. Results indicated the presence of *M. javanica*. This study confirms the need of using the three approaches for an accurate and effective RKN diagnosis.

Keywords: esterase phenotype, *Meloidogyne* spp., molecular identification, perineal patterns, tomato.

RESUMO

Os nemátodes fitoparasitas podem causar grandes estragos em culturas economicamente importantes. Em particular, os nemátodes-das-galhas-radiculares, *Meloidogyne* spp., causam anualmente, prejuízos de milhões de euros, pela redução da quantidade e também da qualidade dos produtos agrícolas numa ampla gama de hospedeiros e distribuição geográfica. Por isso, a identificação correta e exacta destas espécies é essencial para a implementação de estratégias de controlo, efetivas, sustentáveis e amigas do ambiente. Assim, o principal objetivo deste trabalho consistiu na caracterização morfológica, bioquímica e molecular de um isolado proveniente de tomateiros do Norte de Portugal. Para tal, foram realizadas observações e respetivas medições das estruturas morfológicas dos jovens de segundo estádio (comprimento do corpo, estilete, cauda, parte terminal hialina e DGO) e morfologia do padrão perineal das fêmeas. Foram realizados estudos bioquímicos em fêmeas adultas utilizando o padrão enzimático das esterases (EST) e comparados com descrições anteriores. A análise molecular baseou-se em PCR usando *primers* específicos para *M. javanica* (Fjav/Rjav). Os resultados obtidos sugeriram a presença de *M. javanica*. Este estudo permitiu determinar que a identificação precisa destes nemátodes só é possível mediante a conjugação dos três métodos de diagnóstico: morfológico, bioquímico e molecular.

Palavras-chave: fenótipo de esterase, identificação molecular, *Meloidogyne* spp., padrões perineais, tomateiro.

INTRODUCTION

The genus *Meloidogyne*, root-knot nematodes (RKN), comprises more than 90 species (Hunt & Handoo, 2009) and on a worldwide basis includes the most economically damaging plant-parasitic nematodes. *Meloidogyne incognita, M. javanica, M. arenaria, M. chitwoodi, M. fallax* and *M. hapla* account for more than 95% of the occurrences of this genus and are the most widely distributed species. Their wide host range enhances the impact of these species; the most common species are estimated to be able to infect more than 5500 plant species affecting quality and quantity. The direct and indirect damage results in toppling, reduced yields, high costs of production, hence loss of income. (Trudgill & Blok, 2001; Wesemael *et al.,* 2011).

Meloidogyne incognita, M. javanica and *M. arenaria* are highly frequent in tropical climates but also in greenhouses of temperate regions, while *M. chitwoodi, M. fallax* and *M. hapla* are major species in temperate climate. Furthermore, in respect to changing global trade pattern and crop production system, *M. minor* and *M. enterolobii* species are becoming emerging threats for the temperate and tropical regions, respectively (Wesemael *et al*., 2011). As a result, the European and Mediterranean Plant Protection Organization (EPPO) has reported *M. chitwoodi, M. fallax* and *M. enterolobii* as quarantine pests (EPPO, 2016).

In Portugal, species of this genus have been found alone or in mixed populations in different regions of the centre and south, associated with several and important cultivated plants: *M. arenaria, M. chitwoodi, M. hapla, M. hispanica, M. incognita, M. javanica* and *M. lusitanica* (Abrantes & Santos, 1991; Abrantes *et al*., 2008; Conceição *et al*., 2009).

During parasitism, RKN establish and maintain an intimate relationship with their host. The sign of RKN infection is the presence of typical galls on susceptible host plant roots (Figure 1). The degree of root galling generally depends on three factors: nematode population density, *Meloidogyne* species and host plant species/cultivars. Nutrient and water uptake are substantially reduced, because of the damaged root system, resulting in weak and poor-yield (Abad *et al*., 2003).

Figure 1 - Tomato cv. Anaris roots showing abundant root knot nematode-induced galls, from North Portugal.

The soil nematode management is a very challenging task. Over the past century, to minimise crop losses caused by RKN, nematicides were widely used, but due to the adverse impacts on the environment and human health, their use has been reduced resulting on the elimination of several chemicals from the marketplace (Maleita *et al*., 2011).

Nowadays, the most successful approach for nematode management relies on the development of integrated pest management (IPM) programmes that combine control measures to maintain nematode densities below economic threshold levels. These programmes can still be difficult to implement against aggressive and resilient pathogens such as RKN. Nevertheless, a combination of cultural practices (rotations with non-host crops and cover crops that favour the build-up of nematode antagonists), resistant cultivars, and chemical soil treatments if necessary, generally provide an acceptable control of RKN. The extent of success, however, is dependent upon having an accurate identification of the species, definition of damage threshold densities and resistant cultivars.

The present research was undertaken to characterise morphological, biochemical and molecularly one Portuguese isolate of *Meloidogyne* found associated with tomato crops.

MATERIAL AND METHODS

Nematode isolate

Soil and tomato, *Solanum lycopersicum* cv. Anaris, root samples were collected from a field located in Estela – Póvoa de Varzim, Porto, North Portugal. Second-stage juveniles (J2) obtained from soil extraction and mature females extracted from infected roots were used to carry out the morphological, morphometrical, biochemical and molecular characterisation.

Morphological and morphometrical characterisation

Morphological and morphometric studies were conducted on J2 extracted from soil samples by floating, sieving and centrifugal flotation method (Gooris & D'herde, 1972) and females handpicked from infected roots. The J2 were transferred individually to a glass slide, with a drop of water, gently heat killed and observed under an Olympus BX-41 bright field light microscope. The measurements (body length, stylet length, tail length, hyaline terminus length and the distance between the stylet base and dorsal oesophageal gland orifice – DGO) were made using a ProgResSpeed XT core 5 – Jenoptik image software through drawing lines crossing approximately the middle of the specimen's body.

Females extracted from roots were placed in glass blocks with 45% lactic acid for two days, and the perineal patterns were prepared as described by Taylor & Netscher (1974) and observed under a light microscope.

Biochemical characterisation

Young egg-laying females were hand-picked from infected tomato roots under a stereomicroscope, transferred to an isotonic 0.9% sodium chloride solution to prevent osmotic disruption, and then to micro-haematocrit capillary tubes containing 5 µL of extraction buffer (20% sucrose and 1% Triton X-100). The specimens were macerated with a pestle, frozen and stored at -20°C until electrophoresis (no longer than 3 months). Shortly before electrophoresis, the samples were centrifuged at 10000 g for 15 minutes at -5°C.

Native polyacrylamide gel electrophoresis (PAGE) was carried out in vertical polyacrylamide gels, 1 mm thick, in a Mini-Protean II (Bio-Rad Laboratories, Hercules, California, USA) according to Pais *et al*. (1986).

Following electrophoresis, the gels were stained for esterase activity with the substrate α-naphthyl acetate. Protein extracts of females from a reference isolate of *M. javanica* were not possible to include in the gel and so the esterase phenotype was compared with other *Meloidogyne* spp. phenotypes.

Molecular characterisation

For molecular analysis, total genomic DNA was extracted from females using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Before extraction, females were transferred to Eppendorf tubes with 10 µL of sterile water, frozen in liquid nitrogen and then crushed with a micro pestle.

PCR amplification was performed using the SCAR species-specific primers Fjav/Rjav (Zijlstra *et al*., 2000). Primers were synthesised by STAB VIDA Facilities (Lisbon, Portugal). All PCR reactions were performed in a 25 µL final volume using the Promega Go Taq Flexi DNA Polymerase Kit (Promega, Madison), containing 1X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, 1.25 U of Taq DNA Polymerase. Amplifications were carried out in a Biometra TGradient thermo cycler (Biometra, Göttingen, Germany) using the following thermal cycling conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55 ºC for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The products were resolved by electrophoresis at 5 $V.cm^{-1}$ in agarose gel (1.5%) containing 0.5 µg/mL ethidium bromide and 0.5X TBE running buffer. Data analysis was visualised by VersaDoc Imaging System (BioRad, USA).

RESULTS AND DISCUSSION

Morphological and morphometrical characterisation

The J2 were vermiform and slender; head not offset from body. The stylet was slender with a sharp pointed stylet cone, cylindrical stylet shaft and prominent stylet knobs. Stylet knobs transversely elongate and offset from stylet shaft, hyaline tail terminus distinctive, long narrow tapering tail, finely rounded tail tip matching the description given for *M. javanica* species by Williams (1972), Eisenback (1985) and Karssen & Moens (2006). Morphometrics performed on J2 are reported in Table 1.

Table 1 - Morphometric comparison of second-stage juveniles of the Portuguese isolate and *Meloidogyne javanica* reported values (Williams, 1972; Eisenback, 1985; Karssen & Moens 2006)

Characteristic	Portuguese isolate (n=10)	*M. javanica* reported values
Body length	390 - 540 (465)	400 – 560
Stylet length	14.5 – 18.0 (16.2)	14.0 -18.0 (16.0)
Dorsal Oesophageal Gland (DGO)	3.0 – 4.0 (3.5)	3.0 - 4.0 (3.5)
Tail length	49.9 - 60.8 (55.3)	51.0 – 63.0 (57)
Hyaline terminus	10.0 – 18.0 (14.0)	10.0 – 19.0 (14.5)

All measurements in micrometers with range (mean).

Second-stage juveniles' body length averaged 465 µm. The stylet length was 16.2 (14.5 – 18.0) µm and the mean length of the hyaline tail terminus was 14 µm, which is in accordance with previous descriptions of *M. javanica* (Williams, 1972; Eisenback 1985; Karssen & Moens, 2006). However, the values obtained overlap with other *Meloidogyne* species, such as *M. incognita* and *M. arenaria*.

The perineal pattern was typical of *M. javanica* when compared to previous reports (Eisenback, 1985). It had a rounded pattern, striae interrupted laterally by a pair of conspicuous incisures extending on both sides of the tail terminus, low dorsal arch is low and rounded with a whorl in the tail terminal area (Figure 2).

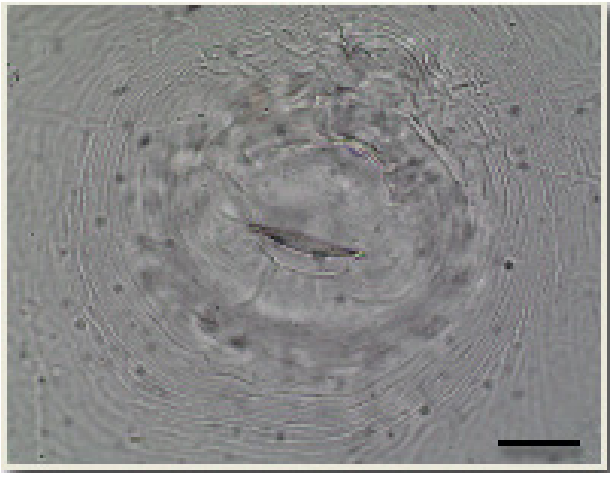

Figure 2 - Figure 2. Perineal pattern of the *Meloidogyne* isolate, from North Portugal (bar = 20 µm).

As noticed, morphological and morphometric studies require great effort and are not always easy even for taxonomists due to the presence of inter and intra-specific variability. The values obtained from morphometrics overlap just as has been previously reported and the morphology of the perineal pattern, although more useful, is still inconclusive due to the variability among individuals, the varied expertise of the people describing the patterns and the increase on the number of species. However, the conjunction of morphology and morphometrics may give a small indication towards the species identification.

Biochemical characterisation

Esterase phenotype was compared to esterase phenotype diagrams previously published due to the difficulty to include a *M. javanica* reference phenotype in the gel. Three bands of esterase activity were detected (Rm: 54, 50 and 41) and the esterase phenotype, apparently, was similar with earlier described phenotypes for *M. javanica* (Dickson *et al*., 1971; Pais & Abrantes, 1989) (Figure 3).

Despite having a positive result, this technique is very restrictive as it can only be applied to a specific developmental stage (mature females) limiting its use since agricultural soils do not contain *Meloidogyne* adult females.

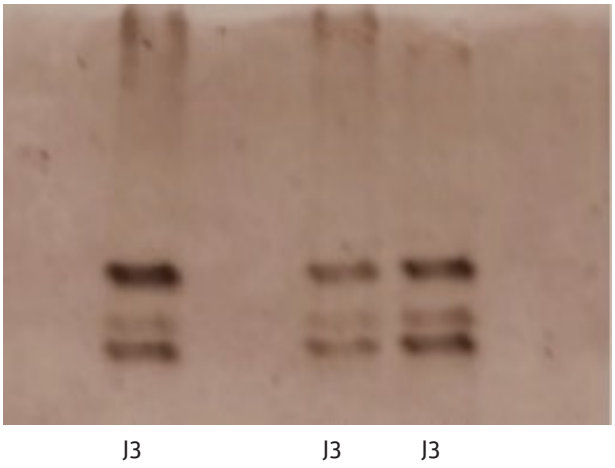

Figure 3 - Esterase phenotype of protein homogenates from three egg-laying females of the Portuguese isolate of *Meloidogyne* sp.

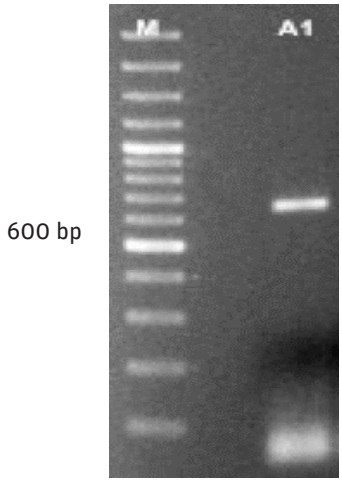

Figure 4 - DNA amplification product obtained of *Meloidogyne* isolate females, using the species-specific primers Fjav/Rjav. M: DNA marker (Gene ruler 100 bp DNA Ladder, Thermo Scientific).

Molecular characterisation

Morphological and biochemical studies indicated that the North Portuguese isolate found associated with tomato belongs to the *M. javanica* species. In order to confirm these results, DNA amplification was carried out using *M. javanica* species-specific primers (Fjav/Rjav). As shown in Figure 4, the fragment size obtained with species-specific primers was 670 bp, which is consistent with previous reports (Zijlstra *et al*., 2000) indicating the identity of the nematode as *M. javanica.*

It is important to highlight that although PCR is fast, straightforward and able to determine the species identity irrespective of the developmental stage and from small amounts of tissue, as reported in this study, its reliability is uncertain due to the intraspecific variability and closeness between species. So, morphology, morphometrics, isozyme analysis and molecular analysis complement each other and provide a more accurate and reliable identification.

CONCLUSIONS

Morphological and morphometric identification is arduous and time consuming. Measurements overlapped within the species and perineal patterns, although useful in the past, showed great inter and intra-specific variability. These difficulties led the investigators to search for other methodologies to confirm and complement RKN species identification. Isozyme analysis has proven to be very useful despite being a very restrictive technique, since it only works with a specific developmental stage (mature females) and requires the inclusion of a reference isolate. Species-specific primers suggested the identity of the North Portuguese population as *M. javanica.*

In conclusion, the diagnosis of species of *Meloidogyne* is challenging because of the poorly defined boundaries among species, intraspecific variability, potential hybrid origin, and polyploidy. Therefore, one single technique cannot be relied upon, since *Meloidogyne* complexity requires all available methodologies in order to get more accurate diagnoses that will lead to better management decisions and control measures.

ACKNOWLEDGEMENTS

We are extremely grateful to Maria João Camacho for her assistance, co-operation and constant support throughout this work. Also, we would like to express our appreciation to Ana Margarida Fontes (Laboratory of Nematology, INIAV, Portugal) for her encouragement and willingness to help us in the identification.

REFERENCES

Abad, P.; Favery, B.; Rosso, M.N. & Castagnone-Sereno, P. (2003) – Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. *Molecular Plant Pathology*, vol. 4, n. 4, p. 217-224. http://dx.doi.org/10.1046/j.1364-3703.2003.00170.x

Abrantes, I.M. & Santos, M.S.N. de A. (1991) – *Meloidogyne lusitanica* n. sp. (Nematoda: Meloidogynidae), a root-knot nematode parasitising olive tree (*Olea europaea* L.). *Journal of Nematology,* vol. 23, n. 2, p. 210-224.

Abrantes, I.M.; Santos, M.C.; da Conceição, I.L.P.M.; Santos, M.S.N. & Volvas, N. (2008) – Root knot and other plant parasitic nematodes associated with fig trees in Portugal. *Nematologia Mediterranea,* vol. 36, n. 2, p. 131-136.

Conceição, I.L.P.M.; Cunha, M.J.M.; Feio, G.; Correia, M.; Vieira dos Santos, M.C.; Abrantes, I.M.; & Santos, M.S.N.; (2009) – Root-knot nematodes, *Meloidogyne* spp., on potato in Portugal. *Nematology*, vol. 11, n. 2, p. 311-313. http://dx.doi.org/10.1163/156854109X415515

Dickson, D.; Huising, D. & Sasser, J. (1971) – Dehydrogenases, acid and alkaline phosphatases and esterases for chemotaxonomy of selected *Meloidogyne, Ditylenchus, Heterodera* and *Aphelenchus* spp. *Journal of Nematology,* vol. 3, n. 1, p. 1-16.

Eisenback, J.D. (1985) – Detailed morphology and anatomy of second-stage juveniles, males, and females of the genus *Meloidogyne* (root-knot nematodes). *In*: Sasser, J.N. & Carter, C.C. (Eds.) – *An advanced treatise on Meloidogyne* Volume I (pp. 47-77) Raleigh, USA, North Carolina State University Graphics.

EPPO (2016) – Diagnostic protocols for regulated pests *Meloidogyne chitwoodi*, *Meloidogyne fallax* and *Meloidogyne enterelobii. Bulletin OEPP/EPPO* vol. 46, p. 171-201.

Gooris, J. & D'herde, C.J. (1972) – *A method for the quantitative extraction of eggs and second stage juveniles of Meloidogyne spp. from soil*. Ghent, Belgium: Agricultural Research Centre, 36 p.

Hunt, D.J. & Handoo, Z.A. (2009) – Taxonomy, identification and principal species. *In*: Perry, R.N.; Moens, M. & Starr, J.L. (Eds) – *Root-knot nematodes*. Wallingford, UK, CABI Publishing, pp. 55-97.

Karssen, G. & Moens, M. (2006) – Root knot nematodes. *In*: Perry, R.N. & Moens, M. (Eds.) – *Plant Nematology*. Wallingford, UK, CABI Publishing, pp. 59-90.

Maleita, C.; Santos, M.C.; Curtis, R., Powers, S., & Abrantes, I.M. (2011) – Effect of the *Mi*gene on reproduction of *Meloidogyne hispanica* on tomato genotypes. *Nematology*, vol. 13, n. 8, p. 939-949. http://dx.doi.org/10.1163/138855411X566449

Pais, C.S. & Abrantes, I.M. (1989) – Esterase and Malate dehydrogenase phenotypes in Portuguese populations of *Meloidogyne* species. *Journal of Nematology*, vol. 21, n. 3, p. 332-346.

Pais, C.S.; Abrantes, I.M.; Fernandes, M.F.M. & Santos, M.S.N. de A. (1986) – Técnica de electroforese aplicada ao estudo das enzimas dos nemátodes-das-galhas-radiculares, *Meloidogyne* spp. *Ciência Biológica Ecology and Systematics*, vol. 6, p. 19-34.

Taylor, D.P. & Netscher, C. (1974) – An improved technique for preparing perineal patterns of *Meloidogyne* spp. *Nematologica,* vol. 20, p. 268-269. http://dx.doi.org/10.1163/187529274X00285

Trudgill, D.L. & Blok, V.C. (2001) – Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annual Review of Phytopathology,* vol. 39, p. 53-77. http://dx.doi.org/10.1146/annurev.phyto.39.1.53

Wesemael, W.M.L.; Viaene, N. & Moens, M. (2011) – Root-knot nematodes (*Meloidogyne* spp.) in Europe. *European Journal of Plant Pathology*, vol. 13, n. 1, p. 3–16. http://dx.doi.org/10.1163/138855410X526831

Williams, K.J.O. (1972) – *Meloidogyne javanica*. *In*: *CIH Descriptions of Plant-Parasitic Nematodes,* Set 1, No 3. Commonwealth Agricultural Bureaux, Farnham Royal, UK.

Zijlstra, C.; Donkers-Venne, D.T.H.M. & Fargette, M. (2000) – Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterized amplified region (SCAR) based PCR assays. *Nematology*, vol. 2, n. 8, p. 847-853. http://dx.doi.org/10.1163/156854100750112798