Biology and Ecology of the Root-Knot Nematode *Meloidogyne hispanica*

A Species of Emerging Importance

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Front cover from left to right: host suitability of *Meloidogyne hispanica* in cultivated plants (pot experiment); females inside a root of tomato, *Solanum lycopersicum*; tomato cv. Easypeel seedlings in Petri dishes (life cycle experiment); DNA amplification products of *Capsicum annuum* cultivars using SCAR_B94 linked to the *Me3* gene; male (light microscope photograph); *Meloidogyne* spp. protein patterns; tomato cv. Easypeel root with a gall and respective egg mass; *Hinf*I and *Alu*I digestion patterns of the amplification products from *Meloidogyne* spp., using $C2F3$ and MRH106 primers; effect of inoculum levels and temperature on *M. hispanica* reproduction in pepper cultivars (pot experiment); esterase phenotypes of *Meloidogyne* spp. females protein homogenates; tomato cv. Easypeel roots inoculated with 5000 eggs; female (light microscope photograph); infected tomato cv. Tiny Tim roots; second-stage juveniles penetration on tomato cv. Easypeel roots; male - posterior region in lateral view (light microscope photograph); tomato genotypes inoculated with 5000 eggs (pot experiment); perineal pattern of female (light microscope photograph); second-stage juveniles inside roots three days after inoculation; infected tomato roots stained with acid fuchsin. All photographs by Carla Maleita.

Thesis submitted to the Faculty of Sciences and Technology, University of Coimbra for the degree of Doctor of Philosophy in Biology-Ecology.

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The present work was financially supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008 and grant SFRH/BD/24275/2005 to Carla Maleita supported by QREN funds through the "Programa Operacional Potencial Humano".

Aos meus pais e irmã pelo seu apoio
incondicional e carinho.
Sem vocês não teria sido possível...

A Ti...
Esta tese também é um pouco tua.

"Na verdade, os meus amigos fizeram a história da minha vida. De mil e uma maneiras, tornaram privilégios as minhas limitações e deram-me a possibilidade de caminhar..."

Hellen Keller (1880-1968)

"Nothing splendid has ever been achieved except by those who dared believe that something inside them was superior to circumstance."

Bruce Barton (1886-1967)

AGRADECIMENTOS

Neste momento não poderia deixar de expressar o meu reconhecimento a todos aqueles que com o seu apoio, interesse, orientação, paciência e amizade contribuíram para a concretização deste trabalho. De todos guardo as mais gratas recordações.

À Fundação para a Ciência e a Tecnologia (FCT) pelo financiamento através da bolsa individual de Doutoramento SFRH/BD/24275/2005 e apoio adicional através do projecto "*Molecular analysis of secretions of the root-knot nematode* Meloidogyne hispanica" (PTDC/AGR-AAM/103873/2008).

O trabalho experimental foi realizado no Instituto do Mar – Centro do Mar e Ambiente (IMAR-CMA), Departamento de Ciências da Vida, da Faculdade de Ciências e Tecnologia da Universidade de Coimbra (FCTUC) em colaboração com *Plant Pathology and Microbiology Department*, *Rothamsted Research*, UK. Deste modo, não posso deixar de agradecer às pessoas responsáveis por estas instituições, nomeadamente ao Professor Doutor João Carlos Marques (Coordenador do IMAR-CMA), ao Professor Doutor António Veríssimo (Director do Departamento de Ciências da Vida, FCTUC) e ao Professor John Lucas (Director *Plant Pathology and Microbiology Department*, *Rothamsted Research*) pelas facilidades concedidas indispensáveis à realização deste trabalho.

À minha orientadora Professora Doutora Isabel Maria de Oliveira Abrantes, que me iniciou na investigação científica e incentivou o interesse pelo estudo da Nematologia, serei sempre grata pela sua orientação científica, supervisão e análise crítica e detalhada desta tese. Pelo seu contínuo apoio, amizade, compreensão e disponibilidade demonstrados ao longo da realização de todo o trabalho experimental, bem como pela atenta revisão da redacção desta tese e que contribuíram para melhorar a sua apresentação final. De igual modo, agradeço à minha co-orientadora Doutora Rosane Hazelman Cunha Curtis pela orientação, apoio e oportunos comentários e sugestões, bem como pela revisão atenta deste trabalho.

À Doutora Conceição Egas, Directora da Unidade de Serviços Avançados do BIOCANT (Cantanhede) e à Doutora Maria José Simões (BIOCANT) pela disponibilidade prestada ao

conceder todas as facilidades indispensáveis à realização do trabalho experimental no âmbito da caracterização molecular de *Meloidogyne hispanica*, bem como pelo seu apoio, orientação, ensinamentos e análise crítica do trabalho.

Ao Doutor Stephen Powers do *Biomathematics and Bioinformatics Department*, *Rothamsted Research*, pelo seu constante apoio na análise estatística dos resultados obtidos.

À Doutora Caroline Djian-Caporalino do INRA, *Unité Intéractions Biotiques et Santé Végétale* (IBSV), França, pelos oportunos comentários e sugestões e disponibilidade em fornecer sementes de pimento. À Germiplanta – Viveiros de Plants Lda., *Pioneer Hi-Bred* Sementes de Portugal, S.A., nomeadamente aos Senhores Engenheiros Joaquim Sousa e Vasco Salgueiro, e ao Senhor Engenheiro Paulo Abreu da *Monsanto Company, Vegatable Seeds Division*, que gentilmente forneceram as sementes e plantas utilizadas nos ensaios realizados.

A todos os meus colegas e amigos do Laboratório de Nematologia, Departamento de Ciências da Vida, FCTUC, que sempre se interessaram pelo progresso do trabalho, por todo o apoio, entusiasmo e palavras amigas. Em especial à Clara Vieira dos Santos gostaria de agradecer por todos os ensinamentos que de forma crítica e amiga sempre me soube transmitir, o constante apoio, interesse, a muita paciência, disponibilidade, compreensão e amizade demonstrados ao longo dos bons e maus momentos. Um muito Obrigado!

A todos os meus amigos(as) que de muitas formas me apoiaram, pelo seu interesse e amizade, em particular à Daniela Oliveira, Cristina Galhano, Mónica Zuzarte, Padre António Domingues e São.

Finalmente, à minha família. Aos meus pais e irmã que sempre me incentivaram, me apoiaram nos momentos mais difíceis e contribuíram de forma activa para a concretização deste trabalho, e a todos os restantes familiares que sempre acreditaram em mim.

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LIST OF PUBLICATIONS RELATED TO THIS PH.D. THESIS

Chapters of this Thesis were written as journal articles as follows:

Chapter 1

MALEITA, C.M., SIMÕES, M.J., EGAS, C., CURTIS, R.H.C. & ABRANTES, I.M. DE O. (2011). Morpho-biometrical, biochemical and molecular diagnosis of Portuguese *Meloidogyne hispanica* isolates. (To be submitted).

Chapter 2

MALEITA, C.M., CURTIS, R.H.C. & ABRANTES, I.M. DE O. (2011). Thermal requirements for the embryonic development and life cycle of *Meloidogyne hispanica*. *Plant Pathology* (Submitted).

Chapter 3

MALEITA, C.M., CURTIS, R.H.C., POWERS, S.J. & ABRANTES, I.M. DE O. (2011). Reproduction and virulence of *Meloidogyne hispanica* in cultivated plants. *European Journal of Plant Pathology* (Submitted).

Chapter 4

MALEITA, C.M., VIEIRA DOS SANTOS, M.C., CURTIS, R.H.C., POWERS, S.J. & ABRANTES, I.M. DE O. (2011). Effect of the *Mi* gene on reproduction of *Meloidogyne hispanica* on tomato genotypes. *Nematology* (In press).

Chapter 5

MALEITA, C.M., CURTIS, R.H.C., POWERS, S.J. & ABRANTES, I.M. DE O. (2011). Effects of inoculum levels of *Meloidogyne hispanica* and *M. javanica* on the nematode reproduction and growth of tomato genotypes. *Phytopathologia Mediterranea* (Submitted).

ABSTRACT

Meloidogyne hispanica Hirschmann, 1986 was originally detected in Spain causing important losses to *Prunus* spp. Since then, the presence of this species has been reported associated to other economically important crops worldwide (Africa, Asia, Australia, Europe, and North, Central, and South America). An accurate and reliable identification of this species is needed for research purposes and also for devising an effective, sustainable and environmentally-safe control strategy. The main goals of this study were to characterize morpho-biometrically, biochemically and molecularly Portuguese *M. hispanica* isolates and to understand better its biology and ecology. The developed work included the assessment of the impacts of temperature on its life cycle; evaluation of host resistance to *M. hispanica* in several cultivated plants and tomato genotypes with the *Mi-1.2* gene, and determination of the effects of the inoculum level on nematode reproduction and growth on various tomato genotypes. A morphobiometrical study was carried out on males, females and juveniles of seven *M. hispanica* isolates and, although, the characters were similar to the original description of the species, the differentiation of *M. hispanica* from other *Meloidogyne* species, mainly *M. arenaria* and *M. incognita* can be very difficult. Reproducible and similar SDS-PAGE protein patterns were found for *M. hispanica* isolates and *M. arenaria*, *M. ethiopica*, *M. hapla*, *M. incognita*, *M. javanica* and *M. mayaguensis*; only *M. chitwoodi* and *M. megadora* revealed distinct profiles. Biochemical and molecular techniques provided a more accurate identification and differentiation of *M. hispanica*, this was achieved by esterase phenotype (Hi4), and mtDNA-PCR-RFLP analysis of the region between *COII* and 16S rRNA genes, with primers C2F3/MRH106. Variability in mtDNA sequences allowed the discrimination of *M. hispanica* (ca. 1,800 bp) from *M. hapla* (ca. 650 bp), *M. chitwoodi* (ca. 650 bp), *M. mayaguensis* (ca. 850 bp) and *M. arenaria* (ca. 1,300 bp) and the species (*M. ethiopica*, *M. javanica* and *M. incognita*) with similar amplified products by *Hinf*I and *Dra*III. Two digestion fragments (ca. 1,700 and 100 bp) were obtained for *Hinf*I and no digestion occurred with *Dra*III to *M. hispanica*. However, more *M. hispanica* isolates need to be tested in order to strengthen the results obtained and to validate the potential applicability of this methodology for diagnosis of this species of root-knot nematode. The life cycle of *M. hispanica* in the tomato cultivars Easypeel highly susceptible, and Rossol, with the

root-knot nematode (RKN) resistance *Mi-1.2* gene, was studied over the temperature range of 10 to 35ºC. A linear relationship between temperature and the rate of embryogenesis and life cycle was obtained from 15 to 30ºC and 20 to 30ºC, respectively. *Meloidogyne hispanica* embryogenesis was faster at 25 and 30ºC and no development occurred at 10 or 35ºC. At temperatures below 35ºC, several second-stage juveniles (J2) failed to establish and develop in roots of tomato cv. Rossol, with the proportion of adult females recorded on roots significantly lower than on cv. Easypeel, and no reproduction occurred at 15 and 25ºC. At 30 and 35ºC, the development was similar on both tomato cultivars. The life cycle in the cv. Easypeel was shorter at 25 and 30ºC, and at 15ºC, the presence of eggs was not observed until 80 days after inoculation. Our results suggest that *M. hispanica* is most suited to soil temperatures around 25ºC. The estimated base temperatures were 11.49ºC and 10.22ºC and thermal constant 76.92 and 515.46 *DD* for embryogenesis and life cycle completion, respectively. Predicted climate changes indicate that *M. hispanica* could spread in Southern Europe and move north. Sixty three cultivars, including 18 plant species from Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae, were assessed for host suitability to *M. hispanica* on the basis of root gall index (GI) and reproduction factor (Rf). All cultivars were susceptible (GI = 3-5; Rf = 1.15-262.86), except cultivar Bacalan of cabbage, Temporão of cauliflower and pepper Zafiro R2 classified as hypersusceptible ($GI \geq 2.4$; $Rf \leq 0.47$); and cultivars Aurelio and Solero of pepper as resistant ($GI \leq 0.4$; $Rf \leq 0.03$). The reproduction of *M. hispanica* on the three pepper cultivars was evaluated using two inoculum levels (2,500 and 5,000 eggs) at four temperatures (24.4±8.2ºC, 25.0±2.7ºC, 29.3±1.8ºC and 33.6±1.2ºC), and these cultivars were considered resistant hosts ($GI \leq 1.8$; $Rf \leq 0.4$) at all conditions. In the cv. Aurelio, at 33.6±1.2ºC, a limited number of J2 developed into females that produced eggs. The eggs from these virulent females were inoculated, by two successive transfers into cv. Aurelio of pepper, in order to bulk up this virulent isolate. This new isolate was able to reproduce on all three pepper cultivars breaking the resistance. Our results suggest that the initial *M. hispanica* isolate is a mixture of virulent and avirulent individuals. These pepper cultivars can be used as an alternative to the chemical control, but should be used in an integrated management context in combination with other control measures to prevent the selection of virulent lines. Twenty five tomato genotypes were screened for the RKN resistance *Mi-1.2* gene, by amplification of markers REX-1 and

Mi23 as sequence-characterised amplified regions. Ten heterozygous tomato genotypes (Mimi), eight homozygous (MiMi) at the *Mi* locus and six (mimi) lacking the *Mi-1.2* gene for resistance to RKN were identified. Only tomato genotype Valouro RZ F1 was homozygous (MiMi) and heterozygous (Mimi) at the *Mi* locus when using the REX-1 and Mi23 markers, respectively. The results of the host suitability suggest a possible dosage effect of the *Mi-1.2* gene and an influence of the genetic background of the plants containing the *Mi-1.2* gene on final population density of *M. hispanica*. The increasing number of *Mi-1.2* alleles (0, 1 or 2) is associated with the decrease of the final population density. Ten tomato genotypes, with *Mi-1.2* gene, out the 25 genotypes are commercially available, but only Rapit (Mimi; IG = 4, Rf = 0.62) can be used to inhibit the increase of *M. hispanica* populations in the soil and to control the three most common *Meloidogyne* species (*M. arenaria*, *M. incognita* and *M. javanica*). Effects of three inoculum levels (2,500, 5,000 and 10,000 eggs/plant) on the reproduction of *M. hispanica* and *M. javanica* isolates and growth of the tomato genotypes Easypeel and Moneymaker (used to maintain the RKN isolates on the laboratory), and Motelle and VFNT-Cherr (with the *Mi-1.2* gene) were evaluated 60 days after inoculation at 25±2ºC. The host status was based on GI and Rf, and shoot/root length and fresh/dry root and shoot weight were also recorded. All tomato plants revealed a trend to show lower values of growth parameters due to the damage caused by the increasing number of nematodes that invaded the roots. However, the genotypes Motelle and VFNT-Cherr remained unchanged regarding shoot and total shoot plus root dry weight. The two RKN species were identified as naturally virulent to tomato plants with the *Mi-1.2* gene, but *M. javanica* showed a higher reproductive and destructive potential than *M. hispanica.* Tomato plants inoculated with *M. hispanica* presented the higher values for growth parameters when compared with plants inoculated with *M. javanica*. *Meloidogyne hispanica* can be considered a polyphagous species of emerging importance with potential economic impact to agricultural areas. These results show that further research should be done to advance the knowledge on new sources of resistance and the identification of plant species and/or cultivars resistant to *M. hispanica.* To improve the management of *M. hispanica* the pathogenicity of the local populations should be assessed before a decision is made on the crop rotation scheme to be used and it is also important to develop preventive measures to avoid the dispersion of this species.

Keywords: base temperature, *Capsicum annuum*, cultivated plants, cytocrome oxidase subunit II, diagnosis, embryonic development, esterase phenotype, host status, inoculum level, life cycle, *Me* genes, *Mi* gene, Mi23 marker, morpho-biometrics, mtDNA, *N* gene, pathogenicity, PCR-RFLP, pepper, post-embryonic development, protein profile, resistance genes, REX-1 marker, root penetration, root-knot nematode, SCAR, SDS-PAGE, *Solanum lycopersicum*, temperature, thermal time requirements, tomato, virulence.

RESUMO

Meloidogyne hispanica Hirschmann, 1986 foi detectada pela primeira vez em Espanha causando perdas importantes em *Prunus* spp. Desde então, esta espécie tem vindo a ser detectada em vários continentes (África, Ásia, Austrália, Europa, e no Norte, Centro e Sul da América) associada a outras culturas economicamente importantes. A identificação correcta desta espécie de nemátodes-das-galhas radiculares (NGR) é fundamental não só para fins de investigação mas também para a concepção de uma estratégia de controlo efectiva, sustentável e amiga do ambiente. Os objectivos principais deste trabalho foram caracterizar morfo-biometrica, bioquímica e molecularmente isolados Portugueses de *M. hispanica* e compreender melhor a sua biologia e ecologia. Os estudos realizados incluíram a avaliação dos impactos da temperatura no seu ciclo de vida, determinação da patogenicidade de *M. hispanica* em plantas cultivadas e genótipos de tomateiro com o gene *Mi-1.2*, e determinação dos efeitos do nível de inóculo na reprodução do nemátode e crescimento de vários genótipos de tomateiro. Os resultados obtidos nos estudos morfobiométricos dos machos, fêmeas e jovens do segundo estádio juvenil (J2) de sete isolados de *M. hispanica* estão de acordo com a descrição original da espécie. Contudo, a diferenciação de *M. hispanica* de outras espécies de *Meloidogyne*, principalmente *M. arenaria* e *M. incognita*, pode ser muito difícil. Os perfis proteicos de *M. hispanica*, obtidos por SDS-PAGE, foram reproduzíveis e semelhantes aos de *M. arenaria*, *M. ethiopica*, *M. hapla*, *M. incognita*, *M. javanica* e *M. mayaguensis*; apenas *M. chitwoodi* e *M. megadora* apresentaram perfis proteicos distintos. A utilização de técnicas bioquímicas e moleculares permitiu uma identificação e diferenciação mais precisa de *M. hispanica*, especificamente através da análise dos fenótipos de esterases (Hi4) e da análise por PCR-RFLP da região entre os genes *COII* e 16S rRNA do ADN mitocondrial (ADNmt), com os *primers* C2F3 e MRH106. A variabilidade encontrada nas sequências de ADNmt permitiu diferenciar *M. hispanica* (ca. 1800 bp) das espécies *M. hapla* (ca. 650 bp), *M. chitwoodi* (ca. 650 bp), *M. mayaguensis* (ca. 850 bp) e *M. arenaria* (ca. 1300 bp) e das espécies (*M. ethiopica*, *M. javanica* and *M. incognita*) com produtos de amplificação semelhantes pelos padrões de restrição obtidos com as endonucleases *Hinf*I e *Dra*III. Em *M. hispanica* foram obtidos dois fragmentos (ca. 1700 e 100 bp) com a enzima de restrição *Hinf*I, não tendo ocorrido

digestão dos produtos amplificados com a enzima *Dra*III. No entanto, um maior número de isolados de *M. hispanica* deverá ser testado a fim de validar os resultados obtidos e a aplicabilidade desta metodologia no diagnóstico desta espécie de NGR. O ciclo de vida de *M. hispanica* foi estudado, num intervalo de temperaturas entre 10-35ºC, em cultivares de tomateiro Easypeel, susceptível a NGR, e Rossol, com o gene *Mi-1.2* que confere resistência a NGR, tendo sido obtida uma relação linear entre a temperatura e a taxa de desenvolvimento embrionário e o ciclo de vida entre 15-30ºC e 20-30ºC, respectivamente. O desenvolvimento embrionário de *M. hispanica* foi mais rápido a 25 e 30ºC, enquanto a 10 e 35ºC não ocorreu desenvolvimento. Nas raízes de tomateiro cv. Rossol, a temperaturas inferiores a 35ºC, alguns J2 foram incapazes de se estabelecer e desenvolver, tendo-se registado uma proporção de fêmeas significativamente menor quando comparada com a cultivar Easypeel; a 15 e 25ºC não houve reprodução. O desenvolvimento foi semelhante em ambas as cultivares de tomateiro a 30 e 35ºC. Na cultivar Easypeel de tomateiro, o ciclo de vida foi mais curto a 25 e 30ºC, e a 15ºC não se observou a presença de ovos até aos 80 dias após a inoculação. Os resultados obtidos sugerem que esta espécie se encontra adaptada a temperaturas do solo à volta de 25ºC. Os valores estimados para a temperatura basal foram 11,49ºC e 10,22ºC e os valores da constante térmica para o desenvolvimento embrionário e ciclo de vida foram 76,92 e 515,46 DD, respectivamente. Considerando as alterações climáticas previstas, *M. hispanica*, tal como as espécies tropicais de NGR, poderá disseminar-se pelo Sul da Europa e mover-se para Norte. O grau de resistência de 63 cultivares, de 18 espécies de plantas das famílias Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae e Solanaceae, a *M. hispanica* foi avaliado com base no índice de galhas (IG) e no factor de reprodução (Rf). Todas as cultivares foram consideradas susceptíveis (IG = 3-5; Rf = 1,15-262,86), excepto as cultivares Bacalan de couve, Temporão de couve-flor e Zafiro R2 de pimento classificadas como hipersusceptíveis (IG $\geq$ 2,4; Rf $\leq$ 0,47); e as cultivars Aurelio e Solero de pimento como resistentes (IG $\leq$ 0,4; Rf $\leq$ 0,03) ao nemátode. Em seguida, foi avaliada a reprodução de *M. hispanica* nas três cultivares de pimento utilizando dois níveis de inóculo (2500 e 5000 ovos) a quatro temperaturas (24,4±8,2ºC, 25,0±2,7ºC, 29,3±1,8ºC e 33,6±1,2ºC). Novamente, estas cultivares de pimento foram consideradas resistentes a *M. hispanica* (IG $\leq$ 1,8; Rf $\leq$ 0,4) em todas as condições testadas. No entanto, na cv. Aurelio, a 33,6±1,2ºC, alguns J2 desenvolveram-se até ao estádio de fêmea adulta que

produziram ovos. Os ovos destas fêmeas virulentas foram inoculados em pimento cv. Aurelio para aumentar a densidade populacional deste isolado virulento. Este novo isolado reproduziu-se nas três cultivares de pimento quebrando a resistência. Os resultados sugerem que o isolado original de *M. hispanica* é uma mistura de indivíduos virulentos e avirulentos. Estas cultivares de pimento podem ser consideradas uma alternativa ao uso de nematodicidas, devendo ser utilizadas com outras medidas de controlo, para evitar a selecção de linhas virulentas. A presença/ausência do gene *Mi-1.2*, responsável pela resistência a NGR, foi verificada em vinte e cinco genótipos de tomateiro através da amplificação dos marcadores REX-1 e Mi23. Dez genótipos de tomateiro foram identificados como heterozigóticos (Mimi) e oito homozigóticos (MiMi) para o *locus Mi* e seis (mimi) não apresentavam o gene *Mi-1.2*. Apenas o genótipo Valouro RZ F1 foi considerado homozigótico (MiMi) ou heterozigótico (Mimi) dependendo do marcador utilizado, REX-1 ou Mi23, respectivamente. Os resultados obtidos nos ensaios de patogenicidade sugerem um possível efeito doseador do gene *Mi-1.2*, bem como uma influência do fundo genético, das plantas que contêm o gene de resistência, na densidade populacional final de *M. hispanica*. O número crescente de alelos do gene *Mi-1.2* (0, 1 ou 2) está associado a uma diminuição da densidade populacional final do nemátode. Dez genótipos de tomateiro com o gene *Mi-1.2*, dos 25 avaliados, estão disponíveis comercialmente, mas apenas o tomateiro Rapit (Mimi; IG = 4, Rf = 0,62) pode ser utilizado para impedir o aumento das populações de *M. hispanica* no solo e controlar as populações das três espécies mais comuns de *Meloidogyne* (*M. arenaria*, *M. incognita* e *M. javanica*). O efeito de três níveis de inóculo (2500, 5000 and 10 000 ovos/planta) na reprodução de *M. hispanica* e *M. javanica* e no desenvolvimento dos tomateiros Easypeel e Moneymaker (usados para manter as populações de NGR em laboratório) e Motelle e VFNT-Cherr (com o gene *Mi-1.2*) foi avaliado, a 25±2ºC, 60 dias após a inoculação. O grau de resistência foi baseado no IG e Rf, tendo-se ainda avaliado o comprimento da raiz/caule e peso seco/húmido das raízes e do caule. De um modo geral, as plantas apresentaram valores baixos nos parâmetros relacionados com o desenvolvimento, devido aos danos causados pelo aumento do número de nemátodes que invadiram as raízes, com excepção dos genótipos Motelle e VFNT-Cherr que apresentaram valores estáveis de peso seco total e do caule. As duas espécies de NGR foram identificadas como naturalmente virulentas ao gene *Mi-1.2*, contudo *M. javanica* apresentou um potencial reprodutivo e destrutivo superior a *M. hispanica*. Os tomateiros inoculados com

M. hispanica apresentaram valores mais elevados para os parâmetros de desenvolvimento das plantas quando comparados com os das plantas inoculadas com *M. javanica*. *Meloidogyne hispanica* pode ser considerada uma espécie polífaga de importância emergente com impacto económico em áreas agrícolas. Os resultados obtidos mostram que uma das linhas de investigação a ser promovida deveria estar relacionada com a descoberta de novas fontes de resistência e com a identificação de espécies de plantas e/ou cultivares resistentes a *M. hispanica*. Para um controlo mais eficiente de *M. hispanica* é fundamental a avaliação da patogenicidade das populações locais antes da incorporação de plantas em esquemas de rotação de culturas e a implementação de medidas preventivas para evitar a dispersão desta espécie.

Palavras-chave: ADNmt, *Capsicum annuum*, ciclo de vida, desenvolvimento embrionário, desenvolvimento pós-embrionário, diagnóstico, fenótipo de esterases, gama de hospedeiros, genes *Me,* gene *Mi,* gene *N,* genes de resistência, marcador Mi23, marcador REX-1, morfobiometria, nemátode-das-galhas-radiculares, nível de inóculo, patogenicidade, PCR-RFLP, penetração, perfis proteicos, pimento, plantas cultivadas, requisitos temperatura-tempo, SCAR, SDS-PAGE, *Solanum lycopersicum*, subunidade II citocromo oxidase, temperatura, temperatura basal, tomate, virulência.

General Introduction

The genus Meloidogyne *Goeldi, 1892*

The Phylum Nematoda comprises a large number of animals adapted to several habitats ranging from free living nematodes to obligate plant/animal parasites. Within this phylum, plant parasitic nematodes cause worldwide damage to crops with great losses annually (Sasser & Freckman, 1987). Some plant parasitic nematodes are ectoparasites and others are migratory or sedentary endoparasites.

Nematodes of the genus *Meloidogyne*, commonly known as root-knot nematodes (RKN), are sedentary endoparasites and the most successful group of parasites. It is estimated that every year they cause worldwide crop losses of about 5%, which constitutes a major obstacle to agricultural production in developing countries (Hussey & Janssen, 2002). The high impact of these nematodes on agricultural areas can be attributed to their global distribution and their ability to parasitize more than 3,000 plant species, including monocotyledons, dicotyledons and herbaceous and wood plants, affecting the production and quality of a number of plants of economic importance (Eisenback & Triantaphyllou, 1991; Abad *et al*., 2003).

The most common species are *M. arenaria*, *M. incognita*, *M. javanica* and *M. hapla*, which represent 95% of the species encountered in agricultural fields (Hussey & Janssen, 2002). Others species, such as *M. chitwoodi*, *M. fallax*, *M. minor* and *M. paranaensis* have shown restricted distribution damaging specific major crops, and are considered as species of emerging importance (Moens *et al*., 2009).

Each population of RKN is influenced by the range of temperatures that characterize its natural environment (Santo & O´Bannon, 1981; Lahtinen *et al*., 1988; Madulu & Trudgill, 1994; Zhang & Schmitt, 1995; Ploeg & Maris, 1999; Yeon *et al*., 2003; Charchar & Santo, 2009; Strajnar *et al*., 2011). *Meloidogyne hapla* is limited to temperate soils with an optimum temperature range of 15 to 25ºC; whereas species found typically in tropical and subtropical areas such as *M. arenaria*, *M. incognita* and *M. javanica* have an optimum temperature range of 25 to 30ºC. Above 40ºC and below 5ºC, very little activity or no development occurs in any *Meloidogyne* spp. (Taylor & Sasser, 1978).

The genus *Meloidogyne* includes more than 90 described species of which 23 have been found in Europe (Hunt & Handoo, 2009; Wesemael *et al*., 2011). In Portugal, several species of this genus have been reported associated with important crops: *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica* and

M. lusitanica (Ibrahim, 1985; Abrantes & Santos, 1991; Abrantes *et al*., 2008; Conceição *et al*., 2009).

Meloidogyne *species identification*

The impact of the genus *Meloidogyne* in agricultural areas reinforces the need for an accurate diagnosis at species level. The development of a rapid and reliable method to identify the species found in the field increase the possibility of nematode control.

In the past, RKN were identified by laborious microscopic examination of morphological and biometric characters which rely on measurements and comparison of morphological structures. According to Hirschmann (1985), the most useful characters in the identification of *Meloidogyne* species were the perineal pattern and the morphology of the female stylet, as well as the head shape, the distance of dorsal esophageal gland orifice to base of stylet and the stylet shape of males. In juveniles due to the small size, head characters were less useful, but tail length and shape were considered good characters because showed low intra-specific variation. However, diagnosis based on morphology is not always easy even for qualified taxonomists due to the great inter and intra-specific morphological and physiological variability and to the frequent occurrence of more than one species in the same sample (Einsenback, 1985). These problems led researchers to search for other methodologies to confirm and complement nematode species identification.

No differences were found in enzyme profiles within the same species even when nematodes were maintained in different hosts and according to Dickson *et al*., (1971) and Hussey *et al.*, (1972) these patterns could be a useful parameter to be used for the taxonomy of the genus *Meloidogyne*. Since then, several studies were conducted with several enzymes and showing that the esterase phenotype of a single or few *Meloidogyne* spp. females is a reliable character for species identification (Esbenshade & Triantaphyllou, 1985, 1990; Fargette, 1987; Pais & Abrantes, 1989). Malate dehydrogenase, superoxide dismutase and glutamate-oxaloacetate enzymes were also often included to confirm species identification. *Meloidogyne incognita* and *M. hapla* can be separated with malate dehydrogenase, due to the difficulty in resolving size variants (Esbenshade & Triantaphyllou, 1985). The rapid and efficient biochemical electrophoretic analysis of non-specific esterases remain the first step in

the RKN species identification process, being very useful in the detection of populations with more than one species that can be easily separated to obtain pure isolates (Carneiro *et al.*, 1996; Carneiro *et al*., 2000, 2004a; Castro *et al*., 2003; Cofcewicz *et al*., 2004; Hernandez *et al*., 2004; Abrantes *et al*., 2008; Brito *et al*., 2008). However, the observation of intra-specific variability, similarity between species and discovery of new esterase patterns make necessary not only the use of more than one enzyme phenotype but also additional information on biology and ecology of the nematode samples to confirm the identification (Esbenshade & Triantaphyllou, 1985; Cenis *et al*., 1992; Blok & Powers, 2009).

With the expansion of DNA-based methodologies, these have been developed and have shown to be useful, not only for nematode identification but also to provide important data on phylogenetic analysis. Methods based on polymerase chain reaction (PCR) are independent of the environmental influence and the nematode's life cycle stage and potentially discriminatory (Zijlstra *et al*., 2000). Random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism variation (RFLP) and sequence characterized amplified regions (SCAR) markers have been developed and different regions of the DNA, including ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA), have been used to identify the RKN isolates (Cenis *et al*., 1992; Powers & Harris, 1993; Zijlstra *et al*., 1995, 2000, 2004; Randig *et al*., 2002; Xu *et al*., 2004; Adam *et al*., 2007).

The PCR-RFLP of mtDNA, namely the region flanked by the *COII* gene and the large (16S) ribosomal gene, has proved extremely useful in studies of characterization and identification of RKN species. This region encompasses partial *COII* and 16S rRNA sequences, the complete tRNA-His sequence, highly conserved in *M. arenaria*, *M. floridensis*, *M. incognita* and *M. javanica*, and an AT-rich non-coding sequence with different size, result of deletions giving rise to unique products of amplification enabling the differentiation of *Meloidogyne* spp. (Powers & Harris, 1993; Hugall *et al*., 1994, 1997; Stanton *et al*., 1997; Jeyaprakash *et al*., 2006). The AT-rich region is completely absent on *M. hapla*, and 167 bp, 573 bp, 603 bp, 963 bp and 1,100 bp products of amplification were found in *M. mayaguensis*, *M. arenaria*, *M. floridensis*, *M. incognita* and *M. javanica*, respectively (Jeyaprakash *et al*., 2006). PCR amplification of the region between *COII* and 16S rRNA genes, and subsequent digestion of the amplified products with restriction endonucleases discriminated five *Meloidogyne* spp. The amplified products of *M. incognita* and *M. javanica* resulted in a fragment of ca.

1,700 bp; *M. arenaria* produced a fragment of ca. 1,100 bp; and *M. chitwoodi* and *M. hapla* a fragment of ca. 520 bp. The restriction patterns produced by enzymes *Hinf*I and *Dra*I discriminated species with similar amplified products (Powers & Harris, 1993). Sequences of this region were also used to distinguish *M. mayaguensis* that produced a unique size product of ca. 705 bp; and *M. floridensis* from *M. arenaria*, *M. mayaguensis*, *M. incognita* and *M. javanica* by the size of amplified PCR products and the restriction pattern produced by enzyme *Ssp*I (Blok *et al*., 2002; Brito *et al*., 2004, Xu *et al*., 2004; Jeyaprakash *et al*., 2006).

This technique has been used widely in phylogenetic analysis, nematode characterization and identification and in various surveys providing not only species discrimination, but also revealing intra-specific variation among isolates (Blok *et al*., 2002; Brito *et al*., 2004; Xu *et al*., 2004; Handoo *et al*., 2005; Powers *et al*., 2005; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006; Skantar *et al*., 2008; Devran *et al*., 2009). For instance, *Meloidogyne arenaria* (ca. 1,300 and 1,700 bp) and *M. incognita* (ca. 1,500 and 1,700 bp) have been reported as having populations that produced two sizes of PCR products (Powers & Harris, 1993; Blok *et al*., 2002; Xu *et al*., 2004; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006).

Life cycle

The interaction between RKN and plants is very complex, because these nematodes manipulate the plant´s gene regulation and metabolism to their own advantage.

The development of the RKN includes four juvenile stages and four moults. The embryonic development results in the first-stage juvenile (J1) which moults in the egg and hatch as a second-stage juvenile (J2), the infective stage. Once hatched, the J2 leave the egg and move through the soil to a new host plant or nearby galled roots. During this period, the J2 depends of their own reserves stored in the intestine and their ability to invade the roots will be reduced after long periods in the soil (Karssen & Moens, 2006).

Infective juveniles enter the plant on the elongation zone and migrate through the intercellular space, separating cells at the middle lamella. During this process, they produce, in the subventral glands, cell wall-degrading enzymes such as β-1,4-endoglucanases (Rosso *et al*., 1999). To surpass the barrier formed by the endodermis,

the J2 migrate towards the root tip and upon reaching the apical meristematic region turn round. Subsequently, they go back up in the vascular cylinder towards the zone of differentiation, where the nematodes induce and maintain a permanent feeding site, in susceptible hosts, that serves as food source for their development and reproduction (Vanholme *et al*., 2004). If feeding site becomes non-functional, the nematodes die or leave the roots, because the access to water and nutrients is limited (Goverse *et al*., 2000). This reaction is characteristic of resistant plants and a localized tissue necrosis or hypersensitive response occurs at or near the anterior end of the nematode (Dropkin *et al.*, 1969). RKN induce the formation of giant cells (2 to 12 cells, each with as many as 100 nuclei) as a result of repeated nuclear divisions, without cytokinesis, and cortical cells proliferation and hypertrophy resulting in formation of typical root galls. These morphological, physiological and molecular changes are induced by nematode secretions produced in the oesophageal gland which are released during feeding, from root cells. Nematodes also release secretions from the oesophageal glands during migration into the root tissue. During the post-embryonic development, the J2 becomes flask-shaped and once inside the root tissue they moult three times into the third (J3) and fourth-stage (J4) and adult. The J3 and J4 do not have stylet and do not feed; it reappears after the fourth moult (Eisenback & Triantaphyllou, 1991; Williamson & Hussey, 1996; Abad *et al*., 2003; Manzanilla-López *et al*., 2004; Karssen & Moens, 2006).

The proportion of males increase under adverse environmental conditions; as reproductive function implies a greater spend of energy, differentiation of females is favoured when food is available (Triantaphyllou & Hirschmann, 1959). Males are vermiform and migrate out of the roots; females are globose and remain sedentary, laying several eggs into a gelatinous matrix on the surface of a galled root or inside the galls.

Most of the *Meloidogyne* spp. reproduces by obligatory mitotic parthenogenesis, including the most widespread species, *M. arenaria*, *M. incognita* and *M. javanica*. A number of species, *M. carolinensis*, *M. kikuyensis*, *M. megatyla*, *M. microtyla*, *M. pini*, *M. spartinae* and *M. subarctica*, reproduce by amphimixis and in this case the presence of males is necessary for fertilization of the females. A third group, that includes *M. chitwoodi*, *M. exigua*, *M. fallax*, *M. graminis* and *M. hapla* reproduce by facultative meiotic parthenogenesis (Chitwoodi & Perry, 2009).

In the presence of *Meloidogyne*, the roots are seriously hampered in their main functions. These nematodes affect water and nutrient absorption and translocation by

the root system, decrease the rate of photosynthesis in leaves and mobilize photosynthates from shoots to roots to support nematode development and reproduction (Hussey, 1985; Carneiro *et al*., 1999). Consequently, the health of the plant species remains compromised and more susceptible to attack by other pathogens. Above-ground symptoms exhibited by infected plants with RKN are unspecific and can be confused with the damage associated with poor nutrition or injury caused by pathogens that attack the root system (bacteria, pathogenic fungi and/or virus) (Hussey, 1985; Whitehead, 1997). These symptoms usually involve stunting, lack of vigour, leaf nutritional deficiencies, such as chlorosis, temporary wilting in periods of water stress and high temperatures, resulting in weak plants depending of the severity of the infestation (Netscher & Sikora, 1990; Williamson & Hussey, 1996; Adab *et al*., 2003).

Management and Control

Once nematodes are established in the soil their eradication is very difficult. The objective of the management strategies is to increase crop yield by reducing the nematode population on soil and, consequently, limiting the damage to a level economically acceptable (Coyne *et al*., 2009).

In the past century, nematicides were used to minimize crop losses caused by RKN. However, the adverse impacts on the environment and human health have reduced their use resulting on the elimination of methyl bromide and others compounds from the market. Nevertheless, nematicides continue to be an alternative for nematode control as part of integrated management programmes, and currently approximately 250,000 t of active compounds are used each year in the world to control nematodes in soil (Haydock *et al*., 2006). Furthermore, for economically important high-value crops, nematicides are the only alternative, often used to prevent infection of established plants. It is a fast way to nematode management though normally implies a re-treatment each year if plants grown are susceptible to RKN (Karsen & Moens, 2006).

The increasing concern of producers and consumers about the risks posed by these chemicals has stimulated research to the development of "natural" nematicides, derived from plant extracts and microorganisms. Non-chemical pest management alternatives are environmentally friendly and cause no risks to humans or animals (Haydock *et al*., 2006).

Root-knot nematodes move only a few meters annually in the soil, but they can be disseminated to different regions through human activities, transport of infected plants and soil adhering to farm implements and in water irrigation. National and international quarantine measures were established to decrease the risk of spread and introduction of a new species into a region where it does not exist (Moens *et al*., 2009). The use and transport of clean, healthy, nematode-free planting material is a prerequisite for limiting spread of nematodes. For example, *M. chitwoodi* and *M. fallax*, two economically important species, due to the high impact on potato, tomato and carrots production and restricted distribution have been included in the list of quarantine species. This status implies an inspection of symptoms of the host plants in the field and in potato tubers or carrots, in order to increase the probability of detection, before they are certificated and authorized to transport (EPPO/OEPP, 2004).

Crop rotation and growing of resistant cultivars are ecologically healthy, effective and widely used strategies for nematode control. In crop rotation fallow periods or non-hosts, resistant or immune plants to RKN species are rotated with susceptible crops, this approach requires the knowledge about the host status of a large number of plants. For some species with a narrow host range is easy but for other important species, with a wide host range, this strategy has its limitation. Also, in soils with more than one *Meloidogyne* species, this approach can lead to a selection and increase the population of particular species as a non-host plant for one RKN species may be a host to another species present in the same field. Nematode management by crop rotation should be performed locally and depend on the nematode species found in the field. Presence of different pests and diseases, soil fertility, presence of weeds, that can be alternative hosts and serve as nematode reservoirs, and the absence/presence of market for the new crop are other parameters to take into account when devising an integrated nematode management strategy (Whitehead, 1997; Halbrendt & La Mondia, 2004). On periods of fallow, the nematode population decrease in the soil due to the lack of susceptible plants and during this period of time there is an increase of the natural flora of non-host plants. Maintaining soil without vegetation is disadvantageous, because it increases the possibility of erosion and loss of soil fertility (Halbrendt & La Mondia, 2004).

The expression of plant resistance is characterized by suppression of nematode development and reproduction, which include programmed cell death and tissue necrosis around the nematode head. In some host plants, as carrot, clover, coffee,

common bean, cotton, cowpea, grape, groundnut, lima bean, lucerne, pepper, potato, *Prunus*, soybean, sugar beet, sweet potato, tobacco, tomato and wheat, multiple resistance genes have been identified but only some are available in cultivated crops. In wild tomato, for example, nine genes that confer resistance to *Meloidogyne* spp. were identified, but only the *Mi-1* gene is available in cultivated tomato (Williamson & Roberts, 2009). Some natural and laboratory-selected *Meloidogyne* isolates, by repeated exposure, can overcome nematode resistance genes and constitute a threat to this strategy of control. Some isolates of *M. arenaria, M. incognita* and *M. javanica*, one isolate of *M. chitwoodi* and species such as *M. enterolobii* (=*M. mayaguensis)*, *M. exigua, M. floridensis* and *M. hapla* can overcome *Mi*-mediated resistance (Roberts *et al*., 1990; Kaloshian *et al*., 1996; Brown *et al*., 1997; Williamson, 1999; Ornat *et al*., 2001; Molinari & Caradonna, 2003; Karajeh *et al*., 2005; Tzortzakakis *et al*., 2005; Brito *et al*., 2007; Silva *et al*., 2008). Incorporation of resistant plants in rotation practices can help to preserve the durability of resistance in the field preventing the selection of virulent nematode populations, reduce the population of RKN and increase the yield of the next crop (Rich & Olson, 2004; Verdejo-Lucas & Sorribas, 2008; Talavera *et al*., 2009).

Combining the use of resistant cultivars and crop rotation can contribute to control RKN and provide a cost-effective and environmentally safe method for managing plant-parasitic nematodes (Roberts, 1992). Others practice more restrictive and specialized have been used: time of planting and harvesting, removal or destruction of infected host plants, flooding, biofumigation, solarization, heat treatment, steaming, use of allelopathic plants that release nematicidal compounds into the rhizosphere, trap crops, green manure and soil amendments and biological control with nematophagous fungi and bacteria. All these practices of nematode management, when available, should be considered as strategies to be use in an integrated management programme (Sasser, 1971; Halbrendt & La Mondia, 2004).

The study organism: Meloidogyne hispanica Hirschmann, 1986

Research on morphological, biochemical and molecular characterization, distribution, life cycle, and host range has been performed for the three tropical species, *M. arenaria, M. incognita* and *M. javanica*, and the temperate species, *M. hapla* and *M. chitwoodi*, but few studies were done with *M. hispanica*, the "Seville root-knot nematode".

A RKN isolate, obtained from peach rootstock, *Prunus persica silvestris* Batsch, Seville, Spain, was included in studies, on the potential application of biochemical polymorphism on taxonomy of *Meloidogyne*, conducted by Dalmasso and Bergé (1978). This isolate had similar enzyme patterns to *M. incognita*, with which it was grouped. Later on, cytological studies were used to clearly differentiate the Seville isolate from *M. incognita*. These studies indicated that the reproduction of this isolate is by mitotic parthenogenesis, the chromosome number is 2n=33-36 and prophase I is not prolonged and the chromosomes do not clump together (Triantaphyllou, 1985). Biochemical investigations revealed that this isolate and other seven from Fiji Island (2), Korea (1), Portugal (1), USA (1) and Australia (2) have a characteristic esterase phenotype (S2-M1=Hi3) (Esbenshade & Triantaphyllou, 1985). Based on these specific characteristics, Hirschmann in 1986 described it as *M. hispanica*. This species is cytologicaly similar to the diploid race of *M. arenaria*, morphologically very close to *M. arenaria*, *M. floridensis* and *M. incognita* and was considered close to *M. incognita* by genomic repetitive DNA analysis (Hirschmann, 1986; Castognone-Sereno *et al*., 1993; Castillo *et al*., 2001; Handoo *et al*., 2004).

Meloidogyne hispanica cannot be differentiated from the most common RKN species even on the basis of the pattern of disease reactions induced in the North Carolina differential hosts. Results of the differential host tests showed that the original population of *M. hispanica* and one isolate from Portugal (F32) gave a response somewhat similar to *M. incognita* race 2 and *M. arenaria* race 1. Only tomato (*Solanum lycopersicum* L.), cv. Rutgers was a good host, tobacco (*Nicotiana tabacum* L.) cv. NC95, pepper (*Capsicum annuum* L.), cv. California Wonder, and water melon (*Citrillus vulgaris* Schrader), cv. Charleston Grey, were slightly infected and therefore rated as poor hosts. Cotton (*Gossypium hirsutum* L.), cv. Deltapine 61, and peanut (*Arachis hypogaea* L.), cv. Florrunner, were non hosts (Hirschmann, 1986; Abrantes *et al*., 2008). The reaction

induced by one isolate from Spain and two from Portugal (F15 and F16) were the same of the *M. arenaria* race 2 or *M. javanica* isolates; whereas isolate F24 gave a response similar to *M. incognita* race 3 (Castillo *et al*., 2001; Abrantes *et al*., 2008).

Meloidogyne hispanica can be regularly confused with other *Meloidogyne* spp. and the differentiation of this species as a distinct species caused same controversy. In order to clarify whether *M. hispanica* was a distinct RKN species, assays with species-specific PCR and phylogenetic analysis of three rDNA regions (ITS1-5.8S-ITS2, 18S and D2-D3 of 28S) demonstrated and supported the differentiation of *M. hispanica* from other RKN species with resemblance in morphology or biological traits. However, *M. hispanica* have an identical ITS sequence to *M. ethiopica*, but these two species were differentiated by their D2-D3 sequences (Landa *et al*., 2008).

Meloidogyne hispanica originally appeared to be restricted to the Southern part of the Iberian Peninsula, associated with *Prunus* crops, causing important losses, but since 1987 several studies have been published on the occurrence of this species (Stalin *et al*., 1998). It was found in Natal, South Africa, parasitizing roots of sugar cane, *Saccharum officinarum* L. (Fargette, 1987) and later, in Pretoria, associated with snapdragon (*Antirrhinum majus* L.), *Amaranthus* sp. and *Pelargonium notatum* (L.) L´Herit, near Worcester, on grape roots (*Vitis vinifera* L.), and at Wilderness on fig-tree (*Ficus carica* L.) (Kleynhans, 1993). In this study, Kleynhans (1993) has also concluded, on the basis of perineal patterns, that the isolate collected, in 1953, from roots of granadilla at Premier Mine, Pretoria, identified as *M. arenaria thamesi* Chitwood, 1952 (Van der Linde, 1956) corresponds to *M. hispanica*.

Meloidogyne hispanica was then found in Australia associated with grapevine (Hugall *et al*., 1994); in Spain on beet (*Beta vulgaris* L.), *Triticum aestivum* L. and grapevines (Karssen & Van Hoenselaar, 1998; Castillo *et al*., 2009); Burkina Faso in tomato, Sahile and Malawi in new cultivated areas (Trudgill *et al*., 2000); Brazil on squash plants (*Cucurbita moshata* Duch ex Lam.) and sugar cane (Carneiro *et al*., 2004b; Chaves *et al.*, 2007); Martinique on banana cv. Cavendish (*Musa* sp.) (Cofcewicz *et al*., 2005); in The Netherlands on cucumber, tomato and sweet pepper organic greenhouse associated with *M. incognita* (van der Wurff *et al*., 2010) and reported in Cape Verde Islands and South France (Fargette, 1987; Karssen, 2004). In Portugal, after the first report of *M. hispanica*, in 1982, originally identified as *M. arenaria* race 2 on the basis of perineal patterns and reactions induced in the North Carolina differential hosts, (Santos & Abrantes, 1982) it was found parasitizing roots of bean (*Phaseolus vulgaris* L.),

carnation (*Dianthus caryophyllus* L.), corn (*Zea mays* L.), fig-trees, and tomato and reported alone or in association with *M. javanica* in potato (*Solanum tuberosum* L.) fields (Santos *et al*., 1992; Abrantes *et al*., 2008; Landa *et al*., 2008; Conceição *et al*., 2009).

The host status of several cultivated plants to *M. hispanica* was evaluated and the following species were recorded as susceptible: bean, corn, cucumber, potato and tomato. Two cultivars of lettuce (*Lactuca sativa* L.) and one of tomato were considered hypersensitive and exhibited galling but did not support reproduction; and one cultivar of pepper was considered as a resistant host (Maleita *et al*., 2005). Also, culivars Felinem, Garnem and Monegro of almond rootstocks were considered resistant hosts and the *Me2* gene from cultivar PM217 of pepper and *Ma* genes from *Prunus cerasifera* L. controlled resistance to *M. hispanica* (Stalin *et al*., 1998, Berthou *et al*., 2003; Finn & Clark, 2008). Five clones of *P. cerasifera*, known as highly resistant to the three most common RKN species, were free from *M. hispanica* galls (Stalin *et al*., 1998).

Objectives

The degree of damage to a particular agricultural area is influenced by the nematode species, level of infestation, crop and abiotic factors that affect the host-parasite system, such as temperature. Thus, in order to reach an informed decision on the development of effective integrated pest management programmes to suppress nematode development and reproduction and prevent their dispersion, an accurate diagnosis and knowledge of the *Meloidogyne* spp. is required.

The general objectives of the present research were to characterize morpho-biometrically, biochemically and molecularly the RKN *M. hispanica* and contribute for the knowledge of its biology and ecology.

The specific objectives were:

• To characterize Portuguese *M. hispanica* isolates by morpho-biometrical, biochemical and molecular characters.

• To evaluate the effects of temperature on the embryonic development, penetration and post-embryonic development of *M. hispanica* in the tomato plants.

• To evaluate the host suitability of *M. hispanica* in cultivated plants.

• To evaluate the ability of a Portuguese isolate of *M. hispanica* to reproduce on *Mi-1.2* tomato genotypes.

• To determine the effects of the inoculum level of *M. hispanica* and *M. javanica* on nematode reproduction and growth of tomato genotypes.

Outline of the thesis

This thesis consists of a general introduction, five chapters, a general discussion and conclusions. Chapter 1 focus on the morpho-biometrical, biochemical and molecular diagnosis of *M. hispanica* isolates and chapters 2 to 5 deal with the biology and ecology of this RKN species.

Chapter 1: Morpho-biometrical, biochemical and molecular diagnosis of Portuguese *Meloidogyne hispanica* isolates

A study was made on the characterization of seven Portuguese *M. hispanica* isolates on the basis of morphological characters, isozyme and protein analysis and PCR-RFLP, including a molecular diagnostic method for the identification and differentiation of *M. hispanica* from other seven *Meloidogyne* spp., by amplification of mtDNA region between *COII* and 16S rRNA gene, with primers C2F3/MRH106. The phylogenetic relationship between *M. hispanica* and other *Meloidogyne* spp., from which sequences of mtDNA were available, is discussed.

Chapter 2: Thermal requirements for the embryonic development and life cycle of *Meloidogyne hispanica*

This chapter provides detailed information on the impacts of temperature on the embryonic development, penetration and post-embryonic development of *M. hispanica* in the tomato plants Easypeel, used to maintain the isolates on the laboratory, and Rossol, with the RKN resistance *Mi-1.2* gene. The value of the base temperature for nematode development and thermal constant values for each temperature analysed were calculated. The results are compared to data published for other *Meloidogyne* spp. and the possible effects of climate change on aggressiveness and the spatial distribution of *M. hispanica* is analysed.

Chapter 3: Reproduction and virulence of *Meloidogyne hispanica* in cultivated plants

The reproduction of *M. hispanica* on 63 plants, comprising 18 plants species of 10 botanical families, was evaluated in pot assays under controlled conditions. Additionally, the effects of temperature (24.4±8.2ºC in the field and 25.0±2.7ºC, 29.3±1.8ºC and 33.6±1.2ºC in a growth chamber) and initial densities (2,500 and 5,000 eggs) on nematode reproduction on three pepper cultivars were determined, and the presence of *Me1*, *Me3* and *N* resistance genes on these pepper cultivars assessed.

Chapter 4: Effect of the *Mi* gene on reproduction of *Meloidogyne hispanica* on tomato genotypes

In this chapter, research was undertaken to screen the RKN resistance *Mi-1.2* gene in 25 genotypes by DNA amplification using REX-1, and Mi23 markers as a SCARs; to evaluate the ability of *M. hispanica* to reproduce on these tomato genotypes, in controlled conditions; and to analyse the influence of the homozygous or heterozygous state at the *Mi* locus on nematode reproduction.

Chapter 5: Effects of inoculum levels of *Meloidogyne hispanica* and *M. javanica* on the nematode reproduction and growth of tomato genotypes

The purpose of this study was to determine the effects of four inoculum levels of *M. hispanica* and *M. javanica* (0, 2,500, 5,000 and 10,000 eggs) on nematode reproduction and growth of the tomato genotypes Easypeel and Moneymaker, which are susceptible to *M. hispanica*, and of Motelle and VFNT-Cherr, which possess the *Mi-1.2* gene, under controlled conditions. Shoot/root length and fresh/dry root and shoot weight were recorded.

1

Morpho-biometrical, biochemical and molecular diagnosis of Portuguese *Meloidogyne hispanica* isolates

1.1 Abstract

Meloidogyne hispanica has been reported associated with economically important crops worldwide. An accurate identification of this pathogen is essential to define efficient and sustainable integrated pest management programmes. Portuguese *M. hispanica* isolates were studied by morpho-biometrical, biochemical and molecular characters. Morpho-biometrical characters of *M. hispanica* females, males and second-stage juveniles were similar to the original description. Biochemical studies revealed a unique enzyme pattern (Hi4) for *M. hispanica* esterases that allowed its differentiation. Molecular analysis of the mtDNA region from *COII* and 16*S* rRNA genes with the described *Hinf*I PCR-RFLP, was unable to discriminate *M. hispanica* from *M. ethiopica* and *M. javanica* with similar amplification products (1,800 bp). Analysis of the mtDNA sequences revealed altered nucleotides among the isolates that created new restriction sites for *Alu*I and *Dra*III. The resulting restriction patterns successfully discriminated the three species providing a new tool for *Meloidogyne* identification. Finally, the phylogenetic relationship between *M. hispanica* and several *Meloidogyne* spp. sequences was analysed using mtDNA, confirming the divergence between meiotic and mitotic species and enlightening the proximity of *M. hispanica* to closely related species. Based on the studies conducted, the application of isozyme or PCR-RFLP analysis would be an efficient methodology to easier *M. hispanica* identification.

Keywords: cytocrome oxidase subunit II, esterase phenotype, mitochondrial DNA, PCR-RFLP, protein profile, root-knot nematode, SDS-PAGE

1.2 INTRODUCTION

The "Seville root-knot nematode", obtained from peach rootstock, *Prunus persica silvestris* Batsch, Spain, was studied for the first time by Dalmasso and Bergé (1978) and described later as *Meloidogyne hispanica* (Hirschmann, 1986). This species has been reported worldwide (Africa, Asia, Australia, Europe, and North, Central, and South America) associated with economically important crops and in South Africa isolates of *M. hispanica* have been identified as *M. arenaria thamesi* (Esbenshade & Triantaphyllou, 1985; Fargette, 1987; Kleynhans, 1993; Trudgill *et al*., 2000; Carneiro *et al*., 2004a; Karssen, 2004; Cofcewicz *et al*., 2005; Abrantes *et al*., 2008; van der Wurff *et al*., 2010).

Meloidogyne hispanica is cytologicaly similar to the diploid race of *M. arenaria*, and morphologically very close to *M. arenaria*, *M. floridensis* and *M. incognita* (Hirschmann, 1986; Castillo *et al*., 2001; Handoo *et al*., 2004). Considering the pattern of the reactions induced in the North Carolina differential host test, the host response to *M. hispanica* isolates is similar to *M. arenaria* race 2 or *M. javanica*, *M. arenaria* race 1 or *M. incognita* race 2, and *M. incognita* race 3, showing an intra-specific variability among the isolates of this species (Hirschmann, 1986; Castillo *et al*., 2001; Abrantes *et al*., 2008). The biochemical electrophoretic analysis of non-specific esterases remain the first stage in species identification (Carneiro *et al*., 2004c; Brito *et al*., 2008; Abrantes *et al*., 2008). The esterase phenotype of *M. hispanica* exhibited some variability in minor and fainter bands. Three phenotypes (Hi2, S2-M1=Hi3 and Hi4) have been detected and all the isolates shared two common major bands that have been used to differentiate this species (Janati *et al*., 1982; Hirschmann, 1986; Abrantes *et al*., 2008; Landa *et al*., 2008).

With the expansion of DNA-based methodologies, new alternatives have been developed and have been shown to be an attractive solution not only for the identification of RKN populations but also to provide important data for phylogenetic analysis. Molecular approaches useful for distinguishing RKN species have included several techniques, such as, RAPD and RFLP, and different regions of the nuclear and mitochondrial DNA have been studied (Powers & Harris, 1993; Zijlstra *et al*., 1995; Stanton *et al*., 1997; Zijlstra, 2000; Zijlstra *et al*., 2000; Blok *et al*., 2002; Randig *et al*., 2002; Wishart *et al*., 2002). However, isolates of *M. hispanica* have been included only in few studies (Cenis *et al*., 1992; Piotte *et al*., 1992; Stanton *et al*., 1997; Landa *et al*., 2008).

In a study based on RFLPs obtained from amplified mtDNA of *Meloidogyne* isolates, digested with *Hinf*I, the patterns of *M. hispanica* consisted in two fragments distinct from those of *M. arenaria*, *M. hapla, M. incognita* and *M. javanica* (Cenis *et al*., 1992). The restriction pattern obtained with the probe, designated as pMiK4, showed two bands common to *M. arenaria, M. hispanica, M. incognita* and *M. javanica* (1.6 and 3 kb). However, all the species could be distinguished by the presence of a characteristic band for *M. arenaria* at 3.5 kb, for *M. incognita* at 2 kb, for *M. hispanica* at 1.5 kb, and another for *M. javanica* at 0.7 kb (Piotte *et al*., 1992). A multiplexed PCR test, based on the amplification of *Meloidogyne* mtDNA with primers MORF, MTHIS, TRNAH and MRH106 and digested with *Hinf*I or *Mnl*I, differentiated *M. hispanica* from *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. javanica*. (Stanton *et al*., 1997). Recently, in order to support the specific identification of *M. hispanica*, species-specific PCR and phylogenetic analyses of sequences from three rDNA regions (18S, ITS1-5.8S-ITS2 and D2-D3 of 28S) were used to characterize three *M. hispanica* isolates from Brazil, Portugal, and Spain (Landa *et al*., 2008).

In this work, seven Portuguese isolates of *M. hispanica* were studied extensively by morpho-biometrical, biochemical and molecular characters. The study also reports a new molecular diagnostic method for the identification and differentiation of *M. hispanica* from other RKN species, based on the mtDNA region between *COII* and 16S rRNA genes, and analysis of the phylogenetic relationship of *M. hispanica* to other *Meloidogyne* spp. from which mtDNA sequences were available.

1.3 MATERIALS AND METHODS

1.3.1 NEMATODE ISOLATES

Seven isolates of *M. hispanica* were used in this study and other eight *Meloidogyne* spp. isolates were included in the biochemical and molecular studies for comparison (Table 1).

Table 1 *Medoidogyne* isolates, hosts, geographic origin and studies where they have been included in this work.

Species (Isolate code)[a]	Host Plant	Geographic origin	Reference	Studies[b]
M. hispanica				
(PtHi1)	*Solanum lycopersicum* L.	Coimbra	—	Mb, B
(PtHi2)	*Ficus carica* L.	Setúbal	Abrantes *et al.*, 2008	Mb, B, M
(PtHi3)	*F. carica* L.	Faro	Abrantes *et al.*, 2008	Mb, B, M
(PtHi4) (selected from PtHi3)	*Capsicum annuum* L.	—	Maleita *et al.*, unpublished	Mb, B, M
(PtHi5)	*Dianthus caryophyllus* L.	Aveiro	Landa *et al.*, 2008	Mb, B, M
(PtHi6)	*S. tuberosum* L.	Santarém	Conceição *et al.*, 2009	Mb, B, M
(PtHi7)	*S. tuberosum* L.	Aveiro	Conceição *et al.*, 2009	Mb, B, M
M. arenaria (PtA)	*Oxalis corniculata* L.	Coimbra	—	B, M
M. chitwoodi (PtCh)	*S. tuberosum* L.	Porto	Conceição *et al.*, 2009	B, M
M. hapla (PtH)	*Alnus glutinosa* (L.) Gaertn.	Viana do Castelo	—	B, M
M. incognita (PtI)	*Cucumis melo* L.	Açores	—	B, M
M. javanica (PtJ)	*S. tuberosum* L.	Guarda	—	B, M
M. mayaguensis (VnM)	*Malpighia glabra* L.	Cabudare	—	B, M
M. megadora (STMe)	*Coffee arabica* L.	S. Tomé	Abrantes *et al.*, 1995 Almeida *et al.*, 1997; 2002	B, M
M. ethiopica (ItE)	*S. lycopersicum* L.	Pontecagnano	—	B, M

[a] Pt- Portugal; Vn- Venezuela; ST- Democratic Republic of S. Tomé and Príncipe; It- Italy.
[b] B = Biochemical studies; M = PCR-RFLP analysis; Mb = morpho-biometrial studies.

All the isolates used in this study were originally obtained from a single egg mass and maintained on tomato, *Solanum lycopersicum* L., cv. Easypeel, by periodic sub-culturing in a growth chamber at 25±2ºC, in the Nematology laboratory (IMAR-CMA, Department of Life Sciences, University of Coimbra).

1.3.2 MORPHO-BIOMETRICAL STUDIES

Morphological and morphometrical studies were conducted on second-stage juveniles (J2), males and females of each of the seven isolates of *M. hispanica* (Table 1). Males were recovered from infected roots, J2 hatched from egg masses in moist chambers and females handpicked from infected tomato roots. Freshly hatched J2, males, females and perineal patterns were prepared for light microscope (LM) studies as previously described in Abrantes and Santos (1991) and measured immediately. Photographs were taken with a Leitz Dialux 20 bright field light microscope.

Freshly hatched J2, males and females were processed for scanning electron microscope (SEM) studies as described earlier (Abrantes & Santos, 1991). Perineal patterns of females and stylets of J2, males and females were also prepared as described in Abrantes and Santos (1989) and Eisenback (1985). The specimens were mounted on stubs, coated with gold (200Å), viewed and photographed with a JEOL JSM-35C. At least 50 specimens of each life stage, perineal patterns, and excised stylets of J2, males and females were examined.

1.3.3 BIOCHEMICAL STUDIES

ISOENZIME ANALYSIS: Young egg-laying females (5/tube or 10/tube for PtCh), handpicked from infected tomato roots, of each *Meloidogyne* species and isolates (Table 1) were transferred to micro-hematocrit tubes with 5 µl of extraction buffer (20% w/v sucrose and 1% v/v Triton X-100). The specimens were macerated, with a pestle, frozen and stored at -20ºC. Electrophoresis was performed according to Pais *et al*. (1986) in vertical gels in a Mini-Protean Tetra System (Bio-Rad Laboratories, Hercules, California, USA). The gels were stained for esterase activity with the substrate a-naphthyl acetate. Protein extracts of females of *M. javanica* isolate were included in each gel as a reference. Phenotypes were designated with a letter(s) suggesting the nematode species, followed by a number indicating the number of bands (Esbenshade & Triantaphyllou, 1985).

PROTEIN PATTERNS: Females from each isolate (Table 1) were dissected from infected roots, transferred to Eppendorf tubes and stored at -20ºC. Before extraction, the nematode pellet was maintained at least 1 h at -80ºC and homogenized in phosphate

buffered saline (PBS - 0.145 M NaCl; 0.0025 M $NaH_2PO_4.2H_2O$ and 0.0075 M $Na_2HPO_4.12H_2O$, pH 7.0) with a pestle, on an ice bath. Then, the homogenates were centrifuged for 4 min at 3,000 rpm and the supernatant recovered to a new Eppendorf tube. The pellet was ressuspended and homogenized again in a solution of 1% v/v Triton X-100 and Tris 0.125M (pH 7.5) and the supernatant transferred to the Eppendorf tube containing the first supernatant (Fioretti *et al*., 2001). Protein concentration was measured with the Bio-Rad Protein Assay Kit according to Bradford (1976) (Bio-Rad Laboratories, Hercules, California, USA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% acrylamide pH 8.8 separating gel and 3.6% acrylamide pH 6.8 stacking gel following the methodology described by Laemmli (1970). Eight micrograms of each female protein extract were mixed with equal volume of sample buffer (0.125 M Tris base, pH 6.8; 4% w/v SDS; 10% v/v glycerol; 0.002% w/v bromophemol blue and 4% v/v β-mercaptoethanol) and boiled for 5 min before the protein extracts were applied on the wells of the gel. Five microliters of SDS-PAGE molecular weight marker were added to each gel (SDS-PAGE Molecular Weight Standards, High Range, Bio-Rad Laboratories, Hercules, California, USA). Separation of proteins was performed with the Mini-Protean Tetra System (Bio-Rad Laboratories, Hercules, California, USA) in a running buffer (0.12 M Tris base; 0.96 M Glycine and 0.017 M SDS) with 15 mA/gel until the bromophenol blue dye reached the separating gel, followed by 20 mA/gel. Slab gels were stained overnight with 0.5 g Comassie Brilliant Blue R-250, 7.5% glacial acetic acid and 45% methanol and destaining with a solution of 7.5% glacial acetic acid and 45% methanol.

1.3.4 MOLECULAR STUDIES

DNA EXTRACTION: Freshly hatched J2 from each isolate (Table 1), obtained from egg masses, were concentrated by centrifugation for 2 min at 3,000 rpm and stored at -20ºC in Eppendorf tubes. Packed juveniles were placed at -80ºC for at least 1 h before genomic DNA extraction and purification according to Randig *et al*. (2001) and Curran *et al*. (1986) with modifications. The juveniles, mechanically squashed with a pestle, were homogenized and 91.7 µl of lysis buffer (0.1 M Tris-HCl, pH 8.0; 50 mM EDTA; 1% w/v SDS; and 0.17 M NaCl) and 8.3 µl of proteinase K (6 µg/µl) were added. After the

incubation of the homogenates at 60ºC for 1 h and 10 min at 95ºC, 1 µl of a solution of 10 mg/ml ribonuclease A (Sigma, St. Louis, Missouri, USA) was added and the tubes were incubated at 37ºC for 15 min. DNA was purified with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) for 1 min, mixed by gentle inversion of the Eppendorf tube, and centrifuged at 15,000 rpm for 1 min at 4ºC. The supernatant was transferred to a new Eppendorf tube, 2.5 volumes of absolute ethanol were added to precipitate the DNA and the mixture was centrifuged at 15,000 rpm for 20 min at 4ºC. The precipitate was washed with 500 µl of ice-cold 70% ethanol, and dried at 45ºC for ±15 minutes. The DNA was ressuspended in 20 µl of sterilised distilled water.

SEQUENCING: Mitochondrial DNA from isolates PtHi3 of *M. hispanica* and ItE of *M. ethiopica* were sequenced with the primer set C2F3 (5'-GGT CAA TGT TCA GAA ATT TGT GG-3') and MRH106 (5'-AAT TTC TAA AGA CTT TTC TTA GT-3') located in the *COII* gene and the 16S rRNA gene (Powers & Harris, 1993; Stanton *et al*., 1997). PCR was performed in a 50 µl volume containing 1x buffer, 2 mM MgSO4, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5 U Platinum Taq DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, California, USA) and 100 ng of nematode DNA. Amplifications were carried using the following conditions: an initial denaturation at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 45 s and extension at 68ºC for 2 min, and a final extension for 10 min at 68ºC. The amplified products were purified with the High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA), according to the manufacturer's instructions. Amplified DNA was sequenced in both directions with the same amplification primers and a new intermediate primer due to the high length of the fragment (5´-GAT CGG GGT TTA ATA ATG GG-3´), by standard procedures at Macrogen, Inc. (Seoul, Korea). Chromatograms were checked and corrected manually by use of the free-available on-line Chromas software (Technelysium Pty Ltd, Brisbane, Australia).

Sequences from the PtHi3 and ItE isolates were aligned with ClustalW (Thompson *et al*., 1994) within BioEdit software (Hall, 1999) with *Meloidogyne* spp. mtDNA sequences between *COII* and the 16S rRNA genes available in GenBank nucleotide database (National Center of Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov). The sequences of PtHi3 and ItE isolates were compared with sequences of *M. ethiopica*,

M. incognita and *M. javanica*, because they showed similar products of amplification. Afterwards, based on altered nucleotides between the isolates that created new restriction sites, their restriction enzyme maps were deduced, using the free-available on-line WebCutter 2.0 software. Enzymes were selected to have digestion products with varied sizes within *Meloidogyne* spp. to allow easier species identification upon visualization by agarose gel electrophoresis.
Additionally, all sequences of *M. chitwoodi* and *M. hapla* available on GenBank nucleotide database were analysed as described above to detect nucleotide variability and find a specific restriction endonuclease able to differentiate these two species.

PCR-RFLP: PCR amplification was conducted with the primer set C2F3 and MRH106 already described (Powers & Harris, 1993; Stanton *et al*., 1997). PCR reactions were performed in 25 µl volume containing 1x buffer, 1.8 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5 U Taq DNA polymerase (Bioline, London, UK) and 50 ng of nematode DNA. The amplifications were carried out in a MyCycle Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) using the following conditions: an initial denaturation at 94ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 45 s and extension at 72ºC for 2 min, and a final extension for 10 min at 72ºC. Five microliters of the PCR reaction were analysed on 1% agarose gel electrophoresis in 1x TAE buffer stained with ethidium bromide.
Products of 650 bp were purified using QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) and digested with 5.0 U of *Bfa*I (New England BioLabs Inc., Ipswich, Massachusetts, USA). Amplification products with ca. 1,800 bp were digested with 5.0 U of *Hinf*I (Amersham Biosciences, Barcelona, Spain). If no digestion occurred with *Hinf*I the amplified products were digested separately with 5 U of *Alu*I (USBiological, Swampscott, Massachusetts, USA) and *Dra*III (New England BioLabs Inc., Ipswich, Massachusetts, USA). All restriction enzymes were applied in 2/3 µl of PCR products according to the manufacturer's instructions. The digestion was conducted at 37ºC for 5 h to *Dra*III and *Bfa*I and for 8 h to *Hinf*I and *Alu*I restriction enzymes. Restriction fragments were separated on 2% agarose gel electrophoresis in 1x TAE buffer stained with ethidium bromide.

1.3.5 PHYLOGENETIC ANALYSIS

The sequences of *Meloidogyne* spp. from different locations included on phylogenetic analysis were obtained by searching the GenBank nucleotide database (Table 2). Sequences from the PtHi3 and ItE isolates were aligned using Muscle (Edgar, 2004) with all sequences or only those of related species with similar product of amplification for the mtDNA region studied (*M. arabicida*, *M. ethiopica*, *M. incognita* and *M. javanica*). Alignment was manually adjusted when necessary. The length of all sequences of *Meloidogyne* spp. was set to 1,673 bp by removing several nucleotides to obtain a common start and end point. Length of sequences used in analysis was enhanced at the region coloured in grey on annex. The phylogenetic relationship was reconstructed using Neigborg-Joining (NJ) (Jukes & Cantor, 1969) and Maximum Likelihood (ML) (Saitou & Nei, 1987) methods. Neigborg-Joining analyses were performed using the Maximum Composite Likelihood model (Tamura *et al*., 2004) with complete deletion. One thousand bootstrap replicates were performed to test the support of each node on the trees (Felsenstein, 1985). Maximum Likelihood analyses were based on the Jukes-Cantor model (Jukes & Cantor, 1969). All positions containing gaps and missing data were eliminated. Alignment and evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Table 2 GenBank accession numbers for the mtDNA region sequences of *Meloidogyne* spp. isolates used for alignment and phylogenetic analysis.

Species	GenBank accession no.	Species	GenBank accession no.
Meloidogyne arabicida	AY942852	*M. haplanaria*	AY757905
M. arenaria	AY635610	*M. incognita*	AY635611
	FJ159615		FJ159614
M. chitwoodi	AY757876		FJ159616
	AY757882	*M. javanica*	AY635612
M. ethiopica	AY942848		FJ159612
M. fallax	AY757883	*M. mayaguensis*	AJ421396
M. floridensis	AY635609		AY635613
M. graminicola	AY757884		AY831967
	GQ865513		AY446975
M. graminis	AY757886	*M. morocciensis*	AY942849
	HM161680	*M. paranaensis*	AY942851
M. hapla	AY539839	*M. partityla*	AY672413
	AY757889		AY757909
	AY757891	*M. thailandica*	EU364883
	AY757904		
	AY942850		

1.4 RESULTS

1.4.1 MORPHO-BIOMETRICAL STUDIES

Morphometrics of females, males and J2 of the seven Portuguese *M. hispanica* isolates are reported in Tables 3-5. Females of *M. hispanica* showed a globose to ovoid shape with a distinguished neck (neck length, 128.33-303.33 µm) and without tail protuberance. The body was rounded posteriorly (Fig. 1A) and the cephalic region was slightly prominent, not annulated and very small when compared with body size (Fig. 1A,B). The stylet length ranged from 11.58 to 18.95 µm with the cone curved dorsally, widening gradually posteriorly, the shaft straight and the knobs with indented anterior margins (Fig. 1C,D). The distance between the stylet base and the dorsal esophageal gland orifice (DGO) was short to long (2.8-6.84 µm) (Fig. 1B-D). Excretory pore position was variable, 13.16 to 77.89 µm from anterior end, sometimes very close to the anterior extremity and on some specimens close to anterior end of metacarpus. Perineal patterns varied from ovoid to rounded (Fig. 1E-G). Dorsal arch was generally low; however some patterns show a higher and quadrangular arch. Dorsal striae were relatively uniform, smooth and straight, sometimes wavy. Ventral pattern area generally shows fine and smooth striae. Lateral lines were distinct; some dorsal and ventral striae connect with each other to originate an angle, while others forked. Perivulval region was not striated. Phasmidial ducts were distinct (Fig. 1E,F).

Males with the length ranging from 1,400.0 to 2,357.14 µm, with the anterior end slightly tapered and the posterior rounded (Fig. 1H-J,L). The labial disc was large, elongated, raised and fused with the medial lips that were narrow, crescent shaped and with smooth outer margins. The head region was smooth (Fig. 1I,J). Stylet was robust and large with a straight cone, pointed and widen gradually to posterior end (Fig. 1K). Excretory pore position was variable (147.5 to 240.0 µm from anterior end). Spicules were long (28.0-41.05 µm) and slight curved and a gubernaculum crescent shape. Tail was short and round with phasmids generally very close to the level of cloaca (Fig. 1L). Lateral field with four longitudinal incisures.

Body length of J2 varied from 320.0 to 514.44 µm with a truncate head region and a narrow tail region (Fig. 1M,Q). Labial disc raised and fused with the medial lips that were crescent shaped. Head region usually smooth occasionally with 1-3 incomplete

annulations (Fig. 1N). Stylet length from 9.21 to 13.0 µm with the cone tip slender, widen gradually to posterior end, the shaft cylindrical, widen gradually to posterior end, and the knobs rounded to ovoid and separated, sloping posteriorly (Fig. 1M,O). Tail with rounded tip and a hialine tail terminus indistinct. The rectal dilation was large (Fig. 1P,Q).

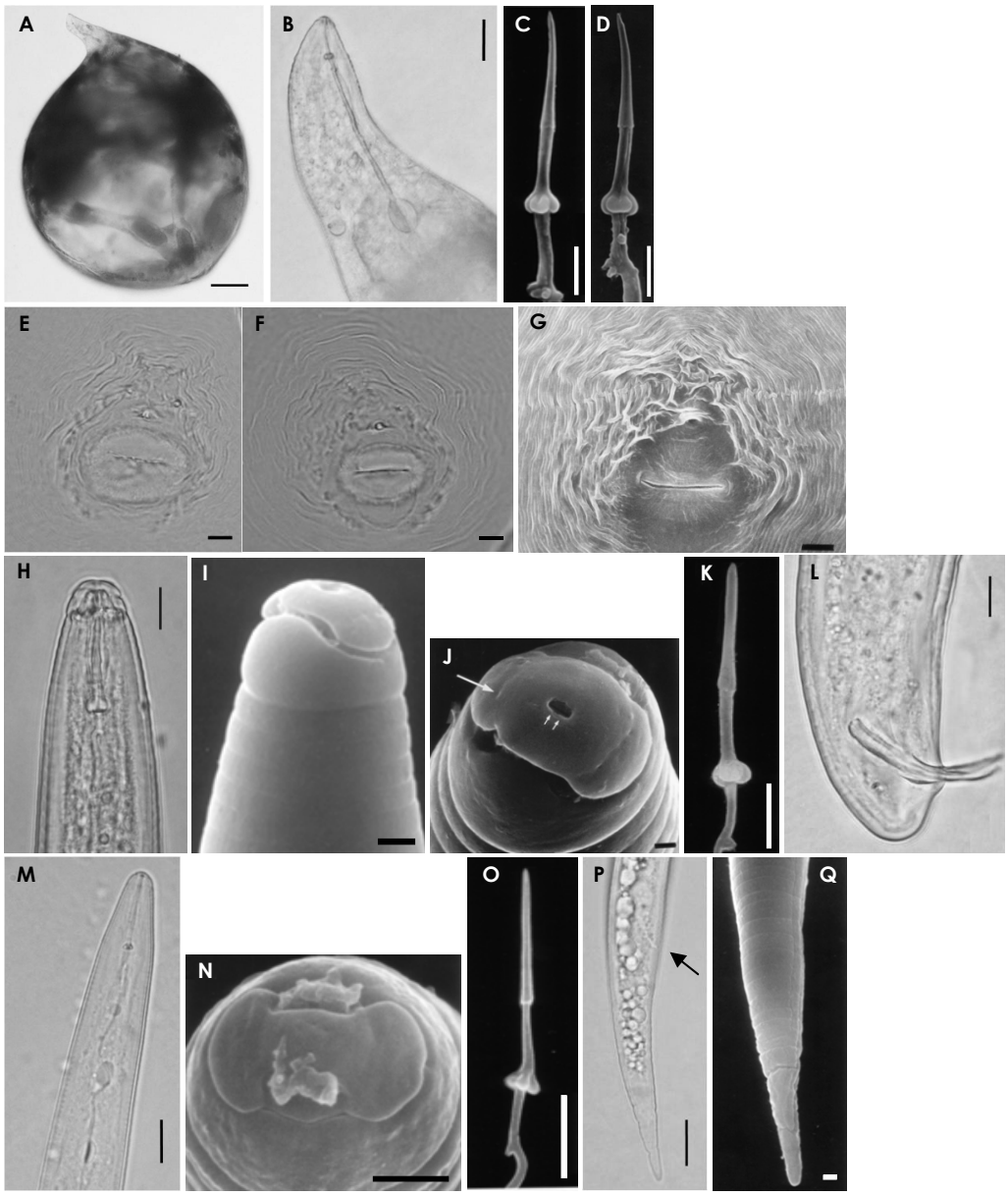

Figure 1 Light (A,B,E,F,H,L,M,P) and scanning electron microscope photographs (C,D,G,I,J,K,N,O,Q) of *Meloidogyne hispanica*. Females (A-G). Whole specimen (A); anterior end and esophageal region (B); excised stylets (C,D); and perineal patterns (E-G). Males (H-L). Anterior region in lateral view (H,I); head region showing cephalic sensilla (arrow) and inner labial sensilla (double arrow) (J); excised stylet (K); and posterior region in lateral view (L). Second-stage juveniles (M-Q). Anterior region (M); head region (N); excised stylet (O); and tail region (P,Q) with anus and inflated rectum (arrow) (P). Scale bars- 100 µm (A), 20 µm (B), 10 µm (E,F,G,H,L,M,P), 5 µm (C,D,K,O), 2 µm (I) and 1 µm (J,N,Q).

Table 3 Morphometrics of females of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PtHi1 (n=30)	PtHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Linear (µm)							
Body length (L)	752.8±15.69 (580.0-920.0)	662.08±61.71 (570.00-770.00)	761.00±52.81 (628.33-841.67)	693.83±69.71 (573.33-833.33)	670.58±58.30 (600.00-780.00)	773.17±65.63 (666.67-900.00)	734.75±72.87 (590.00-893.33)
Body width	483.3±13.19 (370.0-660.0)	473.08±45.88 (388.33-541.67)	545.00±48.20 (436.67-625.00)	481.58±57.87 (365.00-566.67)	505.75±59.39 (446.67-640.00)	552.75±47.52 (465.00-633.33)	495.17±51.08 (405.00-593.33)
Neck length	207.5±5.35 (160.0-270.0)	189.12±32.51 (135.00-261.67)	190.48±28.75 (150.00-263.33)	182.25±39.84 (128.33-280.00)	171.05±20.20 (131.67-203.33)	212.58±40.10 (160.00-295.00)	208.67±38.20 (138.33-303.33)
Neck width	134.2±3.32 (105.0-180.0)	126.67±15.49 (103.33-160.00)	167.00±26.42 (113.33-221.67)	165.67±17.32 (140.00-200.00)	147.17±14.24 (116.67-171.67)	148.75±25.99 (113.33-206.67)	132.67±18.59 (93.33-160.00)
Stylet length	14.3±0.10 (13.0-15.0)	15.49±1.78 (12.11-18.95)	14.75±0.95 (12.63-16.32)	13.97±1.17 (11.58-16.58)	15.12±0.58 (14.21-16.32)	14.41±0.92 (12.11-15.79)	14.87±1.82 (11.05-17.37)
Stylet knob height	2.4±0.05 (2.0-2.8)	2.51±0.23 (2.11-2.89)	2.60±0.24 (2.21-2.89)	2.43±0.19 (2.11-2.63)	2.72±0.23 (2.37-3.16)	2.53±0.26 (2.11-3.16)	2.43±0.38 (1.84-3.68)
Stylet knob width	4.3±0.06 (4.0-5.0)	4.13±0.17 (3.68-4.32)	4.13±0.30 (3.16-4.47)	4.38±0.42 (3.68-5.00)	4.46±0.21 (4.21-4.74)	4.15±0.24 (3.68-4.47)	4.21±0.17 (3.68-4.47)
DGO from base of stylet	4.1±0.10 (2.8-4.5)	5.31±0.77 (4.21-6.84)	5.11±0.77 (3.95-6.58)	4.08±0.75 (3.16-6.32)	4.99±0.81 (3.68-6.84)	5.49±0.64 (4.21-6.32)	5.00±0.75 (3.42-6.58)
L from anterior end to posterior end of metacorpus	101.4±1.27 (91.3-114.5)	103.53±7.48 (89.47-118.68)	101.73±7.97 (90.53-122.11)	88.95±4.48 (82.11-98.42)	99.86±8.63 (87.37-124.74)	105.08±6.98 (93.42-117.89)	105.59±7.12 (94.74-124.74)
Metacorpus length	41.8±0.75 (35.0-50.0)	45.10±3.58 (34.74-52.00)	45.71±1.98 (42.11-49.47)	40.70±2.91 (35.26-45.79)	44.24±2.67 (38.42-50.00)	45.03±3.99 (36.84-52.11)	45.36±4.84 (29.47-52.11)
Metacorpus width	39.4±0.84 (28.7-48.0)	42.76±2.88 (37.89-48.16)	44.69±1.46 (41.84-47.37)	41.16±2.41 (35.26-45.26)	42.62±2.67 (35.00-45.79)	42.93±3.72 (36.05-50.79)	44.03±4.48 (31.58-51.58)
Metacorpus "valve" length	16.6±0.19 (15.0-18.0)	15.49±0.85 (14.47-17.37)	15.61±1.05 (12.63-17.11)	14.58±1.29 (12.63-17.63)	16.45±1.14 (14.47-19.74)	17.05±1.26 (15.00-20.00)	17.72±1.54 (15.26-20.53)
Metacorpus "valve" width	11.7±0.10 (11.0-13.0)	11.29±0.64 (10.26-12.37)	10.92±0.68 (8.95-11.84)	10.28±0.75 (8.95-11.58)	11.00±0.72 (9.74-12.89)	12.01±0.70 (10.79-13.68)	11.16±0.66 (9.74-12.63)
Excretory pore from anterior end	34.5±1.25 (20.0-47.0)	31.18±7.78 (18.16-51.32)	39.61±11.24 (20.53-62.37)	33.05±6.97 (18.95-45.79)	32.30±9.95 (20.00-60.79)	30.46±8.86 (13.16-44.74)	45.96±11.87 (25.79-77.89)
Vulval slit length	23.9±0.39 (20.5-27.5)	27.32±2.70 (20.0-34.29)	25.18±2.45 (19.29-28.57)	25.04±2.28 (20.00-28.57)	24.93±3.19 (14.29-28.57)	29.32±2.85 (22.14-34.29)	25.57±2.89 (19.29-31.43)
Vulva-anus distance	19.5±0.32 (16.5-23.5)	20.89±1.70 (17.14-24.29)	21.00±2.04 (15.71-24.29)	20.96±2.34 (17.14-25.71)	20.64±2.65 (12.86-25.71)	19.71±2.40 (12.14-23.57)	21.07±2.47 (16.43-25.00)
Interphasmidial distance	23.5±0.52 (17.0-28.0)	25.61±4.14 (17.86-31.43)	23.21±2.69 (18.57-27.14)	24.61±5.23 (16.43-35.71)	22.43±3.24 (15.71-27.14)	26.50±2.58 (21.43-30.71)	24.86±2.68 (17.86-31.43)

* Values are mean±standard deviation (range).

Table 3 (CONTINUED). Morphometrics of females of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PtHi1 (n=30)	PtHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Ratio							
a = L/body width	1.6±0.03 (1.3-2.0)	1.41±0.12 (1.24-1.64)	1.40±0.11 (1.22-1.67)	1.45±0.14 (1.24-1.84)	1.33±0.09 (1.19-1.58)	1.40±0.12 (1.22-1.59)	1.49±0.11 (1.30-1.72)
L/L from anterior end to posterior end of metacorpus	7.5±0.17 (5.9-9.2)	6.41±0.57 (5.39-7.69)	7.52±0.73 (6.11-8.61)	7.90±0.87 (6.55-9.99)	6.77±0.87 (4.86-8.20)	7.39±0.84 (5.89-9.22)	7.01±0.99 (5.29-9.43)
Stylet knob width/height	1.9±0.04 (1.6-2.4)	1.66±0.19 (1.33-2.00)	1.61±0.23 (1.09-2.02)	1.81±0.20 (1.40-2.25)	1.65±0.14 (1.42-1.89)	1.66±0.21 (1.30-2.00)	1.77±0.26 (1.14-2.29)
Metacorpus length/width	1.1±0.02 (0.9-1.2)	1.06±0.09 (0.91-1.22)	1.02±0.04 (0.93-1.13)	0.99±0.09 (0.80-1.17)	1.04±0.08 (0.91-1.20)	1.05±0.08 (0.90-1.20)	1.04±0.12 (0.72-1.35)
Metacorpus "valve" length/width	1.4±0.01 (1.3-1.5)	1.38±0.11 (1.23-1.60)	1.43±0.12 (1.17-1.71)	1.42±0.13 (1.14-1.68)	1.50±0.14 (1.24-1.79)	1.43±0.15 (1.14-1.81)	1.59±0.19 (1.32-2.11)
Excretory pore from anterior end/stylet length	2.4±0.08 (1.5-3.1)	2.02±0.48 (1.23-3.25)	2.70±0.77 (1.37-4.11)	2.39±0.57 (1.38-3.17)	2.14±0.69 (1.29-4.28)	2.13±0.66 (0.83-3.24)	3.14±0.88 (1.51-4.77)

* Values are mean±standard deviation (range).

Table 4 Morphometrics of males of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PtHi1 (n=30)	PtHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Linear (µm)							
Body length (L)	1,7740.3±29.11 (1,400.0-2,020.0)	1,856.79±163.42 (1,600.00-2,200.00)	1,913.57±163.54 (1,628.57-2,271.43)	1,790.36±132.02 (1,564.29-2,028.57)	1,793.50±155.02 (1,521.43-2,007.14)	1,951.79±131.32 (1,750.00-2,214.29)	1,930.00±167.36 (1,714.29-2,357.14)
Greatest body width	39.2±0.79 (32.5-48.5)	39.94±3.59 (34.29-46.43)	44.61±6.13 (37.14-58.57)	41.11±2.74 (36.43-47.14)	41.82±4.51 (33.57-47.86)	41.86±4.43 (35.71-53.57)	43.75±5.51 (37.14-55.00)
Body width at stylet knobs	20.5±0.18 (18.5-22.0)	21.68±0.81 (20.00-23.57)	22.52±1.28 (20.00-25.71)	21.64±1.90 (14.29-23.57)	21.79±1.00 (20.00-23.57)	21.50±0.65 (20.00-22.86)	22.64±1.31 (21.43-25.71)
Body width at excretory pore	33.3±0.52 (28.5-40.0)	33.93±3.09 (31.43-41.43)	36.86±3.76 (32.14-48.57)	35.04±2.00 (31.43-39.29)	34.96±2.51 (30.00-39.29)	34.50±1.97 (31.43-37.86)	36.29±3.08 (32.86-44.29)
Head region height	6.1±0.07 (5.5-6.5)	4.61±0.75 (3. 16-5.53)	4.72±1.25 (3.16-8.42)	4.43±1.50 (2.37-9.21)	4.11±0.83 (3.16-5.79)	5.58±0.79 (4.21-6.58)	5.50±1.08 (3.16-6.84)
Head region width	12.3±0.10 (11.0-13.5)	12.83±0.42 (12.11-13.68)	12.89±0.59 (11.58-14.21)	12.16±0.35 (11.58-12.63)	12.33±0.47 (11.58-13.42)	12.89±0.40 (12.11-13.68)	13.32±0.53 (12.37-14.47)
Stylet length	23.7±0.14 (22.0-24.5)	22.80±0.57 (21.58-23.68)	22.89±0.77 (21.05-23.68)	21.99±0.98 (20.00-24.74)	22.54-0.68 (21.05-23.95)	23.63±0.52 (22.89-24.74)	22.93±1.03 (20.00-24.47)
Stylet cone	13.1±0.13 (12.0-14.5)	13.42±0.62 (12.11-15.00)	13.43±1.04 (11.05-15.00)	12.62±1.05 (10.53-15.79)	13.39±0.78 (12.37-15.79)	13.88±0.57 (12.89-15.00)	13.68±0.63 (12.37-15.26)
Stylet shaft and knobs	10.6±0.10 (10.0-11.5)	9.38±0.48 (7.89-10.00)	9.54±0.66 (8.16-10.53)	9.55±0.91 (8.42-12.89)	9.13±0.60 (7.37-10.00)	9.96±1.03 (8.68-13.68)	9.42±0.47 (8.42-10.00)
Stylet knob height	3.2±0.05 (2.5-3.5)	2.97±0.23 (2.37-3.16)	3.04±0.25 (2.63-3.42)	3.08±0.26 (2.37-3.42)	3.22±0.34 (2.63-3.68)	3.47±0.36 (2.63-3.95)	3.30±0.23 (2.89-3.95)
Stylet knob width	5.2±0.04 (5.5-5.6)	4.66±0.26 (4.21-5.26)	5.25±0.30 (4.74-5.79)	5.33±0.25 (5.00-5.79)	4.95±0.20 (4.47-5.26)	5.05±0.35 (4.47-5.79)	5.07±0.34 (4.47-5.79)
DGO from base of stylet	3.6±0.08 (3.0-4.0)	3.41±0.47 (2.63-4.74)	2.92±0.37 (2.37-3.95)	2.82±0.82 (2.11-5.79)	3.87±0.55 (2.89-5.26)	3.46±0.54 (2.63-4.74)	3.12±0.62 (2.37-4.47)
Head end to metacorpus "valve"	93.3±0.98 (86.5-109.0)	91.97±4.38 (81.43-101.43)	90.6±2.45 (85.71-96.43)	90.00±3.33 (85.00-98.57)	91.75±4.61 (84.29-100.00)	96.89±4.96 (92.14-115.71)	90.82±7.31 (80.71-111.43)
Metacorpus width	11.1±0.19 (9.5-13.5)	11.50±0.79 (10.53-12.89)	12.05±1.39 (9.47-15.53)	11.71±1.35 (9.21-15.26)	10.63±1.17 (8.42-12.11)	11.12±0.93 (8.68-12.37)	12.47±0.91 (10.26-13.95)
Metacorpus "valve" length	6.3±0.07 (6.0-7.0)	6.13-0±75 (4.74-7.89)	6.05±0.53 (5.26-7.11)	6.05±0.55 (4.74-6.84)	6.08±0.77 (4.21-7.89)	6.26±0.68 (5.00-7.37)	6.62±0.65 (5.79-8.42)
Metacorpus "valve" width	4.1±0.07 (3.5-5.0)	4.18±0.39 (3.16-5.00)	4.59±0.70 (3.42-6.05)	4.14±0.61 (2.63-5.53)	4.33±0.61 (2.89-5.53)	3.97±0.49 (3.16-4.74)	4.11±0.59 (2.63-5.00)

* Values are mean±standard deviation (range).

Table 4 (CONTINUED). Morphometrics of males of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PtHi1 (n=30)	PtHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Linear (µm)							
Excretory pore to head end	181.7±3.55 (147.5-228.0)	182.43±21.89 (157.86-240.00)	181.46±19.15 (150.71-227.14)	175.04±12.79 (156.43-208.57)	192.52±11.42 (170.71-215.71)	198.43±11.05 (182.86-237.14)	187.71±17.44 (160.00-237.14)
Testis length	855.2±21.91 (650.0-1.130.0)	882.14±61.47 (757.14-1,000.00)	1,049.86±159.49 (757.14-1,357.14)	835.0±148.97 (514.29-1,085.71)	888.93±181.04 (542.86-1,121.43)	1,148.93±124.27 (942.86-1,321.43)	1,065.71±109.50 (928.57-1,357.14)
Tail length	12.8±0.28 (10.5-16.2)	10.67±1.60 (7.37-14.21)	10.36±1.67 (8.42-16.58)	9.50±1.76 (5.79-13.16)	10.14±1.17 (6.84-11.58)	11.26±1.56 (7.89-14.74)	9.91±1.84 (6.05-13.16)
Spicule length	34.6±0.43 (28.0-37.5)	34.26±1.90 (31.05-39.21)	35.32±2.36 (30.53-40.00)	34.26±2.29 (30.79-41.05)	34.33±2.04 (29.74-38.68)	34.87±1.46 (31.84-37.89)	35.16±1.84 (32.37-37.63)
Gubernaculum length	8.4±0.15 (7.0-9.5)	8.28±0.73 (7.37-10.00)	8.22±1.16 (6.05-11.84)	7.80±0.52 (6.84-8.68)	7.91±0.74 (6.32-9.21)	7.95±1.01 (5.79-10.00)	8.24±0.65 (7.11-9.21)
Phasmids to tail end	13.8±0.35 (11.5-19.5)	11.68±2.22 (8.68-16.32)	12.42±2.53 (7.89-17.11)	11.46±3.46 (7.11-20.79)	14.03±2.80 (8.95-20.53)	14.49±2.94 (7.37-18.95)	13.63±3.24 (8.42-19.47)
Ratio							
a = L/body width	44.8±0.82 (37.6-54.6)	46.65±4.00 (40.95-53.75)	43.47±5.68 (36.48-55.37)	43.66±3.41 (38.60-49.63)	43.12±3.68 (36.94-50.00)	46.87±3.11 (40.13-54.00)	44.47±4.20 (36.49-51.58)
L/head end to metacorpus "valve"	18.7±0.32 (15.9-23.4)	20.24±2.15 (17.58-24.91)	20.84±2.02 (16.24-24.65)	19.92±1.62 (16.18-22.28)	19.56±1.61 (16.47-22.42)	20.17±1.39 (18.39-23.48)	21.30±1.68 (18.51-24.82)
c = L/tail length	138.3±2.84 (104.7-167.8)	176.88±24.47 (127.41-228.78)	188.11±27.41 (118.91-227.01)	195.18±45.05 (138.43-350.39)	178.90±25.24 (149.29-256.81)	176.79±29.50 (128.93-240.67)	201.80±43.57 (154.02-297.39)
Head region width/height	2.0±0.02 (1.8-2.2)	2.87±0.53 (2.30-4.00)	2.87±0.60 (1.69-3.83)	3.00±0.87 (1.31-5.11)	3.11±0.58 (2.25-4.08)	2.36±0.35 (2.00-3.06)	2.52±0.56 (1.92-4.00)
Stylet length/body width at stylet knobs	1.2±0.009 (1.1-1.2)	1.05±0.04 (0.96-1.14)	1.02±0.07 (0.85-1.11)	1.03±0.12 (0.93-1.51)	1.04±0.04 (0.99-1.12)	1.10±0.04 (1.01-1.15)	1.02±0.08 (0.80-1.11)
Stylet knob width/height	1.7±0.02 (1.4-2.0)	1.58±0.15 (1.33-1.89)	1.74±0.14 (1.54-2.10)	1.74±0.17 (1.46-2.22)	1.55±0.16 (1.29-1.90)	1.47±0.19 (1.20-2.00)	1.54±0.13 (1.27-1.83)
Metacorpus "valve" width/height	1.5±0.02 (1.4-1.7)	0.69±0.09 (0.52-0.86)	0.76±0.12 (0.56-1.00)	0.69±0.09 (0.45-0.88)	0.72±0.12 (0.46-1.00)	0.64±0.07 (0.50-0.74)	0.65±0.15 (0.36-1.07)
Percentages							
Excretory pore = (excretory pore to head end/L)*100	10.5±0.16 (9.1-12.5)	9.86±1.13 (8.50-13.13)	9.52±1.04 (7.06-11.78)	9.84±1.16 (8.17-13.27)	10.79±0.82 (9.21-12.43)	10.20±0.78 (8.60-12.07)	9.74±0.66 (8.66-11.07)
T = (testis length/L)*100	49.2±1.05 (36.1-61.9)	47.73±3.90 (40.53-53.88)	54.82±6.45 (43.48-64.44)	44.94±10.41 (28.33-69.09)	49.47±8.95 (31.40-61.26)	58.93±5.89 (47.83-69.70)	55.26±3.72 (49.26-64.60)

* Values are mean±standard deviation (range).

Table 5 Morphometrics of second-stage juveniles of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PfHi1 (n=30)	PfHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Linear (µm)							
Body length (L)	403.7±4.84 (360.0-455.0)	412.83±14.87 (365.56-432.22)	397.67±11.53 (368.89-417.78)	410.78±25.21 (381.11-466.67)	443.78±32.98 (401.11-514.44)	438.72±32.39 (320.00-484.44)	428.39±14.38 (408.89-475.56)
Greatest body width	14.9±0.06 (14.5-15.5)	14.11±0.89 (13.33-15.56)	14.67±1.23 (13.33-17.78)	14.83±1.54 (13.33-17.78)	15.44±1.61 (13.33-17.78)	14.56±0.95 (13.33-16.67)	13.78±0.66 (13.33-15.56)
Body width at stylet knobs	9.8±0.08 (9.0-10.5)	9.22±0.23 (8.95-9.74)	9.46±0.23 (8.95-10.00)	9.17±0.34 (8.42-9.74)	8.93±0.23 (8.68-9.47)	9.21±0.30 (8.68-9.74)	9.14±0.25 (8.68-9.47)
Head region height	1.9±0.04 (1.5-2.0)	1.86±0.23 (1.47-2.37)	1.85±0.26 (1.47-2.37)	1.59±0.29 (1.05-2.11)	1.93±0.18 (1.47-2.11)	1.92±0.23 (1.58-2.37)	1.97±0.22 (1.58-2.37)
Head region width	5.3±0.05 (5.0-5.5)	5.17±0.25 (4.74-5.53)	5.07±0.34 (4.47-6.05)	4.92±0.21 (4.74-5.26)	5.03±0.19 (4.74-5.26)	5.09±0.25 (4.47-5.53)	5.00±0.21 (4.74-5.26)
Stylet length	11.8±0.07 (11.0-13.0)	10.25±0.42 (9.21-11.05)	10.46±0.34 (10.00-11.05)	10.08±0.35 (9.47-10.79)	9.99±0.37 (9.21-10.53)	10.32±0.56 (9.21-11.58)	10.53±0.48 (9.74-11.32)
Stylet shaft and knobs	5.1±0.05 (4.5-5.5)	4.91±0.32 (4.21-5.53)	4.78±0.35 (3.95-5.26)	4.80±0.46 (3.68-5.53)	4.62±0.36 (3.68-5.26)	5.05±0.49 (3.95-6.32)	4.70±0.36 (3.68-5.26)
Stylet knob height	1.2±0.03 (1.0-1.5)	1.22±0.15 (1.05-1.58)	1.25±0.12 (1.05-1.32)	1.24±0.15 (1.05-1.58)	1.24±0.12 (1.05-1.32)	1.29±0.17 (1.05-1.58)	1.21±0.16 (1.05-1.58)
Stylet knob width	2.2±0.03 (2.0-2.5)	2.08±0.19 (1.58-2.37)	2.14±0.13 (1.84-2.37)	1.99±0.13 (1.84-2.11)	2.13±0.22 (1.58-2.63)	2.18±0.30 (1.58-2.89)	2.01±0.15 (1.58-2.11)
Stylet base to head end	14.7±0.09 (13.0-15.5)	14.97±0.39 (14.21-15.53)	14.39±0.91 (10.95-15.26)	13.92±0.90 (10.53-14.74)	14.22±0.26 (13.95-14.74)	15.03±0.39 (13.95-15.53)	14.70±0.58 (12.63-15.53)
DGO from base of stylet	3.3±0.05 (2.8-3.5)	3.83±0.54 (2.89-5.00)	3.32±0.32 (2.63-3.95)	3.14±0.46 (2.37-4.21)	3.70±0.30 (3.16-4.21)	3.75±0.32 (3.16-4.47)	4.17±0.36 (3.68-4.74)
Metacorpus "valve" length	4.4±0.05 (4.0-5.5)	4.37±0.32 (3.95-5.26)	4.14±0.28 (3.68-4.74)	4.04±0.26 (3.68-4.74)	4.29±0.37 (3.42-5.00)	4.38±0.29 (3.95-5.26)	4.41±0.28 (3.68-4.74)
Metacorpus "valve" width	3.4±0.04 (3.0-4.0)	3.50±0.23 (3.16-3.95)	3.43±0.31 (2.89-4.21)	3.28±0.20 (2.89-3.68)	3.46±0.26 (2.89-3.95)	3.70±0.20 (3.42-3.95)	3.59±0.29 (3.16-4.47)
Head end to metacorpus "valve"	53.0±0.45 (47.0-57.0)	52.68±1.88 (48.68-55.26)	48.99±1.94 (45.00-53.95)	46.53±3.75 (35.79-52.89)	51.82±2.26 (47.37-55.79)	53.92±2.13 (51.05-58.95)	52.96±1.72 (50.00-55.53)
Body width at excretory pore	14.1±0.09 (13.5-15.0)	13.37±0.63 (12.63-15.26)	13.30±0.67 (11.05-14.47)	12.95±0.41 (12.11-13.68)	13.39±0.50 (12.63-14.74)	13.46±0.54 (12.63-14.74)	13.36±0.35 (12.89-13.95)
Excretory pore to head end	80.0±0.78 (71.5-86.0)	81.05±2.47 (75.26-86.84)	76.20±2.23 (72.63-80.26)	76.78±4.54 (61.58-83.16)	80.30±2.44 (76.32-84.47)	82.55±2.44 (76.84-86.32)	81.68±1.87 (78.42-84.74)
Body width at anus	11.0±0.09 (10.5-12.0)	10.17±0.38 (9.47-11.05)	10.49±0.35 (10.00-11.32)	10.20±0.52 (9.21-11.05)	10.74±0.43 (10.00-11.58)	10.24±0.49 (9.21-11.05)	10.21±0.50 (9.21-11.32)
Tail length	50.2±0.34 (47.0-52.5)	48.12±2.18 (44.21-51.58)	45.34±2.53 (42.11-51.32)	43.74±5.11 (33.16-51.05)	46.95±2.02 (43.68-51.05)	51.13±3.15 (44.74-56.84)	50.53±1.51 (47.37-54.21)

* Values are mean±standard deviation (range).

Table 5 (CONTINUED). Morphometrics of second-stage juveniles of PtHi1, PtHi2, PtHi3, PtHi4, PtHi5, PfHi6 and PtHi7 Portuguese *Meloidogyne hispanica* isolates.*

Character	PfHi1 (n=30)	PfHi2 (n=20)	PtHi3 (n=20)	PtHi4 (n=20)	PtHi5 (n=20)	PtHi6 (n=20)	PtHi7 (n=20)
Ratio							
a = L/body width	27.2±0.38 (23.2-30.3)	29.34±1.69 (26.57-32.42)	27.28±2.21 (23.50-31.00)	27.88±2.31 (22.44-31.00)	28.84±1.45 (26.13-31.25)	30.22±2.64 (24.00-35.25)	31.16±1.85 (28.14-35.67)
L/head end to metacorpus "valve"	7.6±0.06 (7.1-8.2)	7.84±0.35 (7.02-8.40)	8.13±0.31 (7.62-8.99)	8.87±0.77 (7.99-10.87)	8.58±0.79 (7.55-10.19)	8.16±0.73 (5.43-8.81)	8.10±0.43 (7.46-8.90)
c = L/tail length	8.1±0.09 (7.3-8.9)	8.59±0.38 (7.72-9.42)	8.79±0.40 (8.07-9.55)	9.55±1.57 (7.90-12.83)	9.45±0.60 (8.47-10.38)	8.62±0.86 (5.82-9.84)	8.48±0.32 (8.01-9.51)
d = Tail length/body width at anus	4.6±0.06 (3.9-5.0)	4.74±0.26 (4.29-5.16)	4.32±0.21 (4.00-4.67)	4.30±0.54 (3.31-5.17)	4.38±0.24 (3.91-4.87)	5.00±0.33 (4.36-5.63)	4.96±0.26 (4.47-5.54)
Head region width/height	2.9±0.07 (2.5-3.7)	2.81±0.34 (2.22-3.57)	2.79±0.43 (2.22-3.83)	3.19±0.60 (2.25-4.75)	2.76±0.27 (2.38-3.39)	2.68±0.31 (2.22-3.33)	2.57±0.32 (2.00-3.33)
Stylet knob width/height	1.9±0.05 (1.5-2.5)	1.73±0.28 (1.20-2.25)	1.73±0.19 (1.60-2.25)	1.63±0.21 (1.33-2.00)	1.74±0.24 (1.40-2.25)	1.72±0.35 (1.20-2.75)	1.68±0.19 (1.33-2.00)
Metacorpus "valve" width/height	1.3±0.01 (1.1-1.4)	0.80±0.06 (0.65-0.94)	0.83±0.08 (0.67-0.94)	0.81±0.05 (0.73-0.93)	0.81±0.07 (0.72-0.94)	0.85±0.05 (0.75-0.94)	0.82±0.10 (0.72-1.21)
Percentages							
Excretory pore = (excretory pore to head end/L)*100	19.9±0.15 (18.3-21.5)	19.65±0.82 (18.71-21.88)	19.17±0.50 (18.27-19.87)	18.74±1.38 (15.44-20.62)	18.17±1.14 (16.32-19.70)	18.72±2.05 (14.04-25.82)	19.08±0.57 (17.82-19.83)

* Values are mean±standard deviation (range).

1.4.2 BIOCHEMICAL STUDIES

The three esterase bands observed in the *M. javanica* isolate (Rm: 0.37; 0.43; 0.46), were used as a reference phenotype which allowed the determination of the relative position of the bands observed in the *Meloidogyne* isolates (J3, Figs. 2 and 3).

In the *M. hispanica* isolates studied, four bands of esterase activity (two major bands and two minor and fainter bands) were detected (Rm: 0.32; 0.35; 0.38; 0.41) corresponding to the phenotype Hi4 (Fig. 2; PtHi1- data not show). The identification of the other eight *Meloidogyne* spp. isolates used for comparison in the protein and molecular studies was confirmed by the esterase phenotypes (Fig. 3, Table 1). In these isolates, 19 bands of esterase activity were observed, comprising eight phenotypes on the basis of single bands or combinations. All of them contained distinct and species-specific phenotypes. The esterase phenotype of *M. hispanica* was clearly distinct from the other *Meloidogyne* spp. used in this study (Figs. 2 and 3).

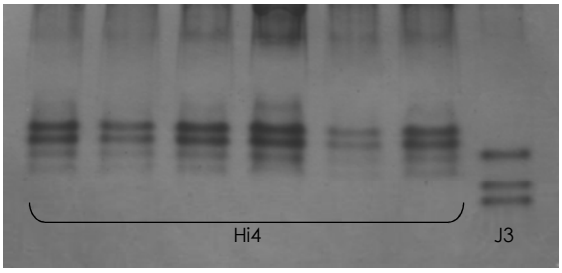

Figure 2 Esterase phenotype (Hi4) of protein homogenates from five egg-laying females of the six Portuguese *Meloidogyne hispanica* isolates (PtHi2, PtHi3, PtHi4, PtHi5, PtHi6 and PtHi7). *M. javanica* (J3) = reference isolate.

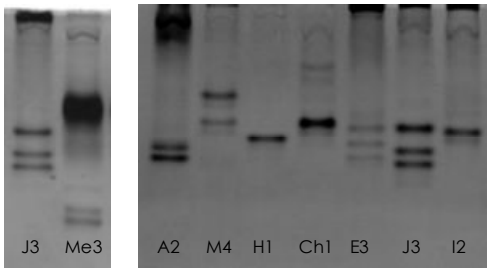

Figure 3 Esterase phenotypes of protein homogenates from five egg-laying females of *Meloidogyne* spp. isolates included in this study. J3- *M. javanica* (reference isolate); Me3- *M. megadora*; A2- *M. arenaria*; M4- *M. mayaguensis*; H1- *M. hapla*; Ch1- *M. chitwoodi* (10 egg-laying females); E3- *M. ethiopica*; I2- *M. incognita*.

The SDS-PAGE technique gave reproducible protein patterns for the homogenates of females of the six *M. hispanica* isolates (PtHi2, PtHi3, PtHi4, PtHi5, PtHi6 and PtHi7) and for all the other *Meloidogyne* spp. isolates (PtA, PtCh, PtH, PtI, PtJ, VnM, STMe and ItE) (Fig. 4). Numerous bands were observed which make difficult to use for diagnostic purposes when compared to esterase phenotypes, therefore only the main bands were considered to characterize and discriminate the isolates. Some of the weakly stained proteins were variable among electrophoretic runs but the protein profiles of *M. hispanica* isolates were similar among the six isolates (211.96, 153.09, 109.73, 63.01 and 53.71 kDa). *Meloidogyne chitwoodi* (PtCh) and *M. megadora* (STMe) were the only two species that showed distinct protein profiles with each other and to all other *Meloidogyne* spp. studied. The protein profiles of these two species were characterized for the presence of 211.96, 144.0 and 116.25 kDa and 156.23, 135.48 and 114.61 kDa proteins, respectively (Fig. 4).

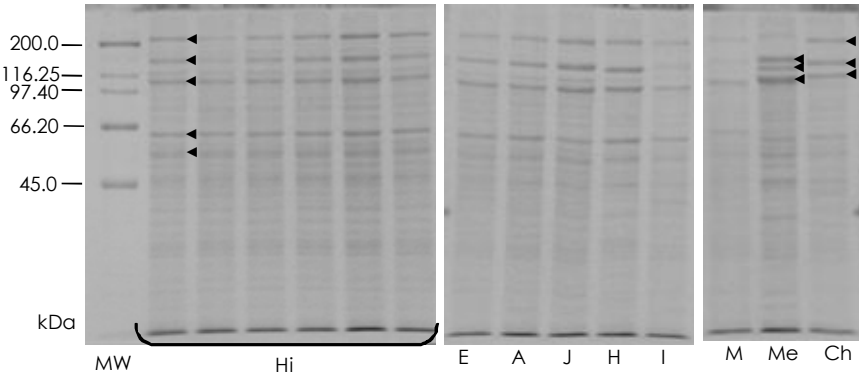

Figure 4 Protein patterns from *Meloidogyne* spp. homogenates of adult females separated by SDS-PAGE (10% polyacrylamide gel). MW- molecular weight markers; Hi- *M. hispanica*; E- *M. ethiopica*; A- *M. arenaria*; J- *M. javanica*; H- *M. hapla*; I- *M. incognita*; M- *M. mayaguensis*; Me- *M. megadora;* Ch- *M. chitwoodi.*

1.4.3 MOLECULAR STUDIES

The region of the mtDNA *COII* and 16S rRNA genes was selected to carry out the molecular characterization of *M. hispanica* isolates. This region, amplified with the primer set C2F3/MRH106 from purified DNA extracted from J2 from each of the *Meloidogyne* isolates, yield single fragments of four different sizes (ca. 650, 850, 1,300 and 1,800 bp) (Fig. 5). Specifically, the isolates of *M. hapla* and *M. chitwoodi* (with esterase phenotype H1 and Ch1), had a PCR product of ca. 650 bp; the *M. mayaguensis* isolate (M4) produced a fragment of 850 bp; the *M. arenaria* isolate (A2) a fragment of 1,300 bp; the isolates of *M. hispanica* (Hi4), *M. ethiopica* (E3), *M. javanica* (J3) and *M. incognita* (I2) gave a fragment of ca. 1,800 bp (Figs. 2, 3 and 5). No amplification occurred with the *M. megadora* isolate (Me3).

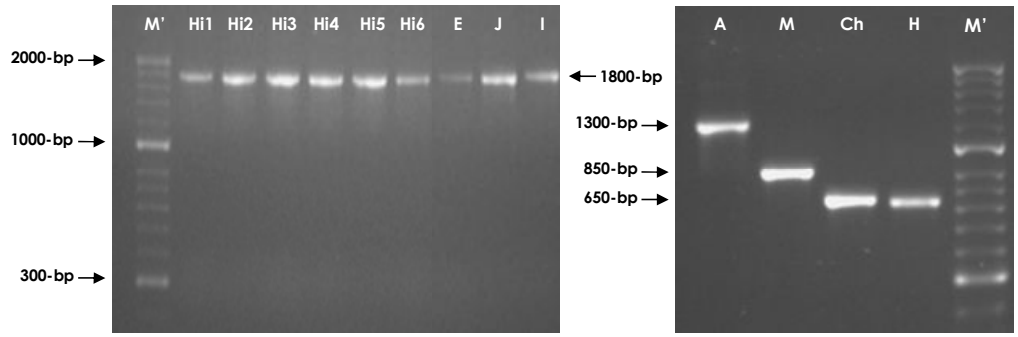

Figure 5 DNA amplification products obtained from 13 isolates of eight *Meloidogyne* spp. using C2F3 and MRH106 primers. Hi1-Hi6- *M. hispanica*; E- *M. ethiopica*; J- *M. javanica*; I- *M. incognita*; A- *M. arenaria*; M- *M. mayaguensis*; Ch- *M. chitwoodi*; H- *M. hapla*; M'- DNA marker (HyperLadder II, Bioline).

When the amplified products of ca. 1,800 bp were digested with the restriction enzyme *Hinf*I, three patterns of digestion were observed, but only clearly differentiated *M. incognita*. Two fragments of ca. 1,300 and 400 bp were produced for this isolate; two fragments of ca. 1,700 and 100 bp were generated in *M. hispanica* isolates and *M. ethiopica* whereas no digestion occurred in *M. javanica* isolate (Fig. 6). Molecular analysis of the mtDNA region, with the described *Hinf*I PCR-RFLP, was unable to discriminate *M. hispanica* from *M. ethiopica* and *M. javanica*. In order to obtain useful information for species discrimination the PCR products of PtHi3 (*M. hispanica*) and ItE (*M. ethiopica*) isolates for mtDNA region were sequenced and compared to equivalent

sequences of *M. ethiopica* and *M. javanica* available on GenBank. Analysis of the nucleotide variation observed in the alignment and the production of the restriction enzyme maps in WebCutter revealed that altered nucleotides between the isolates created new restriction sites for the enzymes *Alu*I and *Dra*III. Digestion of the ca. 1,800 bp mtDNA PCR product with *Alu*I generated three fragments of ca. 1,000, 580 and 240 bp for *M. hispanica* (six isolates, data shown for only one isolate) and *M. javanica*, while two fragments of ca. 1,240 and 580 bp were observed for *M. ethiopica* (Fig. 7A). On the other hand *Dra*III could not digest *M. hispanica* but generated two fragments of ca. 1,000 and 800 bp for *M. ethiopica* and *M. javanica* (Fig. 7B).

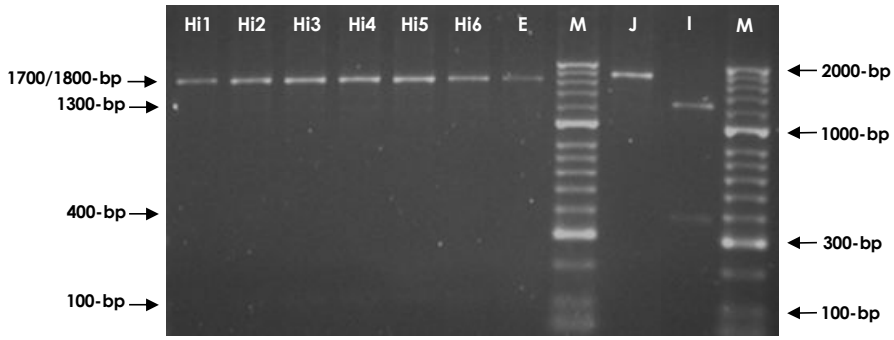

Figure 6 *Hinf*I digestion patterns of the ca. 1,800 bp amplification products from *Meloidogyne* spp. after 8 h of digestion. Hi1-Hi6- *M. hispanica*; E- *M. ethiopica*; J- *M. javanica*; I- *M. incognita*; M- DNA marker (HyperLadder II, Bioline).

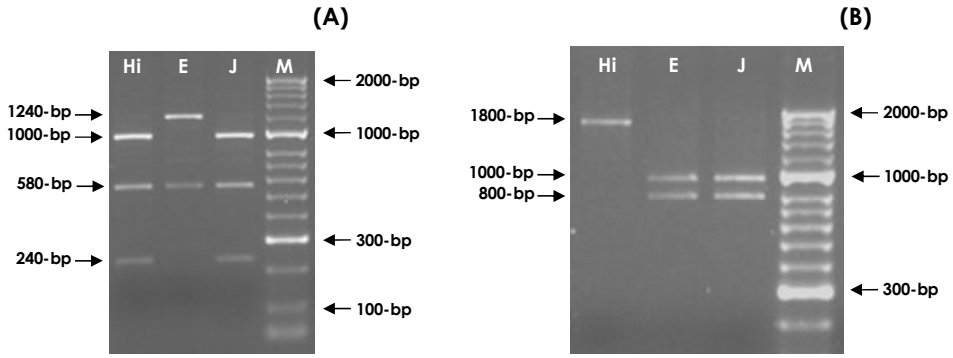

Figure 7 *Alu*I (A) and *Dra*III (B) digestion patterns of the ca. 1,800 bp amplification products from *Meloidogyne* spp. after 8 and 5 hours of digestion, respectively. Hi- *M. hispanica* (similar for all *M. hispanica* isolates); E- *M. ethiopica*; J- *M. javanica*; M- DNA marker (HyperLadder II, Bioline).

Comparison of the *M. chitwoodi* and *M. hapla* mtDNA sequences available on GenBank and analysis of respective restriction enzyme maps also revealed altered nucleotides creating new restriction sites for the *Bfa*I enzyme, which allowed the differentiation of these two species. Restriction digestion of the amplified product of ca. 650 bp with *Bfa*I generated two fragments of ca. 550 and 130 bp in *M. hapla*, whereas no digestion occurred with *M. chitwoodi* (Fig. 8), as expected. *Meloidogyne chitwoodi* identification was further confirmed using the JMV1 (5´-GGA TGG CGT GCT TTC AAC-3´) and JMV2 (5´-TTT CCC CTT ATG ATG TTT ACC C-3´) primers for the intergenic spacer region between the 5S and 18S genes, according to Wishart *et al*. (2002). Amplification of this region yielded the expected fragment of approximately 550 bp. (Fig. 9).

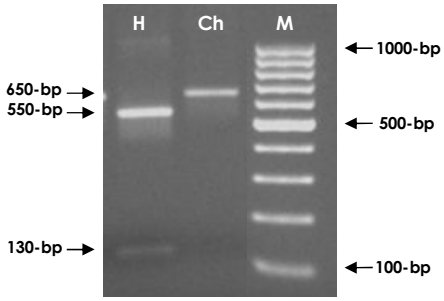

Figure 8 *Bfa*I digestion patterns of the ca. 650 bp amplification products from *M. hapla* (H) and *M. chitwoodi* (Ch) after 5 h of digestion. M- DNA marker (GeneRuler 100 bp, Fermentas).

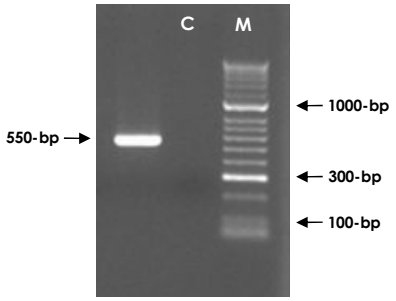

Figure 9 DNA amplification product obtained from *M. chitwoodi* isolate (PtCh) using JMV1 and JMV2 primers. C- negative control; M- DNA marker (HyperLadder II, Bioline).

1.4.4 PHYLOGENETIC ANALYSIS

Mitochondrial DNA sequences, which includes part of the *COII*, a variable intergenic region, tRNAHis and part of the 16*S* rRNA gene, of PtHi3 (*M. hispanica*) and ItE (*M. ethiopica*) isolates were determined and although the sequences were not complete, the sequence size of the PtHi3 isolate was 1,682 bp and 1,684 bp in the ItE isolate. The sequences were compared to the corresponding fragments from closely related species which displayed similar amplification products (*M. arabicida*, *M. ethiopica*, *M. incognita* and *M. javanica*) (annex). The PtHi3 sequence differed by 22

nucleotides positions from *M. ethiopica* (ItE and Me, AY942848), *M. incognita* from Thailand (FY159614) and *M. javanica* (AY635612). The mtDNA sequences of *M. ethiopica* isolates from Italy (ItE) and Brazil (Me) were similar with only seven differences in alignment. The differences included nucleotide changes at positions 781, 1,055, 1,148 and 1,624; two insertions at 987 and 1,340; and finally one deletion at position 58 (annex). Amplified product of *M. arabicida* was slightly longer than those of other species, showing several changes as two insertions between 937 to 951 and 1,068 to 1,128 positions and one deletion between 864 to 892 (annex).

Meloidogyne hispanica displayed sequence divergences ranging from 0.5 to 1.5%, when compared with the other species and *M. ethiopica* (ItE) from 0.1 to 1.5% (Table 10).

Table 10 Pair wise sequence divergences between *Meloidogyne hispanica* (PtHi3), *M. arabicida* (Mar, AY942852), *M. ethiopica* (ItE and Me- AY942848), *M. incognita* (Mi- AY635611; Mi1- FY159614; Mi2- FJ159616) and *M. javanica* (Mj- AY635612; Mj1- FJ159612) sequences of mtDNA using MEGA5. Analyses were conducted using the Maximum Composite Likelihood model. All positions containing gaps and missing data were eliminated.

Species	PtHi3	ItE	Mar	Me	Mi	Mi1	Mi2	Mj	Mj1
PtHi3									
ItE	0.007								
Mar	0.015	0.015							
Me	0.005	0.001	0.013						
Mi	0.008	0.007	0.016	0.006					
Mi1	0.006	0.006	0.014	0.005	0.003				
Mi2	0.006	0.006	0.014	0.005	0.003	0.001			
Mj	0.006	0.005	0.014	0.003	0.007	0.005	0.005		
Mj1	0.005	0.004	0.013	0.003	0.007	0.005	0.005	0.000	

Both NJ and ML trees obtained were similar therefore only the NJ tree is exhibited with bootstrap values (Figs. 10 and 11). The five *Meloidogyne* spp. with similar amplification products clustered separately. *Meloidogyne ethiopica* and *M. javanica* were sister taxa to *M. hispanica* (PtHi3), but with lower bootstrap support (50%) (Fig. 10). The isolate ItE formed a clade with *M. ethiopica* with 94% bootstrap. *Meloidogyne incognita* and *M. arabicida* were the most divergent species (Fig. 10 and Table 10).

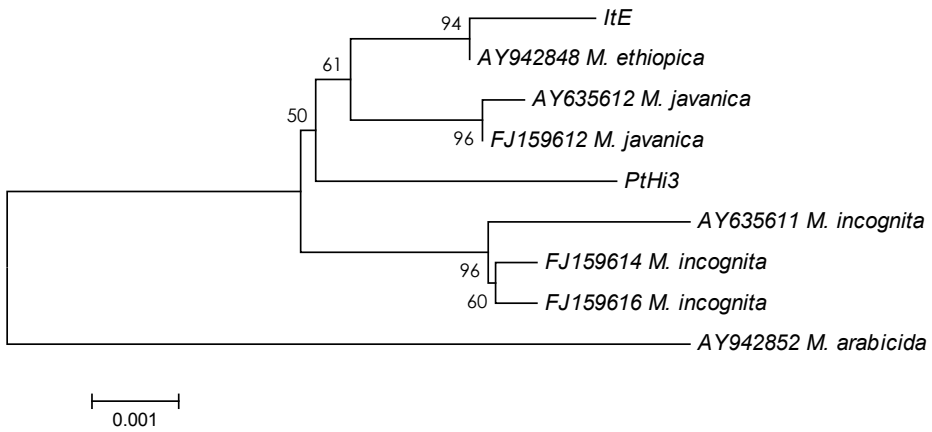

Figure 10 Neighbor-joining tree based on mtDNA sequences of *Meloidogyne* spp. with approximate amplification product sizes to M. *hispanica* (PtHi3) and *M. ethiopica* (Ite). The percentage of replicate trees in which the associated *Meloidogyne* spp. clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and all positions containing gaps and missing data were eliminated.

The tree obtained from NJ analysis of mtDNA sequences of several *Meloidogyne* spp. available on GenBank showed that all *Meloidogyne* species formed two well supported clades (72 and 95%) with the exclusion of *M. graminis* (Fig. 11). First, *M. chitwoodi* and *M. fallax* were clustered together with high bootstrap support (100%) and as a sister taxon to *M. graminicola* (95%). Second, *M. hispanica* formed a clade well supported (99%) with *M. thailandica*, *M. arenaria*, *M. morocciensis*, *M. javanica*, *M. floridensis*, *M. incognita*, *M. ethiopica*, *M. paranaensis* and *M. arabicida*, but the relationship within this clade was poor. This clade was a sister to *M. haplanaria* and *M. mayaguensis*. Another clade was formed by *M. partityla* and *M. hapla* (90%) (Fig. 11).

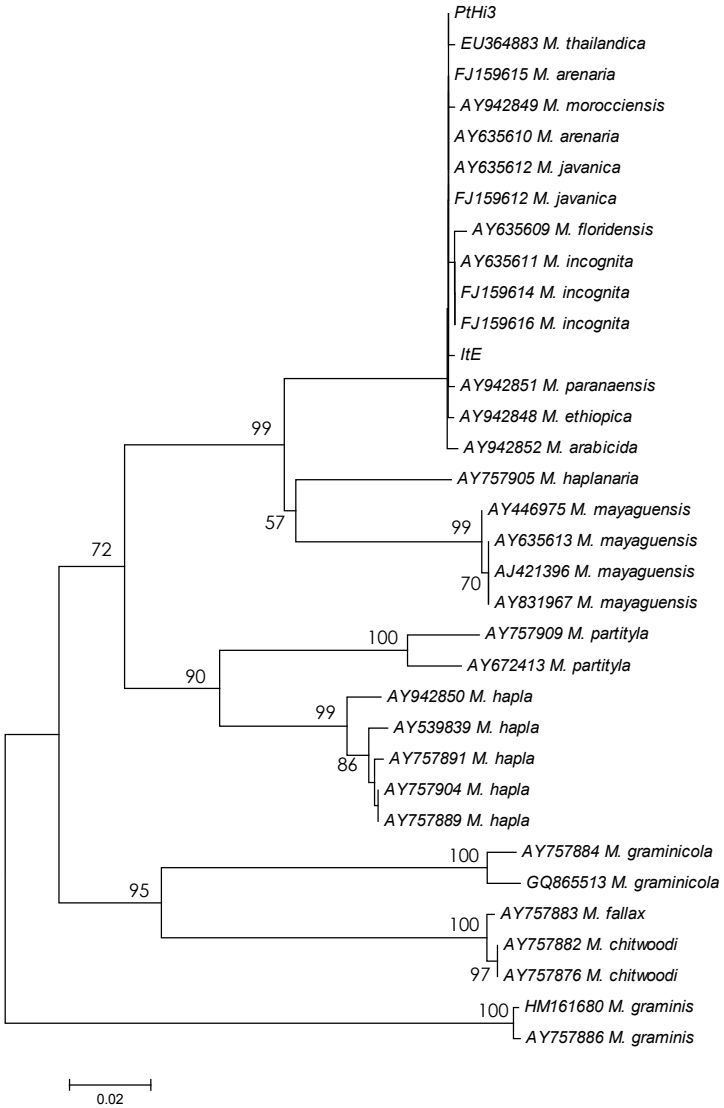

Figure 11 Neigborg-joining tree based on analysis of alignment and adjusting the length of sequences of mtDNA region of *Meloidogyne hispanica* (PtHi3) and *M. ethiopica* (ItE) with available sequences of other *Meloidogyne* spp. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated.

1.5 DISCUSSION

The potential impact of *M. hispanica* on agricultural areas and the need of effective nematode management programs reinforce the importance of an accurate and rapid identification of this nematode species. If the genus *Meloidogyne* is easy to recognize, identification at species level is difficult due to morphological similarity between species, intra-specific variability, and the number of described *Meloidogyne* spp. (Karssen & Van Hoenselaar, 1998).

Range values of most of the morphometric characters for females, males and juveniles of the seven isolates overlapped and are within the expected range according to Hirschmann (1986). Some variability was found in the males head region height values of PtHi1, PtHi2, PtHi5, PtHi6 and PtHi7 isolates that were smaller than described for *M. hispanica* and for isolates PtHi3 and PtHi4 and smaller for all isolates in the J2. The stylet knobs width of the juveniles was smaller than the original description, but the range of values of the seven isolates overlapped. The morphology of the different development stages of the *M. hispanica* isolates was similar among them and to the original description (Hirschmann, 1986).

This species has several morpho-biometrics characters similar to other *Meloidogyne* spp., mainly to *M. arenaria*, *M. floridensis, M. incognita* and *M. thamesi* (Hirschmann, 1986; Kleynhans, 1993; Handoo *et al*., 2004). Therefore, there is the possibility that *M. hispanica* has been undetected due to an incorrect identification. One isolate of *M. hispanica* from South Africa was erroneously associated with *M. arenaria thamesi* on the basis of perineal pattern morphology (Kleynhans, 1993). Perineal pattern morphology, considered one of the characters to discriminate *Meloidogyne* spp., has been found to be variable and the identification of RKN species cannot rely only in this particular character (Hirschmann, 1985; Handoo *et al*., 2005; Carneiro *et al*., 2008). Therefore, the morpho-biometrical diagnostic characters need to be supported by other characters resulting from biochemical and molecular studies.

The esterase phenotypes of the seven *M. hispanica* isolates were similar (Hi4), with the two major bands being used to characterize the isolates of this species as the two fainter bands can vary due to the amount of protein and time of staining. This pattern is unique of *M. hispanica* and is very useful to differentiate this species from the other RKN species (Janati *et al*., 1982; Esbenshade & Triantaphyllou, 1985; Fargette, 1987; Abrantes *et al*., 1995, 2008; Carneiro *et al*., 2004a,b,c; Landa *et al*., 2008). Reproducible protein

patterns, obtained by SDS-PAGE were found for each *M. hispanica* isolate and the other *Meloidogyne* spp. Although, some differences could be detected between *M. megadora* and *M. chitwoodi* and the other species, the large number of protein bands observed made interpretation of the results difficult. Regarding the use of biochemical characters the analysis of proteins did not seem appropriate for species study, whereas enzymes proved to be a reliable character for the identification and differentiation of *M. hispanica* from other *Meloidogyne* spp.

The mtDNA-PCR-RFLP analysis was also an efficient methodology to discriminate this species from the other RKN species. The amplified product of *M. hispanica* (ca. 1,800 bp), using primers C2F3 and MRH106, has been reported in this study for the first time. These primers had been used to identify the major RKN species occurring in China (Xu *et al*., 2004). Taking into consideration that the amplified products, with primers C2F3/MRH106, were ~130 bp larger than those obtained with primers C2F3/1108, our results for *M. hapla* (ca. 650 bp), *M. chitwoodi* (ca. 650 bp), *M. mayaguensis* (ca. 850 bp), *M. javanica* (ca. 1,800 bp) and *M. ethiopica* (ca. 1,800 bp) agree with previous reports (Powers & Harris, 1993; Blok *et al*., 2002; Xu *et al*., 2004; Handoo *et al*., 2005; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006). *Meloidogyne arenaria* isolate produced a fragment of ca. 1,300 bp which agrees with other studies, except with the populations from French West Indies that have produced a PCR product of ca. 1,700 bp (Powers & Harris, 1993; Blok *et al*., 2002; Xu *et al*., 2004; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006). Although *M. incognita* has been reported as having populations that produced two sizes of PCR products (ca. 1,500 and 1,700 bp) with C2F3/1108 primers, the Portuguese isolate produced a product with ca. 1,800 bp (Powers & Harris, 1993; Blok *et al*., 2002; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006).

*Hinf*I restriction patterns of the ca. 1,800 bp amplified products discriminated *M. incognita* from the other species, but no digestion occurred in *M. javanica* which is in agreement with the results reported by Xu *et al*. (2004). *Meloidogyne hispanica* was distinguished from *M. hapla* and *M. chitwoodi*, *M. mayaguensis* and *M. arenaria* by the size of the PCR products, but showed the same size product of amplification as that of *M. ethiopica, M. incognita* and *M. javanica*. It could be differentiated from *M. javanica* and *M. ethiopica* and these from each other by the size of amplified products after digestion with *Dra*III and *Alu*I, as predicted by the restriction enzyme maps. According to Powers and Harris (1993) *Dra*I restriction patterns of the amplified products discriminated *M. chitwoodi* from *M. hapla*, however different profiles were obtained by

Powers *et al*. (2005). Based on altered nucleotides between the two species that created a new restriction site, *Bfa*I restriction endonuclease allowed differentiation of *M. hapla* from *M. chitwoodi*, which is consistent with sequence information obtained from GenBank database. *Meloidogyne chitwoodi* isolate identification was further confirmed by the amplified fragment length using JMV1 and JMV2 primers (Wishart *et al*., 2002). The sequence of the Italian *M. ethiopica* isolate was identical to reported already for this species, which confirmed the results obtained by isozyme analysis (E3). The RFLP strategy implemented required only one to four steps for easy and quick identification of eight important and damaging *Meloidogyne* spp. based on variable mtDNA amplification products and respective nucleotide variations. This methodology could be adapted to single specimens increasing their applicability in routine examination of soil samples where J2 are found.

Low level of recombination, uniparental inheritance and high rate of evolution relative to nuclear genes are important characteristics of mtDNA, which facilitate the analysis at species level and construction of phylogenies (Blok & Powers, 2009). The mtDNA region from *COII* and 16s rRNA genes varies in length and encompasses an AT-rich non-coding sequence with different sizes, result of deletions and insertions (Jeyaprakash *et al*., 2006; Blok & Powers, 2009). The RKN species *M. arabicida*, *M. arenaria, M. ethiopica, M. floridensis*, *M. hispanica, M. incognita*, *M. javanica*, *M. morocciensis* and *M. paranaensis,* formed a well supported clade with the exclusion of *M. mayaguensis* and *M. hapla*. This group includes the mitotic parthenogenetic RKN and that possess the AT-rich region; except for *M. floridensis* that reproduce by facultative meiotic parthenogenesis and *M. thailandica* from which the mode of reproduction has not yet been studied (Handoo *et al*., 2004; Karssen & Moens, 2006). *Meloidogyne mayaguensis* formed a second group with *M. haplanaria* (57% of bootstrap support) and showed an amplified product for the *COII*/16s rRNA region different from all studied species (Blok *et al*., 2002). According to Eisenback *et al*. (2003), *M. haplanaria* was distinct from other species, such as *M. chitwoodi*, *M. fallax*, *M. graminis, M. hapla* meiotic parthenogenetic pathogens and *M. arenaria*, *M. incognita* and *M. javanica* that reproduced by mitotic parthenogenesis and grouped with *M. mayaguensis*. On the other hand, *M. partityla* that reproduce by obligatory mitotic parthenogenesis was included together with species that reproduce by meiotic parthenogenesis and lack the AT-rich region in the amplified product (Jeyaprakash *et al*., 2006; Blok & Powers, 2009). These results support the hypothesis that the ancestral state of the genus is characterized by the absence of

AT-rich region and the evolution of *Meloidogyne* spp. is related with the mode of reproduction and amphimixis is the ancestral reproductive state of the genus (Triantaphyllou, 1985; Blok & Powers, 2009).

Considering the main clades, our results also agree with those obtained by Tigano *et al*. (2005) for the 18S rRNA gene. Relationships between RKN species were not clarified for mtDNA, which limits the confidence of this molecule for species discrimination. Both NJ analyses showed that mtDNA sequences were not enough to differentiate *M. hispanica* and *M. ethiopica* from other species with similarities in morphology, such as *M. arenaria, M. floridensis* and *M. incognita* and similar product of amplification (Hirschmann, 1986; Handoo *et al*., 2004; Blok & Powers, 2009). Thus, the examination of more than one molecular character is very important to aid in the identification and evolution studies of a particular species, when the relationships are not clear. The MP analysis of the D2-D3 region of 28S rDNA and ITS revealed that *M. hispanica* formed a clade with high bootstrap support and distinct from *M. incognita*, *M. konaensis*, *M. paranaensis* and *M. arenaria*. However, *M. hispanica* have been reported to show an identical 18S rDNA sequence to *M. ethiopica* (Landa *et al*., 2008).

The reliable and rapid identification of *M. hispanica*, a potential economically important plant pathogen, is of great importance for the diagnosis of this species and can be useful to monitor its distribution and spread. Isozyme analysis remains an effective methodology for precise identification and differentiation of *M. hispanica* (Hi4). Variability in mtDNA sequences among *Meloidogyne* spp. allowed *M. hispanica* and *M. ethiopica* discrimination from other six studied species by PCR-RFLP, providing a new tool for *Meloidogyne* identification. However, it will be necessary to analyse a broad range of *M. hispanica* isolates to validate the obtained results and strengthen the potential applicability of the mtDNA-PCR-RFLP analysis in diagnosis.

1.6 Acknowledgments

This research was supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008. Carla Maleita was supported by a FCT-MCTES grant (SFRH/BD/24275/2005) supported by QREN funds through the "Programa Operacional Potencial Humano". Rothamsted Research receives grant aided support from the Biotechnological and Biological Sciences Research Council of the United Kingdom.

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2

Thermal requirements for the embryonic development and life cycle of *Meloidogyne hispanica*

2.1 Abstract

The life cycle of *Meloidogyne hispanica* in the tomato cultivars Easypeel and Rossol was studied in growth chambers with temperature ranging from 10 to 35ºC. Embryogenesis was faster at 25 and 30ºC and at 30ºC was about twice faster than at 20ºC and about four times than at 15ºC. No development occurred at 10 or 35ºC. An increase in the invasion of tomato roots by second-stage juveniles (J2) was correlated with an increase in temperature. Several J2 penetrated but failed to establish and develop in tomato Rossol roots, with the *Mi-1.2* gene, at temperatures below 35ºC. Necrotic cells were observed at 5 days after inoculation (DAI), which prevent further development. At 25ºC, *M. hispanica* did not reproduce on Rossol and at 20ºC, the life cycle was slightly longer with the proportion of females lower than on Easypeel. At 30 and 35ºC, the development was similar on both tomato plants. In Easypeel was shorter at 25 and 30ºC and at 20ºC was 1.5 times longer than at 25ºC. At 15ºC egg production was not observed until 80 DAI. *Meloidogyne hispanica* is most suited to soil temperatures around 25ºC and predicted climate change indicates that this nematode species could spread in Southern Europe and move northwards. The estimated base temperatures were 11.49ºC and 10.22ºC and thermal constant 76.92 and 515.46*DD* for embryogenesis and life cycle, respectively. The thermal requirements for *M. hispanica* development are analysed and compared with those of other *Meloidogyne* species.

Keywords: base temperature, post-embryonic development, root penetration, *Solanum lycopersicum* L., thermal time

2.2 INTRODUCTION

Global surface temperature has increased about 0.2ºC per decade in the past three decades and 0.8ºC in the past century (Hansen *et al*., 2006). Global warming constitutes an important climate change with impacts on pathogens and consequently on the occurrence and distribution of plant diseases, that probably intensifies the importance of these problems (Ghini *et al*., 2008). At the population level, the most important prognostic of the magnitude of climate change effects on plant disease is the adaptative potential of the pathogen population (Garrett *et al*., 2006). Assessment of the impacts of climate change on pest infestations in crops is very important, as it enables the adaptation of preventive measures to control their population density and dispersion (Ghini *et al*., 2008).

Root-knot nematodes (RKN), *Meloidogyne* spp., are sedentary endoparasites that have very complex biotrophic relationships with their host plants. They are distributed worldwide and are responsible for significant damage and yield losses in agricultural areas. The embryonic development results in first-stage juveniles who moult in the egg and hatch as second-stage juveniles (J2s), the infective stage. During the post-embryonic development, the infective juveniles moult three times to become an adult and induce the formation of giant cells as a result of repeated nuclear divisions, without cytokinesis, and cortical cells proliferation and hypertrophy leading to the formation of typical root galls where reproduction occurs. In *Meloidogyne* species the sex chromosomes are absent and the sex ratio can be influenced by environmental factors. The production of males may be induced by overcrowding, food shortage, temperature extremes or other adverse environmental stresses. Males do not feed and migrate out the plant; females remain sedentary and lay eggs into a gelatinous matrix (Abad *et al*., 2010).

Root-knot nematodes are poikilothermic organisms and their development usually is dependent on temperature. The embryonic development is a process of differentiation and is possible to study the temperature effect on the absence of other external influences however, the post-embryonic development depends not only on temperature but also on the plant host (Moura *et al*., 1993; Pedrosa *et al*., 1996; Tzortzakakis & Trudgill, 2005; Charchar *et al*., 2009). Variations in response to temperature among RKN species is important to determine the survival of a species in a

particular region, so climate changes affect the aggressiveness and the spatial distribution of these nematodes. In general, it has been demonstrated that the rate of nematode development is correlated linearly with temperature (Trudgill, 1995). Since the first studies on the linear relationship between thermal time and the rate of nematode development which analysed the effects of the temperature on the life cycle of an unknown *Meloidogyne* species (Tyler, 1933), extensive research have showed that rate of RKN embryonic, post-embryonic development and life cycle are strongly influenced by temperature and varies with the species of *Meloidogyne* (Lahtinen *et al*., 1988; Madulu & Trudgill, 1994; Zhang & Schmitt, 1995; Ploeg & Maris, 1999; Yeon *et al*., 2003; Charchar & Santo, 2009; Strajnar *et al*., 2011). The higher values of lower threshold temperature are usually associated with tropical species and the lowers with temperate ones. The optimum temperature range for *M. hapla* and some other cold-climate adapted species is 15 to 25ºC, whereas to *M. javanica* and other warm-climate species is 25 to 30ºC. Above 40ºC and below 5ºC very little activity or no development occurs in any *Meloidogyne* species (Taylor & Sasser, 1978).

Meloidogyne hispanica is one of the lesser known species of RKN and a virulent pathogen of at least 87 different plant species and cultivars, including dicotyledons and monocotyledons in the families Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae, which make it a potential problem to several economically important crops (Maleita *et al*., unpublished results). This species has been recorded in all continents, and in Portugal was found alone or in mixed populations of RKN in different regions of the Center and South, associated with several cultivated plants (Abrantes *et al*., 2008; Conceição *et al*., 2009; Maleita *et al*., 2011).

The present research was undertaken to evaluate the effects of temperature on the embryonic development, penetration and post-embryonic development of *M. hispanica* in the tomato plants cvs Easypeel and Rossol. The results are compared to data published for other *Meloidogyne* species and the possible effects of climate change on the aggressiveness and the spatial distribution of *M. hispanica* are analysed.

2.3 MATERIALS AND METHODS

2.3.1 NEMATODE ISOLATES

Two RKN isolates were used in our experiments. *Meloidogyne hispanica* and *M. arenaria* were obtained from fig-tree (*Ficus carica*, L.) roots, collected in Odeceixe, Faro, Portugal and tomato (*Solanum lycopersicum* L.) roots collected in Coimbra, Portugal, respectively. Both isolates were reared on tomato cv. Easypeel and kept at 25±2ºC in a growth chamber.

2.3.2 EMBRYOGENESIS

The *M. arenaria* isolate was included in this experiment for comparison. Egg masses of both RKN isolates, containing a relatively large proportion of eggs in their earliest stages of development, were collected from tomato infected roots. Thirty eggs (1 or 2 cell stage) of each isolate/treatment were carefully selected from the egg masses, washed in sterilized distilled water and transferred into sterilized Petri dishes containing 2% water agar. The eggs were then incubated at 10, 15, 20, 25, 30 and 35º C and observed every six hours in the first day and then every 24 hours until hatching. The development stages considered were two, four, six and eight cells, multicellular, gastrula, embryo, first-stage juvenile, second-stage juvenile within the egg and hatched juvenile.

2.3.3 PENETRATION, POST-EMBRYONIC DEVELOPMENT AND LIFE CYCLE OF *M. HISPANICA*

The tomato plants used in these experiments were cv. Easypeel, highly susceptible, and cv. Rossol which has the RKN resistance *Mi* gene. The presence of the *Mi* gene was previously confirmed by amplification of the SCAR marker Mi23 (Maleita *et al*., 2011). Tomato seedlings, germinated at 26-27ºC on moist filter paper in Petri dishes, were transplanted singularly in 5.5 cm-diameter plastic pots containing 100 cm^3 of a steam sterilized mixture of sandy loam soil and sand (1:3).

Three-week-old tomato Easypeel and Rossol seedlings were inoculated with freshly

hatched J2s of *M. hispanica* obtained from egg masses (150 J2s/seedling). The plants were transferred to their respective growth chambers (15, 20, 25, 30 and 35°C) with a 12 h photoperiod. Nematode penetration (four seedlings/treatment) was monitored at 3, 6, 9, 12, 18, and 24 h after inoculation (HAI) and every 12 h in the following 2 days. At 15°C, the plants were harvested at the same time point described above for 7 days. Roots were washed and stained with acid fuchsin (Byrd *et al*., 1983) and the numbers of J2s inside the roots were recorded.

To study the post-embryonic development, the seedlings were removed 3/7 days after inoculation (DAI) and the root systems washed gently to remove all J2s from the root surface. Single seedlings were transplanted into 14.5 cm diam. Petri dishes with a hole laterally, containing a mixture of sandy loam soil and sand (1:1), and maintained vertically. Seedlings grown at temperature 15, 20, 25, 30 and 35°C (four seedlings/treatment) were harvested daily between 3/7-10; at 2-days intervals during 20 days and at 5-days intervals thereafter until the end of the experiment. Roots were washed and stained with acid fuchsin (Byrd *et al*., 1983) and nematodes were removed from the root tissues, mounted in glycerin and observed microscopically. The various developmental stages of the nematode (second-stage infective juvenile; swollen, sexually undifferentiated second-stage juvenile; early second-stage juvenile differentiating into a female/male; second-stage female/male juvenile shortly before second molt; fourth-stage female/male juvenile; and adult female/male juvenile shortly after fourth molt) were identified on the basis of body shape and gonad development (Triantaphyllou & Hirschmann,1960). The life cycle was considered complete when hatched J2s were found in the egg masses. When egg masses were observed in the root system, they were removed and placed in glass blocks, containing tap water and maintained at the same temperature to evaluate hatching of J2s.

2.3.4 THERMAL TIME REQUIREMENTS

All data obtained for the embryogenesis, development within the egg until hatched juvenile, post-embryonic development and life cycle completion were expressed as 1/number in days taken for development ($days^{-1}$) to obtain the development rate (R) at each temperature. The value of the base temperature for development (Tb, R=0) that reflects the thermal environment to which the nematode is adapted was obtained by

linear regression of R on the temperature (Te) and extrapolating the regression line to the abscissa. The thermal constant (S) which represents the number of heat units above the Tb required for completion of the embryogenesis, development within the egg until hatched juvenile, post-embryonic development and life cycle was obtained by the reciprocal of slope of the regression and at each temperature according to: S = (Te-Tb)/R expressed in degree (ºC) days (*DD*) (Trudgill, 1995; Trudgill *et al*., 2005).

2.3.5 DATA ANALYSIS

Statistical analysis was performed using Statsoft Statistica version 7 for Windows. Data of the effect of temperature and tomato cultivars on nematode penetration were checked for evidence of a Normal distribution using the Kolmogorov-Smirnov test, and for variance homogeneity using Levene's test. Transformation of the data was performed by square [$\sqrt{(x+0.5)}$]. Following ANOVA, treatment means within each factor were compared using Fisher's LSD test ($P < 0.05$).

2.4 RESULTS

2.4.1 EMBRYOGENESIS

The relationship between temperature and nematode embryonic development was similar in *M. hispanica* and *M. arenaria*. At 10 and 35ºC, embryogenesis did not occur in any of the species. At 15, 20, 25 and 30ºC, the rate of development of both species increased as temperature increased, being slower in *M. arenaria* than in *M. hispanica* (Figs. 1 to 4).

In the two species, the embryogenesis was faster at 25 and 30ºC (6 and 4 days to *M. hispanica* and 7 and 5 days to *M. arenaria*, respectively). At 30ºC, embryogenesis was about twice as faster as at 20ºC (10 days), and about four times as faster as at 15ºC (18 and 21 days for *M. hispanica* and *M. arenaria*, respectively). The daily rate of egg development of the one-cell stage to first-stage juvenile within the egg was related to temperature between 15 and 30ºC (Figs. 1 to 4). Cleavage of the two-cell occurred at 1 and 2 days for *M. hispanica* and *M. arenaria*, respectively, at 15ºC and at 0.5 days for both species at 20 to 30ºC. Cleavage of the eight-cell stage was observed at 4.25, 3, 2.5 and 1 day for *M. hispanica* and 5, 2.5, 2 and 1.5 for *M. arenaria*, respectively at 15, 20, 25 and 30ºC. The embryo was detected at 17, 9, 5.5 and 3.5 days for *M. hispanica* and 20, 9, 6.5 and 4.5 days for *M. arenaria*. Differentiation of embryo on first-stage juvenile was achieved at 18, 10, 6 and 4 days to *M. hispanica* and 21, 10, 7 and 5 days to *M. arenaria* at 15 to 30ºC, respectively (Figs. 1 and 2).

Development time to the second-stage juvenile was faster at 30ºC than that 15 to 25ºC. Newly formed *M. hispanica* J2s were observed in 27, 14, 9 and 7 days at 15 to 30ºC, respectively, whereas *M. arenaria* J2s were observed in 30, 14, 10 and 8 days (Fig. 3). *Meloidogyne hispanica* J2s hatched after 35, 19, 12 and 9 days at 15, 20, 25 and 30ºC, respectively, and *M. arenaria* J2s after 38, 19, 14 and 11 days. The hatching process required 8-2 days at 15 to 30ºC for *M. hispanica* and 8-3 days for *M. arenaria*. At 25 and 30ºC, the hatching process to *M. arenaria* required one day more than *M. hispanica* (Figs. 1 and 2).

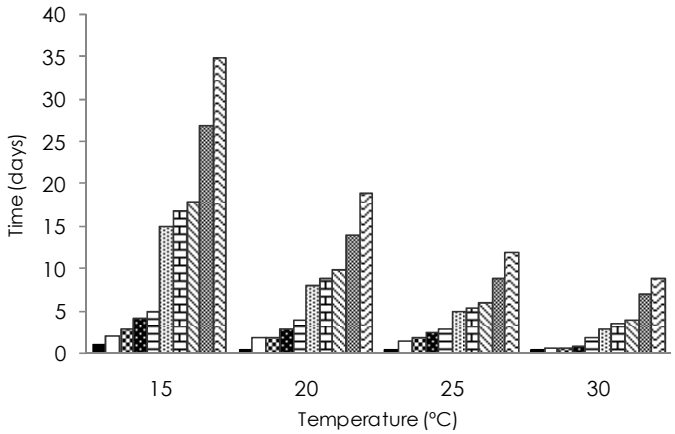

Figure 1 Embryonic development and hatching of *Meloidogyne hispanica* at four temperatures. Duration, in days, of the two (■), four (□), six (⚀) and eight cells stage (▩), multicellular stage (▤), gastrula stage (▥), embryo (▦), first-stage juvenile (▧), second-stage juvenile (▩) within the egg and hatched juvenile (▨).

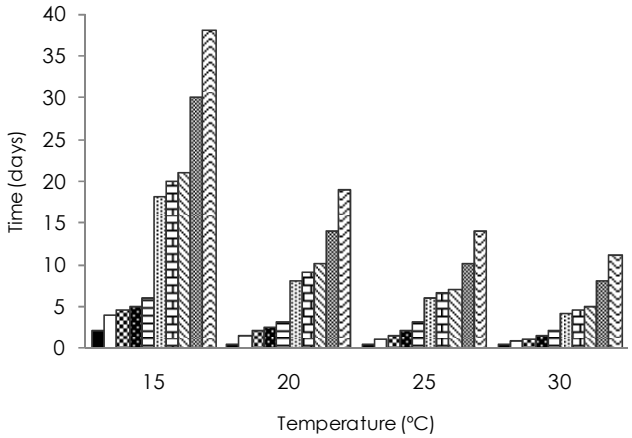

Figure 2 Embryonic development and hatching of *Meloidogyne arenaria* at four temperatures. Duration, in days, of the two (■), four (□), six (⚀) and eight cells stage (▩), multicellular stage (▤), gastrula stage (▥), embryo (▦), first-stage juvenile (▧), second-stage juvenile (▩) within the egg and hatched juvenile (▨).

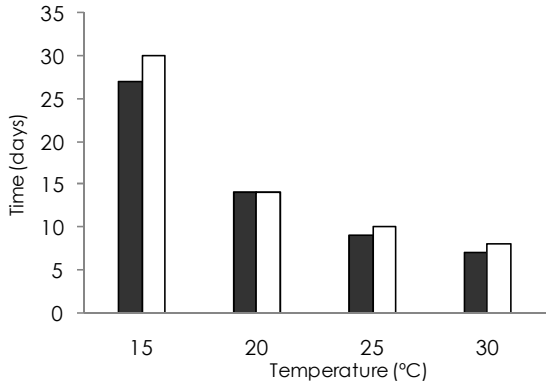

Figure 3 Development of *Meloidogyne hispanica* (■) and *M. arenaria* (□) until the second-stage juvenile within the egg at four temperatures.

The thermal requirement analysis of the embryonic development (until first-stage juvenile) rate yielded a positive linear function with temperature. A linear regression model was obtained for both species and was used to determine Tb for the studied temperatures (Fig. 4). The Tb for the embryonic development of *M. hispanica* was 11.49ºC and 10.24ºC to *M. arenaria* with an S of 76.92 and 100 *DD*, respectively. Thermal constants were estimated for all temperatures (Table 1). For the complete development within the egg including hatching, the Tb of *M. hispanica* was 10.11ºC (y=0.0056x-0.0563, R^2=0.9979) and 8.31ºC to *M. arenaria* (y=0.0043x-0.0353, R^2=0.9934) with S of 179.5 and 235.3 *DD*, respectively (Fig. 7).

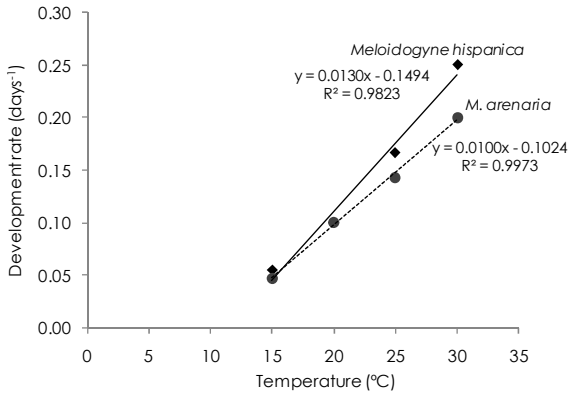

Figure 4 Relationship between *Meloidogyne hispanica* and *M. arenaria* embryonic development rate and temperature. Rate of development for each temperature = 1/total of days required to occur development. R= predicted rate of embryonic development with the limits $15 \leq T \leq 30$ºC.

Table 1 Thermal constants for 1 or 2 cell stage to first-stage juvenile development of *Meloidogyne hispanica* and *M. arenaria* at four constant temperatures, when considering the base temperature as 11.49ºC to *M. hispanica* and 10.24ºC to *M. arenaria*.

Temperature		*M. hispanica*			*M. arenaria*		
		Development period (days)	Thermal constant (S)		Development period (days)	Thermal constant (S)	
ºC	ºF		ºC	ºF		ºC	ºF
10	50	ND	—	—	ND	—	—
15	59	18	63.2	110.5	21	100.0	188.2
20	68	10	85.1	151.4	10	97.6	179.6
25	77	6	81.1	144.8	7	103.3	188.7
30	86	4	74.0	132.6	5	98.8	179.8
35	95	ND	—	—	ND	—	—

ND – no data, no development occurred.

2.4.2 PENETRATION, POST-EMBRYONIC DEVELOPMENT AND LIFE CYCLE OF *M. HISPANICA*

PENETRATION

Three DAI, J2s were found in the root tissues of both tomato cultivars at 20 to 35ºC, suggesting a successful root penetration. However, the number of J2s, inside the roots, was statistically different among temperatures and between the tomato cultivars at certain temperatures (Table 2). At 15ºC, in both cultivars, very few J2s were detected in the roots. On cv. Rossol, the first J2s were observed at 24 HAI and on cv. Easypeel at 72 HAI. At seven DAI, there were no significant differences in nematode penetration in the two cultivars ($P > 0.05$, LSD test); 33.0±7.35 (22%) and 28.0±7.0 (18.7%) in the cultivars Easypeel and Rossol, respectively. At 20ºC, J2s were detected in roots at 18 and 12 HAI on cvs Easypeel and Rossol, respectively. At higher temperatures, there was a decrease in the length of time required for root penetration; 12, 9 and 6 HAI on cv. Easypeel *vs* 9, 6 and 3 HAI on cv. Rossol at 25, 30 and 35ºC, respectively. At 72 HAI, nematode penetration, on both tomato cultivars, increased with temperature, except on cv. Easypeel at 35ºC (23.2%), with maximum penetration at 25ºC (55.5%) and 30ºC (50%). The number of J2s recorded inside the roots 72 HAI on tomato cv. Rossol was statically lower than cv. Easypeel at 20ºC (23.7%) and higher at 15 and 35ºC (8.5% and

56.9%, respectively) ($P \leq 0.05$, LSD test), but no significant differences were found at 25 and 30°C ($P > 0.05$, LSD test) (Table 2).

Table 2 Number of second-stage juveniles (J2s) of *Meloidogyne hispanica* inside the roots of tomato cultivars Easypeel and Rossol, after inoculation with 150 J2s, under five temperatures (15, 20, 25, 30 and 35ºC).

Hours after inoculation	Temperature									
	15ºC		20ºC		25ºC		30ºC		35ºC	
	Easypeel	Rossol	Easypeel	Rossol	Easypeel	Rossol	Easypeel	Rossol	Easypeel	Rossol
3	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.3±0.5b
6	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.8±1.0a	0.5±0.6a	3.5±2.9b
9	0.0±0.0ab	0.0±0.0ab	0.0±0.0ab	0.0±0.0ab	0.0±0.0ab	1.8±2.1a-d	3.5±3.1b-f	5.5±2.9cef	2.5±3.0b-df	5.3±3.8c-f
12	0.0±0.0a-c	0.0±0.0a-c	0.0±0.0a-c	2.8±2.2bdf	0.5±1.0a-c	1.3±1.3a-cf	6.8±4.5bd-f	12.8±5.3de	6.3±7.5b-df	13.3±6.7de
18	0.0±0.0a	0.0±0.0a	1.5±1.7a	10.0±4.2bc	8.5±5.8bc	11.0±6.2bce	14.8±10.3bce	26.5±5.3d	20.5±10.1c-e	59.8±16.5f
24	0.0±0.0a	0.5±1.0a	6.5±2.1bc	17.5±19.7b-d	16.3±6.8b-d	24.5±9.3cd	19.0±10.4cd	51.5±5.1e	23.8±13.4cd	65.8±24.1e
36	0.0±0.0a	9.0±3.0b-d	15.0±5.5b-e	21.0±10.0b-f	58.3±23.9gh	57.5±17.4gh	36.0±15.3d-f	59.8±7.3g-i	28.0±17.9c-f	84.3±7.8hi
48	0.0±0.0a	9.8±4.35b	40.3±7.4de	26.0±15.3e	67.0±16.8c	65.3±15.0c	66.5±16.5c	71.3±18.4c	30.3±5.6de	83.8±4.6c
60	0.0±0.0c	19.0±4.3a	61.8±18.6b	31.8±3.4a	80.8±12.2b	74.3±14.5b	73.5±34.0b	66.8±11.8b	34.5±16.1a	68.5±16.3b
72	0.5±1.0a	12.8±7.6b	69.8±7.3c-g	35.5±7.1h	83.3±15.7c-f	65.5±12.5ce-g	75.0±10.2c-g	59.8±12.0cfg	34.8±2.1h	85.3±9.2c-e
96	10.8±6.2a	14.8±4.1a	–	–	–	–	–	–	–	–
120	5.3±4.99a	15.8±9.8a	–	–	–	–	–	–	–	–
144	17.0±10.6a	31.8±4.0a	–	–	–	–	–	–	–	–
168	33.0±7.35a	28.0±7.0a	–	–	–	–	–	–	–	–

Data are means of four replicates ± standard deviation. Means of each row followed by same letter do not differ significantly at $P > 0.05$, according to Fisher's LSD test.

POST-EMBRYONIC DEVELOPMENT AND LIFE CYCLE

The development of J2s in roots was influenced by tomato cultivars and temperature. The effect of the five temperatures regimes on *M. hispanica* development is presented as percentage of each developmental stage found in the roots at each observation (Figs. 5 and 6).

At 15ºC, a low temperature for tomato, the growth of the plants was strongly retarded, roots development was slow. At 26 DAI, only vermiform J2s were found in roots of tomato cv. Easypeel and two days after swollen J2 were observed (50%, approximately). The earliest sexually differentiated J2s were observed at 45 DAI and mature females (21.74%) at 75 DAI. Gelatinous matrix and egg production was not observed until 80 DAI (Fig. 5A). On tomato cv. Rossol, the J2s were able to penetrate, but did not develope. Twenty four DAI, all the J2s were associated with necrotic cells (data not show). At 20, 25 and 30ºC, necrotic cells were also observed 5 DAI (Fig. 6).

At 20ºC, the entire life-cycle (from J2 to J2) was completed in approximately 53 and 62 days, on cvs Easypeel and Rossol, respectively. Deposition of gelatinous matrix was recorded at 45/50 DAI and eggs were laid at 45 DAI on tomato cv. Easypeel and 5 to 10 days after on cv. Rossol. Adult females were detected at 26 DAI for both tomato cultivars. Fourty five DAI, 89.3% of the nematodes recovered, from tomato cv. Easypeel, were adult females and 5.37% males; in tomato cv. Rossol the proportion of females decreased, with only 9.71% (Figs. 5B and 6A).

The duration of the life cycle of *M. hispanica* on cv. Easypeel at 25ºC was shorter than at 20ºC (35 days *vs* 53 days). The development of the juveniles required 10 days and adult females were first observed at 12 DAI. At 24 DAI more than 80% of the nematodes recovered were females. Males (1.18%) were also observed in the roots at 24 DAI (Fig. 5C). The first gelatinous matrix was recorded on the 22nd day, eggs were laid after 24 days and newly-hatched J2s were present in the egg masses at 35 days. In the tomato cv. Rossol, at 25ºC, *M. hispanica* did not reproduce, more than 90% of the juveniles were associated with necrotic cells and few females were found (Fig. 6B).

Development of *M. hispanica* J2s on both tomato cultivars were similar at 30 and 35ºC, with the presence of necrotic tissue on tomato cv. Rossol at 30ºC. Nematodes developed into adults as early as 10 DAI and began depositing the gelatinous matrix six to eight days later at 30 and 35ºC, respectively. The majority of nematodes reached the adult stage at 12 and 18 DAI to tomato cvs Easypeel and Rossol, respectively, at 30ºC;

and at 12 DAI for both tomato cultivars at 35ºC. Egg production at 30ºC was first observed 18/20 DAI and newly-hatched J2s were present in the egg masses at 26 to 27 days. Eggs were laid at 18/20 and 22 DAI to tomato cvs Easypeel and Rossol, respectively, but no hatching occurred at 35ºC (Figs. 5D,E and 6C,D).

Thermal requirements for the post-embryonic development and life cycle completion were determined on basis of the results for tomato cv. Easypeel. There is a linear relationship between temperature and rate of nematode post-embryonic development (from J2 to adult stage, $y=0.0061x-0.0784$, $R^2=0.9722$) and life cycle completion (from J2 to J2, $y=0.0019x-0.0198$, $R^2=0.9999$, Fig. 8). The Tb for the post-embryonic development and life cycle of *M. hispanica* was 12.85 and 10.22ºC, respectively with a S of 163.93 and 515.46 *DD*. Thermal constants for life cycle were estimated for all the temperatures (Table 3).

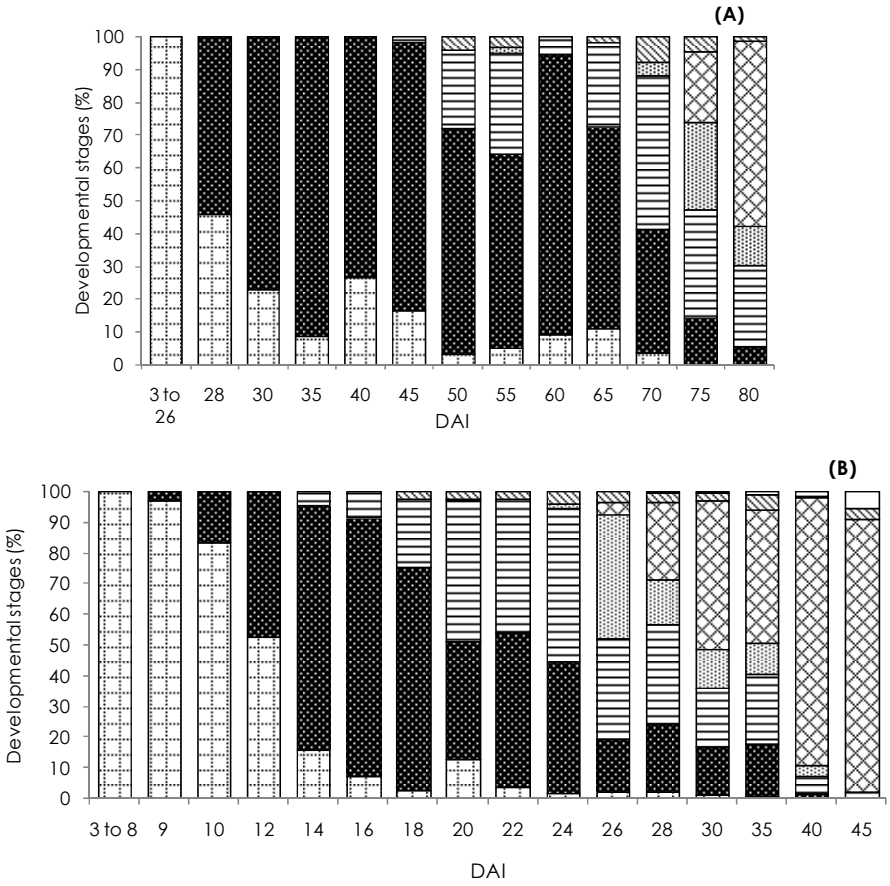

Figure 5 Post-embryonic development of *Meloidogyne hispanica* in roots of tomato, *Solanum lycopersicum*, cv. Easypeel from 3 days after inoculation (DAI) until deposition of eggs in gelatinous matrix, at 15°C (A), 20°C (B), 25°C (C), 30°C (D) and 35°C (E). (▭) Second-stage (J2) infective juvenile; (▩) Swollen, sexually undifferentiated J2; From earliest sexually differentiated J2 until fourth-stage female (▤) /male juvenile (▧); Adult female (▦) /male juvenile shortly after fourth moult (☐); (▣) Adult female (Based on Triantaphyllou & Hirschmann, 1960); (Data are means of four replicates).

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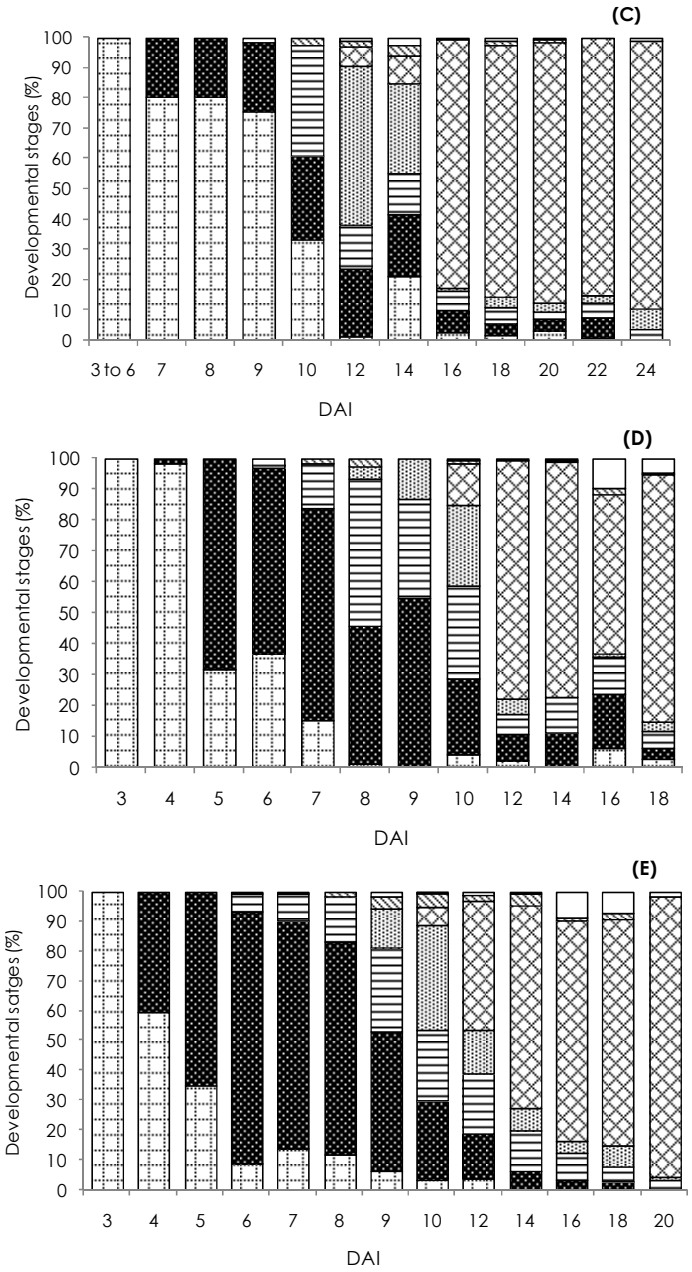

Figure 5 (CONTINUED). Post-embryonic development of *Meloidogyne hispanica* in roots of tomato, *Solanum lycopersicum*, cv. Easypeel from 3 days after inoculation (DAI) until deposition of eggs in gelatinous matrix, at 15ºC (A), 20ºC (B), 25ºC (C), 30ºC (D) and 35ºC (E). (⊡) Second-stage (J2) infective juvenile; (⊠) Swollen, sexually undifferentiated J2; From earliest sexually differentiated J2 until fourth-stage female (⊟) /male juvenile (⧅); Adult female (⊞) /male juvenile shortly after fourth moult (☐); (☒) Adult female (Based on Triantaphyllou & Hirschmann, 1960); (Data are means of four replicates).

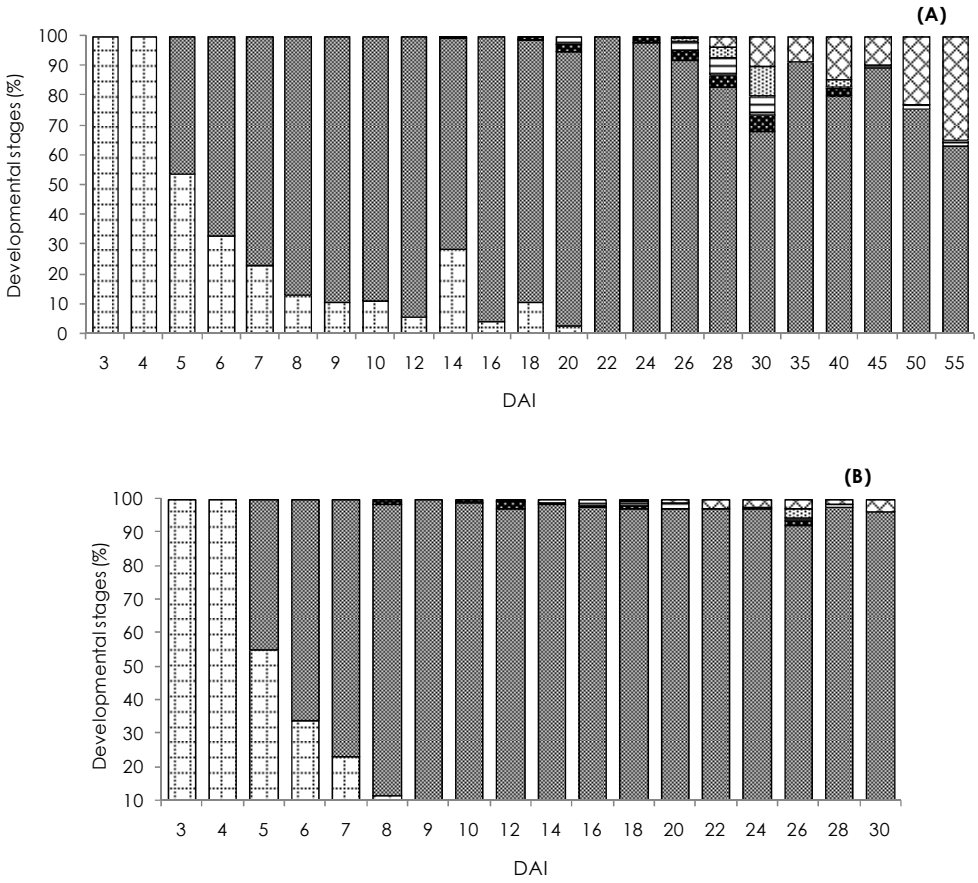

Figure 6 Post-embryonic development of *Meloidogyne hispanica* in roots of tomato, *Solanum lycopersicum*, cv. Rossol from 3 days after inoculation (DAI) until deposition of eggs in gelatinous matrix, at 20ºC (A), 25ºC (B), 30ºC (C) and 35ºC (D). (▨) Second-stage (J2) infective juvenile; (▩) J2 associated with necrosed cells; (■) Swollen, sexually undifferentiated J2; From earliest sexually differentiated J2 until fourth-stage female (▤) /male juvenile (▧); Adult female (▦) /male juvenile shortly after fourth moult (□); (▣) Adult female (Based on Triantaphyllou & Hirschmann, 1960); (Data are means of four replicates).

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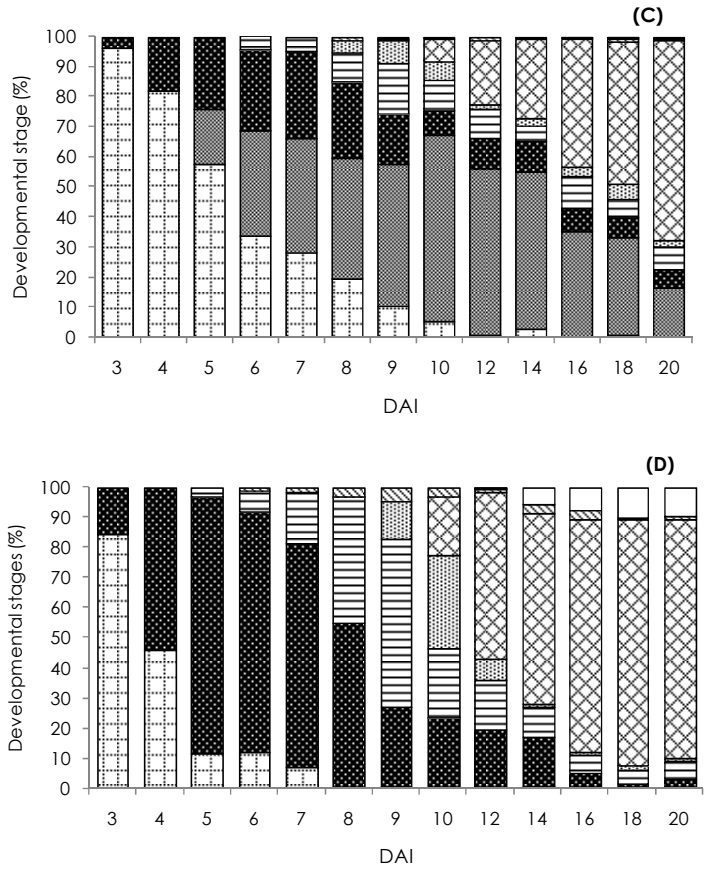

Figure 6 (CONTINUED). Post-embryonic development of *Meloidogyne hispanica* in roots of tomato, *Solanum lycopersicum*, cv. Rossol from 3 days after inoculation (DAI) until deposition of eggs in gelatinous matrix, at 20°C (A), 25°C (B), 30°C (C) and 35°C (D). (▫) Second-stage (J2) infective juvenile; (▩) J2 associated with necrosed cells; (■) Swollen, sexually undifferentiated J2; From earliest sexually differentiated J2 until fourth-stage female (▤) /male juvenile (▧); Adult female (▦) /male juvenile shortly after fourth moult (□); (▨) Adult female (Based on Triantaphyllou & Hirschmann, 1960); (Data are means of four replicates).

Table 3 Thermal constants for the life cycle of *Meloidogyne hispanica* on tomato cultivar Easypeel at three constant temperatures, when considering the base temperature as 10.22°C.

Temperature		Development period (days)	Thermal constant (S)	
°C	°F		°C	°F
20	68	53	513.5	928.2
25	77	35	517.3	933.8
30	86	26	514.3	927.7

2.5 DISCUSSION

Several environmental factors determine the prevalence of a species on a particular region, but temperature is perhaps, the most important for poikilothermic organisms, such as RKN. Previous investigations demonstrated that temperature requirement for embryogenesis and life cycle completion differs significantly among RKN. Despite the agricultural importance of *M. hispanica*, the effect of temperature and host/cultivar preference in relation to its life cycle have not been evaluated. Although, the rate of nematode development may change as the temperature approaches the upper and lower threshold, a linear relationship between temperature and the rate of development was obtained for *M. hispanica*. Consequently, it is possible to estimate the lower threshold temperature value and the thermal environment to which nematodes are adapted and also the thermal constant that shows relative rate of development (Lahtinen *et al*., 1988).

The thermal optimum for *M. hispanica* and *M. arenaria* embryogenesis and development within the egg, until hatching, lies between 25 and 30ºC and the minimal temperature under the conditions of this experiment was 15ºC, since no development occurred at 10ºC, below the Tb obtained for embryogenesis (11.49ºC and 10.24ºC to *M. hispanica* and *M. arenaria*, respectively). These results agree with those of Ferris *et al*. (1978) that showed that below 10ºC there was little development of *M. arenaria*. The thermal optimum for *M. javanica* embryogenesis also lies between 25 and 30ºC, which appears to be the optimum temperature for the development of all the embryogenesis stages, being, the rate of development extremely slow at 15ºC (Bird, 1972). The embryogenesis of *M. exigua* is similar, however the whole process takes longer (Lima & Ferraz, 1985). On the contrary, although very slow, development within the eggs until emergence of J2s occurred at 10ºC for RKN temperate species *M. chitwoodi* (82-84 days) and *M. hapla* (95-97 days) (Inserra *et al*., 1983). In *M. konaensis* the development did not occur or was incomplete at 5ºC and 8-10ºC, respectively, and from 13 to 30ºC a linear relationship was observed, but decreased at 35ºC (Zhang & Schmitt, 1995).

Based on extrapolation of the data using linear regression for temperatures of 15 to 30ºC, the Tb (R = 0) for *M. hispanica* embryogenesis is 11.49ºC and 10.24ºC for *M. arenaria* and S of 76.92 and 100 *DD*, respectively. The Tb for *M. hispanica* is higher than those for *M. arenaria* (10.11ºC and 10.24ºC in this study), *M. konaensis* (10.22ºC),

and *M. exigua* (4.58ºC) and lower than that found in *M. javanica* (13.0ºC) (Ferris *et al*., 1978; Lima & Ferraz, 1985; Trudgill, 1995; Zhang & Schmitt, 1995). The differences found in the estimated Tb of *M. arenaria* can be due to the methodology used or/and to small errors in temperature measurement, which can have great effects on the estimated Tb and S, especially at temperatures close to Tb (Trudgill, 1995).

Considering the development within the egg until hatching, Tb decreased for both species when compared to embryogenesis: 10.11ºC for *M. hispanica* (S=179.5 *DD*) and 8.31ºC for *M. arenaria* (S=235.3 *DD*), which suggests that development from first-stage juvenile until hatching can occur at lower temperatures. The regression lines of development until hatching for the two species intersect at 15.9ºC and below this value, the eggs of *M. arenaria* developed more rapidly than those of *M. hispanica*, whilst *M. hispanica* develop more rapidly at higher temperatures (Fig. 7). The comparison of the intersection of regression lines for the development of *M. incognita* (16.75ºC) and *M. javanica* (20.04ºC) and with the values obtained for *M. hispanica* shows that the development of this species is faster at lower temperatures having a competitive advantage (Fig. 7). In mixed populations of *M. hispanica* and *M. javanica* or *M. incognita*, *M. hispanica* J2s will invade the roots earlier than the J2s of the other two species, although higher temperatures are more suitable for *M. javanica* and *M. incognita* post-embryonic development (Figs. 7 and 8).

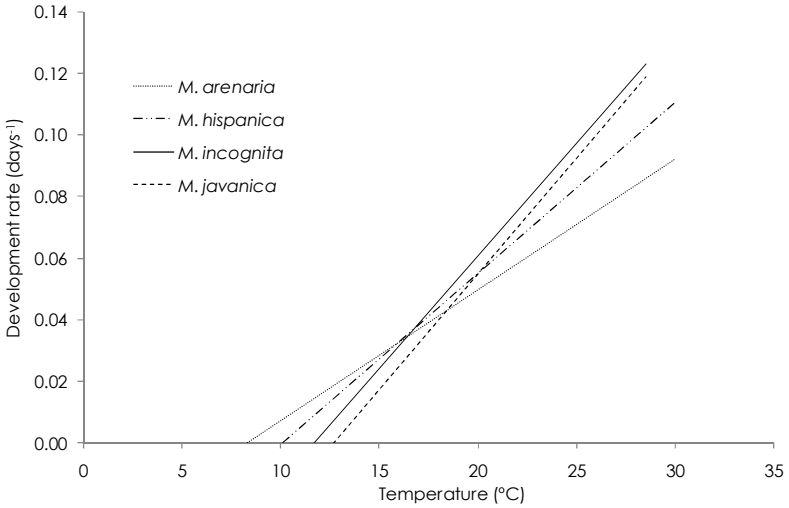

Figure 7 Relationship among *Meloidogyne arenaria*, *M. hispanica, M. incognita* and *M. javanica* development, within the egg until hatched juvenile, and temperature. Regression lines for *M. incognita* and *M. javanica* calculated from Tzortzakakis & Trudgill (2005).

The penetration of *M. hispanica* J2 is related with the temperature and decreased from 72 HAI at 15ºC to 3 HAI at 35ºC, suggesting that the motility of nematodes of this species is stimulated at higher temperatures, being the minimum temperature 15ºC. Other studies conducted with *M. incognita* J2s showed that at 18ºC or below, most of the J2s were inactive in soil, but migrated through soil at 20ºC reaching its maximum at 22ºC (Prot & Van Gundy, 1981).

The number of *M. hispanica* J2s that penetrated on the tomato roots up to 3 DAI increased in higher temperatures, with maximum penetration at 25 and 30ºC on cv. Easypeel and 35ºC on cv. Rossol, decreasing at 20 and 15ºC. Similar results were obtained for *M. hapla* J2s penetration on alfalfa with a maximum penetration at 24 and 28ºC, slight lower at 20 and 32ºC and lowest at 12 and 16ºC (Griffin & Elgin, 1977). In our studies, 3 DAI there was no significant difference in the number of J2s that penetrated in the roots of the cultivars at 25 and 30ºC, but was significantly higher in tomato cv. Rossol at 15 and 35ºC and lower at 20ºC. These results differ from those of Griffin & Elgin (1977), Carneiro *et al*. (2005) and Moritz *et al*. (2008) who stated that penetration of *M. hapla*, *M. incognita* race 3 and *M. paranaensis* J2s was not statistically different between resistant and susceptible plants; but the development in resistant plants was seriously compromised and affected by temperature.

The development of *M. hispanica* from J2 to J2 was influenced by temperature and tomato cultivar and the Tb required for the life cycle completion in tomato cv. Easypeel (10.22ºC) is analogous to that estimated for the development until hatching (10.11ºC), which is in agreement with findings by Trudgill (1995) for Tb values calculated for the development within the egg and the entire life cycle.

Meloidogyne hispanica invade the root system of tomato cv. Easypeel and reproduce at a faster rate at 30ºC than at 25 and 20ºC. Although, *M. hispanica* J2s were able to penetrate roots at 15ºC, they did not develop within 80 days, probably due to the unfavourable conditions for root development, which reduced the availability of the nutrients necessary to nematode development. The effect of the low temperature on the nematode physiology cannot be excluded (Cardin, 1979). No hatching occurred at 35ºC, indicating that this temperature limited the life cycle completion. These results were similar to those obtained for *M. javanica* where the value of optimum temperature was between 27 and 28.5ºC and the life cycle was completed at 31ºC but not at 34.7ºC (Trudgill,1995). However, the upper limit temperature for the life cycle

completion of *M. incognita* lied between 30 and 35.4ºC as no reproduction occurred at 35.4ºC and the optimum temperature placed around 25ºC (Ploeg & Maris, 1999). *Meloidogyne hapla* and *M. chitwoodi* were not able to complete the life cycle at 6ºC in 115 days, and the optimum temperature determined for these species was 30ºC and 12-24ºC, respectively (Charchar & Santo, 2009). *Meloidogyne ethiopica* was not able to reproduce at 13.9ºC and 22.7ºC was considered to be the optimal temperature for reproduction (Strajnar *et al*., 2011). Comparing available data on the effects of temperature on the duration of *M. arenaria*, *M. hapla, M. incognita* and *M. javanica* life cycles with those obtained for *M. hispanica*, the Tb for this species (10.22ºC) lies between values for *M. incognita* (Tb 10.10ºC and S 400 *DD*) and *M. arenaria* (Tb 12.20ºC and S 313 *DD*). However, the S value for *M. hispanica* (515.46 *DD*) lies between *M. incognita* and *M. hapla* (553 *DD*) (Lahtinen *et al*., 1988; Madulu & Trudgill, 1994; Ploeg & Maris, 1999; Yeon *et al*., 2003). Analysis of the relationship between the rate of development and temperature for these species revealed that, at temperatures below 13.89ºC and 18.78ºC, *M. hispanica* has a shorter life cycle than *M. arenaria* and *M. javanica*, respectively, and that *M. hapla* and *M. incognita* has a shorter life cycle than *M. hispanica* at every temperature studied (Fig. 8). According to Trudgill & Perry (1994), for biologically similar nematode species, S tends to decrease as Tb increases and tropical species generally have higher Tb values than temperate ones. Plotting the Tb values against corresponding S values for the *Meloidogyne* species already studied a negative correlation between S and Tb was confirmed (Ploeg & Maris, 1999) (Fig. 9).

Following the initial nematode penetration at temperatures below 35ºC, several J2s failed to establish and develop in roots of tomato cv. Rossol. At temperatures between 15 and 30ºC from 5 DAI, the necrotic cells observed prevent feeding site establishment and further development. At 25ºC no reproduction occurred after inoculation with 150 J2s and at 20ºC the proportion of adult females recorded on roots was significantly lower from those on tomato cv. Easypeel. At 30 and 35ºC *M. hispanica* development was similar on both tomato cultivars. The tomato *Mi* gene only confers resistance at soil temperatures below 28ºC for some *Meloidogyne* spp.

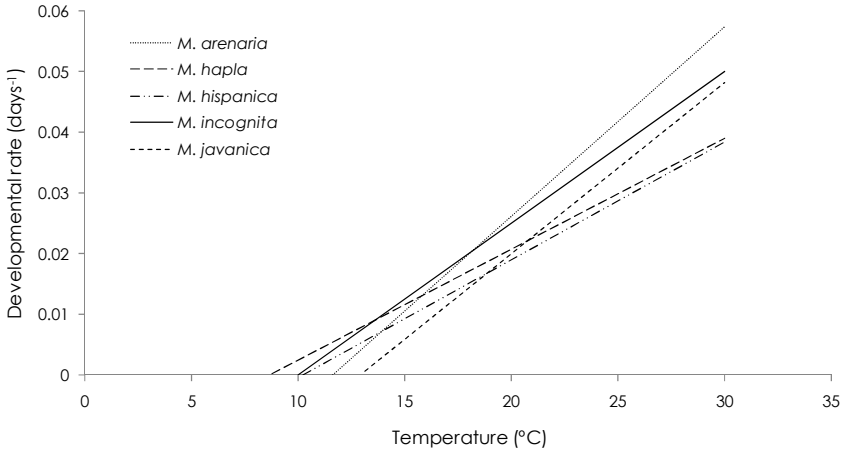

Figure 8 Relationship between the development rate (from J2 to J2) and temperature for five *Meloidogyne* species. Regression lines for *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* calculated from Lahtinen *et al*. (1988), Madulu & Trudgill (1994), Ploeg & Maris (1999), and Yeon *et al*. (2003).

According to our results, *M. hispanica* is a species most suited for soil temperatures around 25ºC. The Tb and S data combined with information on soil temperatures and cropping practices provides a basis for estimating the potential reproductive and aggressiveness and can be used to predict nematode development under different climate conditions (Trudgill, 1995). For example, the distribution and rate of development of *M. incognita*, based on thermal requirements and future climatic scenarios, will increase and the productivity of coffee in Brazil can be compromised (Ghini *et al*., 2008). Thus, predicted climate changes indicate that *M. hispanica* could spread in Southern Europe and move northwards as a tropical RKN.

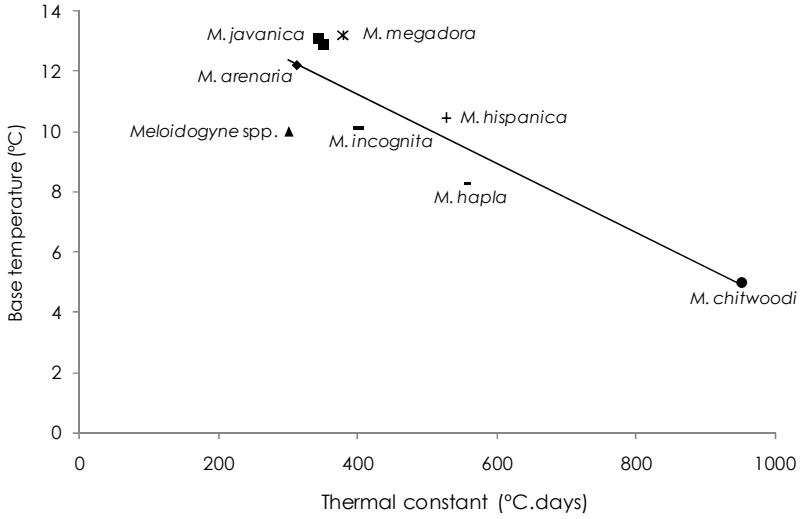

Figure 9 Relationship between the estimated base temperature and thermal constant from J2 to J2 of eight *Meloidogyne* spp. Data for *Meloidogyne* spp., *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica* and *M. megadora* from Tyler (1933), Lahtinen *et al*. (1988), Pinkerton *et al*. (1991), Madulu & Trudgill (1994), Trudgill (1995), Ploeg & Maris (1999), Almeida (A.M.S.F. de Almeida, Escola Superior de Saúde Jean Piaget, personal communication) and Yeon *et al*. (2003).

2.6 Acknowledgments

This research was supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008. Carla Maleita was supported by a FCT-MCTES grant (SFRH/BD/24275/2005) supported by QREN funds through the "Programa Operacional Potencial Humano". Rothamsted Research receives grant aided support from the Biotechnological and Biological Sciences Research Council of the United Kingdom.

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3

Reproduction and virulence of *Meloidogyne hispanica* in cultivated plants

3.1 Abstract

Reproduction of a *Meloidogyne hispanica* isolate from Portugal in 63 plant species/cultivars, from 10 botanical families, was evaluated in pot assays, on the basis of root gall index (GI) and reproduction factor (Rf) 60 days after inoculation. All cultivars of aubergine, bean, beetroot, broccoli, carnation, corn, cucumber, French garlic, lettuce, melon, onion, parsley, pea, potato, spinach, and tobacco and two of cabbage tested were susceptible to *M. hispanica* (GI = 3-5; Rf = 1.15-262.86). Cultivar Bacalan of cabbage, Temporão of cauliflower and Zafiro R2 of pepper were hypersusceptible (Rf < 1; GI > 2). Only the cultivars Aurelio and Solero of pepper were resistant ($0.0 \leq GI \leq 0.4$; $0.00 \leq Rf \leq 0.03$). The reproduction of *M. hispanica* in pepper cultivars was evaluated using two inoculum levels (2,500 and 5,000 eggs) in field conditions (24.4±8.2ºC), and also in a growth chamber at three temperatures (25.0±2.7ºC, 29.3±1.8ºC and 33.6±1.2ºC). At the highest temperature, *M. hispanica* only produced eggs on the cvs Aurelio and Zafiro R2. The eggs obtained from cv. Aurelio were used to produce a selected *M. hispanica* isolate in the same pepper cultivar, by two successive transfers. This new, selected isolate was able to reproduce on all three pepper cultivars, breaking the resistance. Our results suggest that this *M. hispanica* isolate is a mixture of virulent and avirulent individuals and that these pepper cultivars have the potential to contribute to the reduction of *M. hispanica* populations in agro-ecosystems. Nevertheless, the durability of the resistance depends on the frequency of virulent individuals within the population and the continuous exposure of the resistant plants to the nematode.

Keywords: *Me* genes, *N* gene, pathogenicity, pepper, root-knot nematodes

3.2 INTRODUCTION

Meloidogyne hispanica (Hirschmann, 1986) was described for the first time in Seville, Spain, having been isolated from peach rootstock (*Prunus persica silvestris* Batsch). This species has been detected in Australia, Brazil, Burkina Faso, Cape Verde Islands, Fiji Islands, South of France, Korea, Malawi, Martinique, The Netherlands, Portugal, Puerto Rico, Spain, South Africa and United States (Esbenshade & Triantaphyllou, 1985; Fargette, 1987; Trudgill *et al*., 2000; Carneiro *et al*., 2004; Karssen, 2004; Cofcewicz *et al*., 2005; Abrantes *et al*., 2008; van der Wurff *et al*., 2010). In South Africa, *M. hispanica* was found to be associated with sugar cane (*Saccharum officinarum* L.) and was also collected from roots of snapdragon (*Antirrhinum majus* L.), *Amaranthus* sp., *Pelargonium notatum* (L.) L' Herit, grape roots (*Vitis vinifera* L.), and from soil around *Ficus* sp. (Fargette, 1987; Kleynhans, 1993). Kleynhans (1993) concluded, on the basis of perineal pattern, that the isolate collected from roots of granadilla in 1953 at Premier Mine, Pretoria, identified as *M. arenaria thamesi* Chitwood, 1952 (Van der Linde, 1956), corresponded to *M. hispanica*. In Spain, *M. hispanica* was detected on beet (*Beta vulgaris* L.), has reproduced on wheat (*Triticum aestivum* L.), in the greenhouse, and more recently was found on grapevines (Karssen & van Hoenselaar, 1998; Castillo *et al*., 2009). *Meloidogyne hispanica* was also found, in Brazil, in mixed populations with *M. incognita*, *M. javanica* and *Meloidogyne* spp., causing damage on squash plants (*Cucurbita moschata* Duch ex Lam.) and on sugar cane; in Australia on grapevine; in Burkina Faso and Malawi on tomato; in Martinique on banana cv. Cavendish (*Musa* sp.); and in The Netherlands on cucumber, tomato and sweet pepper organic greenhouse associated with *M. incognita* (Hugall *et al*. 1994; Trudgill *et al*., 2000; Carneiro *et al*. 2004; Cofcewicz *et al*., 2005; Chaves *et al*., 2007; van der Wurff *et al*., 2010). In Portugal, *M. hispanica* was found parasitizing roots of fig-trees (*Ficus carica* L.), carnation (*Dianthus caryophyllus* L.) and has been reported alone or in association with *M. javanica* in potato (*Solanum tuberosum* L.) fields (Abrantes *et al*., 2008; Landa *et al*., 2008; Conceição *et al*., 2009). In a previous study, melon (*Cucumis sativus* L.) cvs Gazver and Jazzer, bean (*Phasealus vulgaris* L.) cvs Foicinha and Rajado, corn (*Zea mays* L.) cvs Belgrano and PR35P12, tomato (*S. lycopersicum* L.) cvs Roma, Rutgers and Sinatra, and potato cvs Baraka and Diana, were rated susceptible; lettuce

(*Lactuca sativa* L.) cvs Apulia and Esperie, and tomato Viriato F1 exhibited galls but did not support reproduction; and pepper cv. Galileo was resistant (Maleita *et al*., 2005).

Root knot nematodes (RKN) are usually controlled by the use of nematicides, but these are being phased out due to their harmful effects on human health and the environment (Wesemael *et al*., 2011). Crop rotation including non-host or resistant species can be effective to reduce *Meloidogyne* spp. infections, but to be successful it is important to know the host status of a large number of crops and cultivars thereof.

In pepper, a plant species known to have resistance to RKN, the *N* gene confers dominant resistance to *M. arenaria*, *M. incognita* and *M. javanica*, but has a variable effectiveness depending on the isolate and inoculum level. This gene was found in the cv. Mississipi Nemaheart and was introgressed into two pepper cultivars, thus developing two resistant lines: Carolina Wonder and Charleston Bell (Fery *et al*., 1998). Five genes (*Me1* to *Me5*) were identified in pepper accessions PM217 and PM687 (Hendy *et al*., 1985). *Me1* and *Me3* genes controlled resistance to *M. arenaria, M. incognita and M. javanica*, *Me2* gene to *M. hispanica* and *M. javanica* and *Me4* and *Me5* genes to *M. arenaria*. The *Me* resistance genes, in contrast to *Mi*, were shown to be stable, in terms of conferring resistance at high soil temperatures, which is an advantage in tropical, sub-tropical and warm temperate areas (Dropkin, 1969; Djian-Caporalino *et al*., 2007).

The objectives of this research were: *i*) to evaluate the host suitability of *M. hispanica* in cultivated plants; *ii*) to determine the effects of temperature and initial densities on nematode reproduction in three commercially available pepper cultivars (Aurelio, Solero and Zafiro R2); and *iii*) to assess the presence/absence of *Me1*, *Me3* and *N* resistance genes in these cultivars and in four other pepper accessions.

3.3 MATERIALS AND METHODS

3.3.1 NEMATODE ISOLATE

The *M. hispanica* isolate, originally obtained from infected fig-tree roots collected in Odeceixe, Faro, Portugal, was maintained on tomato, *S. lycopersicum*, cv. Easypeel, in pots containing sterilized sandy loam soil and sand (1:1) in the Nematology laboratory at University of Coimbra, Portugal. The species identification was confirmed by isoesterase phenotype analysis.

3.3.2 HOST STATUS

The resistance of 63 commercial plant species/cultivars (Table 1), comprising 18 plant species and representing 10 botanical families, to *M. hispanica* was evaluated under controlled conditions. Cultivars of bean, corn, cucumber, lettuce, melon, pepper and tomato were grown from seeds. Four-week old seedlings were transplanted into 10 cm-diameter pots filled with a mixture (1:1) of sterilized sandy loam soil and sand. Potato plants were grown from single sprouts in pots with 500 cm^3 of sterilized sandy loam soil and sand (1:2). The plants of the other plant species and cultivars thereof were obtained from a nursery (Germiplanta – Viveiros de Plantas Lda., Portugal) and transplanted into 10 cm-diameter pots containing a mixture (1:1) of sterilized sandy loam soil and sand.

The inoculum was obtained by extraction of eggs from infected cv. Easypeel tomato roots, using 0.52% sodium hypochlorite (NaOCl) (Hussey & Barker, 1973). Five plants from each plant species/cultivar were inoculated with 5,000 eggs (initial population density, Pi). As a positive control, additional pots were planted with tomato cv. Easypeel and inoculated. Also, five pots of each cultivar were not inoculated and served as negative controls. Pots were arranged in a completely randomized design in a growth chamber, at 25±2ºC, with a 12 h photoperiod and 70-75% relative humidity. Plants were watered daily and fertilized weekly with Hyponex® (The Hiponex Co., Inc., Copley Ohio, USA), a water soluble fertilizer (7% N, 6% P and 19% K).

At 60 days after inoculation, the plants were harvested, the root systems washed carefully and rated for galls according to a scale of 0 to 5 (0 = no galls, 1 = 1-2, 2 = 3-10,

3 = 11-30, 4 = 31-100, 5 > 100 galls) (Taylor & Sasser, 1978). Eggs, extracted as described above, were counted to determine the final population density (Pf), and the reproduction factor (Rf = Pf/Pi) was calculated. Host suitability was assessed on the basis of root gall index (GI) and Rf according to the modified quantitative scheme of Canto-Sáenz. Therefore, plants with GI > 2 were considered susceptible (Rf > 1), or hypersusceptible (Rf $\leq$ 1); plants with GI $\leq$ 2 resistant (Rf $\leq$ 1), or tolerant (Rf > 1) (Sasser *et al*., 1984).

3.3.3 Effect of inoculum levels and temperature on *M. hispanica* reproduction in pepper

The cultivars Aurelio, Solero and Zafiro R2 of pepper (Table 1) were used to evaluate the influence of two inoculum densities combined with different temperatures on the reproduction of *M. hispanica*. Five plants of each cultivar were inoculated with 2,500 or 5,000 eggs (Pi) and maintained at 25.0±2.7ºC, 29.3±1.8ºC or 33.6±1.2ºC in a growth chamber, or in field conditions (24.4±8.2ºC), in a completely randomized design. Tomato cv. Easypeel, highly susceptible, was used as a positive control, and cv. Rossol, possessing the *Mi-1* gene for RKN resistance, was used to evaluate the effect of temperature on the efficiency of the *Mi* gene. Also, five pots of each plant species were not inoculated to serve as negative controls. Sixty days later, the plants were uprooted and evaluated for GI and Rf (Sasser *et al*., 1984).

At the highest temperature (33.6±1.2ºC), *M. hispanica* only produced eggs in cvs Aurelio and Zafiro R2. The eggs obtained from cv. Aurelio at this temperature were used to increase the selected *M. hispanica* isolate in the same pepper cultivar by two successive transfers at 25±2ºC. Five four-week-old plants from each of the three cultivars of pepper and tomato cv. Easypeel were then inoculated with 5,000 eggs of the new *M. hispanica* isolate. Experimental conditions, plant maintenance and assessment of the responses were as described above for host status. The species identification of this new isolate was also confirmed by esterase phenotype.

3.3.4 ASSESSMENT OF THE PRESENCE OF GENES *ME1, ME3* AND *N* IN THE PEPPER CULTIVARS

The pepper accessions Yolo Wonder and Doux Long des Landes, lacking the RKN resistance genes *Me*, and the double haploid pepper lines DH149 and DH330, possessing the *Me3* and *Me1* genes respectively, were included in this assessment as controls. Plant DNA extraction was performed as described by Zhang *et al*. (1998) with some modifications. Plant DNA was extracted from leaf tissue, except for the accession DH330 where it was extracted from seeds. Four leaf discs/pepper cultivar were collected opening and closing the lid of a sterile Eppendorf tube to pinch leaf discs directly into the tube. For the accession DH330, the seeds were soaked for 1 h in sterilized distilled water, the seed coat removed, and the embryo and endosperm transferred to a sterile Eppendorf tube. The tubes with leaf or seed samples were then immersed in liquid nitrogen. The tissues were macerated using a pestle and mixed with 800 µl of cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0 and 0.2% mercaptoethanol) at 60ºC. The mixtures were maintained at 60ºC in a water-bath for 20 min and during the incubation they were briefly vortexed several times. After the incubation, 600 µl of phenol:chloroform:isoamyl alchohol (25:24:1) was added and mixed in by vortexing. The extract was centrifuged at 15,441 *g* for 5 min and the supernatant, having been transferred to a new Eppendorf tube, was mixed with equal volume of isopropanol, left in ice for 10 min and centrifuged at 15,441 *g* for 8 min to remove the aqueous phase. The precipitated DNA was washed in 500 µl of 70% ethanol, the pellet dried at 40ºC for 10 min and dissolved in 50 µl of sterilized distilled water.

Amplification of the markers linked to the *Me1* and *Me3* genes as a sequence-characterised amplified region (SCAR) was carried out as described by Djian-Caporalino *et al*. (2007), using the primers CD forward (5´-GAA GCT TAT GTG GTA MCC-3´) and reverse (5´-GCA AAG TAA TTA TAT GCA AGA GT-3´) and B94 forward (5´-GCT TAT CAT GGC TAG TAG GG-3´) and reverse (5´-CGG ACC ATA CTG GGA CGA TC-3´), respectively. Amplification of the marker linked to the *N* gene as a SCAR (forward 5´-AAT TCA GAA AAA GAC TTG GAA GG-3´ and reverse 5´-TAA AGG GAT TCA TTT TAT GCA TAC-3´) was carried out as described by Wang *et al*. (2009). The PCR products were analysed on 1.5-3% agarose gels for *Me1* and *Me3* genes, or on a 15%

polyacrylamide gel in 1× TBE buffer for the *N* gene, stained with ethidium bromide.

3.3.5 DATA ANALYSIS

A few plants died before the end of the host status experiment (Table 1), so some results were missing for cultivars 10044, Arrow, Jaerla, Judia, PR34N43 and PR36B08, these having only four replicates, and cultivars Irazu, Longo da China and PR36R10 having only three (rather than five) replicates. Data (Pf) on host status of cultivated plants were checked for evidence of a Normal distribution using the Kolmogorov-Smirnov test, and for variance homogeneity using Levene's test, and were found to violate these two assumptions of analysis of variance (ANOVA), even after transformations (logarithm and square root). The non-parametric Kruskal-Wallis test was therefore performed to test for differences among the plant species and cultivars. Statistical analysis of the Pf data was performed using Statsoft Statistica version 7 for Windows.

Statistical analysis of data from the experiment considering inoculum levels and temperature on nematode reproduction in the three pepper and two tomato cultivars was performed using GenStat® [2009, 12th edition, ©Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd, Hemel Hempstead, UK]. For this analysis, a square root transformation of the data was used to ensure a Normal distribution and constant variance, as checked by plotting the residuals against the fitted values from the ANOVA. Following ANOVA, least significant difference (LSD) values were used to statistically separate the means of combinations of temperature, inoculum level of *M. hispanica* and cultivar, at 5% level of significance. Pf data for the new isolate of *M. hispanica*, selected in cv. Aurelio and assessed in the three pepper cultivars and tomato Easypeel, were analysed using the Kruskal-Wallis approach as these data also violated the ANOVA assumptions.

3.4 RESULTS

3.4.1 HOST STATUS

Plant species and cultivars thereof varied in their responses to *M. hispanica* and variation in the final population density among replicates was high. *Meloidogyne hispanica* reproduced (Rf > 1) on 58 of the 63 plant species and cultivars (Table 1). These were classified as susceptible and included aubergine, three cultivars of bean, beetroot, one cultivar of broccoli, two of cabbage, carnation, twelve cultivars of corn, two of cucumber, one cultivar of french garlic, five of lettuce, two cultivars of melon, two cultivars of onion, parsley, two cultivars of pea, nineteen cultivars of potato, spinach, tobacco and one cultivar of tomato used as a control, with Rf ranging from 1.15 (beetroot) to 262.86 (potato cv. Vivaldi) (Table 1). Cultivar Zafiro R2 of pepper, Temporão of cauliflower and Bacalan of cabbage were considered hypersusceptible. These cultivars had an average GI, as an indicator of plant damage, greater than 2; however Rf varied from 0.25 (pepper, cv. Zafiro R2) to 0.47 (cauliflower, cv. Temporão) (Table 1). Cultivars Temporão and Bacalan each presented more than 100 galls per plant and an average of 46.40 and 28.80 egg masses (EM), respectively. Pepper Zafiro R2 had a lower number of galls (11.0) and of egg masses (11.4) per plant. In this case, second-stage juveniles (J2) were able to penetrate the roots and establish a feeding site, but only a few completed the life cycle. Cultivars Aurelio and Solero of pepper were resistant ($0.0 \leq GI \leq 0.4$; $0.0 \leq Rf \leq 0.03$) (Table 1). In these pepper cultivars, the J2 invaded the roots, cell necrosis was observed around the nematode head, and only a reduced number of nematodes were able to establish a feeding site, complete the life cycle (EM = 0.4; 0.0, respectively) and produce eggs (Pf = 153±221; 0.0±0.0, respectively). Tomato cv. Easypeel, used as a control, was highly infected with GI = 5 and Rf = 72.42 (Table 1). The uninoculated plants were not galled.

Significant differences in *M. hispanica* reproduction were detected between the plants ($P \leq 0.05$, Kruskal-Wallis test). The Pf across plant species and cultivars thereof indicated that the most susceptible hosts were (in order of susceptibility): Vivaldi > Latona > Agria > Adora > Picasso > Jaerla > Red Scarlet > Safira > Progresso 9 > Monalisa > Sylvana > Bartina > Innovator. The resistant and the *least* susceptible hosts were (in order of susceptibility) PR38R92 > Afiction > Tobacco > Carnation > Verde > Lancelot > Beetroot

> Temporão > Bacalan > Zafiro R2 > Aurelio > Solero. All the other plant species/cultivars outside these two groups did not differ statistically from each other ($P > 0.05$, Kruskall-Wallis test).

Table 1 Host status of cultivated plants to the root-knot nematode *Meloidogyne hispanica*, 60 days after inoculation with 5,000 eggs per plant in a pot experiment conducted in a growth chamber (25±2°C, photoperiod 12 h, 70-75% relative humidity).

Family Plant species (common name)	Cultivar	GI[a]	Pf[b]	Rf[c]	Host status[d]
Alliaceae					
Allium sp. (French garlic)	Lancelot	3.6	6,228±4,292a-i, n-q	1.25	S
Allium cepa L. (Onion)	10044*	4.5	31,292±18,684a-q	6.26	S
	Canataxique	4.2	22,704±13,336a-q	4.54	S
Apiaceae					
Petroselinum crispum (P.Mill.) Nyman ex A.W.Hill (Parsley)	—	5.0	44,193±31,247a-q	8.84	S
Asteraceae					
Lactuca sativa L. (Lettuce)	Irazu**	4.0	63,267±55,823a-q	12.65	S
	Rolina	4.0	53,000±38,240a-q	10.60	S
	Esperie	5.0	15,683±2,964a-q	3.14	S
	Invicta	4.0	15,290±5,272a-q	3.06	S
	Afiction	4.0	13,269±7,814a-i, k-q	2.65	S
Brassicaceae (Cruciferae)					
Brassica oleracea L. var. *botrytis* (Cauliflower)	Temporão	5.0	2,328±871a-i, o-q	0.47	HS
Brassica oleracea L. var. *capitata* (Cabbage)	Tronchuda Portuguesa	5.0	15,367±2,805a-q	3.07	S
	Coração de boi	5.0	14,880±7,981a-q	2.98	S
	Bacalan	5.0	2,207±1,874a-i, o-q	0.44	HS
Brassica oleracea L. var. *italica* (Broccoli)	Verde	5.0	6,923±3,439a-i, n-q	1.38	S
Caryophyllaceae					
Dianthus caryophyllus L. (Carnation)	—	5.0	11,390±4,754a-i, m-q	2.28	S
Chenopodiaceae					
Beta vulgaris L. (Beetroot)	—	3.8	5,773±2,481a-i, n-q	1.15	S
Spinacia oleracea L. (Spinach)	—	5.0	37,933±10,771a-q	7.59	S

(*Continued on next page*)

Table 1 (CONTINUED). Host status of cultivated plants to the root-knot nematode *Meloidogyne hispanica*, 60 days after inoculation with 5,000 eggs per plant in a pot experiment conducted in a growth chamber (25±2ºC, photoperiod 12 h, 70-75% relative humidity).

Family Plant species (common name)	Cultivar	GI[a]	Pf[b]	Rf[c]	Host status[d]
Cucurbitaceae					
Cucumis melo L.	Fiesta	5.0	216,480±68,212a-q	43.30	S
(Melon)	Branco Ribatejo	5.0	20,920±4,551a-q	4.18	S
Cucumis sativus L.	Inglês Comprido	5.0	147,080±58,651a-q	29.42	S
(Cucumber)	Longo da China**	5.0	141,467±124,439a-q	28.29	S
Fabaceae					
Phaseolus vulgaris L.	Bencanta Trepar	5.0	512,520±153,370a-q	102.50	S
(Bean)	Judia*	5.0	234,133±72,849a-q	46.83	S
	Helda	5.0	205,093±132,174a-q	41.02	S
Pisum sativum L.	Progresso 9	5.0	625,920±249,344a-q	125.18	S
(Pea)	Rondo	5.0	370,280±97,383a-q	74.06	S
Poaceae					
Zea mays L.	PR34N43*	5.0	267,900±32,142a-q	53.58	S
(Corn)	Lambada	5.0	169,733±76,882a-g, j-q	33.95	S
	PR34B23	5.0	160,240±35,667a-q	32.05	S
	PR39T84	5.0	138,893±62,094a-q	27.78	S
	PR33Y56	5.0	137,600±39,943a-q	27.52	S
	PR33A46	5.0	132,440±70,047a-q	26.49	S
	PR36Y03	5.0	125,680±23,803a-q	25.14	S
	PR33N09	5.0	98,773±25,313a-q	19.75	S
	PR36R10**	4.0	29,233±15,922a-q	5.85	S
	PR34G13	4.0	28,780±8,747a-q	5.76	S
	PR36B08*	3.0	17,871±11,789a-q	3.57	S
	PR38R92	5.0	14,375±11,695a-i, k-q	2.88	S
Solanaceae					
Capsicum annuum L.	Zafiro R2	2.4	1,238±862a-i, p-q	0.25	HS
(Pepper)	Aurelio	0.4	153±221a-i, q	0.03	R
	Solero	0.0	0.00±0.00a-i	0.00	R
Nicotiana tabacum L. (Tobacco)	—	4.0	12,343±6,796a-i, l-q	2.47	S
Solanum lycopersicum L. (Tomato)	Easypeel	5.0	362,080±44,699a-q	72.42	S
Solanum melongena L. (Aubergine)	—	5.0	77,560±34,109a-q	15.51	S

(*Continued on next page*)

Table 1 (CONTINUED). Host status of cultivated plants to the root-knot nematode *Meloidogyne hispanica*, 60 days after inoculation with 5,000 eggs per plant in a pot experiment conducted in a growth chamber (25±2ºC, photoperiod 12 h, 70-75% relative humidity).

Family Plant species (common name)	Cultivar	GI[a]	Pf[b]	Rf[c]	Host status[d]
Solanaceae					
Solanum tuberosum L.	Vivaldi	5.0	1,314,307±144,312a, j-q	262.86	S
(Potato)	Latona	5.0	1,224,973±119,409a, b, j-q	244.99	S
	Agria	5.0	1,160,400±217,046a-c, j-q	232.08	S
	Adora	5.0	1,015,200±161,033a-d, j-q	203.04	S
	Picasso	5.0	804,120±393,967a-e, j-q	160.82	S
	Jaerla*	5.0	716,867±342,187a-h, j-q	143.37	S
	Red Scarlet	5.0	694,640±194,175a-e, j-q	138.93	S
	Sifra	5.0	673,440±101,789a-e, j-q	134.69	S
	Monalisa	5.0	586,373±39,479a-f, j-q	117.27	S
	Sylvana	5.0	584,400±142,530a-g, j-q	116.88	S
	Bartina	5.0	551,280±290,098a-q	110.26	S
	Innovator	5.0	535,200±110,145a-h, j-q	107.04	S
	Arrow*	5.0	510,300±171,412a-q	102.06	S
	Marfona	5.0	509,520±48,830a-q	101.90	S
	Carrera	5.0	496,560±149,079a-q	99.31	S
	Pekaro	5.0	474,000±340,495a-q	94.80	S
	Kondor	5.0	459,360±227,325a-q	91.87	S
	Red Baron	5.0	438,267±201,523a-q	87.65	S
	Asterix	5.0	433,440±100,041a-q	86.69	S

[a] GI = Gall Index (0-5): 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 > 100 galls per root system.

[b] Pf = final population density. Data are means of three **, four * or five (all other cultivars) replicates ± standard deviation. Means in this column followed by the same combination of letters do not differ significantly at $P > 0.05$, according to the Kruskal-Wallis test.

[c] Rf (reproduction factor) = Pf / initial population density.

[d] Host status category: R = resistant (Rf $\leq$ 1 and GI $\leq$ 2); HS = hypersusceptible (Rf $\leq$ 1 and GI > 2); S = susceptible (Rf > 1 and GI > 2).

3.4.2 EFFECT OF INOCULUM LEVELS AND TEMPERATURE ON *M. HISPANICA* REPRODUCTION IN PEPPER

The response of the nematode varied across the pepper (Aurelio, Solero and Zafiro R2) and tomato (Easypeel and Rossol) cultivars and with respect to temperature and inoculum level (Table 2), as indicated by a significant cultivar by temperature by inoculum interaction for Pf ($P < 0.001$, F-test). For tomato cv. Rossol, the GI and Rf values were greater than or equal to 4.0 and 5.42, respectively, at all temperatures and at the two inocula. The greatest Pf was observed in field conditions (24.4±8.2ºC) for tomato

cv. Easypeel. In controlled conditions, this cultivar showed the greatest Pf at 25±2.7°C. However, cv. Rossol showed the greatest Pf in controlled conditions at 29.3 ± 1.8ºC even when inoculated with 2,500 (Pf = 220,440) rather than 5,000 (Pf = 295,813) eggs, significantly different from Easypeel ($P \leq 0.05$, LSD test, Table 2). Rossol also showed the highest Pf at 33.6±1.2 °C; at this temperature tomato cv. Easypeel plants died before the end of the experiment. Significant differences were observed in the Pf of *M. hispanica* produced for each of the tomato cultivars between the two inocula ($P \leq 0.05$, LSD test); except in field conditions for cv. Rossol, and at 25±2.7ºC for cv. Easypeel. Final population density of *M. hispanica* increased with increasing Pi. The pepper cultivars were largely resistant at all temperatures, but at lower temperatures Pf tended to be greater than at high temperatures. At 29.3±1.8ºC no reproduction occurred, and at 33.6±1.2ºC the Rf varied from 0.00 to 0.01 (cv. Aurelio when inoculated with 5,000 eggs). No differences were detected in Pf of *M. hispanica* among pepper cultivars at each temperature ($P > 0.05$, LSD test) and the two inoculum densities (Table 2).

Table 2 Effect of temperature, in the field (24.4±8.2ºC) and in the growth chamber (25.0±2.7ºC, 29.3±1.8ºC, 33.6±1.2ºC, photoperiod 12 h, 70-75% relative humidity), and initial nematode density (2,500 and 5,000 eggs) on reproduction of *Meloidogyne hispanica* on pepper and tomato cultivars.

Plant species			Pepper (*Capsicum annuum* L.)						Tomato (*Solanum lycopersicum* L.)			
Cultivar			Aurelio		Solero		Zafiro R2		Easypeel		Rossol	
Inoculum level			2,500	5,000	2,500	5,000	2,500	5,000	2,500	5,000	2,500	5,000
Temperature	Field (24.4 ± 8.2ºC)	GI[a]	0.80	1.60	0.60	1.40	0.60	0.40	5.00	5.00	5.00	5.00
		Pf[b]	125±128 (8.6)	436±386 (18.7)	153±222 (7.7)	336±359 (15.5)	77±122 (5.4)	44±62 (4.2)	166,800±42,710 (405.5)	473,413±60,992 (686.9)	19,256±10,889 (134.2)	27,120±8,848 (163.0)
		Rf[c]	0.05	0.09	0.06	0.07	0.03	0.01	66.72	94.68	7.70	5.42
	25 ± 2.7ºC	GI[a]	1.40	1.6	1.80	1.8	0.40	1.2	5.00	5.00	4.00	4.60
		Pf[b]	490±399 (21.0)	793±441 (27.1)	992±557 (30.4)	880±308 (29.3)	133±183 (7.3)	478±485 (18.6)	132,853±23,289 (363.4)	139,387±32,149 (371.1)	19,187±5,479 (137.2)	35,653±14,914 (185.9)
		Rf[c]	0.20	0.16	0.40	0.18	0.05	0.10	53.14	27.88	7.67	7.13
	29.3 ± 1.8ºC	GI[a]	0.00	0.00	0.00	0.00	0.00	0.00	5.00	5.00	5.00	5.00
		Pf[b]	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	79,600±18,035 (280.7)	138,693±33,856 (370.2)	220,440±59,100 (466.2)	295,813±60,046 (541.6)
		Rf[c]	0.00	0.00	0.00	0.00	0.00	0.00	31.84	27.74	88.18	59.16
	33.6±1.2ºC	GI[a]	0.00	1.00	0.00	0.00	0.00	1.00	*	*	5.00	5.00
		Pf[b]	0.00 (0.0)	30±67 (2.4)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	12±279 (1.5)	*	*	44,668±13,576 (209.4)	74,398±40,033 (263.6)
		Rf[c]	0.00	0.01	0.00	0.00	0.00	0.0024	*	*	17.87	14.88

[a] GI = Gall Index (0-5): 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 > 100 galls per root system.

[b] Pf = final population density, evaluated 60 days after inoculation. Data are means of five replicates ± standard deviation. Means on the square root scale for statistical comparison following ANOVA are given in parenthesis; SED = 17.88 on 152 degrees of freedom; LSD (5%) = 35.32.

[c] Rf (reproduction factor) = Pf / initial population density.

*****Plants died before the end of the experiment.

When the selected *M. hispanica* isolate conditioned in cv. Aurelio at 33.6±1.2°C was used to inoculate the three pepper cultivars, the Rf values ranged from 11.57 (cv. Zafiro R2) to 21.42 (cv. Solero), with GI = 5 (Table 3). Cultivars Aurelio and Solero had slightly higher Pf values than cv. Zafiro R2, but the difference was not significant ($P > 0.05$, Kruskal-Wallis test). However, Zafiro R2 had Pf values significantly different ($P \leq 0.05$, Kruskal-Wallis test) and lower than the tomato cv. Easypeel, which was used as a control. No differences were observed between the Pf of *M. hispanica* for cvs Aurelio and Solero and the tomato cv. Easypeel (Table 3).

These pepper cultivars, found to be hosts of the selected *M. hispanica* isolate in this study (Table 3), were considered previously as hypersusceptible or resistant (Tables 1 and 2).

Table 3 Reproduction of three cultivars of pepper, *Capsicum annuum* L., to the selected isolate of *Meloidogyne hispanica* (conditioned on cv. Aurelio at 33.6±1.2°C), 60 days after inoculation with 5,000 eggs per plant in a pot experiment conducted in a growth chamber set at controlled conditions (25±2°C, photoperiod 12 h, 70-75% relative humidity). Tomato Easypeel was used as a control.

Plant species (Common name)	Cultivar	GI[a]	Pf[b]	Rf[c]
Capsicum annuum L. (Pepper)	Aurelio	5	105,720.00±16,534.87ab	21.14
	Solero	5	107,100.00±19,650.57ab	21.42
	Zafiro R2	5	57,840.00±11,076.46b	11.57
Solanum lycopersicum L. (Tomato)	Easypeel	5	139,560.00±65,530.05a	27.91

[a] GI = Gall Index (0-5): 5 > 100 galls per root system.

[b] Pf = final population density. Data are means of five replicates ± standard deviation. Means followed by the same letter do not differ significantly at $P > 0.05$, according to Kruskal-Wallis test.

[c] Rf (reproduction factor) = Pf / initial population density.

3.4.3 ASSESSMENT OF THE PRESENCE OF GENES *ME1*, *ME3* AND *N* IN THE PEPPER CULTIVARS

Amplification of SCAR_CD using DNA from the cultivars of pepper Aurelio, Doux Long des Landes, Solero, Yolo Wonder and Zafiro R2 resulted in a single DNA fragment of approximately 160 bp, indicating the absence of the *Me1* gene. Amplified DNA from pepper DH330, used as a positive control, displayed two bands, a weak band of approximately 160 bp and a strong band of approximately 100 bp confirming the presence of the *Me1* gene (Fig. 1). Amplification of the SCAR_B94 marker presented a band of approximately 240 bp, for the pepper accession DH149, indicating the presence of the *Me3* gene, and a band of approximately 220 bp for the other pepper cultivars, indicating the absence of this gene (Fig. 1). Amplification of the SCAR marker linked to the *N* gene resulted in a band of approximately 330 bp in the three pepper cultivars tested (cvs Solero, Zafiro R2 and Aurelio) and in the cultivars used as negative controls (cvs Doux Long des Landes and Yolo Wonder), indicating the absence of this gene (Fig. 2).

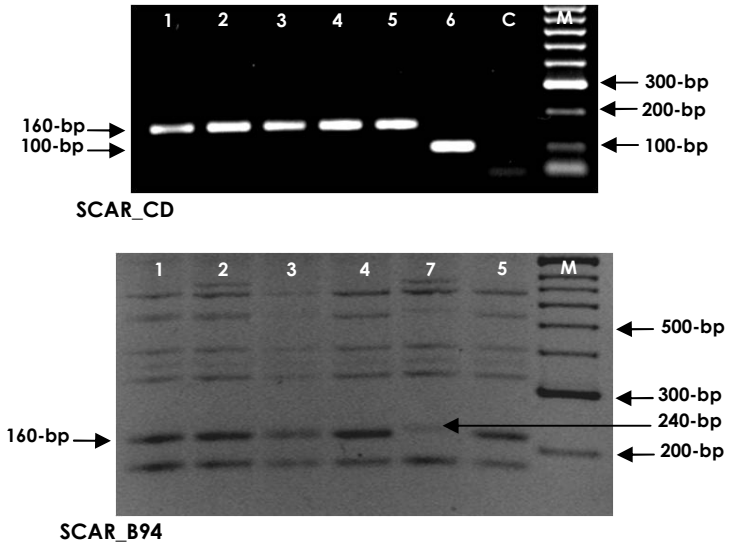

Figure 1 DNA amplification products of *Capsicum annuum* L. cultivars using SCAR_CD and SCAR_B94 linked to the *Me1* and *Me3* genes, respectively. 1- Solero; 2- Zafiro R2; 3- Aurelio; 4- Doux Long des Landes; 5- Yolo Wonder; 6- DH330; 7- DH149; C- negative control; M- DNA marker (HyperLadder II, Bioline).

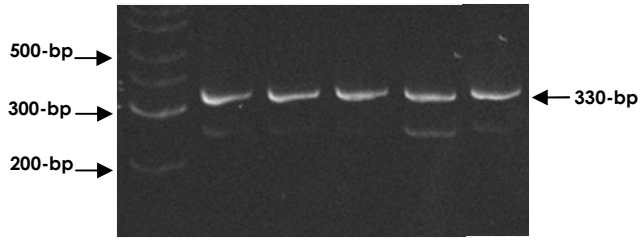

Figure 2 DNA amplification products of *Capsicum annuum* L. cultivars using SCAR linked to the *N* gene. 1- Solero; 2- Zafiro R2; 3- Aurelio; 4- Doux Long des Landes; 5- Yolo Wonder; M- DNA marker (HyperLadder II, Bioline).

3.5 DISCUSSION

Although substantial variation existed among the various plant species and cultivars thereof, with regard to the host suitability parameters measured, only the pepper cvs Aurelio and Solero were resistant to *M. hispanica* (Table 1). Fifty eight of the 63 plant species and cultivars thereof were found to be good or excellent hosts for *M. hispanica* with GI > 2 and Rf > 1. The Rf values ranged from 1.15 (beetroot) to 262.86 (potato cv. Vivaldi). Cultivars of potato exhibited the highest Rf, from 86.69 (cv. Asterix) to 262.86 (cv. Vivaldi) (Table 1). Many species of *Meloidogyne* are known to infect potato and cause significant losses (Brodie *et al*., 1993). In Portugal, only *M. chitwoodi* is considered as a quarantine organism but *M. hispanica* and other *Meloidogyne* spp. have been found frequently in potato fields. Although, their exact involvement in afflicting potato production has not been determined, reduced tuber quality has been observed (Conceição *et al*., 2009).

According to Canto-Sáenz (1985), cabbage cv. Bacalan, cauliflower cv. Temporão and pepper cv. Zafiro R2 were all hypersusceptible to *M. hispanica*, a term proposed by Canto-Sáenz (1985) instead of "intolerant", to indicate a non-efficient host that suffers significant damage (GI > 2) despite the nematode not actually reproducing (Rf < 1).

Cultivar Esperie of lettuce was found to be susceptible in the present study but was listed previously as hypersusceptible. Also, cultivar Zafiro R2 of pepper was found to be hypersusceptible but had been reported as resistant (Maleita *et al.*, 2005).

Research into host status for species such as *M. graminis*, *M. ichinohei, M. kralli, M. naasi* and *M. partityla* showed that the introduction of crop rotations for nematode control is advantageous due to the restriction on good hosts appearing too regularly in rotation (Karssen & Moens, 2006). However, our findings show that *M. hispanica* is a polyphagous species with a wide host range, such as *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*, including plants in several botanical families, suggesting that there are very few options for the control of this nematode by crop rotation. Consequently, it would be wise to avoid these crops in areas known to be infested with this nematode and it is essential to take all measures possible to limit the spread of *M. hispanica* from known infested to non-infested areas. If susceptible plants are successively planted in the same field, the nematode population will increase to levels that will affect plant growth and crop quantity and quality. The host range of

M. hispanica includes Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae. The plant species from these families, except certain plant species and cultivars of families Brassicaceae and Solanaceae, proved to be good hosts for *M. hispanica*. Only the resistant pepper cultivars Aurelio and Solero, appear to be suitable as rotation crops for management of *M. hispanica* populations. These cultivars could be included in rotation in areas where crops highly susceptible to this nematode are currently grown, and so help to reduce nematode population density and prevent possible losses due to *M. hispanica*. Previous studies have shown that the use of non-host, immune or resistant crops, for example grasses, barley and marigold, was highly effective in controlling populations of *Meloidogyne* species. However, for farmers, crop rotation including non-host plant species does not fit in with intensive agricultural practices and also requires investment in machinery and cultural practices. Also, there may not be a market for the non-host crop (Wesemael *et al*., 2011). Furthermore, the duration of resistance in non-host plant species and cultivars thereof can be limited by the breaking of resistance due to changes in environmental conditions and/or the selection to virulence by continuous exposure of the particular nematode species to resistant plants, when they are not included in a crop rotation scheme (Dropkin, 1969; Jarquin-Barberena *et al*., 1991).

According to Dropkin (1969), *Mi*-mediated resistance breaks down at soil temperatures above 28ºC for some *Meloidogyne* species, thus planting during the hot season should be avoided. *Meloidogyne hispanica* can reproduce on tomato cultivars possessing the *Mi* gene (Maleita *et al*., 2011), and, in the present study, the highest reproduction values were recorded at 29.3±1.8ºC and 33.6±1.2ºC for tomato cv. Rossol, which has the *Mi* gene for resistance to RKN. At lower temperatures, the inability of the nematodes to reproduce at the same levels on tomato cv. Rossol as on susceptible tomato cv. Easypeel suggests an incomplete virulence of the nematode or resistant cultivar retain some of their capability to limit nematode development and reproduction (Ornat *et al*., 2001). At 33.6±1.2ºC, the plants of tomato cv. Easypeel died before the end of the experiment, probably due to this high and constant temperature regime being unfavourable for the growth of this cultivar.

Good resistance was shown at higher temperatures for the pepper cultivars, but at lower temperatures Pf tend to increase, 25±2.7ºC being most favorable for reproduction. Thus, at least for the pepper cultivars in the present study, high

temperature may be not a critical factor in the expression of resistance, as soil may reach 33.6±1.2ºC. Consequently, resistance may remain effective in the tropics and warm climates. However, the resistance conferred by the peppers Aurelio, Solero and Zafiro R2 to *M. hispanica* reproduction was not total and a proportion of nematodes were able to reproduce. The selected *M. hispanica* isolate, obtained from plants of cv. Aurelio maintained at the highest temperature (33.6±1.2ºC), was able to reproduce on the three pepper cultivars, breaking the resistance. The variation in reproduction (Pf) among the pepper cultivars and across replicate plants within cultivars suggests that the original isolate of *M. hispanica* was a mixture of virulent and avirulent individuals. Therefore, it will be important to monitor the performance of these cultivars against local populations of the nematode to assess their potential for use in infested soils, because different populations of the same *Meloidogyne* spp. can react differently on the same plant species/cultivar. Moreover, their use over long periods of time may result in the selection of virulent *M. hispanica* isolates able to overcome the resistance. The durability of the resistance depends on the frequency of virulent individuals within the particular local population, and the continuous exposure of the resistant plants to the nematode (Sorribas *et al*., 2005). Pepper cultivars can be an alternative to *M. hispanica* control because it provides a high level of nematode suppression. However, caution should be taken and resistant cultivars should be used in an integrated management context in combination with other control measures, such as nematicides, fallow periods and solarisation or included in crop rotation schemes.

Our studies reveal that *M. hispanica* poses a major threat to many crops due to its ability to attack several plant species and cultivars thereof. Therefore, the inclusion of plant species and/or cultivars resistant to *M. hispanica* will be essential for the efficacy of nematode control through crop rotation.

3.6 Acknowledgments

This research was supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008. Carla Maleita was supported by a FCT-MCTES grant (SFRH/BD/24275/2005) supported by QREN funds through the "Programa Operacional Potencial Humano". The authors would like to thank Dr. Caroline Djian-Caporalino from INRA, Unité Interactions Biotiqes et Santé Végétale (IBSV), France, for the helpful comments and suggestions and for providing the seeds of the pepper accessions Yolo Wonder, Doux Long des Landes, DH149 and DH330 and *Germiplanta – Viveiros de Plantas Lda.*, Portugal and *Pioneer Hi-Bred Sementes de Portugal, S.A.*, Portugal, for providing some plants and seeds. Rothamsted Research receives grant aided support from the Biotechnological and Biological Sciences Research Council of the United Kingdom.

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4

Effect of the *Mi* gene on reproduction of *Meloidogyne hispanica* on tomato genotypes

4.1 Abstract

Root-knot nematode (RKN) resistance *Mi* gene was screened in 25 tomato genotypes of *Solanum lycopersicum*, by amplification of REX-1 and Mi23 markers. Ten heterozygous tomato genotypes (Mimi), nine homozygous (MiMi) at the *Mi* locus and six lacking the *Mi* gene for resistance to RKN were identified using the marker REX-1. The results obtained with Mi23 marker confirmed the *Mi* gene status of the tomato genotypes, except for genotype Valouro RZ F1 that was homozygous (MiMi) and heterozygous (Mimi) at the *Mi* locus when using the REX-1 and Mi23 markers, respectively. The pathogenicity of *Meloidogyne hispanica* on the 25 tomato genotypes was assessed 60 days after inoculation with 5,000 eggs on the basis of root gall index (GI) and reproduction factor (Rf). All the tomato genotypes were susceptible (excellent or good hosts), with GI $\geq$ 4 and Rf > 2, except the genotype Rapit (Mimi) considered as resistant/hypersensitive (poor host). In this genotype, the nematode induced galls (GI = 4) on its roots and a small number of eggs were produced (Pf = 3,085 ± 485). Significant differences in reproduction were detected between the *Mi* allelic conditions and genotypes within *Mi* allelic conditions. The increasing number of *Mi* alleles (0, 1 or 2) is associated with decreasing Rf which suggest a possible dosage effect of the *Mi* gene. The variability observed in the Rf values for MiMi tomato genotypes may reflect an influence of the genetic background of the plants containing the *Mi* gene. Ten of the 25 tomato genotypes with *Mi* gene are commercially available. However, only Rapit can be used to control the three most common *Meloidogyne* spp. and inhibit the increasing of *M. hispanica* populations and may have potential to be included in an integrated pest management programme. However, is advisable to evaluate the pathogenicity of the local populations of this nematode species, associated with different environmental factors.

Keywords: host status, Mi23 marker, resistance gene, REX-1 marker, root-knot nematode, SCAR, *Solanum lycopersicum*

4.2 INTRODUCTION

Root-knot nematodes (RKN), *Meloidogyne* spp., are amongst the world's most damaging agricultural pest, attacking nearly all crops grown. They are distributed worldwide and are parasites of a wide range of economically important plants, especially causing serious damage to tomato crops in warm temperate areas where conditions are favourable for a rapid growth in nematode populations (Netscher & Sikora, 1990; Abad *et al*., 2003; López-Pérez *et al.,* 2006). The most economically destructive species of RKN are *M. arenaria, M. incognita* and *M. javanica*. They are important pathogens in tropical, sub-tropical and warm climates as well as temperate areas having relatively mild winters (Sasser, 1977; Lamberti, 1979; Eisenback & Triantaphyllou, 1991; Abad *et al*., 2003).

Meloidogyne hispanica is one of the lesser know species of RKN, but its ability to attack several plant species and different cultivars thereof makes it a potential threat to several crops (Van der Linde, 1956; Van der Linde *et al.,* 1959; Coetzee, 1968; Stalin *et al*., 1998; Carneiro *et al*., 2004; Maleita *et al*., 2005). *Meloidogyne hispanica* was detected for the first time in Spain from peach rootstock, *Prunus persica silvestris* Batsch (Hirschmann, 1986), and has been reported in Africa, Asia, Europe, North, Central and South America and Australia (Janati *et al*., 1982; Esbenshade & Triantaphyllou, 1985; Fargette, 1987; Abrantes, 1989; Abrantes *et al*., 1994; Karssen & van Hoenselaar, 1998; Trudgill *et al*., 2000; Carneiro *et al*., 2004; Cofcewicz *et al*., 2005). In Portugal, *M. hispanica* was found parasitizing roots of fig-trees, *Ficus carica* L., and carnation, *Dianthus caryophyllus* L., and reported associated with *M. javanica* in fields of potato, *Solanum tuberosum* L. (Abrantes, 1989; Santos *et al*., 1992; Abrantes *et al*., 1994, 2008; Conceição *et al*., 2009).

Plant-parasitic nematodes are generally controlled by chemical nematicides, cultural practices and the growing of resistant cultivars. The use of methyl bromide to control RKN was, until recently, a common practice. However, nematicides include some of the most hazardous compounds used in agriculture and, because of the adverse effects they may have on human health and the environment, specific nematicides are banned in some countries. Therefore, growing resistant cultivars may be the only economically practical management, certainly in developing countries, to control

these pests. Such management is safe and non-polluting, and their use does not require major adaptations in farming practices (Roberts, 1992; Oka *et al*., 2000).

There is an increasing market for organically grown tomatoes, where the use of chemical pesticides is forbidden and alternative methods of nematode control are used (López-Pérez *et al*., 2006). Tomato is one of the crops in which genetic resistance against RKN has been investigated and the *Mi-1* gene found to play an important role. In the 1940s, the RKN resistance gene was introgressed into the cultivated tomato *Solanum lycopersicum* L. from the wild species *S. peruvianum* L. accession PI-128657 using embryo rescue (Smith, 1944). The *Mi-1* gene is located on the upper part of chromosome 6 near the centromere (Kaloshian *et al*., 1998) and belongs to the nucleotide binding site leucine-rich repeat group of genes that are characteristic of a family of plant proteins, including several that are required for resistance against viruses, bacteria and fungi (Milligan *et al*., 1998). Today, many tomato varieties that carry the *Mi-1* gene are available commercially. This gene is the first example of a plant resistance gene active against three very different organisms, namely root-knot nematodes, some isolates of potato aphid, *Macrosiphum euphorbiae*, and whitefly, biotypes B and Q of *Bemisia tabaci*. These pests are among the most important on tomato crops worldwide, making *Mi-1* a valuable resource in integrated pest management programmes (Rossi *et al*., 1998; Nombela *et al*., 2003). Nevertheless, cultivars of tomato with the *Mi-1* gene are not extensively used, probably because markets demand fruits with specific characteristics and qualities that are not matched by the resistant cultivars (Verdejo-Lucas & Sorribas, 2008).

The tomato gene *Mi-1* confers resistance to the three most common RKN species, *M. arenaria*, *M. incognita* and *M. javanica,* namely *Mi-1.2,* one of three genes (*Mi-1.1*, *Mi-1.2*, *Mi-1.3*) identified at the *Mi* locus (Milligan *et al*., 1998; Williamson, 1999). This gene does not confer protection against nematode invasion, but the nematode fails to produce a functional feeding site able to support the development and reproduction of a female (Williamson & Kumar, 2006). Therefore, cultivars possessing the gene can be grown in most infected fields without incurring significant yield and quality losses (Rich & Olson, 1999). However, newly emerged natural isolates of the three nematode species and other *Meloidogyne* spp. can overcome this resistance gene, and so its usefulness for managing these nematodes may be limited (Tzortzakakis & Gowen, 1996; Tzortzakakis *et al*., 1999, 2005; Karajeh *et al*., 2005; Silva *et al*., 2008). In the present context, natural virulence is defined as the ability of nematodes to reproduce on a host

plant that possesses one or more resistance genes without previous exposure, and selection by repeated exposure, to *Mi-1* (Ornat *et al*., 2001; Jacquet *et al*., 2005). Nonetheless, tomato cultivars containing the *Mi-1* gene should be used as part of integrated pest management. For example, used in rotations, such cultivars can help to preserve the durability of resistance in the field, reduce the population of RKN, increase the yield of the subsequent crop and prevent the selection of virulent nematode populations (Rich & Olson, 2004; Verdejo-Lucas & Sorribas, 2008; Talavera *et al*., 2009). Otherwise, expression of the *Mi*-resistance gene can be affected by gene zygosity, depending on whether the resistance gene is in a homozygous or heterozygous condition and temperature (Tzortzakakis *et al*., 1998).

Mi-mediated resistance breaks down at soil temperatures above 28ºC for some *Meloidogyne* species (Dropkin, 1969); however, other *Mi* heat-stable resistance genes to RKN have been identified in the related wild-type species *S. peruvianum*. These genes segregate independently of *Mi*-1 and are mainly located in chromosomes 6 and 12 of tomato. The *Mi-3* gene confers resistance to populations of RKN that are virulent on *Mi*-carrying tomato cultivars, and is effective at 32ºC, a temperature at which *Mi-1* is not effective; *Mi-2, Mi*-5 and *Mi*-6 and *Mi-4* and *Mi*-9 confers heat stability resistance to *Mi*-avirulent *M. incognita, Mi*-avirulent *M. arenaria* and *Mi*-avirulent isolates of the three most common root-knot nematode species, respectively (Cap *et al*., 1993; Veremis & Roberts, 1996a, b; Yaghoobi *et al*., 2005).

The effect of the *Mi-1* gene on the reproduction of *M. hispanica* is unknown, and knowledge about the resistance and host-response of selected tomato genotypes to this nematode is essential for the development of vegetable cropping systems as this species of root-knot nematode is a potential threat to several crops. The objectives of this study were: *i*) to screen the root-knot nematode resistance *Mi-1.2* gene (referred to from now on as *Mi*) in 25 genotypes by DNA amplification using REX-1, and Mi23 markers; *ii*) to evaluate the ability of a Portuguese isolate of *M. hispanica* to reproduce on these tomato genotypes, in controlled conditions; and *iii*) to analyse the influence of the homozygous or heterozygous state at the *Mi* locus on nematode reproduction.

4.3 MATERIALS AND METHODS

4.3.1 DETECTION OF *MI* GENE PRESENCE

PLANT MATERIAL: Twenty five tomato genotypes of *S. lycopersicum* (Table 1) were grown from seeds. The seeds were germinated in a growth chamber at 26-27ºC in Petri dishes with filter paper soaked in distilled water. Then the seedlings were transplanted to 5 cm diam. plastic pots filled with sterilized sandy loam soil and sand (1:1) and maintained in a growth chamber at 25±2ºC, with a 12 h photoperiod and ±60% relative humidity. Plants were watered daily and fertilized weekly with Hiponex® (Hiponex, Copley, OH, USA), a water soluble fertilizer (7% N, 6% P and 19% K).

DNA EXTRACTION: Plant DNA was extracted from leaf tissue as described by Edwards *et al*. (1991) with some modifications. Samples were collected using the lid of a sterile Eppendorf tube to pinch out a disc of material into the tube. The tissue was macerated, using a plastic homogeniser (Biomedix), with 100 µl of extraction buffer (200 mM Tris HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS) in the original tube, at room temperature for 15 s. Then, 300 µl of extraction buffer was added and the sample vortexed for 5 s. The extract was centrifuged at 11,148 *g* for 1 min and 300 µl of the supernatant transferred to a new 1.5 ml Eppendorf tube. The supernatant was mixed with 300 µl isopropanol and left at room temperature for 2 min. Following centrifugation at 11,148 *g* for 5 min, the supernatant was removed and the pellet dried at 60ºC for 20 min and dissolved in 100 µl of sterilised distilled water.

Table 1 Origin and resistances of tomato plants used to screen the root-knot nematodes resistance *Mi* gene, using the PCR-based co-dominant markers REX-1 and Mi23, and to evaluate the reproduction of a Portuguese isolate of *Meloidogyne hispanica*.

Genotype	Seed Company	Resistance[a)]
Avignon	De Ruiter Seeds	Ff:1-5, Fol:0-1, N (MaMiMj), ToMV, Va, Vd
Bond	Seminis	F2, N (MaMiMj), TMV, V
Corianne	De Ruiter Seeds	Fol:0-1, N (MaMiMj), ToMV, Va, Vd
Dixie	Zeta seeds	F2, TMV, V
Dundee	De Ruiter Seeds	C5, For, F2, N, TMV, V
Easypeel	United Genetics Seed CO	Bsp, F1, F2, V
Firamo	Fitó	Ff:1-5, Fol:0-1, N (MaMiMj), ToMV, V
Genaros	De Ruiter Seeds	C5, Ff:1-5, Fol:0-1, N (MaMiMj), ToMV, Va, Vd
Mercurio	De Ruiter Seeds	Fol:0-1, N (MaMiMj), ToMV, TSWV, Va, Vd
Moneymaker	TGSC* (acession LA2706)	———
Monita	TGSC (acession LA2819)	———
Motelle	TGSC (acession LA2823)	———
Optima	Seminis	F1, F2, N, ToMV, V
Oria	Fitó	Ff:1-5, Fol:0-1, N(MaMiMj), ToMV, V
Rapit	Seminis	Fol:0-1, N (MaMiMj), V
Reconquista	De Ruiter Seeds	Fol:0-1, V, ToMV
Reus	Fitó	Fol:0-1, N (MaMiMj), ToMV, V
Rossol	Agri Obtentions	———
Santa Cruz	Seminis	———
Tiny Tim	TGSC (acession LA0154)	———
Tores	Fitó	Fol:0-1, ToMV, V
Valouro RZ F1	Rijk zwaan	Ff:1-5, Fol:0-1, For, Sbl, N (MaMiMj), ToMV, TSWV, TYLCV, Va, Vd
VFN-8	TGSC (acession LA1022)	———
VFNT-Cherr	TGSC (acession LA1221)	———
Viriato	De Ruiter Seeds	C5, F2, N, ToMV, V

[a)] Information collected from product catalogue provided by the seed companies: C5- *Cladosporium fulvum* races A-E; F1- *Fusarium oxysporum* race 1; F2- *Fusarium oxysporum* race 2; Ff:1-5- *Fulvia fulva* races 1-5 ; Fol:0-1- *Fusarium oxysporum* f.sp. *lycopersici*; For- *Fusarium oxysporum* f.sp. *radicis-lycopersici*; N- Nematodes; N (MaMiMj)- Nematodes (*Meloidogyne arenaria*, *M. incognita*, *M. javanica*); Sbl- *Stemphylium botryosum* f.sp. *lycopersici*; TMV- *Tobacco mosaic virus*; ToMV- *Tomato mosaic virus*; TSWV- *Tomato spotted wilt virus*; TYLCV- *Tomato yellow leaf curl virus*; V- *Verticilium* sp.; Va- *Verticillium albo-atrum*; Vd- *Verticillium dahliae*.

*Tomato Genetics Stock Center, University of California, Davis, USA.

ANALYSIS OF TOMATO DNA FOR THE REX-1 AND MI23 ALLELES: The presence of the *Mi* resistance gene was determined using the markers REX-1, frequently used to detect the presence of the *Mi* gene in hybrids of *S. lycopersicum* x *S. peruvianum*, and Mi23, more tightly linked with the *Mi-1.2* gene (Seah *et al*., 2007) as a sequence-characterised amplified region (SCAR). DNA amplification using REX-1 and Mi23 markers was carried out as described by Williamson *et al*. (1994) with some modifications, and Seah *et al*. (2007), respectively. REX-1 primers were: REX-F1 (5'-TCG GAG CCT TGG TCT GAA TT-3') and REX-R2 (5'-GCC AGA GAT GAT TCG TGA GA-3') and the Mi23 primers Mi23F (5'-TGG AAA AAT GTT GAA TTT CTT TTG-3') and Mi23R (5'-GCA TAC TAT ATG GCT TGT TTA CCC-3'). Specifically, for REX-1, 20 µl of PCR reaction mix containing 10 mM Tris (pH 9.0), 50 mM KCl, 2.0 mM MgCl, 0.1% Triton X-100, 0.4 mM dNTPs, 0.5 µm of each primer and 1.0 U *Taq* DNA polymerase (Q-Biogene, Cambridge, UK) were added to 5 µl DNA samples. A similar PCR reaction was used for Mi23, except that 0.25 mM dNTPs, 1.0 µm each of the primers and 0.5 U *Taq* DNA polymerase were used. Amplifications were carried out in a GeneAmp PCR System 2700 Thermalcycler (Applied BioSystems, Carlsbad, CA, USA) using the following conditions: 5 min at 94ºC, 30 cycles at 94ºC for 1 min; 55ºC for 0.5 min and 72ºC for 2 min with a final extension at 72ºC for 7 min for REX-1 marker, and 3 min at 94ºC, 35 cycles at 94ºC for 0.5 min; 57ºC for 1 min and 72ºC for 1 min with a final extension at 72ºC for 10 min for Mi23 marker. Then 5 µl of the PCR reaction was analysed on a 1.0% agarose gel in 1× TBE buffer stained with ethidium bromide.

Following amplification, 5 µl of the REX-1 amplified products were transferred to a new tube and digested at 65ºC with 5.0 U of *Taq*I (Promega, Madison, WI, USA), as recommended by the manufacturer, in a total volume of 10 µl. Initially, for optimisation, the digestion was conducted at different times (1, 2, 4 h or overnight) at 65ºC, so that the time used in experiments was 2 h. Evaluation was performed in a 1% agarose gel in 1× TBE buffer stained with ethidium bromide.

4.3.2 NEMATODE ISOLATE

An isolate of the root-knot nematode *M. hispanica,* obtained from infected fig-tree roots collected in Odeceixe, Faro, Portugal, was maintained on tomato, *S. lycopersicum*, Easypeel, in pots containing sterilized sandy loam soil and sand (1:1) in

the Nematology laboratory at University of Coimbra, and characterized according to perineal pattern morphology and isoesterase phenotype (Abrantes *et al*., 2008).

4.3.3 PATHOGENICITY TESTS

The reproduction of *M. hispanica* on homozygous tomato plants, at the *Mi*/*mi* locus (MiMi, mimi), and heterozygous (Mimi) tomato plants was compared. For this, 4-week old tomato seedlings were transplanted, one per pot, to 10 cm diam. pots (500 cm^3) filled with sterilised sandy loam soil and sand (1:1).

The inoculum was obtained by extracting eggs from the infected Easypeel tomato roots, using 0.52% sodium hypochlorite (NaOCl) (Hussey & Barker, 1973). Five plants from each tomato cultivar were inoculated with 5,000 eggs (the initial population density, Pi). Tomato Easypeel was used as a control. Pots were arranged in a completely randomised design in a growth chamber.

The plants were uprooted after sixty days and the roots were washed free of soil. The number of galls/root system were recorded, and an index of 0-5 (0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 = > 100 galls) assigned (Taylor & Sasser, 1978). Eggs were extracted from each root system with 0.52% NaOCl (Hussey & Barker, 1973) and counted to determine the final population density (Pf). Host suitability was assessed on the basis of root gall index (GI) and reproduction factor (Rf = Pf/Pi), according to the modified quantitative scheme of Canto-Sáenz. Therefore, plants with GI > 2 were considered susceptible (Rf > 1) or resistant/hypersensitive (Rf $\leq$ 1); plants with GI $\leq$ 2 were resistant (Rf $\leq$ 1) or tolerant (Rf > 1) (Sasser *et al*., 1984; Huang, 1985; Almeida & Santos, 2002).

4.3.4 DATA ANALYSIS

A few plants died before the end of the experiment, so some results were missing for the tomato genotypes Monita, Motelle and VFNT-Cherr, whit these having only four (rather than five) replicates. Statistical analysis of the Pf data was performed using GenStat® (2009, 12th edition, Lawes Agricultural Trust (Rothamsted Research), VSN International, Hemel Hempstead, UK). *Mi* allelic condition, based on REX-1 profiles, and tomato genotypes within *Mi* allelic condition were compared using analysis of variance

(ANOVA), with no blocking, as appropriate for the completely randomised design. For this analysis, a natural logarithmic transformation of the data was used to ensure a normal distribution and constant variance, as checked by plotting the residuals against the fitted values from the ANOVA. Following ANOVA, least significant difference (LSD) values were used to separate statistically the means of the three allelic conditions, at 5% level of significance. Similarly, means of genotypes within and across *Mi* allelic conditions may be compared.

4.4 RESULTS

4.4.1 DETECTION OF *MI* GENE PRESENCE

The REX-1 amplified products, using DNA from each tomato genotype, resulted in a single DNA fragment of 750 bp (data not shown). One, 2 or 4 h of digestion of the amplified PCR product from tomato genotype Rossol was sufficient time to digest the totality of the DNA (data not show), 2 h subsequently being used for all cultivars. Restriction of the amplified PCR products from each tomato genotype with *Taq*I enzyme resulted in one, two or three bands of approximately 750, 570 and 160 bp (Fig. 1 A-D).

In the amplified PCR products from Dixie, Easypeel, Moneymaker, Santa Cruz, Tiny Tim and Tores tomato samples (750 bp), no *Taq*I restriction site was present, indicating the absence of the *Mi* gene (mimi). Amplified PCR products from Monita, Motelle, Reconquista, Reus, Rossol, Valouro RZ F1, VFN-8, VFNT-Cherr and Viriato displayed two bands of approximately 570 and 160 bp, and were classified as homozygous at the *Mi* locus (MiMi). Avignon, Bond, Corianne, Dundee, Firamo, Genaros, Mercurio, Optima, Oria and Rapit gave three bands of 750, 570 and 160 bp and were classified as heterozygous at the *Mi* locus (Mimi) (Fig. 1 A-D).

The Mi23 marker allows the amplification of a 380 bp fragment for tomato genotypes without the *Mi* gene (mimi), a fragment of 430 bp for homozygous tomato genotypes at the *Mi* locus (MiMi) and two fragments for heterozygous genotypes at the *Mi* locus (Mimi) (Fig. 1 E, F). No differences were detected between the amplification of the two markers (REX-1 and Mi23), except for tomato Valouro RZ F1 that was homozygous (MiMi) and heterozygous (Mimi) at the *Mi* locus when using the REX-1 and Mi23 markers, respectively (Fig. 1 A,E).

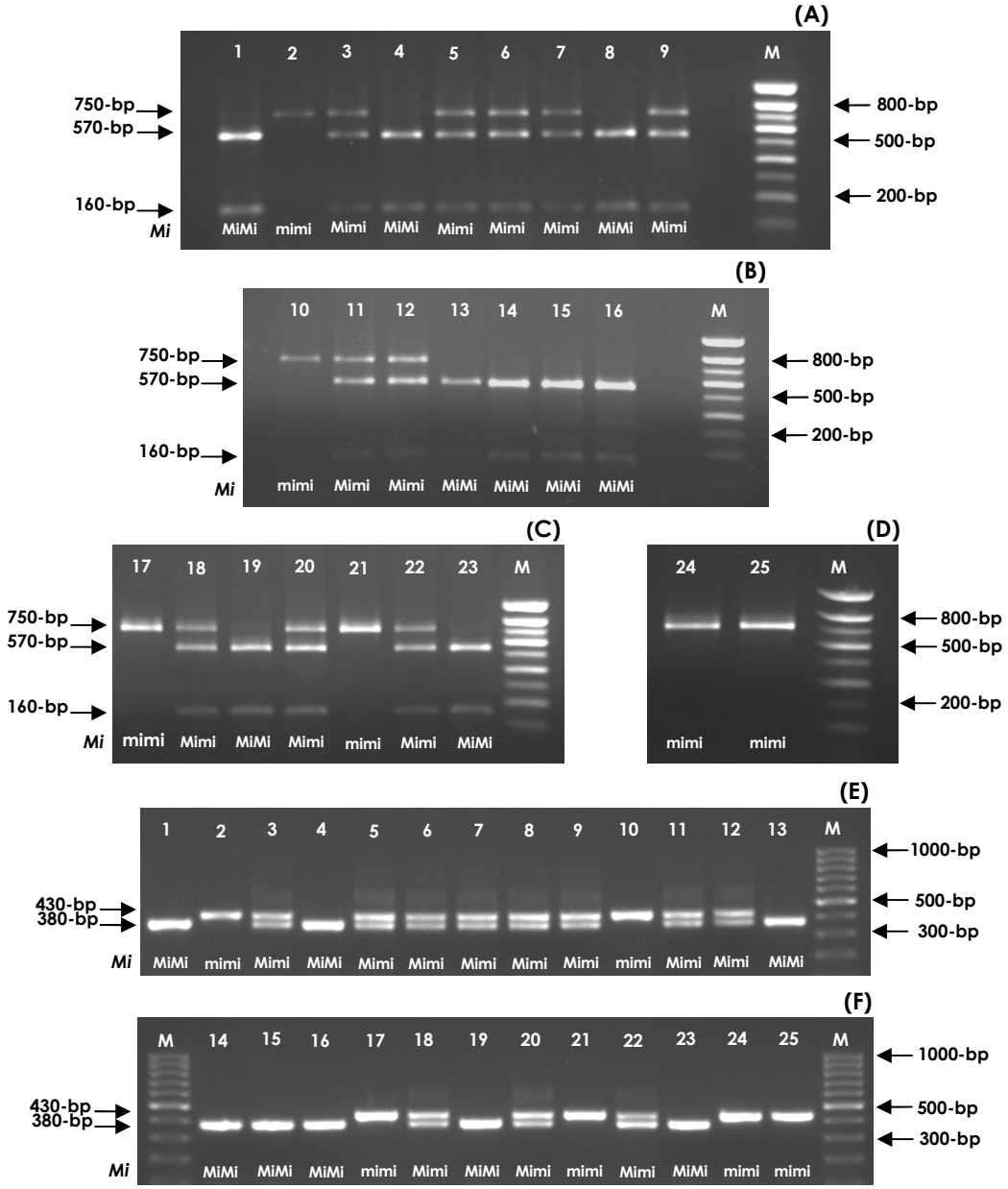

Figure 1 Patterns of amplified DNA of tomato genotypes, using primers REX-F1 and REX-R2, after 2 h of digestion with the restriction enzyme *Taq*I (A-D) and primers Mi23F and Mi23R (E-F). 1-Viriato; 2-Tores; 3- Dundee; 4- Reconquista; 5- Avignon; 6- Bond; 7- Firamo; 8- Valouro RZ F1; 9- Corianne; 10-Dixie; 11- Optima; 12- Genaros; 13- VFN-8; 14- VFNT-Cherr; 15- Monita; 16- Rossol; 17- Easypeel; 18-Oria; 19- Reus; 20- Mercurio; 21- Santa Cruz; 22- Rapit; 23- Motelle; 24- Tiny Tim; 25- Moneymaker; M-DNA marker (A-D: SmartLadder SF 100 bp, Eurogentec; E-F: GeneRuler 100 bp, Fermentas); MiMi-Homozygous at the *Mi* locus; Mimi- Heterozygous at the *Mi* locus; mimi- Homozygous at the *mi* locus.

4.4.2 PATHOGENICITY TESTS

All the tomato genotypes were susceptible except the genotype Rapit (Mimi). In this genotype, the nematode induced galls on its roots (galls = 44.2; GI = 4) with an average of 13 egg masses/root system and a small number of eggs was produced (Table 2). The Rf values of *M. hispanica* for the remaining MiMi, Mimi and mimi tomato genotypes were greater than one (Rf $\geq$ 2.92). The Rf values of *M. hispanica* varied from 0.62 (Rapit) to 102.60 (Dixie). The Rf of *M. hispanica* on genotypes MiMi ranged from 2.92 (Motelle) to 8.08 (Viriato), whereas for those genotypes lacking the *Mi* gene, Rf ranged from 33.67 (Santa Cruz) to 102.60 (Dixie). In heterozygous tomato genotypes (Mimi), the Rf ranged from 0.62 (Rapit) to 41.90 (Mercurio). Genotype Easypeel, used as a control, was highly infected with GI = 5 and Rf = 85.39 (Table 2).

To evaluate statistically the influence of the status of the *Mi* gene (MiMi, Mimi or mimi) on nematode reproduction, the tomato genotypes were separated into three groups according to the REX-1 allelic profile, since the tomato genotype Valouro RZ F1 seems to perform more like a MiMi than a Mimi genotype given its Pf value. Significant differences in *M. hispanica* reproduction were detected between the *Mi* allelic conditions (MiMi, Mimi and mimi) ($P < 0.001$, F-test) and genotypes within *Mi* allelic conditions ($P < 0.001$, F-test) (Table 3). This indicates that not only the status of the *Mi* gene (homozygous *vs* heterozygous) but also the genetic background of the genotypes influence the reproduction of the nematode. The final population density shows that reproduction of the nematode was higher (significantly different, $P < 0.05$, LSD test) on genotypes lacking the *Mi* gene than on those heterozygous and homozygous at the *Mi* gene locus (means of final population on the log scale for *Mi* allelic conditions: MiMi 9.935 (sample size (n) = 42), Mimi 10.727 ($n = 50$), mimi 12.666 ($n = 30$); LSD (5%) = 0.1613 (MiMi *vs* mimi), 0.1558 (Mimi *vs* mimi), 0.1412 (Mimi *vs* MiMi). Tomato genotypes within MiMi revealed that Pf and Rf of Motelle, VFNT-Cherr, Monita, Reconquista and VFN-8 are not significantly different ($P > 0.05$, LSD test) (Table 2).

Table 2 Host status of tomato plants, *Solanum lycopersicum*, for *Meloidogyne hispanica*, 60 days after inoculation with 5,000 eggs per plant and respective REX-1 and Mi23 profile.

Genotype	REX-1 profile	Mi23 profile	GI[a]	Pf[b]	Rf[c]	Host status[d]
Motelle	MiMi	MiMi	5	14,621±3,690 (9.478)	2.92	S
VFNT-Cherr	MiMi	MiMi	4	15,825±3,953 (9.593)	3.17	S
Monita	MiMi	MiMi	5	16,238±1,342 (9.685)	3.25	S
Reconquista	MiMi	MiMi	5	17,546±3,620 (9.693)	3.51	S
VFN-8	MiMi	MiMi	5	17,840±1,328 (9.778)	3.57	S
Reus	MiMi	MiMi	5	24,346±3,120 (10.057)	4.87	S
Rossol	MiMi	MiMi	5	35,653±6,670 (10.420)	7.13	S
Viriato	MiMi	MiMi	5	40,387±7,393 (10.509)	8.08	S
Valouro RZ F1	MiMi	Mimi	5	22,186±1,861 (9.994)	4.44	S
Rapit	Mimi	Mimi	4	3,085±485 (7.987)	0.62	R^H
Oria	Mimi	Mimi	5	25,440±2,668 (10.122)	5.09	S
Firamo	Mimi	Mimi	5	40,081±7,629 (10.524)	8.02	S
Corianne	Mimi	Mimi	5	45,586±7,657 (10.676)	9.12	S
Bond	Mimi	Mimi	5	46,960±4,907 (10.730)	9.39	S
Dundee	Mimi	Mimi	5	69,000±5,202 (11.130)	13.80	S
Genaros	Mimi	Mimi	5	69,787±10,971 (11.103)	13.96	S
Optima	Mimi	Mimi	5	86,546±12,611 (11.313)	17.31	S
Avignon	Mimi	Mimi	5	104,694±11,970 (11.531)	20.94	S
Mercurio	Mimi	Mimi	5	209,470±41,417 (12.158)	41.90	S
Santa Cruz	mimi	mimi	5	168,360±21,190 (12.003)	33.67	S
Moneymaker	mimi	mimi	5	254,266±42,070 (12.379)	50.85	S
Tiny Tim	mimi	mimi	5	308,053±25,259 (12.622)	61.61	S
Tores	mimi	mimi	5	403,362±12,262 (12.906)	80.67	S
Easypeel	mimi	mimi	5	426,967±28,506 (12.956)	85.39	S
Dixie	mimi	mimi	5	513,002±44,714 (13.132)	102.60	S

[a] GI = Gall Index (0-5): 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 = >100 galls per root system.

[b] Pf = final population density. Data are means of four (Monita, Motelle, VFNT-Cherr) or five (all other genotypes) replicates±standard errors. Log data means are given in parenthesis for comparison (REX-1 profile) using LSD (5%) values: 0.4772 (means with four replicates), 0.4268 (means with five replicates), 0.4527 (means with four *vs* means with 5 replicates).

[c] Rf (reproductive factor) = Pf / initial population density.

[d] Host status categories: R = resistant (Rf $\leq$ 1 and GI $\leq$ 2); R^H = resistant/hypersensitive (Rf $\leq$ 1 and GI > 2); S = susceptible (Rf > 1 and GI > 2).

Table 3 Analysis of variance (ANOVA) testing the effects of *Mi* allelic conditions and tomato genotypes within *Mi* allelic conditions (REX-1 profile) on *Meloidogyne hispanica* final population density.

Source	df	*F*-value	*P* > F-value[a)]
Mi allelic condition	2	579.75	< 0.001
Mi allelic condition/tomato genotypes	22	25.78	< 0.001
Residual	97		
Total	121		

[a)] Probability of obtaining an *F*-value greater than that calculated for an *F*-distribution on the degrees of freedom for the respective source and residual terms in the ANOVA.

4.5 DISCUSSION

The reproduction of *M. hispanica*, a pathogen of many crops in five continents, on 25 tomato genotypes with the *Mi* gene for resistance is reported. One major DNA band was amplified, using REX-1 marker, for all tomato genotypes; the detection of one, two or three bands after 2 h of digestion with restriction enzyme *Taq*I indicated the presence or absence of the *Mi* gene and heterozygous or homozygous tomato plants to the *Mi* locus were easily distinguishable (Williamson *et al*., 1994). The Rex-1 marker very often gave false positives for root-knot nematode resistance in tomato hybrids with introgressions of *S. habrochaites* and the Mi23 marker has been specifically useful for tomato breeding programmes (El Mehrach *et al*., 2007; Seah *et al*., 2007). The results obtained with Mi23 confirmed the results obtained with REX-1, except for tomato genotype Valouro RZ F1 (Fig. 1 A,E). Ten of the 25 tomato genotypes with *Mi* gene (two homozygous and eight heterozygous at the *Mi* locus according to the REX-1 profile) are commercially available and all were considered highly resistant to *M. arenaria*, *M. incognita* and *M. javanica* according to the information collected from the product catalogue. Tomato Reconquista was considered homozygous at the *Mi* locus; however no information is available in the product catalogue about resistance to nematodes.

The ability of some *Meloidogyne* spp. and isolates to overcome the tomato *Mi* gene has been reported in earlier experiments, and has been suggested to occur naturally or to have been induced by a selection pressure by repeated exposure to the *Mi* gene in field and laboratory conditions in relatively few generations (Molinari & Caradonna, 2003; Karajeh *et al*., 2005; Tzortzakakis *et al*., 2005; Brito *et al*., 2007; Abd-Elgawad & Molinari, 2008; Silva *et al*., 2008).

Our results indicate that 19 tomato genotypes of the 25 evaluated presented the *Mi* gene and all were considered susceptible or excellent (Rf > 5.0) and good hosts (Rf = 1.1 - 5.0) according to Almeida and Santos (2002) and Ibrahim *et al.* (1993), respectively, except genotype Rapit (Rf < 1). This genotype can be considered as resistant/hypersensitive or a poor host. Statistically, the reproduction of *M. hispanica* on MiMi tomato plants was lower than on Mimi and mimi plants. Therefore, the *Mi* gene does not prevent but limits *M. hispanica* reproduction. The increasing number of *Mi* alleles (0, 1 or 2) is associated with decreasing Rf, which suggest a possible dosage effect of the *Mi* gene. Reproduction of three isolates of *M. javanica* with partial

virulence and some isolates of *M. incognita* was much greater on tomato genotypes heterozygous for the *Mi* gene than on MiMi genotypes, but no difference was observed in highly virulent isolates of *M. javanica* (Tzortzakakis *et al*., 1998; Jacquet *et al*., 2005).

Significant differences in reproduction of *M. hispanica* between MiMi tomato genotypes were observed. Thus, tomato genotypes should be carefully selected, particularly in badly infested soils and especially when the tomato crop will be followed by a nematode-susceptible crop. The variability observed in the Pf and Rf values for these tomato genotypes may reflect an influence of the genetic background of the plants containing the *Mi* gene.

Tomato plants with the *Mi* gene can be grown as an alternative to (or in conjunction with) the use of chemical nematicides, but should be included in an integrated pest management programme as repeated exposure to *Mi* tomato genotypes could lead to a selection of virulent isolates (Eddaoudi *et al*., 1997; Sorribas *et al*., 2005; Verdejo-Lucas *et al*., 2009). It has been shown that a cropping cycle with *Mi* tomato genotypes can reduce initial population density for the next crop, and the effect was similar to the use of nematicides on a susceptible tomato crop (Tzortzakakis *et al*., 2000; Rich & Olson, 2004; Talavera *et al*., 2009).

The use of tomato genotypes with the *Mi* gene (MiMi or Mimi) can be optimised in a rotation sequence of a cropping system. Tomato homozygous genotypes (Reus and Valouro RZ F1) or heterozygous (Rapit, Oria, Firamo, Corianne, Bond, Genaros, Avignon and Mercurio) at the *Mi* locus, available to farmers, can be used to control the three most common *Meloidogyne* spp. but only Rapit will inhibit the increasing of *M. hispanica* populations and may have potential to be included in an integrated pest management programme. However, it is advisable to evaluate the pathogenicity of the local populations of this nematode species, associated with different environmental characteristics. Response to temperature regimes, or other abiotic factors, and system compatibility, including undesirable associations with other pests, disease or agronomic traits, should also be assessed (Roberts, 1992).

In order to understand the parasitism of *M. hispanica* and host response, further research is needed on the biology and ecology of this nematode.

4.6 Acknowledgments

This research was supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008. Carla Maleita was supported by a FCT-MCTES grant (SFRH/BD/24275/2005). The authors would like to thank *Germiplanta – Viveiros de Plantas Lda.*, Portugal and Engenheiro Paulo Abreu from *Monsanto Company, Vegetable Seeds Division*, Portugal for providing information about plant seeds used in this work. Rothamsted Research is an Institute of the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

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5

Effects of inoculum levels of *Meloidogyne hispanica* and *M. javanica* on the nematode reproduction and growth of tomato genotypes

5.1 Abstract

A pot experiment was conducted to determine the effects of three inoculum levels (2,500, 5,000 and 10,000 eggs/plant) on the reproduction of *Meloidogyne hispanica* and *M. javanica* isolates and growth of the susceptible tomato genotypes Easypeel and Moneymaker, used to maintain the *Meloidogyne* isolates, and genotypes Motelle and VFNT-Cherr, which possess the *Mi* gene, under controlled conditions at 25±2°C. Sixty days after inoculation, roots were assessed for gall index (GI) and reproduction factor (Rf). Shoot/root length and fresh/dry root and shoot weight were also recorded. All four tomato plants were susceptible to both isolates of *Meloidogyne* spp. ($4 \leq GI \leq 5$ and $3.44 \leq Rf \leq 317.30$) at all inoculum levels. The *M. javanica* isolate, obtained from an infected potato field, was identified as naturally virulent to the *Mi*-gene, which emphasizes the need of new sources of resistance to root-knot nematodes and for more assessment of *Mi*-tomato plants for their susceptibility to local populations. Otherwise, the use resistant cultivars with the *Mi* gene in crop rotation schemes could be compromised. Reproduction on Motelle and VFNT-Cherr was significantly lower than on Easypeel and Moneymaker; and VFNT-Cherr was the least infected, which suggest a dosage effect of the *Mi* gene and an influence of the genetic background of the plants. For Easypeel and Moneymaker when inoculated with either isolate, there was a trend of decrease in plant growth parameters with higher inoculum level due to damage caused by the increasing number of nematodes that invaded their roots. However, Motelle and VFNT-Cherr remained relatively stable regarding shoot and total shoot plus root dry weight. The reproductive rate of the virulent *M. javanica* isolate was higher than of *M. hispanica,* for all four genotypes tested. Tomato plants inoculated with *M. hispanica* presented the lowest Rf values and the highest values for growth parameters when compared with plants inoculated with *M. javanica*.

Keywords: *Mi gene, root-knot nematodes, Solanum lycopersicum, virulence*

5.2 INTRODUCTION

Nematodes of the genus *Meloidogyne*, commonly known as root-knot nematodes (RKN), belong to a group of plant parasitic nematodes that is widely dispersed around the world. Root-knot nematodes can parasitize virtually all crops, affecting production and quality. This constitutes a major threat to agriculture in temperate and tropical regions and an obstacle to agricultural production in developing countries (Sasser, 1977; Hussey & Janssen, 2002; Abad *et al*., 2003). During their development inside the roots these nematodes release secretions and some cells of the vascular parenchyma tissue become hypertrophied, with intense cellular multiplication and hyperplasia, leading to formation of giant cells and galls. *Meloidogyne* species, as obligate sedentary endoparasites, require healthy plants to support nematode development and reproduction. Differentiation of females is favoured when food is available, since the reproductive function requires a greater expenditure of energy. Alternatively, nematodes differentiate into larger number of males when food availability is reduced (Triantaphyllou & Hirschmann, 1959; Eisenback & Triantaphyllou, 1991).

Meloidogyne infection affects water and nutrient absorption and their translocation by the root system, decreases the rate of photosynthesis in leaves, which is negatively correlated with inoculum levels, and mobilizes photosynthates from shoots to roots, more specifically to giant cells, in order to support nematode development and reproduction (Hussey, 1985; Carneiro *et al*., 1999). Symptoms exhibited by plants infected with RKN lead to suppression of plant yields and is caused by an altered plant metabolism, usually involving debilitation of the root system, leaf nutritional deficiencies, such as chlorosis with temporary wilting in periods of water stress and high temperatures. However, some of these symptoms differ greatly according to plant species and cultivars and can be confused with the damage associated with poor nutrition or injury caused by bacteria, pathogenic fungi and/or viruses (Hussey, 1985; Whitehead, 1997). Stunting, yellowing, internal potato tissue necrosis and brownish and severe galling in potato tubers caused by *M. chitwoodi* and *M. fallax*, forking and hairiness of carrots due to *M. hapla*, yellow patch disease caused by *M. minor*, in grass on golf courses, and stunting, wilting and severe galling of the hosts of *M. ethiopica* are some examples of the effects of these economically important RKN (EPPO/OEPP, 2004, 2011; Moens *et al.*, 2009; Wesemael *et al.*, 2011). The extent of the damage caused by

nematodes is directly proportional to the number of second-stage juveniles (J2) penetrating and becoming established in the root tissue, and their reproduction rate in the plant (Barker & Olthof, 1976; Karssen & Moens, 2006). By increasing the initial population density of *M. javanica* and *Heterodera shachtii* a negative correlation was observed with tomato and pepper, and sugarbeet growth, respectively (Griffin, 1981; Mekete *et al*., 2003). According to Wong and Mai (1973) and Vrain (1982), the greatest reduction in foliage and root weight was observed in lettuce and carrots, respectively, inoculated with the highest inoculum level of *M. hapla*, in growth chamber and field experiments. There was a decline in leaf area and root, shoot, leaf and total plant dry weight with higher inoculum levels of *M. incognita* on tomato (Fortnum *et al*., 1991). High population densities of *M. incognita* resulted in severe reduction of growth or in death, of tomato Roma VF plants, while resistant tomato plants showed negligible growth reduction even at high population densities (Di Vito *et al.*, 1983).

Research of the relationship between initial population densities and plant damage has been performed for the RKN species which are more widely distributed in agricultural areas, but no studies to date have been done with *M. hispanica*. This RKN species was isolated for the first time in Seville, Spain, from peach rootstock (*Prunus persica silvestris*) (Hirschmann, 1986) and there are some records of their occurrence in Africa, Asia, Europe, North, Central and South America and Australia (Landa *et al.*, 2008). This species has the ability to infect and reproduce in a wide range of plant species and cultivars. It presents a great concern for tomato producers due to its ability to reproduce on *Mi*-1.2 gene resistant cultivars (Maleita *et al*., 2011; Maleita unpublished results). The purpose of this study was to determine the effects of the inoculum level of *M. hispanica* and *M. javanica* on nematode reproduction and growth of the tomato genotypes Easypeel and Moneymaker, which are susceptible to *M. hispanica*, and of Motelle and VFNT-Cherr, which possess the *Mi-1.2* gene (referred to from now on as *Mi*).

5.3 MATERIALS AND METHODS

5.3.1 NEMATODE ISOLATES

One isolate of each species of the RKN *M. hispanica* and *M. javanica* obtained from infected fig-tree roots collected in Odeceixe, Faro, and a potato field in Celorico da Beira (Espinheiro), Portugal, respectively, were maintained on tomato, *Solanum lycopersicum* L., cv. Easypeel, in pots containing sterilized sandy loam soil and sand (1:1) in the Nematology laboratory at University of Coimbra, Portugal. The isolates were characterized according to perineal pattern morphology and isoesterase phenotype (Hi4 and J3, respectively) (Abrantes *et al.*, 2008). The isolate of *M. javanica* was included in this experiment for comparison and control.

5.3.2 PLANT MATERIAL

The susceptible (mimi) genotypes, Easypeel and Moneymaker, and the resistant (MiMi) genotypes Motelle and VFNT-Cherr (obtained from C. Rick, Tomato Genetics Stock Center, University of California, Davis, USA) were used in this study.
Tomato seedlings, grown from seeds in a Petri dish with filter paper soaked in distilled water and placed in a growth chamber at 26-27ºC, were transplanted to 5 cm diameter plastic pots filled with sterilized sandy loam soil and sand (1:1) and maintained in the growth chamber at 25±2ºC, with a photoperiod 12 h and ±60% relative humidity. Plants were watered daily and fertilized weekly with Hiponex® (The Hiponex Co., Inc., Copley Ohio, USA), a water soluble fertilizer (7% N, 6% P and 19% K).

5.3.3 POT EXPERIMENT

Four-week old seedlings of each tomato genotype were transplanted to 10 cm diameter pots (500 cm^3), one per pot, filled with sterilized sandy loam soil and sand (1:1). The inocula (eggs) were obtained from infected Easypeel tomato roots, using 0.52% sodium hypchlorite (NaOCl) (Hussey & Barker, 1973). Four plants from each genotype were inoculated with 0, 2,500, 5,000 and 10,000 eggs (initial population density, Pi) of *M. hispanica* or *M. javanica* corresponding to 28 treatments with four

replicates/treatment (four tomato genotypes × two species of *Meloidogyne* × four inoculum levels including controls). Pots were arranged in a completely randomized design in a growth chamber at the same conditions referred above. Some plants of the tomato genotype Motelle died before the end of the experiment, and the number of replicates was two (control) or three in the plants inoculated with *M. javanica*.

Sixty days after inoculation, the plants were uprooted and the roots were washed free of soil. The number of galls/root system was recorded, and an index of 0-5 (0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 > 100 galls per root) assigned (Taylor & Sasser, 1978). Final population densities (Pf) were determined as the total number of eggs+J2 extracted from each root system with 0.52% NaOCl (Hussey & Barker, 1973) and from soil by the Whitehead and Hemming tray method (Hooper, 1986). Second-stage juveniles that migrated to the water were collected one week later, concentrated on a 20 µm pore sieve and counted. Host suitability was assessed on the basis of root gall index (GI) and reproduction factor (Rf = Pf/Pi) according to the modified quantitative scheme of Canto-Sáenz (Sasser *et al*., 1984). Shoot/root length and fresh/dry root and shoot weight were also recorded.

5.3.4 DATA ANALYSIS

Analysis of Variance (ANOVA), with no blocking, as appropriate for the completely randomised design, was applied to the data in order to assess the statistical significance of the main effects and interactions between the three experimental factors. For all analysis, a logarithmic (to base *e*) transformation of the data was used to ensure a Normal distribution and constant variance for the data, as checked by plotting the residuals against the fitted values from the ANOVA. Following the extraction of the significant interaction terms from the ANOVA (using the F-test), means of interest in these terms were compared using the appropriate least significant difference (LSD) values, at the 5% level of significance. Statistical analysis was performed using GenStat® (2009, 12th edition, VSN International, Hemel Hempstead, UK).

5.4 RESULTS

Both isolates of *Meloidogyne* reproduced on all the tomato genotypes, with high numbers of eggs and J2 recovered from roots and soil, so that all four would be considered as susceptible (GI > 2 and Rf > 1) (Table 1). The Rf values for *M. hispanica* varied from 3.44 (VFNT-Cherr inoculated with 5,000 eggs) to 137.65 (Moneymaker inoculated with 2,500 eggs); and for *M. javanica* from 4.29 (VFNT-Cherr inoculated with 10,000 eggs) to 317.30 (Easypeel inoculated with 2,500 eggs). With increasing inoculum of the two *Meloidogyne* isolates, the Rf values decreased in all genotypes, except for tomato VFNT-Cherr inoculated with 10,000 eggs (Table 1, Fig. 1).

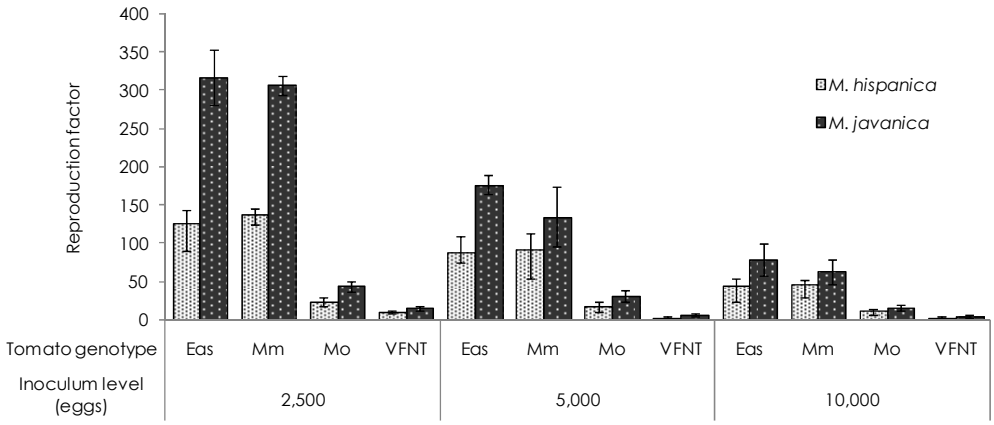

Figure 1 Reproduction of *Meloidogyne hispanica* and *M. javanica* on tomato genotypes Easypeel (Eas) and Moneymaker (Mm), and two other tomato genotypes, Motelle (Mo) and VFNT-Cherr (VFNT) possessing the *Mi* gene, 60 days after the inoculation with either 2,500, 5,000 or 10,000 eggs, at 25±2ºC. Bars represent the standard deviation.

Although the interaction between the tomato genotypes and the *Meloidogyne* isolates was not significant ($P > 0.05$, F-test) for Pf, there was a trend for all genotypes to show higher levels of Pf when inoculated with *M. javanica* than with *M. hispanica*. For all genotypes, the population recovered from roots (Pfr) was significantly higher for *M. javanica* than *M. hispanica* [$P < 0.05$, LSD test; means on log scale from significant ($P = 0.024$, F-test) genotype by isolate interaction: Easypeel *M. hispanica* 12.770, *M. javanica* 13.553; Moneymaker *M. hispanica* 12.819, *M. javanica* 13.357; Motelle *M. hispanica* 11.263, *M. javanica* 11.697; VFNT-Cherr *M. hispanica* 10.029, *M. javanica*

10.344; SED = 0.1090 on 69 df; LSD (5%) = 0.2175]. Genotype VFNT-Cherr was the least infected, with average Pfr ranging from 15,825 to 37,067 eggs and significantly different ($P < 0.05$, LSD test) from Motelle, as the next most infected genotype. The final population recovered from soil (Pfs) was higher for *M. javanica* for all genotypes except Moneymaker, with significant differences between Motelle and VFNT-Cherr [$P < 0.05$, LSD test; means on log scale from significant ($P < 0.001$, F-test) genotype by isolate interaction: Easypeel *M. hispanica* 10.421, *M. javanica* 10.531; Moneymaker *M. hispanica* 10.610, *M. javanica* 10.401; Motelle *M. hispanica* 7.832, *M. javanica* 9.375; VFNT-Cherr *M. hispanica* 7.266, *M. javanica* 8.395; SED = 0.2604 on 69 df; LSD (5%) = 0.5194].

Higher Pf and Pfr were recorded for *M. javanica* than *M. hispanica* at all inoculum levels [$P < 0.05$, LSD test; Pf means on log scale from significant ($P = 0.013$, F-test) inoculum by isolate interaction: 2,500 eggs - *M. hispanica* 11.616, *M. javanica* 12.298; 5,000 eggs - *M. hispanica* 11.754, *M. javanica* 12.338; 10,000 eggs - *M. hispanica* 12.036, *M. javanica* 12.357; SED = 0.0869 on 69 df; LSD (5%) = 0.1733. Pfr means on log scale from significant ($P = 0.033$, F-test) inoculum by isolate interaction: 2,500 eggs - *M. hispanica* 11.550, *M. javanica* 12.204; 5,000 eggs - *M. hispanica* 11.662, *M. javanica* 12.246; 10,000 eggs - *M. hispanica* 11.949, *M. javanica* 12.264; SED = 0.0944 on 69 df; LSD (5%) = 0.1884]. However, the increasing trend for Pf and Pfr over inoculum levels was stronger for *M. hispanica*; for *M. javanica* they only increased slightly. The tomato genotypes were differently affected as inoculum levels increased, as shown by a significant ($P < 0.001$, F-test) genotype by inoculum interaction for both Pf and Pfr. Tomato Motelle had the greatest response, Pf increasing on average by 78% and Pfr by 76%, compared with Pi 10,000 to Pi 2,500. Tomato VFNT-Cherr showed the lowest Pf, with GI $\geq$ 4, for all inoculum levels and Easypeel and Moneymaker the highest (GI = 5). Genotype Moneymaker was relatively slightly affected by the increase in the inoculum level (Table 1).

Table 1 Effects of inoculum levels (2,500, 5,000 and 10,000 eggs) on the reproduction of *Meloidogyne hispanica* and *M. javanica* on tomato, *Solanum lycopersicum* L., genotypes Easypeel, Moneymaker, Motelle and VFNT-Cherr, 60 days after the inoculation, at 25±2ºC. See results section for data means on natural log (to base *e*) scale to consider for statistical comparison.

Nematode isolate	Tomato genotype	Inoculum level	GI[a]	Soil[b] (J2)	Root[b] (eggs)	Pf[c]	Rf[d]
M. hispanica	Easypeel	2,500	5	26,832	287,400	314,232±43,590	125.69
		5,000	5	45,267	392,533	437,800±112,176	87.56
		10,000	5	39,733	405,000	444,733±103,719	44.47
	Moneymaker	2,500	5	33,775	310,350	344,125±21,950	137.65
		5,000	5	50,433	409,867	460,300±106,386	92.06
		10,000	5	46,767	412,267	459,033±59,961	45.90
	Motelle	2,500	5	1,407	57,650	59,057±13,658	23.62
		5,000	5	3,178	85,400	88,578±32,917	17.72
		10,000	5	9,933	105,550	115,483±22,744	11.55
	VFNT-Cherr	2,500	4	1,498	23,975	25,473±5,145	10.19
		5,000	4	1,373	15,825	17,198±7,763	3.44
		10,000	5	1,967	33,950	35,917±3,165	3.59
M. javanica	Easypeel	2,500	5	52,850	740,400	793,250±90,108	317.30
		5,000	5	56,242	827,700	883,942±59,860	176.79
		10,000	5	28,983	764,100	793,083±206,614	79.31
	Moneymaker	2,500	5	46,642	722,100	768,742±31,046	307.50
		5,000	5	31,183	643,800	674,983±191,408	135.00
		10,000	5	53,742	579,300	633,042±161,892	63.30
	Motelle	2,500	5	21,047	88,400	109,447±16,458	43.78
		5,000	5	11,960	141,600	153,560±37,345	30.71
		10,000	5	11,793	148,333	160,127±44,634	16.01
	VFNT-Cherr	2,500	4	3,803	34,667	38,470±6,034	15.39
		5,000	4	4,560	28,200	32,760±10,990	6.55
		10,000	4	5,867	37,067	42,933±20,704	4.29

[a] GI = Gall Index (0-5): 0 = no galls, 1 = 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 = >100 galls per root

[b] Number of second-stage juveniles (J2) and eggs extracted from soil and roots, respectively.

[c] Pf = final population density on soil+roots. Data are means of three (Motelle inoculated with *M. javanica*) or four (all other plants) replicates±standard deviation.

[d] Rf (reproduction factor) = Pf/initial population density.

Root length was only affected by the *Meloidogye* isolates (P = 0.002, F-test). Longer roots were observed in plants infected with *M. javanica* (P < 0.05, LSD test; means on log scale: *M. hispanica* 3.068, *M. javanica* 3.174; SED = 0.0332 on 79 df; LSD (5%) = 0.0661). There was an apparent reduction of root length for inoculated compared with non-inoculated plants [means on log scale for non-inoculated plants by genotype: Easypeel 3.259, Moneymaker 3.687, Motelle 3.242, VFNT-Cherr 3.210; inoculated plants by isolate: *M. hispanica* 3.068, *M. javanica* 3.174; SED = 0.0847 on 79 df; LSD (5%) = 0.1685 comparing non-inoculated to inoculated; SED = 0.1151 on 79 df; LSD (5%) = 0.2290 comparing non-inoculated]. Significant differences in shoot and total shoot plus root length were detected between the genotypes and *Meloidogyne* isolates, and between the genotypes and inoculum levels (significant interactions, P < 0.001, F-tests). Shoot and total shoot plus root length were more reduced in Moneymaker and Motelle when inoculated with *M. javanica* than with *M. hispanica*, whereas genotype VFNT-Cherr inoculated with *M. javanica* had longer shoot and total shoot plus root length than when inoculated with *M. hispanica*. No substantial differences were observed in shoot and total shoot plus root length when comparing the two species of nematode for genotype Easypeel. Shoot and total shoot plus root length decreased with increasing inoculum levels in all genotypes, but most dramatically for Moneymaker, with a reduction, on average, of 42% and 44%, respectively, compared to control. Shoot and total shoot plus root length of genotype VFNT-Cherr was not significantly affected by the inoculum level, comparing the results using Pi 2,500 to those using Pi 10,000 [means on log scale of shoot length for VFNT-Cherr by inoculum levels: 2,500 eggs: 4.080, 5,000 eggs: 4.069, 10,000 eggs: 3.996; SED = 0.0655 on 79 df; LSD (5%) = 0.1304; total shoot plus root length by inoculum levels: 2,500 eggs: 4.414, 5,000 eggs: 4.407, 10,000 eggs: 4.358; SED = 0.0539 on 79 df; LSD (5%) = 0.1072] (Fig. 2).

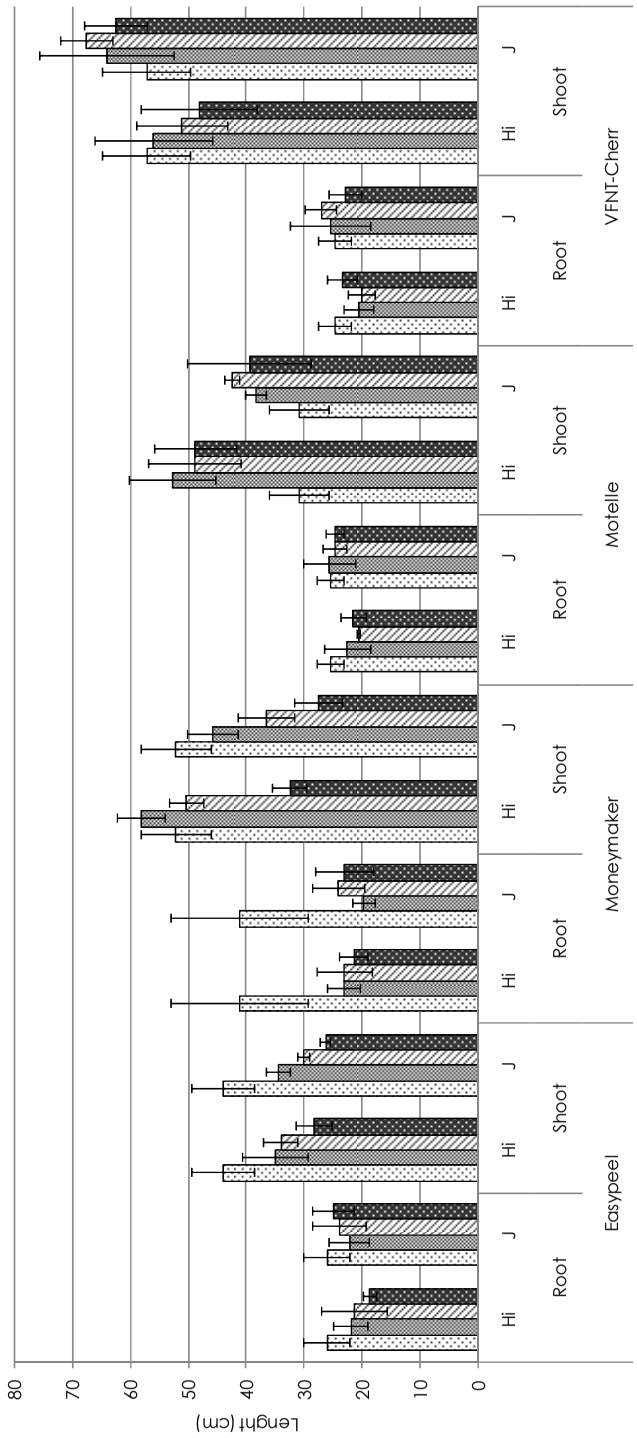

Figure 2 Effects of the initial inoculum level (☒ 0, ▩ 2,500, ▨ 5,000 and ■ 10,000 eggs) of *Meloidogyne hispanica* (Hi) and *M. javanica* (J) on plant length of tomato genotypes Easypeel, Moneymaker, Motelle and VFNT-Cherr. Bars represent the standard deviation. See results section for data means on natural log (to base e) scale to consider for statistical comparison.

Lower root fresh weight (RFW) and dry weight (RDW) was observed in all tomato genotypes infected with *M. javanica* compared to *M. hispanica*, with significant genotype by isolate interactions ($P < 0.001$, F-tests) for both variables (Figs 3 and 4). Although the difference was not significant ($P > 0.05$, LSD test) for tomato Easypeel, it was significant ($P < 0.05$, LSD test) for all other genotypes [means on log scale of RFW: Easypeel *M. hispanica* 1.9037, *M. javanica* 1.8334; Moneymaker *M. hispanica* 1.7167, *M. javanica* 1.5405; Motelle *M. hispanica* 1.6791, *M. javanica* 1.5859; VFNT-Cherr *M. hispanica* 1.7722, *M. javanica* 1.3861; SED = 0.04087 on 79 df; LSD (5%) = 0.08136; means on log scale of RDW: Easypeel *M. hispanica* -0.441, *M. javanica* -0.627; Moneymaker *M. hispanica* -0.739, *M. javanica* -0.943; Motelle *M. hispanica* -0.742, *M. javanica* -1.003; VFNT-Cherr *M. hispanica* -0.596, *M. javanica* -1.241; SED = 0.0593 on 79 df; LSD (5%) = 0.1181]. Easypeel had the greatest RFW in both inoculated and non-inoculated conditions. There were also significant genotype by inoculum interactions for RFW ($P = 0.026$, F-test) and RDW ($P = 0.036$, F-test). When nematode inoculum level increased, RFW and RDW decreased for all genotypes, except Easypeel, for which the two variables remained stable or increased only slightly [means on log scale of RFW by inoculum levels: 2,500 eggs - Easypeel 1.8412, Moneymaker 1.6284, Motelle 1.7320, VFNT-Cherr 1.5991; 5,000 eggs - Easypeel 1.8702, Moneymaker 1.6693, Motelle 1.6377, VFNT-Cherr 1.5728; 10,000 eggs - Easypeel 1.8943, Moneymaker 1.5881, Motelle 1.5279, VFNT-Cherr 1.5657; SED = 0.05006 on 79 df; LSD (5%) = 0.09964; means on log scale of RDW by inoculum levels: 2,500 eggs - Easypeel -0.556, Moneymaker -0.778, Motelle -0.781, VFNT-Cherr -0.769; 5,000 eggs - Easypeel -0.501, Moneymaker -0.797, Motelle -0.909, VFNT-Cherr -1.040; 10,000 eggs - Easypeel -0.546, Moneymaker -0.947, Motelle -0.927, VFNT-Cherr -0.946; SED = 0.0727 on 79 df; LSD (5%) = 0.1446]. Genotype Motelle had the greatest decrease (9%) in RFW comparing non-inoculated plants to those at the highest inoculum level, although both genotypes Motelle and VFNT-Cherr appeared to show a slight increase compared to control for the lowest inoculum level [means on log scale of RFW by inoculum levels: non-inoculated Motelle: 1.6195, VFNT-Cherr 1.5707; 2,500 eggs - Motelle 1.7320, VFNT-Cherr 1.5991; 5,000 eggs - Motelle 1.6377, VFNT-Cherr 1.5728; 10,000 eggs - Motelle 1.5279, VFNT-Cherr 1.5657; SED = 0.06131 on 79 df; LSD (5%) = 0.12204] (Fig. 3).

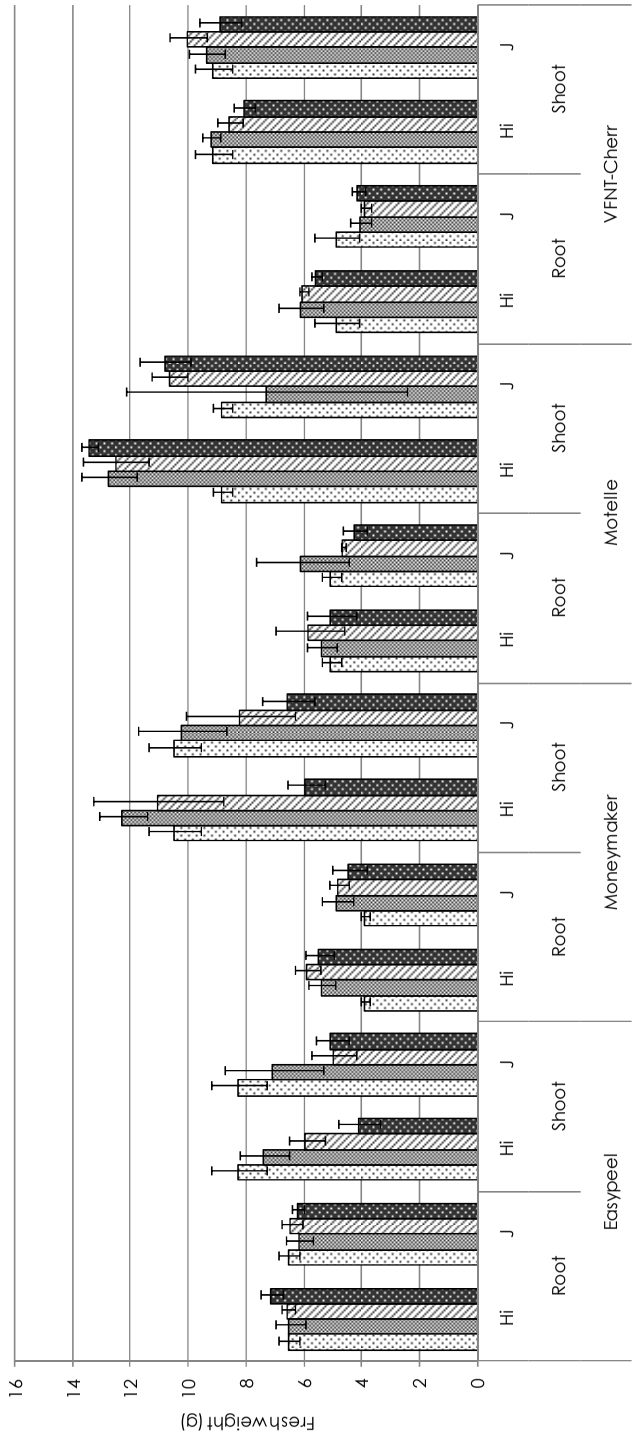

Figure 3 Effects of the initial inoculum level (0, 2,500, 5,000 and 10,000 eggs) of *Meloidogyne hispanica* (Hi) and *M. javanica* (J) on plant fresh weight of tomato genotypes Easypeel, Moneymaker, Motelle and VFNT-Cherr. Bars represent the standard deviation. See results section for data means on natural log (to base e) scale to consider for statistical comparison.

For shoot fresh weight (SFW), there were genotype by isolate and genotype by inoculum interactions ($P < 0.001$, F-tests). SFW was greater for tomato genotypes Easypeel, Moneymaker and Motelle inoculated with *M. hispanica* than with *M. javanica*, the difference being significant ($P < 0.05$, LSD test) for Motelle [means on log scale by genotype: Easypeel *M. hispanica* 1.722, *M. javanica* 1.713; Moneymaker *M. hispanica* 2.223, *M. javanica* 2.091; Motelle *M. hispanica* 2.555, *M. javanica* 2.155; VFNT-Cherr *M. hispanica* 2.152, *M. javanica* 2.241; SED = 0.0832 on 79 df; LSD (5%) = 0.1656]. SFW decreased with higher inoculum levels for all genotypes, except Motelle [means on log scale of SFW by inoculum levels: 2,500 eggs - Easypeel 1.961, Moneymaker 2.411, Motelle 2.133, VFNT-Cherr 2.227; 5,000 eggs - Easypeel 1.685, Moneymaker 2.234, Motelle 2.445, VFNT-Cherr 2.226; 10,000 eggs - Easypeel 1.506, Moneymaker 1.825, Motelle 2.488, VFNT-Cherr 2.136; SED = 0.1019 on 79 df; LSD (5%) = 0.2028]. For shoot dry weight (SDW), there was a genotype by inoculum interaction ($P < 0.001$, F-test) and an isolate by inoculum interaction ($P < 0.001$, F-test). SDW decreased more rapidly with higher inoculum levels of *M. hispanica* than with *M. javanica*, but SDW was greater for tomato plants inoculated with *M. hispanica* [means on log scale of SDW by inoculum levels: 2,500 eggs - *M. hispanica* 0.505, *M. javanica* 0.358; 5,000 eggs - *M. hispanica* 0.337, *M. javanica* 0.142; 10,000 eggs - *M. hispanica* -0.033, *M. javanica* 0.044; SED = 0.0496 on 79 df; LSD (5%) = 0.0986]. SDW decreased dramatically for genotypes Easypeel and Moneymaker with higher inoculum levels, but remained relatively stable for Motelle and VFNT-Cherr [means on log scale of SDW by inoculum levels: 2,500 eggs - Easypeel 0.170, Moneymaker 0.410, Motelle 0.646, VFNT-Cherr 0.501; 5,000 eggs - Easypeel -0.195, Moneymaker 0.090, Motelle 0.593, VFNT-Cherr 0.471; 10,000 eggs - Easypeel -0.595, Moneymaker -0.461, Motelle 0.654, VFNT-Cherr 0.425; SED = 0.0701 on 79 df; LSD (5%) = 0.1395] (Figs 3 and 4).

For total (shoot plus root) fresh weight (TFW) there were significant genotype by isolate ($P = 0.008$, F-test) and genotype by inoculum ($P < 0.001$, F-test) interactions. TFW was less for plants infected with *M. javanica* than for those infected with *M. hispanica* [means on log scale of TFW by genotype: Easypeel *M. hispanica* 2.522, *M. javanica* 2.475; Moneymaker *M. hispanica* 2.709, *M. javanica* 2.551; Motelle *M. hispanica* 2.907, *M. javanica* 2.655; VFNT-Cherr *M. hispanica* 2.674, *M. javanica* 2.597; SED = 0.0449 on 79 df; LSD (5%) = 0.0894]. Also, TFW decreased with higher inoculum levels for all genotypes except Motelle [means on log scale of TFW by inoculum levels: 2,500 eggs - Easypeel

2.601, Moneymaker 2.790, Motelle 2.712, VFNT-Cherr 2.661; 5,000 eggs - Easypeel 2.477, Moneymaker 2.689, Motelle 2.817, VFNT-Cherr 2.656; 10,000 eggs - Easypeel 2.417, Moneymaker 2.411, Motelle 2.813, VFNT-Cherr 2.589; SED = 0.0550 on 79 df; LSD (5%) = 0.1094]. There were significant genotype by inoculum ($P < 0.001$, F-test) and isolate by inoculum ($P = 0.039$, F-test) interactions for total (shoot plus root) dry weight (TDW). Genotypes inoculated with *M. hispanica* showed higher TDW than those inoculated with *M. javanica* at all inoculum levels ($P < 0.05$, LSD test). However, the greatest decline was observed for plants inoculated with *M. hispanica* [means on log scale for TDW by inoculum levels: 2,500 eggs - *M. hispanica* 0.8041, *M. javanica* 0.6317; 5,000 eggs - *M. hispanica* 0.6746, *M. javanica* 0.4654; 10,000 eggs - *M. hispanica* 0.4543, *M. javanica* 0.3777; SED = 0.03728 on 79 df; LSD (5%) = 0.07421]. TDW was substantially lower for genotypes Easypeel and Moneymaker with higher inoculum levels, reductions of up to 70% compared to control being observed. There was a only slightly decrease in TDW for genotype VFNT-Cherr and TDW remained relatively stable for Motelle [means on log scale for TDW by inoculum levels: 2,500 eggs - Easypeel 0.5676, Moneymaker 0.6798, Motelle 0.8652, VFNT-Cherr 0.7590; 5,000 eggs - Easypeel 0.3590, Moneymaker 0.4419, Motelle 0.8006, VFNT-Cherr 0.6786; 10,000 eggs - Easypeel 0.1329, Moneymaker 0.0260, Motelle 0.8437, VFNT-Cherr 0.6614; SED = 0.05273 on 79 df; LSD (5%) = 0.10495] (Figs 3 and 4).

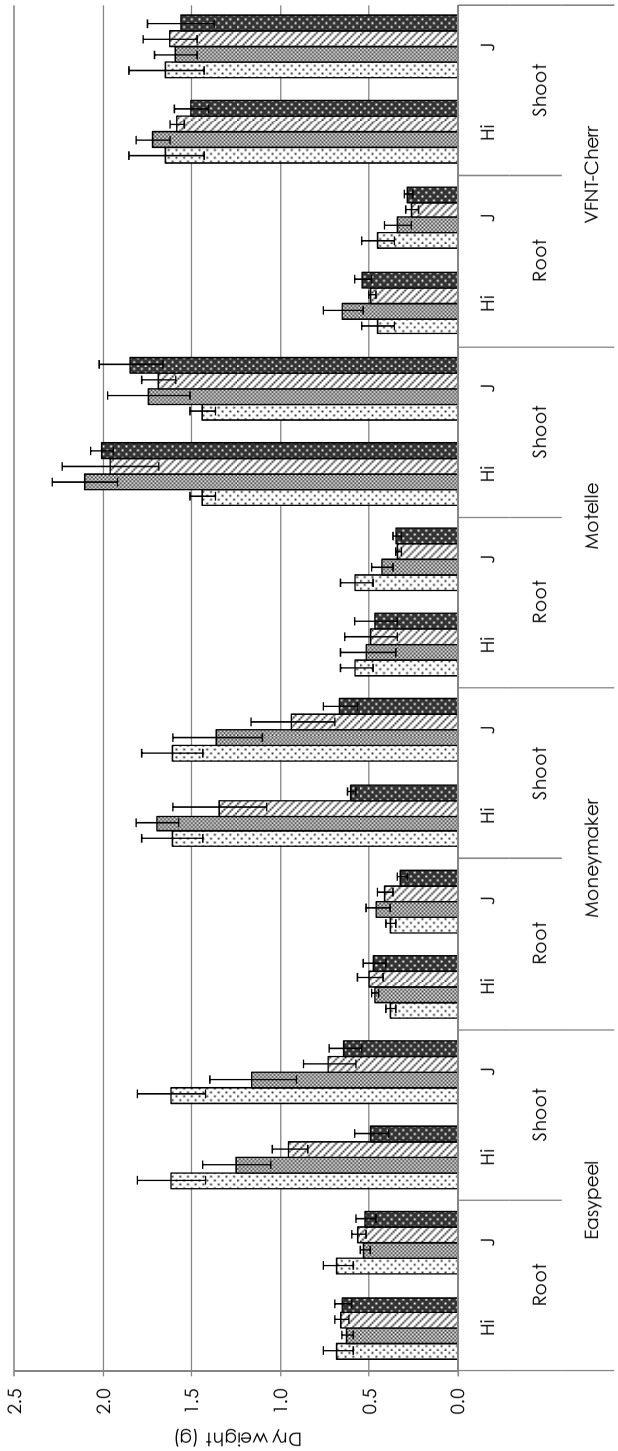

Figure 4 Effects of the initial inoculum level (0, 2,500, 5,000 and 10,000 eggs) of *Meloidogyne hispanica* (Hi) and *M. javanica* (J) on plant dry weight of tomato genotypes Easypeel, Moneymaker, Motelle and VFNT-Cherr. Bars represent the standard deviation. See results section for data means on natural log (to base e) scale to consider for statistical comparison.

5.5 DISCUSSION

Meloidogye hispanica and *M. javanica* isolates were able to reproduce (Rf > 1) on the two tomato homozygous at the *Mi* locus, Motelle and VFNT-Cherr, at all inoculum levels. The tomato *Mi* gene confers resistance to the three most common warm-climate RKN, *M. arenaria*, *M. incognita* and *M. javanica* (Williamson, 1999). However, some isolates of these species, and species of *M. enterolobii*, *M. exigua*, *M. floridensis*, *M. hapla* and *M. hispanica*, can reproduce on tomato genotypes possessing the *Mi* gene (Brown *et al*., 1997; Brito *et al*., 2007; Abd-Elgawad & Molinari, 2008; Silva *et al*., 2008, Maleita *et al*., 2011).

Our results reveal that the Portuguese isolate of *M. javanica* can be considered as virulent, as it breaks resistance and reproduces (Rf > 1) on *Mi* tomato plants. This isolate was obtained, in 1980, from an infected potato field with high yield losses, and has been maintained in the laboratory on susceptible tomato genotypes. The crop rotation scheme used in this field was potato and bean rotated with rye-grass (*Lolium multiflorum* Lam.) and rye (*Secale cereale* L.). Thus, this *M. javanica* population seems to have an inherent ability to reproduce in *Mi* tomato plants as there was no prior exposure of the nematode to the *Mi* gene, at least in the recent cropping history, although the possibility of previous exposure in evolutionary time could be considered (Roberts, 1995; Ornat *et al*., 2001). According to Williamson (1998), nematode virulence to plants with *Mi* gene is stably inherited even after extended growth on susceptible plants. Natural and selected resistance-breaking populations of *M. javanica* have been already reported in Cyprus, Egypt, Greece, Jordan, Morocco, Spain and Tunisia. Selected virulent populations can take place due to pressure exerted on the nematode by their frequent infection or inoculation on tomato plants with the *Mi* gene in field and laboratory conditions, respectively (Philis & Vakis, 1977; Eddaoudi *et al*., 1997; Tzortzakakis *et al*., 1999, 2005; Ornat *et al*., 2001; Molinari & Caradonna, 2003; Verdejo-Lucas *et al*., 2009). The occurrence of nematode populations able to overcome the *Mi* resistance gene in tomato genotypes emphasizes the need for the development of new sources of resistance to RKN, and also for screening of local populations of *Meloidogyne* for their ability to break *Mi* resistance. Otherwise, the successful employment of tomato plants with the *Mi* gene in integrated control strategies such as crop rotation could be compromised.

The reproduction of both *Meloidogyne* isolates on tomato genotypes Motelle and VFNT-Cherr, with *Mi* gene, was lower ($3.44 \leq Rf \leq 43.78$) than on Easypeel and Moneymaker ($45.90 \leq Rf \leq 317.30$); and genotype VFNT-Cherr was the least infected. These results suggest that *Mi* gene provides a partial protection to the development and reproduction of nematodes, which is influenced by the genetic background of the plants (Maleita *et al.*, 2011).

For all tomato plants inoculated with either isolate and with increasing levels of inoculum, there was a decrease in root length and shoot/total (shoot plus root) length. This was most likely due to damage caused by the increasing number of nematodes that invaded their roots, which results in reduced nutrient and water uptake (Karssen & Moens, 2006). According to Carneiro *et al*. (1999), reduction in shoot length is strongly influenced by increased partitioning of carbohydrates to the roots in nematode-infected soybean plants and may vary with nematode species. Mobilization and accumulation of photosynthetic products from shoots to roots reaches a maximum level when the adult females start egg laying (Karssen & Moens, 2006). Substantially lower shoot and total shoot plus root length, and shoot dry weight, were observed on genotype Moneymaker with higher inoculum levels. However, genotype VFNT-Cherr was not greatly affected and Motelle remained relatively stable for as regards shoot dry weight. Total shoot plus root dry weight was also much lower (70%) for genotypes Easypeel and Moneymaker with higher inoculum levels, only slightly lower for VFNT-Cherr, but remained relatively stable for Motelle. The response of these genotypes can be related to the presence of the *Mi* gene and consequently the inability of the nematodes to reproduce at the same levels as on the more susceptible genotypes, which suggest an incomplete or partial nematode virulence (Ornat *et al*., 2001). The results for root and shoot dry weights confirmed the differences between isolates and genotypes and are considered a more realistic measure of the effects of the nematode on the plant growth (Fortmun *et al*., 1991). As well as the relationship between the initial nematode density and corresponding plant damage, the reproduction factor declined as initial nematode density increased.

Both genotypes Motelle and VFNT-Cherr showed a slight increase in root fresh weight for the lowest inoculum level compared to control. This increase, at low levels of inoculum, has been attributed to gall formation and secondary root proliferation (Abrão & Mazzafera, 2001; Carneiro *et al*., 1999).

The tomato genotypes responded differently to *M. javanica* than to *M. hispanica* for Rf and all growth parameters analysed. Although the increase in the level of inoculum lead to a reduction in growth parameters, tomato plants inoculated with *M. hispanica* showed higher growth values than plants inoculated with *M. javanica*, and lower values of Rf. The virulent *M. javanica* isolate presented a higher reproductive and destructive potential than *M. hispanica*, and both nematode species seriously affected the growth of the tomato genotypes tested. This study shows that *M. hispanica* is a real threat to tomato production and suggests that it will be important to monitor the performance of tomato plants with the *Mi* gene in fields infested with *M. javanica*.

5.6 Acknowledgments

This research was supported by FEDER funds through the "Programa Operacional Factores de Competitividade – COMPETE" and by national funds through FCT – Fundação para a Ciência e a Tecnologia under the project PTDC/AGR-AAM/103873/2008. Carla Maleita was supported by a FCT-MCTES grant (SFRH/BD/24275/2005) supported by QREN funds through the "Programa Operacional Potencial Humano". Rothamsted Research receives grant aided support from the Biotechnological and Biological Sciences Research Council of the United Kingdom.

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General Discussion and Conclusions

The first challenge of the research on root-knot nematode (RKN), given the current concerns in relation to climate change and food security, is to find management strategies that can be efficient and sustainable to control the RKN, *Meloidogyne* spp., and increase quality and productivity of important crops.

Although, *M. hispanica* is one of the lesser known RKN, it has been reported in 14 countries. In Portugal, the first report of this nematode was in 1982, initially identified as *M. arenaria* race 2, after that it has been found associated with important crops alone or in mixed populations in the Center and South of the country (Santos & Abrantes, 1982; Santos *et al*., 1992; Abrantes *et al*., 2008; Landa *et al*., 2008; Conceição *et al*., 2009).

In the past, the identification of the most common and economically important *Meloidogyne* spp. was carried out by microscopic examination of morphological characters, mainly perineal patterns of the females, and by the North Carolina differential host test (Hartman & Sasser, 1985). Identification of the RKN species based only in these characters is not reliable even for trained researchers. The morphological characters of the seven Portuguese *M. hispanica* isolates were similar to the described species isolates, and very difficult to differentiate from other *Meloidogyne* spp., mainly from *M. arenaria* and *M. incognita*. Morphometric studies were also performed on females, males and second-stage juveniles (J2) and most of the values were similar among isolates and to the original description. The variability found in the morphological and morphometrical characters confirms that a reliable identification of this RKN should include J2, females and males and must be supplemented by other characters. (Hirschmann, 1985). The problems associated with the morphological characters analysis were overcome through the species-specific enzyme phenotypes obtained by electrophoresis. The seven *M. hispanica* isolates studied exhibited an esterase phenotype characterised by two major and two fainter bands (Hi4) which agrees with the phenotype reported for this species that differentiate it from other *Meloidogyne* spp. (Janati *et al*., 1982; Esbenshade & Triantaphyllou, 1985; Fargette, 1987; Abrantes *et al*., 1995, 2008; Carneiro *et al*., 2004a,b,c; Landa *et al*., 2008). Isozyme analysis by electrophoresis requires the use of adult females and despite some variability observed within *Meloidogyne* spp., has been proved to be an accurate and

rapid technique for species identification and can be suitable on surveys to evaluate RKN frequency and distribution, species characterisation and detect mixed populations (Carneiro *et al*., 1996; Carneiro *et al*., 2000; Carneiro *et al*., 2004a; Cofcewicz *et al*., 2004; Brito *et al*., 2008). PCR techniques are more flexible and can be performed with any nematode stage; therefore it can be used for RKN identification in routine examination of soil samples and also for genetic diversity studies, eliminating the need to use mature females from infected roots obtained from field samples or from established cultures in the laboratory. Recently, much effort has been given to the development of specific markers for *Meloidogyne* spp. using variations in the nuclear and mitochondrial genome (Blok & Powers, 2009). In this study, *M. hispanica* isolates could also be successfully differentiated, from other seven *Meloidogyne* spp., by the amplification products of the mtDNA region that includes part of the *COII*, a variable intergenic region, tRNAHis and part of the 16S rRNA gene, using primers C2F3 and MRH106, and respective nucleotide variations. The amplified product of *M. hispanica* isolates (ca. 1,800 bp) is reported for the first time and is similar to *M. ethiopica*, *M. incognita* and *M. javanica* and is different of *M. hapla* (ca. 650 bp), *M. chitwoodi* (ca. 650 bp), *M. mayaguensis* (ca. 850 bp) and *M. arenaria* (ca. 1,300 bp) (Powers & Harris, 1993; Blok *et al*., 2002; Xu *et al*., 2004; Handoo *et al*., 2005; Tigano *et al*., 2005; Jeyaprakash *et al*., 2006; Devran *et al*., 2009); no amplification occurred with the *M. megadora* isolate. *Meloidogyne* spp. isolates with similar amplified products can be differentiated through the restriction endonucleases *Hinf*I, *Alu*I, *Dra*III and *Bfa*I. *Hinf*I PCR-RFLP only differentiated *M. incognita* from *M. ethiopica, M. hispanica* and *M. javanica.* A detailed analysis of their mtDNA sequences revealed the presence of altered nucleotides among the isolates that created new restriction sites. The restriction patterns generated by enzymes *Dra*III and *Alu*I successfully discriminated *M. hispanica* from *M. ethiopica* and *M. javanica* and these from each other, respectively, as predicted by the restriction enzyme maps. *Bfa*I differentiates *M. hapla* from *M. chitwoodi*, which agree with the sequence information obtained from GenBank database. However, it will be necessary to validate the results by analysing a broad range of *M. hispanica* isolates.

Phylogenetic analysis revealed that *M. hispanica* grouped with mitotic parthenogenetic RKN, but the relationship between this species and species with similar morphological and ecological traits remains unclear. These data limit the confidence of mtDNA sequences to phylogenetic studies and strengthen the importance to take in

account more than one molecular character in the identification and characterization of a particular species (Tigano *et al*., 2005).

As RKN are poikilothermic organisms, the length of their life cycle and distribution are greatly influenced by temperature, but the host plant also play an important role. *Meloidogyne hapla* and *M. chitwoodi* are cryophils and occur most frequently in cooler climates, while *M. arenaria*, *M. incognita* and *M. javanica* are termophils and most common in warmer conditions (Taylor & Sasser, 1978). An increase in temperature can be correlated with an increase of the number of J2 that penetrated roots and with a decrease of time for invasion to take place. Nevertheless, the number of J2 that penetrated in the roots were significantly higher in tomato cv. Rossol, resistant to *M. arenaria*, *M. incognita* and *M. javanica*, at 15 and 35ºC and lower at 20ºC, than on tomato cv. Easypeel, used to maintain *Meloidogyne* spp. on the laboratory. These results differ from those of Griffin & Elgin (1977), Dropkin & Nelson (1960), Carneiro *et al*. (2005) and Moritz *et al*. (2008) who stated that penetration of *Meloidogyne* spp. J2 was not statistically different between resistant and susceptible plants; but the development in resistant plants was seriously compromised and affected by temperature. Post-embryonic development of *M. hispanica* was greatly affected in tomato cv. Rossol. At temperatures below 35ºC, several J2 failed to establish a feeding site and develop (Dropkin, 1969). *Meloidogyne hispanica* is most adapted to temperatures between 25 and 30ºC, a result similar to the already obtained for *M. javanica* and *M. incognita* (Trudgill, 1995; Ploeg & Maris, 1999). No hatching occurred at 10ºC and 35ºC, indicating that these temperatures limit embryogenesis and life cycle completion. At 15ºC, J2 were able to penetrate roots but did not develop within 80 days, thus *M. hispanica* can be considered a thermophil species. Data available on the effects of temperature on the development, within the egg until hatching and life cycle completion, of several *Meloidogyne* spp. were compared with those obtained for *M. hispanica*. At temperatures below 15.9ºC, the eggs of *M. hispanica* develop more slowly than those of *M. arenaria* and more rapidly than *M. javanica* and *M. incognita* at temperatures below 20.04 and 16.75ºC, respectively (Tzortzakakis & Trudgill, 2005) Considering the development from J2 to J2, *M. hispanica* has a shorter life cycle than *M. arenaria* and *M. javanica* at temperatures below 13.89 and 18.78ºC, respectively, and a longer life cycle than *M. hapla* and *M. incognita* at all temperatures (Lahtinen *et al*., 1988; Madulu & Trudgill, 1994; Ploeg & Maris, 1999; Yeon *et al*., 2003). The estimated base temperatures of *M. hispanica* were 10.11 and 10.22ºC and thermal constant 179.5 and

515.46 DD for the development within the egg until hatching and life cycle completion, respectively. These values combined with information of soil temperatures can be used to predict *M. hispanica* development and spread with climate changes. It is possible to predict that *M. hispanica* could establish northwards of Portugal and Europe, spread in southern Europe, and have more generations per year.

Therefore, the knowledge about the host status of a larger number of cultivated plants to *M. hispanica* is required in order to select plants with potential in managing this RKN species. Fifty eight (92%), out of 63 commercial plants, comprising 18 plant species and representing 10 botanical families, were susceptible to *M. hispanica* ($1.15 \leq Rf \leq 262.86$ and $3 \leq GI \leq 5$). Rotations can be effective in minimizing crop losses due to *M. hispanica*, and cultivars Aurelio, Solero and Zafiro R2 of pepper, Temporão of cauliflower, Bacalan of cabbage are less suitable hosts for *M. hispanica*, than the other plants tested, and may offer some options for the management of this species. However, the use of resistant cultivars (Aurelio and Solero of pepper) can be limited due to the break of resistance by the environmental conditions. Effects of the temperature and initial population densities on the nematode reproduction on resistant cultivars and cultivar Zafiro R2 of pepper, previously found as resistant (Maleita *et al*., 2005), did not alter with inoculum level and temperature (24.4±8.2ºC to 33.6±1.2ºC), and consequently resistance may remain effective in tropics and warm climates. Nevertheless, after repeated exposure to resistant plants, a new *M. hispanica* isolate, selected at higher temperatures, was obtained. This isolate was able to reproduce ($11.57 \leq Rf \leq 21.42$) on the three pepper cultivars breaking the resistance, suggesting that the original *M. hispanica* isolate was a mixture of virulent and avirulent nematodes. According to Sorribas *et al*. (2005), the durability of resistance depends on the frequency of virulent individuals and the continuous exposure of resistant plants to the nematode. Performance of these cultivars should be monitored against local populations of the nematode before being used in infested soils and integrated on pest management programmes. The selected *M. incognita* isolates, obtained by repeated exposure to resistant tomato, reproduced less efficiently on susceptible cultivars. The rotation of resistant with susceptible cultivars can limit the selection of virulent isolates and delay the durability of the plant resistance (Castagnone-Sereno *et al*., 2007).

Research on host status showed that rotations with non-host species for the management of *M. hispanica* are difficult. Another important control strategy is the use of plant resistance, probably the oldest and the most effective, environmentally safe

and economically practical management strategy in developing countries, which use does not require major adaptations in farming practices (Roberts, 1992; Oka *et al*., 2000). The most important and studied example of resistance is conferred by the *Mi-1* gene introduced into cultivated tomato *Solanum lycopersicum* L. from the wild species *S. peruvianum* L. The tomato gene *Mi-1*, namely *Mi-1.2*, confers resistance to the three most common species of RKN, *M. arenaria*, *M. incognita* and *M. javanica* (Williamson, 1999). Several tomato cultivars that carry the *Mi-1.2* gene are commercially available, but are not often used (Verdejo-Lucas & Sorribas, 2008). In this work, 25 tomato genotypes were screened to the RKN resistance *Mi-1.2* gene using REX-1 and Mi23 markers. No differences were obtained with both markers, except for tomato Valouro RZ F1 that was homozygous and heterozygous at the *Mi* locus when using REX-1 and Mi23 markers, respectively. The Rex-1 marker very often gave false positives for RKN resistance in tomato hybrids with introgressions of *S. habrochaites* and the Mi23 marker has been specifically useful for tomato breeding programmes (El Mehrach *et al*., 2007; Seah *et al*, 2007). According to the information collected from the supplier's product catalogue, ten of the 14 tomato genotypes with the *Mi-1.2* gene, commercially available and used by farmers and nurseries (two homozygous and eight heterozygous at the *Mi* locus, according to the REX-1 profile), are considered highly resistant to *M. arenaria*, *M. incognita* and *M. javanica*; and three (Viriato, Dundee and Optima) resistant to nematodes in general. Tomato Reconquista was considered homozygous (MiMi), but in the supplier's catalogue no information is provided about the resistance to nematodes. All these tomato genotypes and another five not available to the farmers possess the *Mi-1.2* gene (MiMi and Mimi), but do not suppress *M. hispanica* infection and reproduction and were considered good or excellent hosts, with exception for genotype Rapit. Only this genotype can be used to control the three most common RKN and inhibit the increase of *M. hispanica* populations. Even so, *Mi-1.2* gene limits *M. hispanica* reproduction, because the increasing number of *Mi* alleles (0, 1 or 2) is associated with decreasing of reproduction factor suggesting a possible dosage effect of the gene (Tzortazakakis *et al*., 1998; Jacquet *et al*., 2005).

Damage extension caused by nematodes is directly proportional to initial population density in the soil and their reproduction on plant (Wong & Mai, 1973; Griffin, 1981; Vrain, 1982; Hussey, 1985; Fortnum *et al*., 1991; Mekete *et al*., 2003; Karssen & Moens, 2006). To understand the possible effect of *Mi-1.2* gene on growth of tomato genotypes and the inoculum level on final population densities, tomato genotypes Motelle and

VFNT-Cherr, homozygous at the *Mi* locus, and Easypeel and Moneymaker, susceptible to *Meloidogyne* spp., used for comparison, were inoculated with 2,500, 5,000 and 10,000 eggs of *M. hispanica* or *M. javanica*. These tomato genotypes revealed a trend to show lower values of growth parameters (shoot/root length and fresh/dry root and shoot weight) due to the damage caused by *M. hispanica* and *M. javanica* as the number of nematodes that penetrated their roots increase. However, genotypes with *Mi-1.2* gene remained relatively stable regarding shoot and total shoot plus root dry weight. Reproduction factors on tomato genotypes with *Mi-1.2* gene were lower than on the susceptible ones; and genotype VFNT-Cherr was the least infected. *Mi-1.2* gene provides a partial protection to development and reproduction of nematodes, which is influenced by the genetic background of the plants (Roberts, 1995). *Meloidogyne javanica* isolate from Portugal, identified as naturally virulent to *Mi-1.2* gene, showed a higher reproduction and destructive potential than *M. hispanica*. The current use of *Mi-1.2* can be limited and the results reinforce the need for the evaluation of the pathogenicity of the local populations before the use of resistant plants in the field.

The work developed in this research increased the knowledge of the biology and ecology of *M. hispanica*. Early sampling and diagnostic analysis of the species found in the soil is the first procedure to prevent the damage on the crops and although it implies extra-costs, the farmers will be compensated by the reduction of the crop losses due to the possibility of developing a specific integrated pest management program. Isozyme analysis remains the first stage for the RKN identification, when females are available, but a molecular diagnostic method is presented to have a rapid and accurate identification of *M. hispanica* based on J2. The RFLP strategy implemented requires only three steps for the identification of *M. hispanica* isolates and one to four for the differentiation of eight important and damaging RKN species (*M. arenaria*, *M. chitwoodi*, *M. ethiopica*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. mayaguensis*) (Chapter 1).

The ability of *M. hispanica* to spread in Europe and move northwards (Chapter 2), to attack at least 87 economically important plant species and cultivars, from Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae (Chapters 3 and 4), to overcome the tomato *Mi-1.2* gene (Chapter 4), and to cause damage on tomato

crops (Chapter 5) make it to be regarded as a polyphagous species of emerging importance for economically important crops, difficult to control by crop rotations and resistance.

These findings combined with the wide geographical distribution of *M. hispanica* and the diminishing availability of effective nematicides, demonstrate the potential economic impact of this species to the agricultural areas where it is present and the need for the development of preventive measures to avoid its spread.

Conclusions

►The morphobiometrical characters of seven Portuguese *M. hispanica* isolates were similar to the original description. However, the differentiation of the isolates of this species, based only on these characters, can be misinterpreted with other *Meloidogyne* spp., mainly with *M. arenaria* and *M. incognita* (Chapter 1).

►The esterase phenotype (Hi4) is a reliable character for the identification of *M. hispanica* isolates, differentiating them from other *Meloidogyne* spp. (Chapter 1).

►The amplification of the *M. hispanica* mtDNA region, between *COII* and 16S rRNA genes, produced a product of ca. 1,800 bp similar to that obtained with *M. ethiopica, M. incognita* and *M. javanica* and distinct from *M. hapla* (650 bp), *M. chitwoodi* (650 bp), *M. mayaguensis* (850 bp) and *M. arenaria* (1,300 bp) (Chapter 1).

►*Hinf*I and *Dra*III restriction patterns discriminated *M. hispanica* from *M. ethiopica*, *M. incognita* and *M. javanica* (Chapter 1).

►Phylogenetic analysis using the mtDNA sequences was not enough to differentiate *M. hispanica* from other species with similar morphological or ecological traits (Chapter 1).

►The estimated base temperatures were 11.49ºC and 10.22ºC and thermal constant 76.92 and 515.46 *DD* for embryogenesis and life cycle completion, respectively (Chapter 2).

►The number of *M. hispanica* J2 that penetrated on the tomato cvs Easypeel and Rossol roots increased as temperature increase but several J2 failed to establish and develop in roots of tomato Rossol, with the *Mi-1.2* gene, at temperatures below 35ºC (Chapter 2).

►*Meloidogyne hispanica* is most suited to soil temperatures around 25ºC and with climate change may spread in Southern Europe and move northwards (Chapter 2).

►*Meloidogyne hispanica* has a wide host range including economically important plant species and cultivars (87) from Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Poaceae and Solanaceae botanical families, and can overcome *Mi*-mediated resistance (Chapters 3 and 4).

►Only the resistant cultivars Aurelio, Solero and Zafiro R2 of pepper can be used to inhibit the increasing of *M. hispanica* populations in the soil and can be considered as an alternative to the use of chemical nematicides (Chapters 3 and 4).

►Pepper cultivars initially resistant were susceptible to a *M. hispanica* isolate selected at higher temperatures and by repeated exposure of the resistant plants to the nematode (Chapter 3).

►Ten heterozygous tomato genotypes (Mimi), nine homozygous (MiMi) at the *Mi* locus, according to the REX-1 profile, and six lacking the *Mi-1.2* gene for resistance to RKN were identified and the reproduction was higher on genotypes lacking the *Mi-1.2* gene and in Mimi genotypes (Chapter 4).

►*Meloidogyne hispanica* showed a lower reproductive potential than *M. javanica* isolate identified as naturally virulent to *Mi-1.2* gene (Chapter 5).

►Tomato genotypes Easypeel, Moneymaker, Motelle and VFNT-Cherr revealed a trend to show lower values of growth parameters due to the damage caused by *M. hispanica* related with the increase of the number of nematodes that invaded their roots (Chapter 5).

►Shoot and total shoot plus root dry weight of genotypes Motelle and VFNT-Cherr, with the *Mi-1.2* gene, were not affected by infection by *M. hispanica* (Chapter 5).

►*Meloidogyne hispanica* can be considered a species of emerging importance and the results obtained limit the use of non-hosts and *Mi-1.2* gene tomato plants to manage these nematodes.

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ANNEX: MITOCHONDRIAL DNA SEQUENCES FROM *Meloidogyne hispanica* AND *M. ethiopica*

Mitochondrial DNA sequences from *Meloidogyne hispanica* (PtHi3) and *M. ethiopica* (ItE) aligned with *M. ethiopica* (Me- AY942848), *M. arabicida* (Mar- AY942852), *M. incognita* (Mi- AY635611; Mi1- FY159614) and *M. javanica* (Mj- AY635612) sequences obtained from GenBank, using Muscle. A dot denotes a matching nucleotide and a hyphen a deletion. Nucleotides are numbered starting with the first base in the 5' primer sequence.

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                10        20        30        40        50        60        70        80        90
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      ------------------------------------------ATTTTGGTTG-AATTACTTTATTTGATTTTTTTAAGTTAAATTTA
ItE        ------------------------------------------C..........-..............................
Me         GGTCAATGTTCAGAAATTTGTGGTATTAATCATTCATTTATACCA..........A..............................
Mar        GGTCAATGTTCAGAAATTTGTGGTATTAATCATTCATTTATACCA..........A..............................
Mi         GGTCAATGTTCAGAGATTTGTGGTATTAATCATTCATTTATACCA..........A..............................
Mi1        GGTCAATGTTCAGAAATTTGTGGTATTAATCATTCATTTATACCA..........A..............................
Mj         GGTCAATGTTCAGAAATTTGTGGTATTAATCATTCATTTATGCCA..........A..............................

               100       110       120       130       140       150       160       170       180
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      TTAACTAATTGATTATTTTATTTTTGTTGAAGCGAGAGAAAATATTAGTTGTTTAAATTTTTTTTATTAGTTTTAAAAATAATAAAAAAT
ItE        ................................A.............................................................
Me         ................................A.............................................................
Mar        ..............-.................A.............................................................
Mi         ................................A..............C..............................................
Mi1        ................................A..............C..............................................
Mj         ................................A..............C..............................................

               190       200       210       220       230       240       250       260       270
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      GTTTTTAAGTGATTAGGGATTAGATTAACAATTGTATTATTTATTATTATAAGTTTTTTGAATATTATTTTGATTTTTTGTTGTTTAATT
ItE        ...............................................................................................
Me         ...............................................................................................
Mar        ...............................................................................................
Mi         ....................A..........................................................................
Mi1        ...............................................................................................
Mj         ...............................................................................................

               280       290       300       310       320       330       340       350       360
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      TCTTTTTTTTGTATTAAATTATTTTTATATTTTTTGTTAATTTTAATTTTTTATATTTGTAATTTTATCTATAATAATTTGTTAAAATTT
ItE        ...............................................................................................
Me         ...............................................................................................
Mar        ...............................................................................................
Mi         ...............................................................................................
Mi1        ...................................................------------------------------T....
Mj         ...............................................................................................

               370       380       390       400       410       420       430       440       450
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      TTTTCTAATTTGGGTGAATTAAAATTAGAGATGAATTGATTAGATTTTTTTTTTGTATTACTAATATCATTTTTATTTATTATTAGGATA
ItE        ..................T.....................................T.............................
Me         ..................T.....................................T.............................
Mar        ............T.....................................T.............................
Mi         ...................................................-.....T.............................
Mi1        ...................................................-.....T.............................
Mj         ...................................................--....T....................G.....

               460       470       480       490       500       510       520       530       540
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PtHi3      TTTTCAATTGGTTGTGTTAATCATTCTTTATTAGATCGGGGTTTAATAATGGGTTCTTTATTGTGTTAATTATTAAAATATTATTTTGGT
ItE        ...............................................................................................
Me         ...............................................................................................
Mar        ................T..T......C.....................................A.....................
Mi         ...............................................................................................
Mi1        ...............................................................................................
Mj         ...............................................................................................
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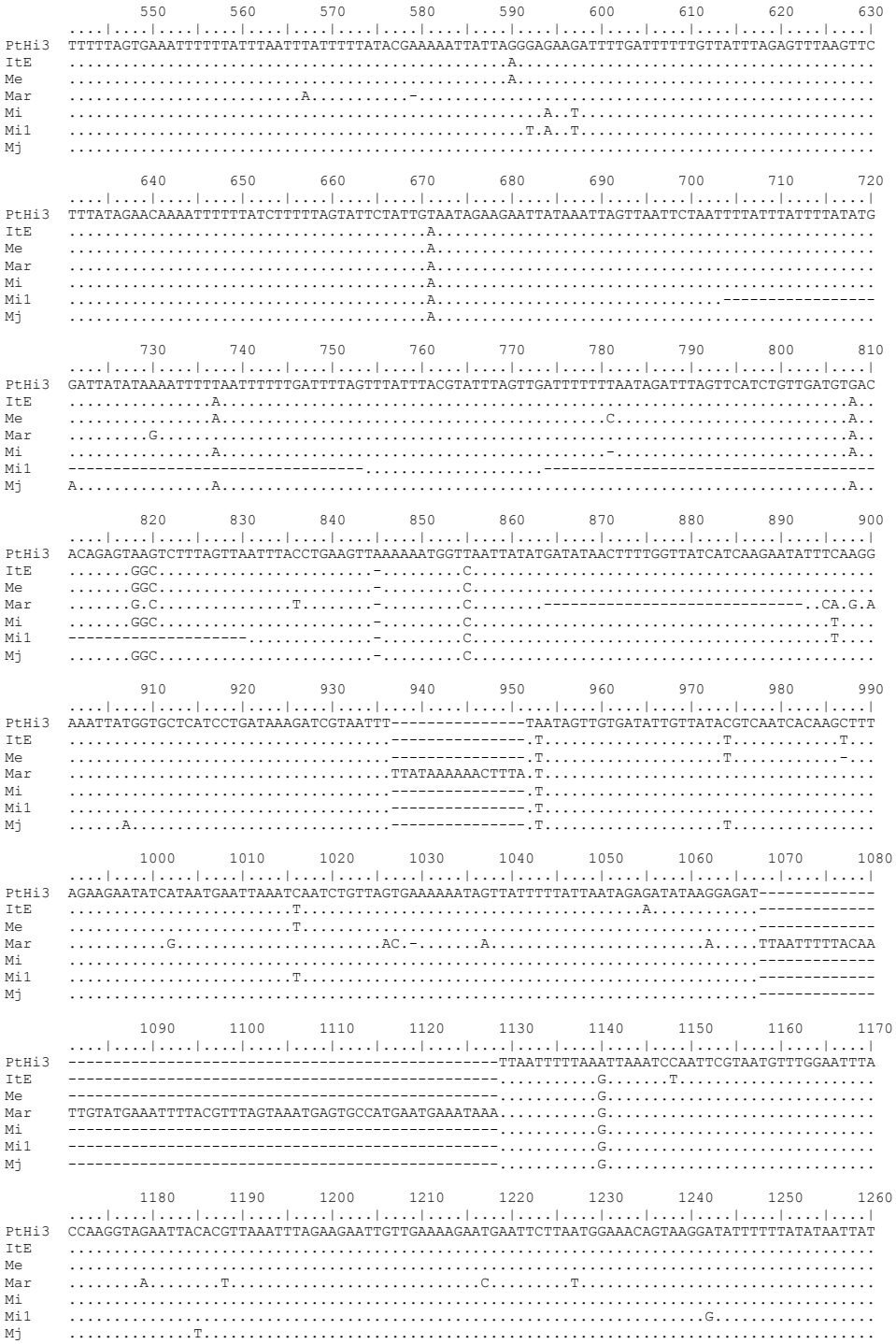
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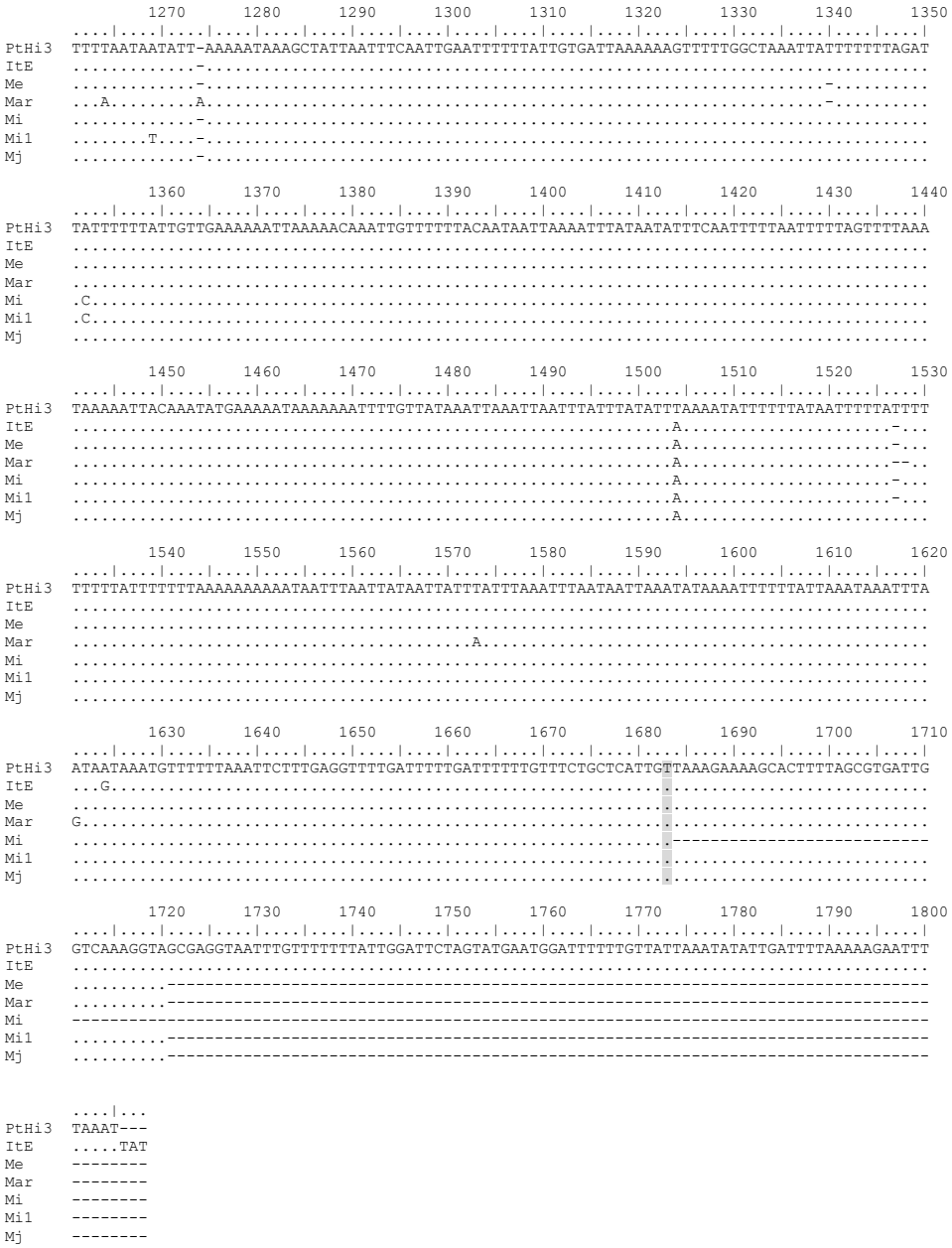
1270 1280 1290 1300 1310 1320 1330 1340 1350
PtHi3 TTTTAATAATATT-AAAAATAAAGCTATTAATTTCAATTGAATTTTTTATTGTGATTAAAAAAGTTTTTGGCTAAATTATTTTTTTAGAT
ItE .....-.....
Me .....-.....-.....
Mar ...A.....A.....-.....
Mi .....-.....
Mi1 .....T....-.....
Mj .....-.....
1360 1370 1380 1390 1400 1410 1420 1430 1440
PtHi3 TATTTTTTATTGTTGAAAAAATTAAAAACAAATTGTTTTTTACAATAATTAAAATTTATAATATTTCAATTTTTAATTTTTAGTTTTAAA
ItE .....
Me .....
Mar .....
Mi .C.....
Mi1 .C.....
Mj .....
1450 1460 1470 1480 1490 1500 1510 1520 1530
PtHi3 TAAAAATTACAAATATGAAAAATAAAAAAATTTTGTTATAAATTAAATTAATTTATTTATATTTAAAATATTTTTTATAATTTTTATTTT
ItE .....A.....-...
Me .....A.....-...
Mar .....A.....--..
Mi .....A.....-...
Mi1 .....A.....-...
Mj .....A.....
1540 1550 1560 1570 1580 1590 1600 1610 1620
PtHi3 TTTTTATTTTTTTAAAAAAAAAATAATTTAATTATAATTATTTATTTAAATTTAATAATTAAATATAAAATTTTTTATTAAATAAATTTA
ItE .....
Me .....
Mar .....A.....
Mi .....
Mi1 .....
Mj .....
1630 1640 1650 1660 1670 1680 1690 1700 1710
PtHi3 ATAATAAATGTTTTTTAAATTCTTTGAGGTTTTGATTTTTGATTTTTTGTTTCTGCTCATTGTTAAAGAAAAGCACTTTTAGCGTGATTG
ItE ...G.....
Me .....
Mar G.....
Mi .....----------
Mi1 .....
Mj .....
1720 1730 1740 1750 1760 1770 1780 1790 1800
PtHi3 GTCAAAGGTAGCGAGGTAATTTGTTTTTTTATTGGATTCTAGTATGAATGGATTTTTTGTTATTAAATATATTGATTTTAAAAAGAATTT
ItE .....
Me .....----------
Mar .....----------
Mi ----------
Mi1 .....----------
Mj .....----------
PtHi3 TAAAT---
ItE .....TAT
Me --------
Mar --------
Mi --------
Mi1 --------
Mj --------