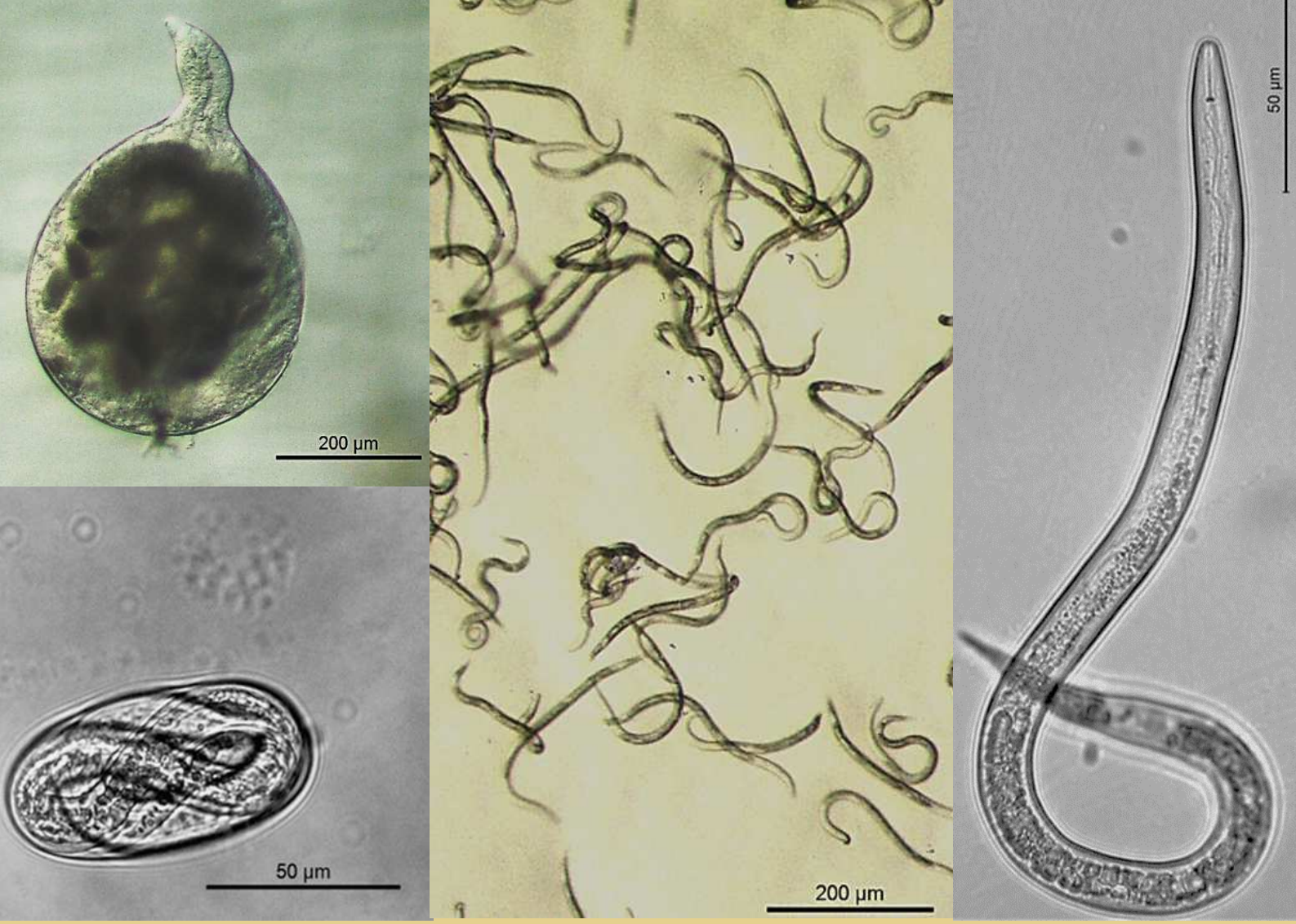

Aida Maria Dâmaso Duarte

MELOIDOGYNE HISPANICA PARASITISM GENES: MOLECULAR AND FUNCTIONAL CHARACTERIZATION

Tese de Doutoramento em Biociências, ramo de especialização em Ecologia, orientada pelas Professora Doutora Isabel Maria de Oliveira Abrantes e Doutora Rosane Hazelmann Cunha Curtis e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Setembro de 2014

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UNIVERSIDADE DE COIMBRA

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Front cover: *Meloidogyne hispanica* developmental stages (light microscope photographs) from left to right: female (top); egg (bottom); second-stage juveniles; and second-stage juveniles. All photographs by Aida Duarte.

Tese de Doutoramento em Biociências, especialidade em Ecologia, orientada pelas Professora Doutora Isabel Maria de Oliveira Abrantes do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra e Doutora Rosane Hazelmann Cunha Curtis do *Bionemax UK Limited, Rothamsted Centre for Research and Enterprise*, Reino Unido e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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"The fairest thing we can experience is the mysterious. It is the fundamental emotion that stands at the cradle of true art and true science."[1]

Albert Einstein, 1931

1 Einstein, A. (1931). The World As I See It. *Forum and Century* 84, 193-194.

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Ao meu querido PAI

Table of Contents

Abstract

The root-knot nematode (RKN), *M. hispanica* (*Mhi*), has been found in all continents associated with a wide range of plant hosts. The main goal of this study was to investigate the functions of *M. hispanica* effector genes, in the host plant-nematode interactions and to contribute for the development of new management strategies. As no genomic data is available for *M. hispanica,* a number of putative orthologs were selected in the National Center for Biotechnology using gene models from the genome of *M. incognita* and *M. hapla.* Primers were designed to the conserved regions of the selected putative effectors of *M. incognita* and *M. hapla* and used to amplify these genes in *M. hispanica*. This approach led to the successful amplification of the *M. hispanica* effectors genes: annexin-2 (*nex-2*); *β*-1,4-endoglucanase-1 and 2 (*eng-1* and *eng-2*); cathepsin L cysteine protease (*cpl-1*); calreticulin (*crt-1*); fatty acid and retinol binding protein (*far-1*); glutathione-S-transferase (*gsts-1*); manganese superoxide dismutase (*mnsod*); pectase lyase 3 (*pel-3*); polygalacturonase (*gp-1*); venom allergen-like protein 1 and 2 (*vap-1* and *vap-2*) and 14-3-3a (*14-3-3a*). The *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1*, *Mhi-far-1, Mhi-mnsod* and *Mhi-vap-1* genes were shown to be differentially expressed during *M. hispanica* development. The cDNA was amplified from mRNA from eggs, second-stage juveniles (J2) and females. The *Mhi-cpl-1*, *Mhi-crt-1, Mhi-far-1* and *Mhi-vap-1* genes were present in all developmental stages, suggesting having a role in various aspects of the life cycle. However, *Mhi-vap-1* showed the highest level of expression in J2. The *Mhi-eng-1* gene was expressed in eggs and J2 and may have a function in the early events of infection. The expression in eggs of *Mhi-mnsod* indicates that this gene could have a role in the embryogenesis and hatching of J2. *Meloidogyne arenaria*, *M. hapla*, *M. hispanica*, *M. incognita* and *M. javanica cpl-1*, *crt-1*, *far-1*, *eng-1*, *mnsod* and *vap-1* genes were sequenced and phylogenetic studies revealed that *M. hapla is* the most divergent species. Phylogenetic analysis of the FAR-1 and VAP-1 predicted protein sequences between *M. hispanica*, other RKN species and other plant-parasitic nematodes indicated towards a high degree of conservation between *M. hispanica* and the other species. The temporal and spatial expression patterns of *far-1* and *vap-1* effector genes were analyzed in J2 by *in situ* hybridization. These transcripts were localized within the subventral oesophageal glands which suggest that these proteins are secreted by J2 and could play a crucial role in the early stages

of the infection process. RNA interference (RNAi) was used to evaluate the putative role of the *Mhi-vap-1* gene in the parasitism, the transcript level of *vap-1* gene in J2 decreased after incubation with dsRNA for 48 h, indicating the susceptibility of this gene to RNAi. It was showed for the first time that a significant reduction in nematode attraction and penetration of tomato roots was observed when *M. hispanica* J2 were treated with dsRNA to silence the *Mhi-vap-1* gene. Therefore, the nematode effector *Mhi-vap-1* gene seems to play an important role in the infection process. It was also reported for the first time that tomato root exudates induce changes in the gene expression of some nematode candidate parasitism genes. Gene expression of *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-far-1* and *Mhi-vap-1* genes were up-regulated in the pre-parasitic J2 after exposure to tomato root exudates. Possibly, these candidate parasitism genes have a strategic function during the early events of infection and their up-regulation prior to root infection may contribute to their successful parasitism. Nematode infection differentially changes the gene expression of numerous plant genes and this study showed differential expression of *PR-1* and *WRKY1* upon nematode infection in the susceptible tomato cv. Easypeel and in the resistant pepper cv. Solero. The *PR-1* gene in tomato may also contribute to the process of regulation of primary host plant defence pathways and in the early events of the compatible plant-nematode interaction. Its differential expression during parasitism might be co-related with the presence of different nematode development stages. Several *M. hispanica* effector genes were identified and a detailed insight of their molecular characterisation, during the infection process and function during parasitism, was provided. This study also analysed the effect of nematode infection on two important plant defence genes and the signalling occurring in the rhizosphere before nematode penetration. The results contribute to further understanding the plant-nematode interaction and offer some possible avenues for the development of novels strategies for the management of *M. hispanica*.

Keywords: effectors, gene expression, *in situ* hybridization, phenotype, plant defence, plant-nematode interactions, plant-parasitic nematodes, RNA interference, root exudates, root-knot nematodes, secretions, subventral oesophageal glands.

Resumo

O nemátode-das-galhas-radiculares (NGR), *Meloidogyne hispanica* (*Mhi*), tem sido detetado em todos os continentes associado a uma grande diversidade de plantas hospedeiras. Os objetivos principais deste estudo foram determinar as funções de genes efetores de *M. hispanica* na interação entre a planta hospedeira e o nemátode e contribuir para o desenvolvimento de novas estratégias de controlo.

Em virtude de não existirem dados genómicos disponíveis para *M. hispanica*, alguns genes ortólogos foram selecionados, a partir de genes modelo do genoma de *M. incognita* e *M. hapla*, no "National Center for Biotechnology". Foram desenhados primers para as regiões conservadas dos efetores selecionados de *M. incognita* e *M. hapla* e usados para amplificar estes genes em *M. hispanica*. Este processo levou à amplificação dos genes efetores de *M. hispanica*: anexina-2 (*nex-2*); *β*-1,4-endoglucanase-1 e 2 (*eng-1* e *eng-2*); cisteína protease L-catepsina (*cpl-1*); calreticulina (*crt-1*); proteína de ligação de ácidos gordos e retinol (*far-1*); transferase glutationa-S (*gsts-1*); dismutase superóxido de manganésio (*mnsod*); liase pectase 3 (*pel-3*); poligalacturonase (*gp-1*); proteína semelhante à alérgica do veneno 1 e 2 (*vap-1* e *vap-2*) e 14-3-3a (*14-3-3a*). Os genes *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1*, *Mhi-far-1*, *Mhi-mnsod* e *Mhi-vap-1* foram diferencialmente expressos durante o desenvolvimento de *M. hispanica*. O cDNA foi amplificado a partir do mRNA de ovos, jovens do segundo estádio (J2) e fêmeas. Os genes *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-far-1* e *Mhi-vap-1* foram detetados em todas as fases do desenvolvimento, sugerindo terem um papel em vários aspectos do ciclo de vida. No entanto, o nível de expressão mais elevado de *Mhi-vap-1* foi nos J2. O gene *Mhi-eng-1* foi expresso nos ovos e J2 e pode ter uma função nas primeiras fases da infecção.

A expressão de *Mhi-mnsod* nos ovos indica que este gene poderá ter uma função na embriogénese e eclosão dos J2. Os genes *cpl-1*, *crt-1*, *far-1*, *eng-1*, *mnsod* e *vap-1* foram sequenciados em *M. arenaria*, *M. hapla, M. hispanica*, *M. incognita* e *M. javanica* e os resultados da análise filogenética mostraram que *M. hapla* é a espécie mais divergente. A análise filogenética das sequências previstas para as proteínas FAR-1 e VAP-1 entre *M. hispanica*, outras espécies de NGR e outros nemátodes fitoparasitas revelou existir um elevado grau de conservação entre *M. hispanica* e as outras espécies. Os padrões de expressão temporal e espacial dos genes efetores *Mhi-vap-1* e *Mhi-far-1* foram analisados nos J2 através de hibridização *in situ*. Estes

transcritos foram localizados nas glândulas esofágicas subventrais, o que sugere que estas proteínas são produzidas pelos J2 e que poderão ter um papel importante nas primeiras fases do processo de infeção. Para avaliar a função do gene *Mhi-vap-1* no parasitismo, foi utilizada a metodologia associada ao RNA de interferência (RNAi). O nível dos transcritos do gene *vap-1* diminuiu às 48 h, indicando ser um gene susceptível ao RNAi. Na atração e penetração de J2 nas raízes de tomateiro, após tratamento com dsRNA para o silenciamento do gene *Mhi-vap-1*, foi possível observar, pela primeira vez, uma redução significativa do número de J2, confirmando a função deste gene no processo de infeção. Também foi detetado, pela primeira vez, que os exsudatos de raízes de tomateiro induzem alterações na expressão de alguns genes. A expressão dos genes *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-far-1* e *Mhi-vap-1* aumentou nos J2 após a exposição aos exsudatos de tomateiro. Estes genes, provavelmente, têm uma função estratégica nas fases iniciais do processo de infeção e sua sobreexpressão antes da infecção poderá contribuir para o sucesso do seu parasitismo. A infeção altera diferencialmente a expressão génica de numerosos genes de plantas e este estudo mostrou a expressão diferencial de *PR-1* e *WRKY1* no tomateiro suscetível cv. Easypeel e no pimentão resistente cv.Solero após inoculação de J2 de *M. hispanica*. O gene *PR-1* no tomateiro pode também contribuir para o processo de regulação das primeiras vias metabólicas de defesa da planta hospedeira e nos primeiros eventos da interação nemátode-planta. A sua expressão diferencial durante o parasitismo pode estar correlacionada com os diferentes estágios de desenvolvimento dos nemátodes. Para além da identificação de vários genes efetores em *M. hispanica*, procedeu-se à sua caracterização molecular durante o processo de infeção e possível função durante o parasitismo. Este estudo também analisou o efeito da infeção por nemátodes em dois genes importantes de defesa das plantas e as vias de sinalização que ocorrem na rizosfera antes da penetração do nemátode. Os resultados contribuem para o conhecimento e compreensão da interação nemátode-planta e indicam algumas perspetivas para o desenvolvimento de estratégias para o controlo de *M. hispanica*.

Palavras-chave: efetores, expressão génica, exsudatos de raízes, fenótipos, genes de defesa de plantas, glândulas esofágicas subventrais, hibridação *in situ*, interação nemátode-planta, nemátodes-das-galhas-radiculares, nemátodes fitoparasitas, RNA de interferência, secreções.

General Introduction

Introduction

Nematodes comprise a large phylum of animals that includes plant and animal parasites as well as many free-living species (Decraemer & Hunt, 2006). They are among the most ample groups of animals on earth, due to their ability to adapt to hostile and changing environmental conditions (Abad & Williamson, 2010). Among the plant-parasitic nematodes (PPN), the genus *Meloidogyne*, known as root-knot nematodes (RKN), has a worldwide distribution and is considered as the most important genus among the top 10 PPN with major scientific and economic importance (Jones *et al*., 2013). This genus has more than 90 recognized species and thirteen new species have been described in the last decades (Hunt & Handoo, 2009; Moens *et al*., 2009). The four most common species of RKN are *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*, undoubtedly of immense economic importance because of their wide geographical distribution and wide host range (Moens *et al*., 2009). In Portugal, species of this genus have been found alone or in mixed populations in different regions of the center and south, associated with several and important cultivated plants (Abrantes *et al.*, 2008; Conceição *et al*., 2009; Maleita *et al*., 2011).

These nematodes are obligate endoparasites and their common name is due to the formation of characteristic galls in the roots or root-knots in the host plants. Distinct species of RKN attack different plant species and cultivars (Garcia & Sanchez-Puerta, 2012). They can also parasitize tubers and corms of several plant species, causing devastating adverse effects on the crops quality and yield (Wesemael *et al*., 2011; Jones *et al*., 2013).

The RKN spend most of their lives within plant roots, feeding on dramatically changed host cells (Caillaud *et al*., 2008). Their life cycle involves four juvenile stages

and four moults and adults (Fig. 1). The embryonic development results in the first-stage juvenile (J1) and the first moult takes place inside the egg giving rise to the second-stage juvenile (J2), the infective stage which after hatching, moves through the soil in direction to a host plant. Initially, the J2 moves toward the apex of the root and then turn round to penetrate the root at the zone of elongation. The parasitic J2 migrates intercellularly between the root cells to reach the plant vascular cylinder where the nematode feeding site is formed (Abad *et al*., 2008; Abad & Williamson, 2010). During the migration, the nematodes release secretions from the oesophageal glands into the root tissue, causing dramatic physiological changes in the parasitized cells. The stylet, a specialized feeding structure connected to the oesophagus, is used to inject oesophageal secretions into the plant cells and to take up nutrients. Nematode secretions are considered responsible for elaborate developmental and morphological changes in root cells which lead to the formation of feeding cells which provide the nutrients for the nematode. (Jaouannet & Rosso, 2013). The J2 induces the formation of five to seven multinucleate and hypertrophied feeding cells, the giant cells (Caillaud *et al.*, 2008). With the onset of feeding, the nematode becomes sedentary and goes through three moults before becoming mature adult (female or male). The third-juvenile stage (J3) and the fourth-juvenile stage (J4) do not have a functional stylet and do not feed and the combined time for the development of the J3 and J4 is much shorter than for the J2 or the adult, typically 4 - 6 days. Males are vermiform and migrate out of the roots when the development is completed. As the majority of the RKN species are parthenogenetic, males do not have a role in reproduction (Castagnone-Sereno, 2006). The pear-shaped female remain sedentary, produces eggs that are usually released on the root surface in a protective gelatinous matrix. Nematode growth and reproduction depend on the

successful establishment and maintenance of the giant cells, the specialized feeding sites within the root.

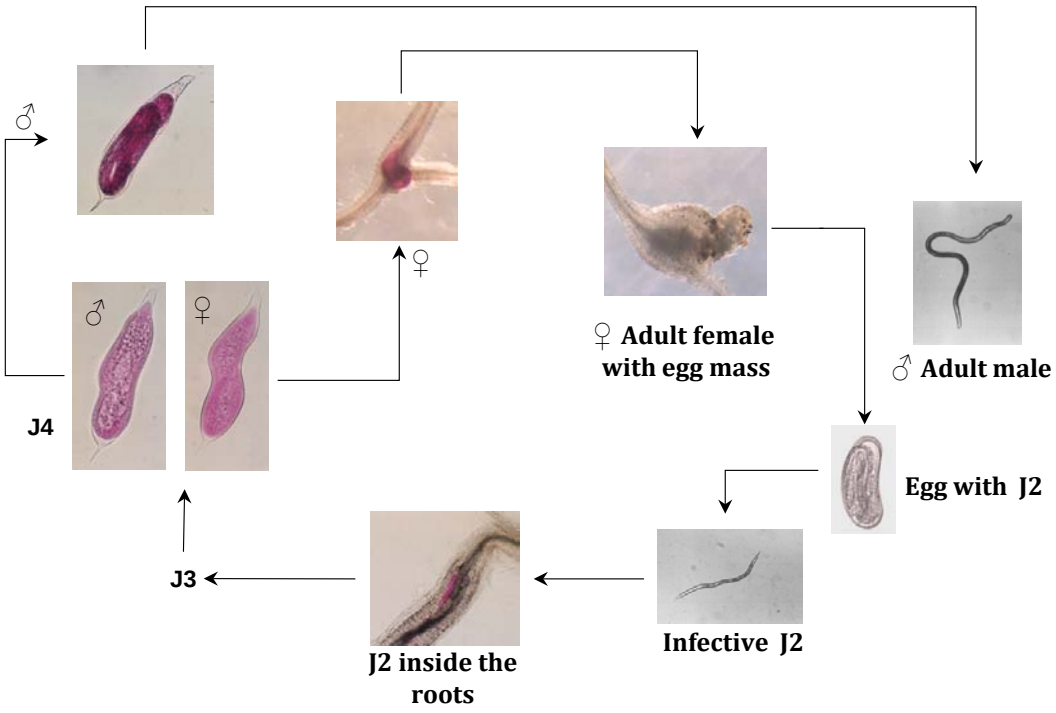


Figure 1 Life cycle of root-knot nematodes, *Meloidogyne* spp. J2, J3, J4 - Second-, third- and fourth-stage juvenile (Adapted from Abrantes *et al*., 2007).

Meloidogyne hispanica

Meloidogyne hispanica Hirschmann 1986, detected for the first time in Spain from peach rootstock (*Prunus persica silvestris* Batsch), has a worldwide distribution, being found in all continents (Europe, Africa, Asia, Australia and North, Central and South America) associated with a wide range of plant hosts (Hirschmann, 1986; Maleita *et al.*, 2012a).

In Portugal, the nematode was found in roots of fig-trees (*Ficus carica* L.) and 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). Recent studies for the evaluation of the host status of 76 cultivated plants to *M. hispanica* showed that most of the plants were susceptible, only five were hypersusceptible or poor hosts and two were resistant (Maleita *et al*., 2012a).

Differentiation of *M. hispanica* from other RKN species using morphological and biological characters can be very difficult. Therefore, biochemical and molecular markers should be used for differentiating this species from the other *Meloidogyne* species. It was first studied biochemically by Dalmasso and Bergé (1978) who found certain enzyme patterns similar to those of *M. incognita*. Later, the analysis of nonspecific esterase showed that *M. hispanica* can be differentiated from other RKN species by esterase phenotypes, when adult females are available. Three phenotypes (Hi2, S2-M1=Hi3 and Hi4) have been reported and all the isolates shared two common major bands characteristic of this species (Maleita *et al*., 2012b). The molecular analysis of three rDNA regions (18S, ITS1-5.8S-ITS2, and D2-D3) and the phylogenetic analysis of the sequences of three *M. hispanica* isolates, from different geographical origin (Brazil, Portugal and Spain), demonstrated that *M. hispanica* can

be differentiated from *M. incognita*, *M. javanica*, *M. arenaria* and other known *Meloidogyne* species (Landa *et al*., 2008). In previous studies, seven Portuguese *M. hispanica* isolates were investigated by biometrical, biochemical and molecular characteristics and a new molecular diagnostic method, based on the mtDNA region between COII and 16S rRNA genes, was developed (Maleita *et al*., 2012b). Tomato resistance against RKN has been explored and the *Mi* gene was found to play an important role. However, the emergence of *M. hispanica* virulent biotypes, able to overcome this resistance gene, might be a limitation for the control of this nematode (Maleita *et al*., 2011). The impact of this nematode in agricultural areas reinforces the urgent need for new approaches to develop new control strategies. The study of secretor genes present in the secretions of the nematodes and their roles in early stages of parasitism will contribute to the understanding of how plant-parasitic nematodes infect theirs hosts, leading to the identification of novel pathogenicity genes.

Secretor genes involved in the parasitism

The most important nematode organs producing secretions are the oesophageal glands, the hypodermis, which deposits secretions on the cuticle surface and the amphids (Haegeman *et al*., 2012). The RKN possess two specialized structures, stylet and oesophageal secretory glands, that are thought to be essential for many aspects of parasitism (Huang *et al*., 2006; Davis *et al*., 2008; Jaouannet & Rosso, 2013). Changes in the content and activity of the RKN oesophageal gland cells occur throughout the parasitic cycle with strong activity in the subventral gland cells during nematode penetration and migration in roots and a transition to dominance in dorsal gland cell activity as feeding cells are formed and maintained throughout

the sedentary nematode life stages (Davis et al., 2004). Secretions from the dorsal gland are transported through a cytoplasmic extension and are released close to the base of the stylet, while the two subventral glands empty their granules behind the pump chamber (Curtis, 2007). They are released into the plant tissue and may play a role in the pathogenicity. These secretions are thought to contain the biochemical trigger (s) and important substances that mat have a role in the penetration and migration of the J2 in the plant tissue, in the digestion of host cell contents to facilitate nutrient acquisition by the nematodes, in the modification and maintenance of the feeding sites, and in the suppression of host responses (Caillaud *et al*., 2008; Quentin *et al*., 2013). Secretions from the nematode oesophageal glands, surface cuticle and the amphids, the important nematode chemosensory organs, are the first signals at the host interface and they are thought to contain nematode pathogenicity factors. These secretions are the products of genes that are expressed exclusively in the nematode oesophageal gland cells and are considered the genetic determinants of nematode parasitism and, therefore, are called parasitism genes, although the current nomenclature prefers the term effector gene (Hussey, 1989; Davis *et al*., 2004). Nematode effectors are a collective of proteins and other molecules secreted by the nematode that can affect the host defence response, facilitating the penetration of the host tissue and are thought to contain pathogenicity factors that induce feeding site formation as well as those that are associated with triggering defence responses and immunity (Vanholme *et al*., 2004; Davis *et al*., 2008; Hassan *et al*., 2010; Hewezi & Baum, 2013). However, the precise role of these effectors during root penetration or during giant cell induction remains unknown (Rosso *et al*., 2011). The cuticle is a potential important source of nematode effectors that consist of a dynamic structure covered with a surface coat

composed of proteins, carbohydrates and lipids (Curtis, 2007). Effector genes codify enzymes, such as β-1,4 endoglucanase, pectase lyase, xylanase, polygalacturonase and expansin, that can modify and weaken the plant cell wall in order to facilitate migration through the plant root. In sedentary nematodes, these are mostly expressed in the subventral oesophagel gland cells, and showed greatest activity during the migration phase (Haegeman *et al*., 2012; Michum *et al*., 2013; Quentin *et al*., 2013).

The identification of genes encoding candidate effector proteins has been achieved using a range of different techniques including cDNA-AFLP (Qin *et al*., 2000; Tytgat *et al*., 2004) and microarrays (De Boer *et al*., 2002; Elling *et al*., 2009). The first proteins related with parasitism were identified using antibodies (Curtis, 1996; Smant *et al*., 1998). Nevertheless, the most widely used technique is the analysis of expressed sequence tags (ESTs), which has been performed for various nematode species (*Ditylenchus africanus*, *M. graminicola*, *Pratylenchus coffeae*; *Radopholus similis*, *Xiphinema index*) (Furlanetto *et al*., 2005; Jacob *et al*., 2008; Haegeman *et al*., 2009; 2011; 2013). Proteomic and bioinformatics studies through the use of peptide mass spectometry identified nearly 500 proteins from secretions of *M. incognita* and *M. hapla* (Bellafiore *et al*., 2008; Bellafiore & Briggs, 2010; Mbeunkui *et al*., 2010). Using several approaches, a long list of genes encoding candidate effector proteins have been produced but experimental studies are still required to confirm that these genes have a role in parasitism, which means that many effectors remain to be investigated (Haegemen *et al*., 2012). Although the function of the majority of identified effector genes remains speculative at the present time, the adoption of novel reverse genetic techniques, such as RNA interference (RNAi), opens the way for the direct assessment of nematode gene function and will greatly enhance our

understanding of the molecular basis of the nematode pathogenicity (Chen *et al*., 2005; Mitchum *et al*., 2013). The knowledge of the genome sequencing of *M. incognita* and *M. hapla* (Abad *et al*., 2008; Opperman *et al*., 2008), the first genome data for phytoparasitic nematodes, will generate new opportunities for studying the interaction between RKN and plants.

Plant parasitism interaction

The life style of the sedentary endoparasitic RKN requires a close and prolonged biotrophic interaction with heir host plants. This interaction starts in the soil when hatched infective J2 are attracted to a host plant root system. The RKN infection process can be considered a much more complex interaction than that of other parasitic nematodes (Hewezi & Baum, 2013). The nematodes nervous system is the channel between stimulus, reception and behavioural output and multicellular animals use their chemical senses to trigger complex behaviour and developmental processes (Curtis, 2008). The root exudates contain compounds that induce nematode hatching, attraction and repellence to roots but also induce nematode behaviour as for example the release of secretions, produced by the specialized oesophageal gland cells and released through the stylet (Quentin *et al*., 2013). Understanding host defence responses at the molecular level is primarily based on the interaction between plant and pathogenic microorganisms. A typical plant defence response against a pathogen or parasite begins with the perception by the host at the local site of initial contact. This initiates a signaling process that can include multiple exchanges between various pathogen or parasite-associated molecules and different host proteins (Goto *et al*., 2013). The RKN manipulate host cellular processes to their benefit through specific interactions with certain host

proteins. Genes that are induced in defence responses against other pathogens are also up-regulated after inoculation with RKN (Lambert *et al*., 1999). A large number of genes that are induced by infection are likely to contribute for the establishment of the parasite interaction (Gheysen & Fenoll, 2002). During nematode infection, genes, such as endoglucanase and polygalacturonase that encode host cell wall degrading enzymes are up-regulated. The putative pectin acetylesterase gene is up-regulated in pre-giant cells, but not in the mature giant cells, suggesting its possible role in the formation of the feeding cells (Williamson & Gleason, 2003). Previous studies reported some genes that have a role in metabolic pathways, cell-cycle progression and water transport whose expression is increased around and in the feeding cells (Potenza *et al*., 2001; Gheysen & Fenoll, 2002). However, several genes are down-regulated after nematode infection. Many of these genes are involved in pathogen defense responses, suggesting that the nematode actively suppresses the host defense response (Gheysen & Fennol, 2002). For example a transcription factor of the ethylene-responsive element binding protein (EREBP) family that contribute for the regulation of the defence gene response is down-regulated after infection in *Arabidopsis* with the sugar beet cyst nematode and after susceptible infection with the soybean cyst nematode (Williamson & Gleason, 2003). The up-regulation or down-regulation of gene transcription may reflect the complex functions of these genes in the plant-nematode interactions.

Plant defence genes

Abiotic and biotic stresses cause major losses in crop productivity. The plant responses to stress are regulated by multiple signaling pathways and there is significant overlap between the patterns of the gene expression that are induced in

plants in response to different stresses (Singh *et al*., 2002; Atkinson & Urwin, 2012). Each stress elicits a complex cellular and molecular response system implemented by the plant in order to prevent damage and ensure survival, but often at the detriment of growth and yield (Atkinson & Urwin, 2012). Defence signaling pathways are typically first activated at the local site of infection. During defence responses, plants have a common feature, after pathogen infection they activate a large number of genes, leading to the complex changes in plant gene expression. After an infection with RKN, the genes that are induced in defence responses against other pathogens are also up-regulated (Williamson & Gleanson, 2003). However, genome analyses provide an important overview and have shown that similar numbers of genes are up and down-regulated and it is suggested that gene down-regulation is essential for the feeding site formation (Jammes *et al*., 2005). The process of nematode feeding cell formation involves changes in the differentiation of normal cells to produce a highly metabolically active cell type altered in cell cycle, hormone regulation, cell wall architecture and cytoskeleton. In general the gene expression during plant-nematode interactions has shown the suppression of plant defences is associated with the nematode feeding site development (Caillaud *et al*., 2008; Abad & Williamson, 2010). Several genes conferring resistance to RKN (*R* genes) in annual and perennial crops have been reported (Williamson & Roberts, 2009). The tomato gene *Mi-1* encodes a nucleotide binding, leucine-rich repeat protein and is currently the best characterized and most widely used resistance against the most diffused RKN species: *M. incognita*, *M. javanica* and *M. arenaria* (Molinari *et al*., 2014). The effect of the *Mi* gene on reproduction of *M. hispanica* on tomato plants was also studied and the results showed *Mi* gene confers resistance to the three most common RKN species, however *M. hispanica* can overcome this

resistance gene trigger (Maleita *et al*., 2011). The plant defence response is a complex process. Several hormones are involved in the regulation of defense response, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and indole acetic acid (IAA) (Wubben *et al.*, 2008; Matthews *et al*., 2014). The SA accumulation, following pathogen infection, induces the expression of multiple pathogenesis-related (*PR*) genes, which are markers of the onset of the so-called systemic acquired resistance (SAR) (Durrant & Dong, 2004). The pathogenesis-related proteins (PR), implicated in the active plant defence restricting pathogen development and spread, were classified as proteins encoded by the host plant and induced by various types of pathogens: oomycetes, fungi, bacteria, viruses, parasitic nematodes and phytophagous insects (Antoniw *et al*., 1980; Van Loon *et al*., 2006; Hamamouch *et al*., 2011). The PR-1 class of proteins, the most abundant in plants has been detected in tobacco, tomato, barley, maize, parsley and other plants and their role was associated in acquired resistance induced in association with necrotic lesions in plants (Sudisha *et al*., 2012). Other proteins that have shown enhanced expression and/or DNA-binding activity following induction by several pathogens, defence signals and wounding are the WRKY proteins. They comprise a novel family of transcription factors, exclusive to plants and appear to be involved in the regulation of the plant defence reaction (Eulgem *et al*., 2000; Singh *et al*., 2002; Ulker & Somssich, 2004; Pan *et al*., 2009). The WRKY family of transcription factors, was classified into three major groups and was associated with various development processes in plants, such as seed development, senescence, dormancy, germination and also in the regulation of the defence against biotic and abiotic stresses (Eulgem *et al.*, 2000, Huang *et al*., 2012; Wang *et al*., 2014). Significant progress in identifying targets for WRKY factors has been made in the last years. Recently the WRKY

transcription factors were identified in tomato (*S. lycopersicum)*, and grapevine (*Vitis vinifera)* (Huang *et al*., 2012; Wang *et al*., 2014). *WRKY* genes have been identified from *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*Glycine max*), pine (*Pinus monticola*), barley (*Hordeum vulgare*), tobacco (*Nicotiana attenuata*) and green alga (*Chlamydomonas reinhardtii*) (Wu *et al*., 2005; Shen *et al*., 2007; Skibbe *et al*., 2008; Zhou *et al*., 2008; Liu & Ekramoddoullah, 2009; Tao *et al*., 2009; Bhattarai *et al*., 2010; Rushton *et al*., 2010). Several studies have shown that WRKY proteins have regulatory functions in plant response to pathogen infection. *WRKY* genes from a number of plants are rapidly induced by pathogens, pathogen elicitors, or treatment with SA (Chen & Chen, 2002).

Defence gene expression depends on the pathogen and the genes activated in response to a particular aggressor.

Aims of this study

The sedentary endoparasitic RKN, *Meloidogyne* spp., with a wide geographical distribution and host range, are amongst the world's most damaging agricultural pests. The interaction between RKN and plants is very complex and the nematode secretions are involved in host penetration, migration and formation of the feeding site to manipulate host plant cells to own advantages. Therefore, the identification of the nematode genes encoding parasitism proteins is important to provide a better understanding of the plant-nematode interactions and develop new control strategies such as the introduction of novel genes into crop plants that confer enhanced resistant. The RKN, *M. hispanica*, detected for the first time in Seville, Spain, has been found in all continents associated with a wide range of plant hosts

and was recently considered a polyphagous species of emerging importance, for economically important crops, difficult to control.

The main goals of this Thesis were to investigate the functions of *M. hispanica* effector genes, in the host plant-nematode interactions and to contribute for the development of new management strategies.

The specific objectives were:

- To identify *M. hispanica* effector genes, to determine whether these genes are differentially expressed during *M. hispanica* development and to study the phylogenetic relationship with other *Meloidogyne* spp. (Chapter 1).

- To identify and isolate the effector genes venom allergen-like protein (*vap-1*) and fatty acid and retinol binding protein (*far-1*) in *M. hispanica*, to analise the temporal and spatial expression patterns of the identified gene sequences, to study the degree of conservation of theses effector sequences genes between other plant-parasitic nematodes by phylogenetic studies (Chapter 2).

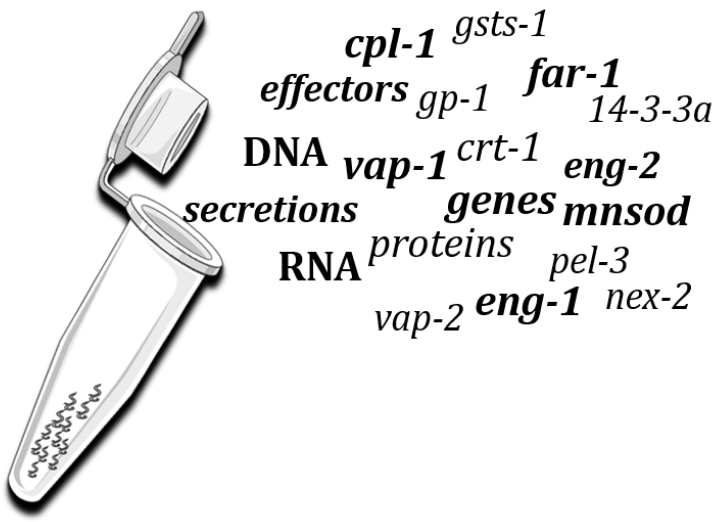
- To evaluate the putative role of the effector gene venom allergen-like protein (*Mhi-vap-1*) in the parasitism by RNAi (Chapter 3).

- To assess the effect of tomato root exudates in *M. hispanica* genes *cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1* expression (Chapter 4).

- To evaluate the effects of the *PR-1* and *WRKY1* plant defence genes in tomato and pepper plants after *M. hispanica* infection. (Chapter 5).

Chapter 1

Molecular characterization of putative parasitism genes in the plant-parasitic nematode *Meloidogyne hispanica*



Witten as a journal article:

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1.1 Abstract

Meloidogyne hispanica (*Mhi*) is a polyphagous root-knot nematode species of emerging importance for economically valuable crops and difficult to control. Nematode secretions are likely to be the first signals perceived by the plant and are thought to be involved in various aspects of the plant-nematode interation. These molecules are considered parasitism genes and are also known as effectors which are thought to facilitate the penetration of the second-stage juveniles (J2) in the host plant, the nematode development inside the roots and to affect the host defence response. The aims of this work were to identify and characterize *M. hispanica* parasitism genes. As there is no genomic data available for *M. hispanica*, primers were designed to the conserved regions of eleven selected putative effectors of *M. incognita* and *M. hapla* and used to amplify these genes in *M. hispanica*. This approach led to recognize the presence of the successful amplification of *M. hispanica* genomic DNA of β-1,4-endoglucanase-1 and 2 (*eng-1*) and (*eng-2*), annexin-2 (*nex-2*), manganese superoxide dismutase (*mnsod*), venom allergen-like protein-2 (*vap-2*), cathepsin L cysteine protease (*cpl-1*), pectase lyase 3 (*pel-3*), polygalacturonase (*gp-1*), 14-3-3a (*14-3-3a*), glutathione-S-transferase (*gsts-1*) and calreticulin (*crt-1*). Partial gene sequences were also obtained for *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica cpl-1*, *crt-1*, *eng-1* and *mnsod* and their phylogenetic relationship analysed.

In order to determine whether these genes are differentially expressed during *M. hispanica* development, cDNA was amplified from mRNA isolated from eggs, J2 and females. Amplification products were observed from cDNA of all developmental stages for the *Mhi-cpl-1* and *Mhi-crt-1* genes. However, the gene *Mhi-crt-1* exhibited intense amplification bands in females whilst the *Mhi-eng-1* gene was equally amplified in eggs and J2 and the *Mhi-mnsod* gene was only expressed in eggs. In comparison to the other RKN species, the genes *Mhi-eng-1* and *Mhi-mnsod* showed transcription in different nematode developmental stages.

Keywords: effectors, *Meloidogyne* spp, parasitism genes, root-knot nematodes, secretions.

1.2 Introduction

Meloidogyne hispanica, one of the lesser-known species of root-knot nematodes (RKN), was detected for the first time in Spain, from peach rootstock, *Prunus persica silvestris* Batsch and has been also found in 14 other countries (Hirschmann, 1986; Maleita *et al*., 2012a). For survival, as an obligate biotrophic pathogen, *M. hispanica* relies on its ability to successfully locate and infect several plant species and cultivars. Previous studies showed that this species can spread to other regions of Europe and overcome *Mi*-mediated resistance which emphasizes its potential economic impact (Maleita *et al*., 2011, 2012a, 2012b).

Current approaches for the management of RKN are the use of nematicides, cultural methods and resistant cultivars that may be used in an integrated manner. However, new approaches are required urgently, due to the emergence of RKN virulent biotypes (Bleve-Zacheo *et al*., 2007) and health and environmental concerns associated with the use of toxic nematicides. The successful parasitism of RKN depends on a complex interaction with the host plants and these nematodes manipulate the plant´s gene regulation and metabolism to their own advantage by delivering a wide variety of secreted molecules to the apoplast and cytoplasm of the host cells via the stylet (Hussey, 1989; Davis *et al*., 2008). After receiving signals from the host plant, nematode migration, penetration of the plant root and feeding site formation are accompanied by a number of secretory processes from the nematode surface, amphids, rectal glands, excretory pore and oesophageal glands (Gheysen & Fenoll, 2002; Davis *et al.*, 2004; Abad & Williamson, 2010). Some of these secreted molecules are thought to be localized to host nuclei or interact with host nuclear proteins (Rivas, 2012; Haegeman *et al*., 2013; Quentin *et al*., 2013) and others seem able to recruit proteins involved in nucleo-cytoplasmic movement and

nuclear dynamics during the parasitism of their hosts (Hewezi & Baum, 2013). Therefore, the identification and characterization of the nematode genes encoding secretions is important to provide a better understanding of the plant-nematode interaction and may increase the possibility of the development of new approaches for nematode control. The first nematode molecule involved in parasitism identified was the enzyme cellulase which was recognized from cyst nematodes using monoclonal antibodies (Smant *et al*., 1998). Ever since, the study of nematode parasitism genes has been a relevant subject of research and a number of proteins involved in parasitism were discovered by extensive genome, transcriptome and proteomics studies with the most common RKN species (Abad *et al*., 2008; Bellafiore *et al*., 2008; Opperman *et al*., 2008; Hassan *et al*., 2010; Haegeman *et al*., 2012, 2013). The function of several parasitic genes has been predicted to be related with food digestion, plant cell wall degradation, protection and suppression of host defenses, targeting plant signaling pathways and ubiquitination (Abad & Williamson, 2010; Haegeman *et al*., 2012). Nevertheless, the nature, molecular functions and the mode of action of most nematode effectors are still to be determined (Bellafiore *et al*., 2008; Bellafiore & Briggs, 2010; Hassan *et al*., 2010; Haegeman *et al*., 2012; Postma *et al*., 2012).

The cysteine proteases were first identified in homogenates of *Globodera pallida* females, in the intestine of *Heterodera glycines* and later in *M. hapla* females (Lilley *et al.,* 1996; Michaud *et al*., 1996). In plant-parasitic nematodes, these proteases, have been associated with digestion and transgenic plants expressing specific proteases inhibitors showed a high level of defence against nematode infection (Atkinson *et al*., 2003). The calreticulin (CRT) protein was described in *Bursaphelenchus xylophilus*, *Ditylenchus destructor*, *H. glycines*, *M. hapla* and *M.*

incognita (Jaubert *et al*., 2002, 2005; Ithal *et al.,* 2007; Opperman *et al*., 2008; Lin *et al*., 2011; Peng *et al*., 2013). *Meloidogyne incognita* studies showed that the knockdown of Mi-CRT in pre-parasitic infective second-stage juveniles (J2) reduced the ability of nematodes to induce galls on tomato, highlighting the importance of this protein in nematode infection (Dubreil *et al*., 2009). The enzyme *β*-1,4-endoglucanase is involved in the invasion of root tissues by pre-parasitic J2 and in the migration of nematodes (Davies & Curtis, 2011), however this enzyme may also play other important roles, such as feeding site formation and suporting the expansion of RKN induced giant cells (Quentin *et al*., 2013). The anti-oxidant enzyme manganese superoxide dismutase (MNSOD), identified in *M. incognita*, was suggested to protect plant-parasitic nematodes from host-induced oxidative damage (Rosso, 2009) and facilitate the nematodes development on resistant tomato plants (Molinari *et al*., 2005). Several studies have been conducted to characterize Portuguese *M. hispanica* isolates using morpho-biometrical, biochemical and molecular methods to understand its biology and ecology (Maleita *et al*., 2011, 2012a,b,c,d). Recently, the venom allergen-like protein (*vap-1*) and the fatty acid and retinol binding protein (*far-1*) genes were isolated from *M. hispanica* and designated as *Mhi-vap-1 and Mhi- far-1* and a higher expression of the *Mhi-vap-1* transcript was observed in the oesophageal gland cells of J2 when compared with the expression obtained in eggs and females (Duarte *et al*., 2014). This gene is believed to be associated with resistance as it triggers a Cf-2/Rcr3pim dependent programmed cell death in tomato plants (Lozano-Torres *et al*., 2012).

This paper describes the identification of four *M. hispanica* putative parasitism genes (cathepsin L cysteine protease, *cpl-1*; calreticulin, *crt-1*; *β*-1,4-endoglucanase-1, *eng-1*; and manganese superoxide dismutase, *mnsod*) and the

differential expression from mRNA isolated from eggs, J2 and females using semi-quantitative PCR. The phylogenetic relationship of *M. hispanica* putative parasitism genes and predicted amino acid sequences *M. hispanica* to genes with other RKN species was also evaluated.

1.3 Materials and methods

1.3.1 Nematode isolates

The isolates of *M. hispanica* (PtHi3), *M. arenaria*, *M. javanica* and *M. hapla* (Maleita *et al*., 2012c) and one of *M. incognita*, provided by Rothamsted Research, UK, were selected and reared on tomato, *Solanum lycopersicum* L., cv. Tiny Tim, in a growth chamber. Two months after the inoculation with 10 eggs masses (EM), the eggs were extracted with 0.52% sodium hypochlorite (Hussey & Barker, 1973); the freshly hatched J2 were obtained from EM placed on a 25 µm mesh sieve and the females extracted from galled roots. The isolates identity was confirmed by isoesterase phenotypes (Pais *et al*., 1986; Abrantes *et al*., 2008).

1.3.2 Bioinformatics and sequence analysis

Eleven putative effector genes, annexin-2 (*nex-2*), β-1,4-endoglucanase-1 and 2 (*eng-1* and *eng-2*), cathepsin L cysteine protease (*cpl-1*), calreticulin (*crt-1*), glutathione-S-transferase (*gsts-1*), manganese superoxide dismutase (*mnsod*) pectase lyase 3 (*pel-3*), polygalacturonase (*gp-1*), venom allergen-like protein -2 (*vap-2*) and 14-3-3a (*14-3-3a*), were searched in the National Center for Biotecnology information (Table 1.1) and gene models were attributed to expressed sequence tag (EST) contigs using BLAST searches against the predicted proteins from the genome of *M. incognita* (http://www.inra.fr/meloidogyne_incognita). The same criteria were used

to attribute gene models from the *M. hapla* genome (http:www.pngg.org/cbnp/index.php). Putative orthologs were searched by reciprocal best-hit comparison, using gene models from the genome of *M. incognita* and *M. hapla* (Table 1.1). Alignments were inspected in the program Multiple Sequence Alignment by Florence Corpet (MultAlin, Hosted by the Plateforme Bioinformatique Genotoul). After the alignment, the sequences for each gene in *M. incognita* and *M. hapla* were used to design primers (MIHA) to the conserved regions using the program Vector NTI (Invitrogen, UK) (Table 1.2).

Table 1.1. Category and name of selected effectors and homologous predicted proteins retrieved from the *Meloidogyne incognita* and *M. hapla* genome sequences.

Category	Effector name (gene code)	GenBank	*M. incognita*		*M. hapla*	
			Contig	Position	Contig	Position
Defence against local ROS production	Manganese superoxide dismutase (*mnsod*)	CAO78623.1	MIV1ctg543	33122-34277	MhA1_Contig1330	13461-14286
Food digestion	Cathepsin L cysteine protease (*cpl-1*)	CAD89795.1	MiV1ctg688	627-1923	MhA1_Contig1380	5354-6366
Host cell wall degradation	*β*-1,4–endoglucanase-1 (*eng-1*)	AAD45868.1	MiV1ctg652	34739-37126	MhA1_Contig344	4481-6245
	β-1,4–endoglucanase-2 (*eng-2*)	AAK21883.2	MiV1ctg2217	1161-3874	MhA1_Contig188	563-2063
	Pectase lyase 3 (*pel-3*)	AAW56829.1	MiV1ctg24	109367-111322	MhA1_Contig418	1229-2522
	Polygalacturonase (*gp-1*)	AAM28240.1	MiV1ctg2057	1783-5051	MhA1_Contig1443	5637-8213
Host defense suppression	Calreticulin (*crt-1*)	AAL40720.1	MiV1ctg188	55929-58098	MhA1_Contig309	111554-112581
	Glutathione-S-transferase (*gsts-1*)	ABN64198.1	MIV1ctg612	16153-17172	MhA1_Contig1372	581-1485
Targeting plant signaling pathways	Annexin-2 (*nex-2*)	CAC33829.1	MiV1ctg94	102978-106250	MhA1_Contig3394	269-1769
	14-3-3a (*14-3-3a*)	AAL40719.1	MiV1ctg1197	11882-14562	MhA1_Contig618	35755-37527
Unknown	Venom allergen-like protein-2 (*vap-2*)	ABO38110.1	MiV1ctg1347	11170-13062	MhA1_Contig2874	4892-6143

Effector category designation based on Hassan *et al.* (2010), Haegeman *et al.* (2012) and Jaounnet *et al.* (2013).

1.3.3 DNA extraction

Genomic DNA was extracted from J2 of each *Meloidogyne* isolate, using an adaptation of the protocol described by Orui *et al*. (1999). Nematodes were homogenized in liquid nitrogen with 400 µL of extraction buffer (200 mM Tris-HCl pH 8; 250 mM NaCl and 25 mM EDTA) and centrifuged at 20,000 x g for 5 min. The supernatant was transferred to a new tube and an equal volume of isopropanol was added. After swirling the tube, the mixture was incubated at room temperature for 30 min and centrifuged at 20,000 x g for 15 min. The supernatant was removed and the pellet washed with 500 µL of 70% ethanol and centrifuged for 5 min at 20,000 x g. Afterwards, the supernatant was removed and the pellet dried. The DNA was resuspended in 30 µL of Tris-EDTA (10 mM Tris-HCl pH 8 and 1 mM EDTA) and the concentration determined in a Nanodrop ND-1000 Spectrophotometer (Labtech International, UK).

1.3.4 Amplification of the genes

PCR amplifications were performed in a mixture containing 25 ng of RKN DNA as template, 5 units of Taq DNA polymerase (Promega, UK), 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's and 10 µM of each primer (Table 1.2). Amplifications were carried out using the following conditions: 3 min at 95°C, 30 cycles at 95°C for 30 s, 40°C for 30 s and 72°C for 2 min and a final extension at 72°C for 5 min. The PCR reaction was analyzed on a 1.0% agarose gel in 1x TAE buffer stained with GreenSafe (NZYTech, Portugal). The amplified products to *cpl-1*, *crt-1*, *eng-1* and *mnsod* were purified with the QIAquick Gel Extraction Kit (QIAGEN, UK) and sequenced by standard procedures at Eurofins MWG Operon (Ebersberg, Germany) and Macrogen (Europe). The sequences were deposited in GenBank as KF679112,

KF679109, KF679110, KF679108, KF679111 for CPL-1 in *M. arenaria*, *M. hapla*, *M. hispanica*, *M. incognita* and *M. javanica*; KF679115, KF679113, KF679116, KF679114 for CRT-1 in *M. arenaria*, *M. hapla*, *M. hispanica* and *M. javanica*; KF679117, KF679118, KF679121, KF679120, KF679119 for ENG-1 in *M. arenaria*, *M. hapla*, *M. hispanica*, *M. incognita*, and in *M. javanica*; and KF679122, KF679125, KF679124, KF679123, KF679126 in *M. arenaria*, *M. hapla*, *M. hispanica*, *M. incognita* and *M. javanica* for MNSOD, respectively. To continue the study and analyze the gene expression in different *M. hispanica* developmental stages, the sequences of *cpl-1*, *crt-1*, *eng-1* and *mnsod* genes were aligned with the program MultAlin with M. incognita sequences available in *M. incognita* (consortium) (Table 1.1). *Meloidogyne hispanica* specific primers (MHI) were designed, using the program Vector NTI, from conserved sequences between this species and *M. incognita* (Table 1.2).

Table 1.2. Primers used in this study.

Effector name	Primer name	Primer sequence 5'→3'
Annexin-2	MIHA-NEX-2f	TGAGTTACTACGCAAAGCTA
	MiHA-NEX-2r	GTTGATATGCTGAACGAATA
β-actin	*β*-actinf	GATGGCTACAGCTGCTTCGT
	β-actinr	GGACAGTGTTGGCGTAAAGG
β-1,4-endoglucanase-1	MIHA-ENG-1f	GAATTCTTCACATATTTTGCCC
	MIHA-ENG-1r	AGTGTAGCAAAGGTTTGTGC
	MHI-ENG-1f	TGAGACTTTCAATGAGCCACT
	MHI-ENG-1r	TGTCAGTGTAGCAAAGGTTTGTG
β-1,4-endoglucanase-2	MIHA-ENG-2f	CAATGGTATTCTCCTGAAG
	MIHA-ENG-2r	GTTCATTCCAAAGCTCCAAA
Calreticulin	MIHA-CRT-1f	ACCTATGAAGTCCAAATTGA
	MIHA-CRT-1r	TTAAAGCTCATCATGCTCCT
	MHI-CRT-1f	CTGATTGGGAGTTGTTGCCG
	MHI-CRT-1r	GTCACAATAATATTGTCGAA
Cathepsin L cysteine	MIHA-CPL-1f	CAGATTGCTGTTGCCACACA
	MIHA-CPL-1r	CCGCATTGATTGTCTTTGTT
	MHI-CPL-1f	GTTTATGATGAGGAGGCTTG
	MHI-CPL-1r	CGCATTGATTGTCTTTGTTT
Glutathione-S-transferase	MIHA-GSTS-1f	GAAAAATGGCCAGCCGAGAA
	MIHA-GSTS-1r	TCCTTTCCAGCCAAACCTTT
Manganese superoxide dismutase	MIHA-MNSODf	CATGCTTTGCCTGATCTTCC
	MIHA-MNSODr	CATCAATTCCAAAGAGAGGC
	MHI-MNSODf	CATCATGGTTTGTCTGTTGGA
	MHI-MNSODr	AAAATAAACCTTTGGCAAGAGC
Pectase lyase 3	MIHA-PEL-3f	GGTGCTTTAAACGGTTTTCA
	MIHA-PEL-3r	TTGAGTTTTCAATGGTAACG
Polygalacturonase	MIHA-GP-1f	TAAATACTTTGTCTGGCCCT
	MIHA-GP-1r	AATATTTTTATTATAAACTG
Venom allergen-like protein-2	MIHA-VAP-2f	TGTTGTTAAATTCCGAGGTT
	MIHA-VAP-2r	ATAAGTGCATTTATCAGCCC
14-3-3a	MIHA-14-3-3Af	TCAAATTATTCTTTCGGTTT
	MIHA-14-3-3Ar	CCCTTTCATACCATTCTTTA

1.3.5 RNA extraction and expression analysis

Total RNA was extracted from *M. hispanica* eggs, J2 and females. The specimens were placed in liquid nitrogen and homogenized, separately, using the Sample Preparation System M.P Fast Prep-24, speed at 4.0 M/s (MP Biomedicals, California, USA) and the RNA isolated using the RNeasy Mini Kit including RNase-Free DNase Set (QIAGEN, UK). The concentration and purity of the RNA was determined in a Nanodrop ND-1000 Spectrophotometer. Total RNA (180 ng) of each developmental stage was reverse transcribed into cDNA using the SuperScript II Reverse Transcriptase (Invitrogen, UK), according to the manufacturer's instructions, and the concentration determined. After the reverse transcriptase reaction, a standard PCR was performed with the *M. hispanica* specific primers (MHI-CPL-1f/r, MHI-CRT-1f/r, MHI-ENG-1f/r and MHI-MNSOD-f/r, Table 1.2). Actin genes were amplified from each sample as positive control (Table 1.2). The PCR mixture, containing 25 ng of synthesized cDNA template and 5 units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's and 10 μM of each primer, was first heated for 3 min at 95°C, and then submitted to 30 temperature cycles (95°C for 30 s, 40°C for 30 s and 72°C for 1.5 min) with a final extension at 72°C for 5 min or 10 min for *Mhi-mnsod*.

1.3.6 Phylogenetic analysis

The sequences of *cpl-1*, *crt-1*, *eng-1* and *mnsod* genes of *M. hispanica*, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* isolates were successfully amplified and sequenced, with the primers designed in this work (Table 1.2), except the *crt-1* gene sequencing of the *M. incognita* isolate that was obtained from the GenBank nucleotide database. Sequences were aligned and truncated to obtain a common

start and end point. The alignment allowed the identification of the additional coding sequence of each sequence and the removal of introns. The amino acid sequences were deduced from the 380 bp, 970 bp, 220 bp and 580 bp DNA sequences for the partial *cpl-1*, *crt-1*, *eng-1* and *mnsod* sequences, respectively. Protein phylogenetic trees were constructed using the Neighbor-Joining (Saitou & Nei, 1987) and Maximum-Likelihood (Jones *et al*., 1992) algorithms, and topology of the trees were generated from evolutionary distances computed using the Poison correction method, included in MEGA5 (Zuckerkandl & Pauling, 1965; Tamura *et al*., 2011). The topology of the trees generated was evaluated by performing bootstrap analysis (Felsenstein, 1985) of 500 resamplings of the data set. All positions with less than 75% site coverage were eliminated. The protein alignment was used to determine the nucleotide position in the DNA sequence alignments that were further used to perform DNA sequences and phylogenetic analysis as described above. Evolutionary distances were computed using the Jukes-Cantor model correction method included in MEGA5 (Jukes & Cantor, 1969; Tamura *et al.,* 2011).

1.4 Results

1.4.1 Bioinformatics and sequence analysis

Based on the analysis of ESTs from *M. incognita* and *M. hapla*, a pair of primers was designed to the conserved regions of the selected putative parasitic genes, which permitted the successful amplification of genomic DNA of all *Meloidogyne* isolates. The amplification of the DNA from J2 of the selected RKN species resulted in a fragment of approximately 380 bp for *cpl-1*; 970 bp for *crt-1*; 220 bp for *eng-1*; 670 bp for *eng-2*; 2150 bp for *gp-1*; 280 bp for *gsts-1*; 580 bp for *mnsod*; 1220 bp for *nex*-

2; 130 bp for *pl-3*; 250 bp for *vap-2* and 820 bp for *14-3-3a* (Fig. 1.1, data for *M. hispanica* and *M. incognita*).

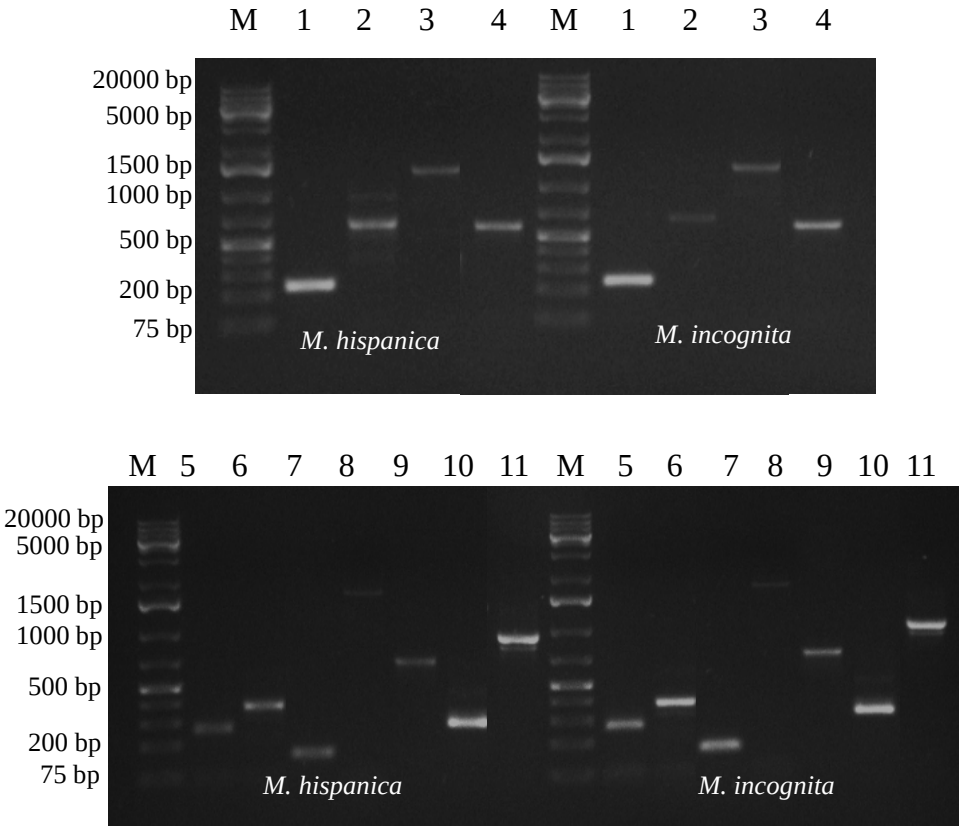


Figure 1.1 DNA amplification products of *Meloidogyne hispanica* and *M. incognita* (positive control) obtained from the selected nematode genes β-1,4-endoglucanase-1 (1), β-1,4-endoglucanase-2 (2), annexin-2 (3), manganese superoxide dismutase (4), venom allergen-like protein-2 (5), cathepsin L cysteine protease (6), pectase lyase 3 (7), polygalacturonase (8), 14-3-3a (9), glutathione-S-transferase (10) and calreticulin (11). M - DNA marker (GeneRuler 1 kb Plus DNA ladder, Fermentas).

1.4.2 Transcription analysis

Reverse transcription polymerase chain reaction (RT-PCR), using the specific primers for the *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1* and *Mhi-mnsod* genes, shows the gene expression of these genes in *M. hispanica* developmental stages (eggs, J2 and females). The specific bands obtained have a molecular weight of approximately 180,

420, 170 and 160 bp for *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1* and *Mhi-mnsod*, respectively. Amplification of the *β-actin* gene was used as a positive control (Fig. 1.2). Although the cDNA fragments of *Mhi-cpl-1* and *Mhi-crt-1* were amplified in all of the developmental stages, there was a clear difference in band intensity: in *Mhi-cpl-1*, the bands were equally intense in all the developmental stages while in *Mhi-crt-1*, the band was more intense in females when compared with those obtained in eggs and J2 (Fig. 1.2 A, B). The cDNA fragments were equally amplified in eggs and J2 for the gene *Mhi-eng-1* (Fig. 1.2 C) and the *Mhi-mnsod* gene was only expressed in eggs (Fig. 1.2 D).

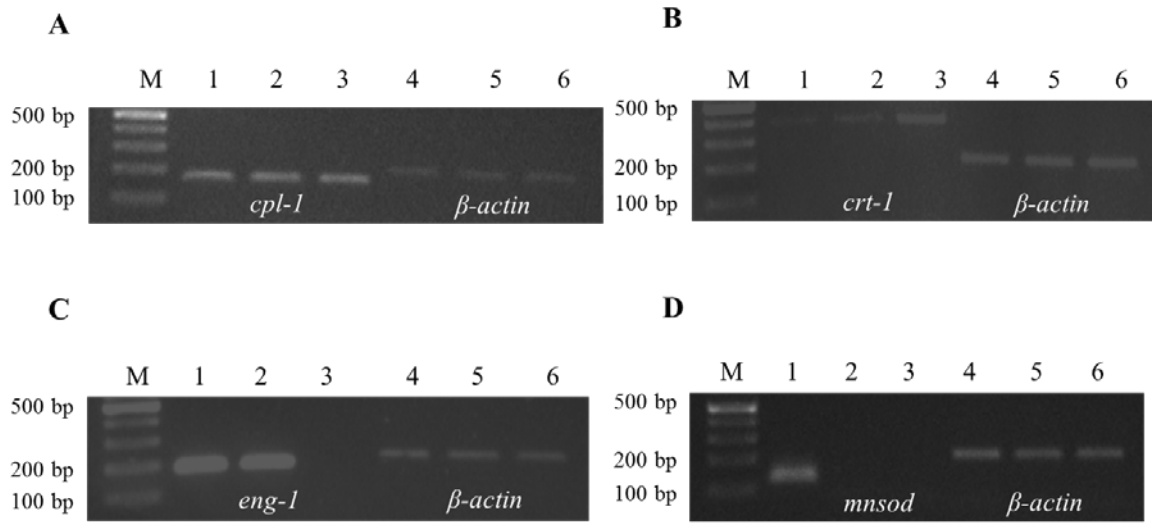


Figure 1.2 Expression of the genes putative cathepsin L cysteine protease, *cpl-1* (A), calreticulin, *crt-1* (B), β-1,4 endoglucanase-1, *eng-1* (C) and manganese superoxide dismutase, *mnsod* (D) by reverse transcriptase mediated PCR amplification of cDNAs from *Meloidogyne hispanica* eggs (1), second-stage juveniles (J2, 2) and females (3). M - DNA marker. As positive control, cDNA templates of eggs, J2 and females were amplified with the primers of *β-actin* gene (4 to 6, respectively).

1.4.3 Phylogenetic analysis

The *M. hispanica cpl-1*, *crt-1*, *eng-1* and *mnsod* partial gene sequences and the corresponding fragments from *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* were used for the phylogenetic analysis of the partial gene and predicted amino acid sequences (Figs. 1.3 and 1.4). Both Neighbor-Joining (NJ) and Maximum-Likelihood algorithms yielded similar topologies for DNA and protein trees, so only NJ trees are shown. Analyzing the multiple DNA and protein alignments, significant sequence conservation within the *Meloidogyne* species was detected, despite the fact that DNA sequences showed slightly more differences (Fig. 1.5). *Meloidogyne hispanica* displayed amino acid sequence similarity ranging from 88 to 100% when compared with the other species CPL-1, CRT-1, ENG-1 and MNSOD sequences, being the lowest values always to those obtained for *M. hapla* (data not shown). Similar results were obtained for the gene sequences similarity values. The results of the topology of the MHI-CPL-1, MHI-CRT-1, MHI-MNSOD and MHI-ENG-1 trees showed slight differences to the obtained from DNA sequences, but the overall phylogenetic relations were essentially the same (Figs. 1.3 and 1.4). Indeed, *M. hapla* appeared as an outgroup and all the other RKN species clustered together (Fig. 1.3). The similarity values of DNA sequences decreased when compared with the values obtained from amino acid sequences (data not shown).

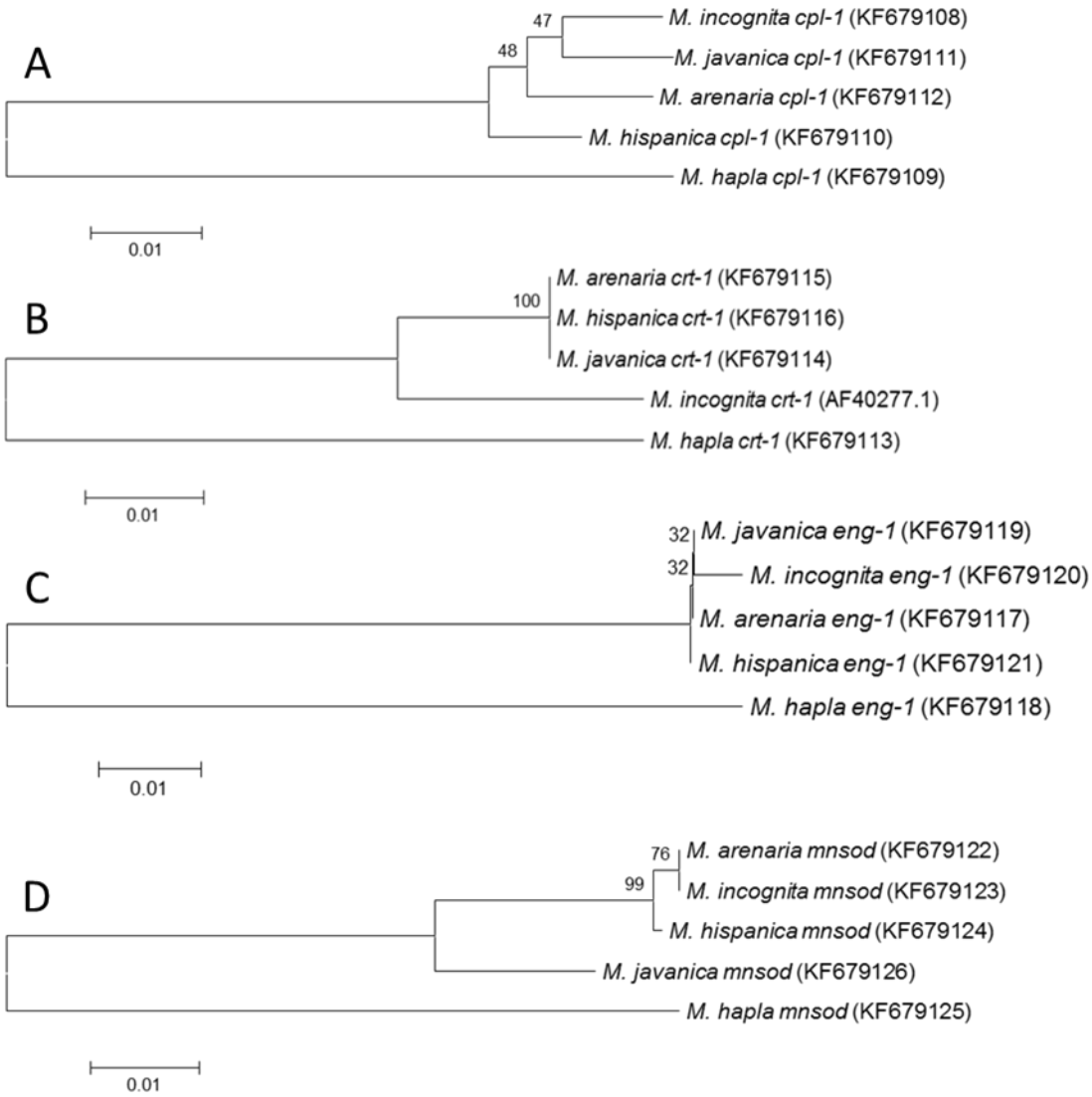


Figure 1.3 Neighbor-joining phylogenetic trees constructed from gene sequences of the cathepsin L cysteineprotease, *cpl-1* (A), calreticulin, *crt-1* (B), β-1,4 endoglucanase-1, *eng-1* (C) and manganese superoxide dismutase, *mnsod* (D) from *Meloidogyne hispanica*, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. *Meloidogyne incognita crt-1* (AF40277.1) sequence was obtained from GenBank nucleotide database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

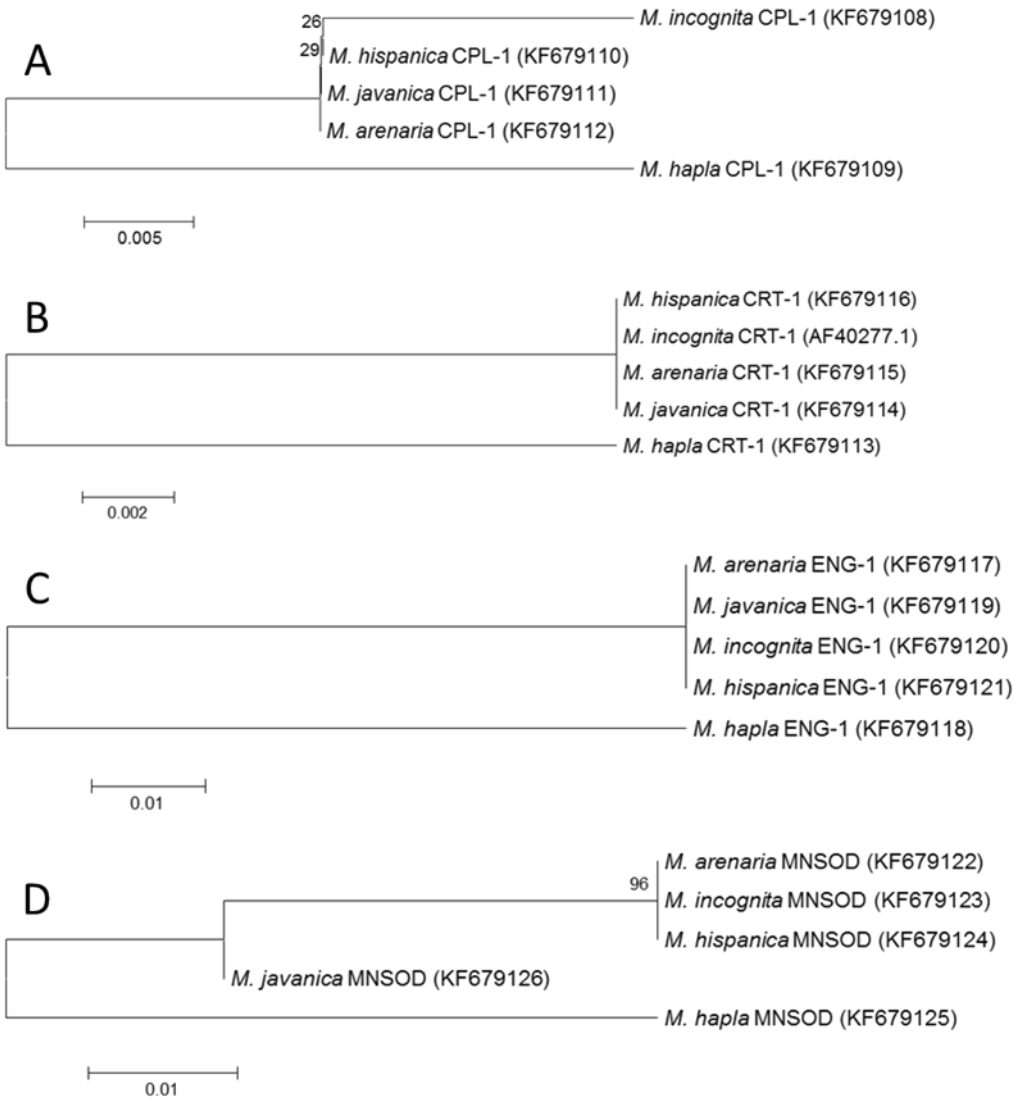


Figure 1.4 Neighbor-joining phylogenetic trees constructed from amino acid sequences of the predicted cathepsin L cysteine protease, CPL-1 (A), calreticulin, CRT-1 (B), β-1,4-endoglucanase-1, ENG-1 (C) and manganese superoxide dismutase, MNSOD (D) from *Meloidogyne hispanica*, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

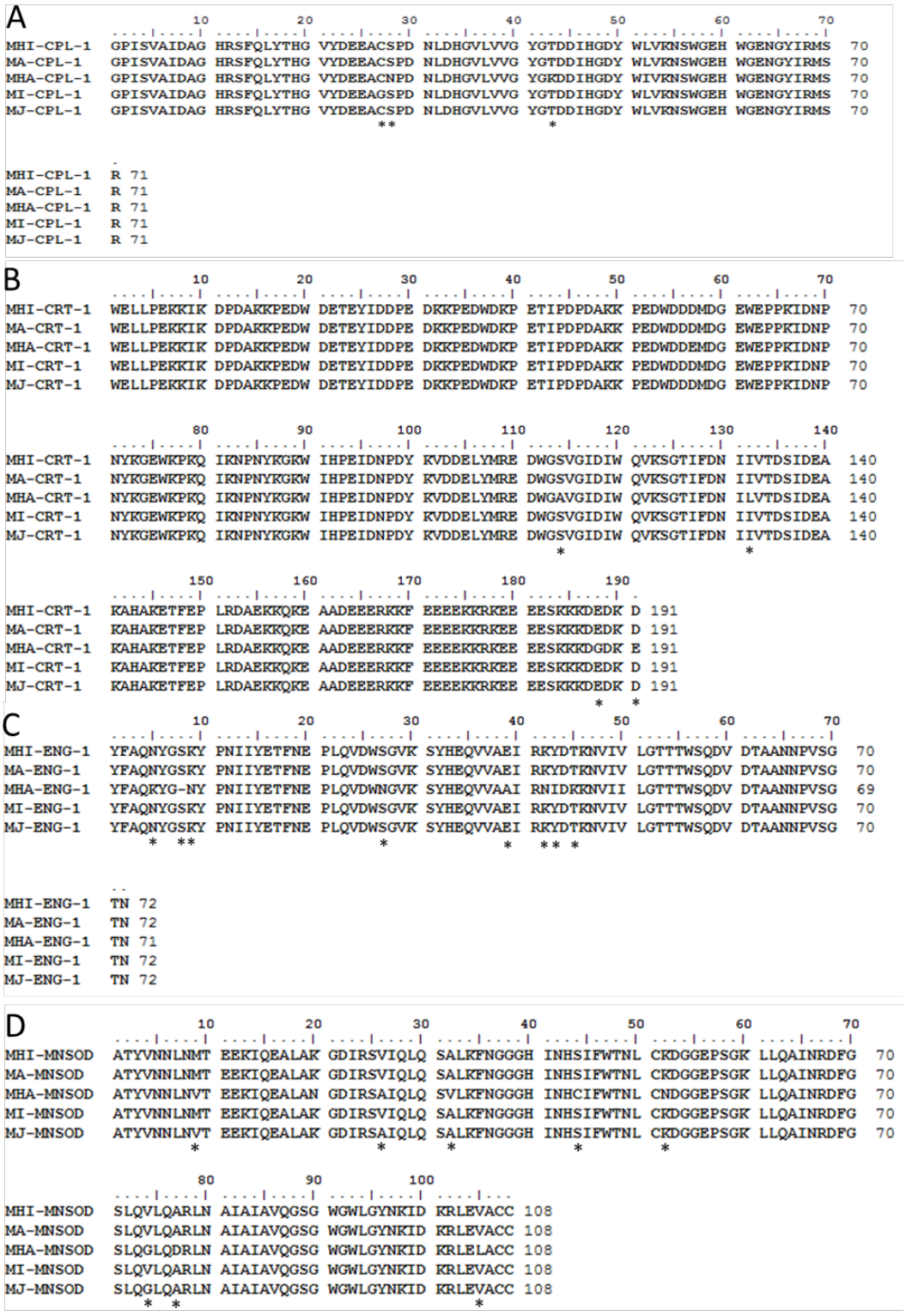


Figure 1.5 Multiple sequence alignment of predicted *Meloidogyne hispanica* putative cathepsin L cysteine protease, MHI-CPL-1 (A) (KF679110), calreticulin, MHI-CRT-1 (B) (KF679116), β-1,4-endoglucanase-1, MHI-ENG-1 (C) (KF679121), and manganese superoxide dismutase, MHI-MNSOD (D) (KF679124), amino acid sequences with homologues from other *Meloidogyne* spp. Differences between amino acids are indicated by asterisks.

1.5 Discussion

Seven Portuguese *M. hispanica* isolates were studied by biometrical, biochemical and molecular characteristics, and a new molecular diagnostic method, based on the mtDNA region between COII and 16S rRNA genes, was developed (Maleita *et al*., 2012c). However, to date little is known about the *M. hispanica* genes and their possible roles in the parasitism. The recent availability of the *M. incognita* and *M. hapla* genome sequences will lead to a better understand of plant-parasitic nematodes (Abad *et al*., 2008; Opperman *et al*., 2008) and allowed the isolation of eleven *M. hispanica* genes. *Meloidogyne incognita* and *M. hapla* are considered optimal RKN species for genome comparison (Abad *et al*., 2008; Opperman *et al*., 2008; Bird *et al*., 2009) and their genome information was instrumental for the successful of isolation of twelve *M. hispanica* genes. These were amplified using primers designed to the most conserved regions of the sequences of proteins potentially involved in the parasitism of *M. incognita* and *M. hapla*. The partial sequences of four selected genes (*cpl-1, crt-1*, *eng-1* and *mnsod* genes) encoding secreted proteins potentially involved in the early events of nematode infection and during nematode development was obtained and their differential expression in eggs, J2 and adult females was analyzed. The expression of the *cpl-1* gene occurred equally in eggs, J2 and females of *M. hispanica* however Shingles *et al.* (2007) report that in *M. incognita* the expression of this gene was slightly lower in mature females. The CPL-1 enzyme, identified in secretions of *M. incognita* belong to a family of proteins that affect a broad range of biological processes including nutrition, digestion, and tissue penetration and may influence the host-parasite relationship (Neveu *et al*., 2003; Ultaigh *et al*., 2009). In plant-parasitic nematodes this enzyme is specifically expressed in the intestinal cells and is associated with food digestion and

protein degradation (Hassan *et al*., 2010; Haegeman *et al*., 2012). Urwin *et al*. (1995) showed that transgenic expression of a cysteine proteinase inhibitor in plant roots confers resistance to *G. pallida*, by causing detrimental effects in the growth and development of the nematode with reduction in female size to levels that compromise fecundity.

The expression of the calreticulin gene in all the developmental stages of *M. hispanica*, is consistent with other RKN species, however, the stronger band intensity of *Mhi-crt-1* in females of *M. hispanica*, might suggest that this gene plays an important role in the later events infection (possibly during eggs deposition in the gelatinous matrix). The CRT protein, identified in *M. hapla* and *H. glycines* (Ithal *et al*., 2007; Opperman *et al*., 2008), is synthesized in the subventral oesophageal glands of pre-parasitic J2 and in the dorsal oesophageal gland of the parasitic stages. During feeding cell induction and maintenance, this protein accumulates along the wall of the giant cells and might play an important role in the suppression of plant basal defences and in targeting plant signaling pathways (Jaubert *et al*., 2002, 2005; Hassan *et al*., 2010; Haegeman *et al*., 2012; Jaouannet *et al*., 2012, 2013). Furthermore, the knockdown of the *M. incognita crt* gene by RNA interference (RNAi) affected the infection process (Jaouannet *et al*., 2013).

β-1,4-endoglucanases, secreted into the apoplast during nematode intra or intercellular migration (Wang *et al*., 1999), interact with specific cytoplasmic or nucleus targeted proteins (Hewezi & Baum, 2013). The hydrolysis of cellulose by RKN is catalysed through β-1,4 endoglucanase (Mitreva-Dautova *et al*., 2006). The gene *eng-1* was transcribed in the migratory path of J2, males and adult females of *M. incognita* inside the roots (Rosso *et al*., 1999) however our data demonstrate that in *M. hispanica*, this gene was transcribed only in the eggs and J2 but not in adult

females, suggesting that for *M. hispanica* this gene might function only in the early events of infection. This gene was also shown to be highly expressed in *Rotylenchulus reniformis* J2 and a strong decline of the expression was observed in the sedentary females (Wubben *et al*., 2010).

The MNSOD enzyme has been previously identified and characterized in *M. incognita* J2 and localized in the intestine, which suggest a putative function as a detoxification enzyme. Plants produce ROS in response to nematode penetration, however J2 seems to quickly react to the oxidative stress and metabolize these compounds. This enzyme could play an important role in the establishment and maintenance of the nematode inside the host (Rosso, 2009). Nevertheless, the *M. hispanica mnsod* gene was only expressed in eggs which imply another function for this gene during the infection process and life-cycle of this RKN.

The RT-PCR assays confirmed the expression of *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1* and *Mhi-mnsod* genes in the different developmental stages, revealing differences in the expression of these genes when compared with other RKN species. Therefore, these genes can be involved in multiple important functions during parasitism of RKN. In earlier experiments, the molecular analysis of three rDNA regions (18S, ITS1-5.8S-ITS2, and D2-D3) and the phylogenetic analysis of the sequences of three *M. hispanica* isolates from different geographical origin (Brazil, Portugal and Spain), demonstrated that *M. hispanica* can be differentiated from *M. incognita*, *M. javanica*, *M. arenaria* and other known *Meloidogyne* species (Landa *et al.*, 2008).

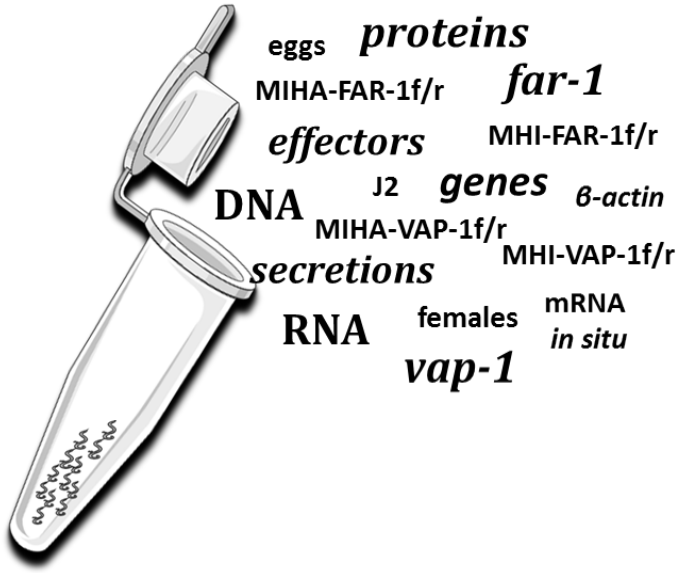
The multiple alignments, obtained for *cpl-1*, *crt-1*, *eng-1* and *mnsod* parasitic genes and the respective predicted amino acids sequences, confirmed the close phylogenetic relationship among the *Meloidogyne* species analyzed, with *M. hapla* the outgroup. High level of identity and similarity values among DNA and protein

sequences was also observed. The presence of silent mutations on DNA sequences alignments were scrutinized and are probably responsible for the discrete differences between DNA and protein trees topologies.

This paper reports for the first time the amplification of 11 putatively parasitism-related genes from *M. hispanica*, four of these genes were characterized in *M. hispanica*. In comparison to the other RKN species, two of the genes in particular, *Mhi-eng-1* and *Mhi-mnsod*, showed transcription in different nematode developmental stages indicating that these genes might play different roles in the life-cycle processes of *M. hispanica*. Further studies are needed to understand their biological functions in this nematode which is considered a species of emerging importance for economically valuable crops and difficult to control.

Chapter 2

Characterization of the venom allergen-like protein (*vap-1*) and the fatty acid and retinol binding protein (*far-1*) genes in *Meloidogyne hispanica*



Written as a Journal article:

Duarte, A., Curtis, R., Maleita, C., Tiago, I. & Abrantes, I. (2014). Characterization of the venom allergen-like protein (*vap-1*) and the fatty acid and retinol binding protein (*far-1*) genes in *Meloidogyne hispanica*. European Journal of Plant Pathology 139, 825-836.

2.1 Abstract

The root-knot nematode (RKN) *Meloidogyne hispanica* has been found in all continents associated with a wide host range, including economically, important plants and can be considered a species of emerging importance. Considerable progress has been made to identify nematode effector genes as they are important targets for the development of novel control strategies. The effector genes, venom allergen-like protein (*vap-1*) and fatty acid and retinol binding protein (*far-1*), were identified, isolated and sequenced in *M. hispanica* (*Mhi-vap-1* and *Mhi-far-1*) using the genome information available for the RKN *M. incognita* and *M. hapla*. These genes are differentially expressed during *M. hispanica* development and their amplification products were observed from cDNA of the eggs, second-stage juveniles (J2) and adult females. However, *Mhi-vap-1* showed the highest level of expression in J2. *In situ* hybridization analysis revealed that the *Mhi-vap-1* and *Mhi-far-1* transcripts are accumulated within the J2 subventral oesophageal glands. The specific expression in the subventral oesophagel glands and presence of the secretion signal peptide for both genes suggests that these proteins are secreted by the J2 and may play a role in the early parasitic stage of the infection process. These genes were also isolated and sequenced in *M. arenaria*, *M. incognita* and *M. javanica*; and phylogenetic analysis revealed that the predicted protein sequences belonging to *M. hispanica* and several other species of plant-parasitic nematodes have a high degree of conservation.

Keywords: nematode effectors, root-knot nematodes, secretions, subventral oesophageal glands.

2.2 Introduction

The sedentary endoparasite root-knot nematode (RKN) *Meloidogyne hispanica* Hirschmann (1996), detected for the first time in Seville, Spain, from the peach rootstock, *Prunus persica silvestris* Batsch, has a worldwide distribution, being found in all continents (Europe, Africa, Asia, Australia and North, Central and South America) associated with a wide range of host plants (Hirschmann 1996; Maleita *et al*. 2012a). Recent studies showed that *M. hispanica* can spread in Europe and move northwards, can overcome the tomato *Mi*-1.2 gene and can attack economically important plant species and cultivars, including commercial tomato crops. These are characteristics of a polyphagous species of emerging importance, which is difficult to control with crop rotation and with the use of resistant cultivars (Maleita *et al*. 2011, 2012a,b,c).

Like other plant-parasitic nematodes, RKN are known to secrete effector proteins into the host tissues which can alter plant physiology and assist the infection process (Rosso & Grenier, 2011). These molecules are secreted from the nematode oesophageal gland cells, as well as from amphids and nematode surface cuticle (Davis *et al*., 2008; Davies & Curtis, 2011; Rosso *et al.,* 2012). A number of candidate effector genes, from both cyst nematodes and RKN have been identified, using cDNA libraries from RNA extracted specifically from the oesophageal gland cells of these nematodes (Wang *et al*., 2001; Gao *et al*., 2003; Huang *et al*., 2003; Hussey *et al*., 2011). So far, the functions for several potential effector proteins have been predicted and showed that various cellular processes can be targeted by the nematode for successful manipulation of the host response. These include the cell wall structure, manipulation of cell fate, protein synthesis and alteration of signaling pathways. Functional tests, using RNAi, have supported the putative role of some of

these nematode effectors in pathogenesis (Bellafiore *et al*., 2008; Roze *et al*., 2008; Bellafiore & Briggs, 2010). The venom allergen-like proteins (VAPs) are homologues of the plant and animal cystein-rich secretory proteins (CRISPs). Although, the VAPs are part of a family of effectors considered to be conserved in all parasitic nematodes of plants and animals, its function is still unknown (Haegeman *et al*., 2012). They have been identified and characterized from the RKN *M. incognita*, *M. hapla* and *M. chitwoodi*, the cyst nematodes *Heterodera glycines*, *H. schachtii* and *Globodera pallida*, the root-lesion nematode *Pratylenchus coffeae* and the pinewood nematode *Bursaphelenchus xylophilus* (Ding *et al*., 2000; Gao *et al.,* 2001; Vanholme *et al*., 2006; Wang *et al*., 2007; Opperman *et al*., 2008; Roze *et al.,* 2008; Jones *et al*., 2009; Kang *et al*., 2010, 2012; Haegeman *et al*., 2011, 2012). Various allergen proteins were described as being highly transcribed during plant nematode parasitism (Ding *et al*., 2000; Gao *et al*., 2001; Wang *et al*., 2007). Some are proposed to be involved in the defence response mediated by extracellular innate immune receptors and host invasion (Hawdon *et al*., 1999; Murray *et al*., 2001). The *vap-1* gene is recognized as being potentially associated with the *M. incognita* infection process, with the induction of a host immune response and resistance since it triggers a Cf-2/Rcr3pim dependent programmed cell death in tomato plants (Gao *et al*., 2001; Haegeman *et al*., 2009; Chen *et al*., 2010; Lozano-Torres *et al*., 2012). The protein FAR-1 is a member of the nematode specific fatty-acid and retinol binding (FAR) family of proteins and was detected for the first time in *G. pallida*. This protein is present in the surface coat of potato cyst nematode species and binds fatty acids, including linoleic acids that are precursor of plant defence compounds (Prior *et al*., 2001). These fatty acids are metabolized by lipoxygenase as part of the signalling pathway leading to the production of jasmonic acid and FAR-1 inhibited this

biochemical process *in vitro* thus may have a role in the suppression of jasmonate synthesis and of the downstream signalling pathways, reducing host defences (Curtis, 2007; Haegeman *et al*., 2012). A study with *M. javanica* suggests that the MJ-FAR-1 protein has an important role in the infection process. FAR-1 induces susceptibility to RKN through the manipulation of jasmonate-dependent defence response (Iberkleid *et al*., 2013). FAR-1 has also been identified in *H. schachtii*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *P. coffeae* and *Radopholus similis* (Vanholme *et al*., 2006; Bellafiore *et al*., 2008; Jacob *et al.,* 2008; Opperman *et al*., 2008; Roze *et al.*, 2008; Haegeman *et al.,* 2009a, 2011).

The main goals of this research were to isolate, to characterize the expression and to localize the *vap-1* and *far-1* genes in *M. hispanica*, which may be good targets for the development of novel control strategies for this species, and also to investigate the phylogenetic relationship of *M. hispanica* with other plant-parasitic nematodes.

2.3 Materials and methods

2.3.1 Nematode isolates

The *M. hispanica* isolate used in this study was originally obtained from infected fig tree (*Ficus carica* L.) roots collected in Odeceixe, Faro, Portugal; the *M. incognita* isolate was provided by Rothamsted Research, UK; and the Portuguese isolates of *M. arenaria* and *M. javanica* were originally obtained from *Oxalis corniculata* L. and *Solanum tuberosum* L roots, respectively. All the isolates were maintained on tomato, *S. lycopersicum* L., cv. Tiny Tim, in pots containing sterilized sandy loam soil and sand (1:1), in a growth chamber, at 25±2°C, with approximately 75% relative humidity. Two months after the inoculation with 10 egg masses (EM), the eggs were extracted using 0.52% sodium hypochlorite (NaOCl) solution (Hussey & Barker

1973), the freshly hatched J2 were obtained from the egg masses placed on a 25 µm mesh sieve, and the females extracted from galled roots. Species identification were confirmed by esterase phenotype analyses (Pais *et al.,* 1986; Abrantes *et al.,* 2008).

2.3.2 Bioinformatics and sequence analysis

Homolog proteins VAP-1 and FAR-1 sequences were searched in the National Center for Biotecnology information (Genbank accessions Nº. ABL61274.1 for VAP- 1 in *M. arenaria* and Nº. CAA70477.2 for FAR-1 in *G. pallida*).

Gene models were attributed to expressed sequence tag (EST) contigs using tblastn searches against the predicted proteins from the genome of *M. incognita* (http://www.inra.fr/fr/meloidogyne_incognita). The same criteria were used to attribute gene models from the *M. hapla* genome (http://www.pngg.org/cbnp/index.php). Putative orthologs were searched by reciprocal best-hit comparison, using gene models from the genome of *M. incognita* (Minc17158) and *M. hapla* (Mh10g200708_contig2874) for *vap-1* and from the genome of *M. incognita* (Minc08986) and *M. hapla* (Mh10g200708_contig113) for *far-1*.

Alignments were analysed in the program Multiple Sequence Alignment by Florence Corpet (MultAlin Hosted by the Plateforme Bioinformatique Genotoul). After the alignment, the conserved regions of the DNA sequences of *vap-1* and *far-1* in the two species of RKN, *M. incognita* and *M. hapla,* were used to design primers using the program Vector NTI (Invitrogen, UK). For *vap-1* gene, the primers were MIHA-VAP-1f/MIHAVAP-1r and for *far-1* MIHA-FAR-1f/MIHA-FAR-1r (Table 2.1).

Table 2.1. Primers used in this study.

Primer name	**Primer sequence 5'→3'**
MIHA-VAP-1f	TGGGCTGATAAATGCACTTA
MIHA-VAP-1r	GTGTCCAATGTCCAATACCT
MIHA-FAR-1f	GGCTAGGGTTAATAAGATTTG
MIHA-FAR-1r	CCTTCTGGTTTCAACAAGCT
MHI-VAP-1f	TTATGGAGAGATTTCTATGC
MHI-VAP-1r	GTGTCCAATGTCCAATACCT
MHI-FAR-1f	GATTTGGTCCGCCTGAGGTT
MHI-FAR-1r	CGGTAATCTTGGGGAAGTTG
β-actinf	GATGGCTACAGCTGCTTCGT
β-actinr	GGACAGTGTTGGCGTAAAGG
M13f	CGCCAGGGTTTTCCCAGTCACGAC
M13r	TCACACAGGAAACAGCTATGAC

2.3.3 DNA extraction

Genomic DNA was extracted from *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* J2, using an adaptation of the protocol described by Orui (1999). Nematodes were homogenized in liquid nitrogen with 400 µl of extraction buffer (200 mM Tris–HCl pH 8; 250 mM NaCl and 25 mM EDTA) and centrifuged at 20,000 × g for 5 min. The supernatant was transferred to a new tube and equal volume of isopropanol was added. After swirling the tube, the mixture was incubated at room temperature for 30 min and centrifuged at 20,000 × g for 15 min. The supernatant was removed and the pellet washed with 500 µL of 70% ethanol. After centrifugation for 5 min at 20,000 × g, the supernatant was removed and the pellet dried. The DNA was then resuspended in 30 µl of Tris-EDTA (10 mM Tris– HCl pH 8 and 1 mM EDTA) and the concentration determined in a Nanodrop ND-1000 Spectrophotometer (Labtech International, UK).

2.3.4 Amplification of *vap-1* and *far-1* genes

PCR amplifications were performed in a mixture containing 25 ng of *M. hispanica*, *M. arenaria, M. incognita*, or *M. javanica* DNA as template and five units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's, and 10 µM of each primer, *vap-1* primers were MIHA-VAP- 1f/MIHA-VAP-1r and MIHA-FAR-1f/MIHA-FAR-1r for *far-1* (Table 2.1). Amplifications were carried out using the following conditions: 3 min at 95°C, 40 cycles at 95°C for 30 s, 40°C for 30 s and 72°C for 2 min and a final extension at 72°C for 5 min. The PCR reaction was analysed on a 1.0% agarose gel in 1x TAE buffer stained with GreenSafe (NZYTech, Portugal). The amplified products were purified with the QIAquick Gel Extraction Kit (QIAGEN, UK) and sequenced by standard procedures at Eurofins MWG Operon (Ebersberg, Germany). The sequences designated as *Ma-vap-1*, *Mhi-vap-1*, *Mi-vap-1*, *Mj-vap-1*, *Ma-far-1*, *Mhi-far-1*, *Mi-far-1* and *Mj-far-1* were deposited in GenBank as KF030969, KF030970, KF030971, KF030972, KF030973, KF030974, KF030975 and KF030976, respectively. *Meloidogyne hispanica* specific primers (MHI-VAP-1f/MHI-VAP-1r and MHI-FAR- 1f/MHI-FAR-1r) were designed from conserved sequences between this species and *M. incognita*, located in the 3'UTR region, as described above (Table 2.1).

2.3.5 RNA extraction and developmental expression analysis

Total RNA was extracted from *M. hispanica* eggs, J2 and adult females. The specimens were placed in liquid nitrogen and homogenized, separately, using the sample preparation system MP Fast Prep-24, speed at 4.0 m/s (MP Biomedicals, USA). Afterwards, the RNA was isolated using the RNeasy Mini Kit including RNase-Free DNase Set (QIAGEN, UK). The concentration and purity of the RNA was

determined in a Nanodrop ND-1000 Spectrophotometer. Total RNA (180 ng) of each developmental stage was reverse transcribed into cDNA using the SuperScript II Reverse Transcriptase (Invitrogen, UK), according to the manufacturer's instructions, and the concentration determined. After the reverse transcriptase reaction, a standard PCR was performed with the *M. hispanica vap-1* and *far-1* specific primers (Table 2.1). Actin genes were amplified from each sample as positive control (Table 2.1). The PCR mixture, containing 25 ng of synthesized cDNA template and five units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's and 10 μM of each primer, was first heated for 3 min at 95°C, and then submitted to 39 temperature cycles (95°C for 30 s, 40°C for 30 s, and 72°C for 1.5 min) with a final extension at 72°C for 5 min.

2.3.6 Genomic clone

Amplified *M. hispanica* cDNA fragments were purified, as described above, cloned into pGEM-T Easy vector (Promega, UK), and transformed into *Escherichia coli* DH5-alfa by electroporation, in a Micro-Pulser (Bio- Rad, Hercules, USA). One positive clone of each *Mhi-vap-1* and *Mhi-far-1* genes was selected and amplified with the primers M13f/M13r (Table 2.1).

2.3.7 mRNA *in situ* hybridization

For *in situ* hybridization, the DNA fragment used as probe was amplified from the cloned cDNA of *M. hispanica* J2 with the designed specific primers MHI-VAP-1f/MHI-VAP-1r and MHI-FAR-1f/MHI-FAR-1r. Ten ng of each purified PCR product (QIAquick PCR purification Kit, QIAGEN, UK) was the template in an asymmetric PCR to synthesize digoxigenin (DIG)-labelled sense and antisense single-stranded cDNA

probes with PCR DIG Probe Synthesis kit (Roche Applied Science, USA) (Lee & Schedl, 2006). *In situ* hybridization was performed with *M. hispanica* J2 as described by De Boer *et al.* (1998).

2.3.8 Phylogenetic analysis

The *vap-1* and *far-1* sequences of *M. hapla* were obtained from *M. hapla* genome website (http://www.pngg.org/cbnp/index.php?option=com_wrapper&Itemid=45) and *far-1* sequence of *G. pallida* was obtained from GenBank nucleotide database. The *vap-1* and *far-1* sequences of the *M. arenaria*, *M. hispanica*, *M. incognita* and *M. javanica* isolates were aligned with the ones from *M. hapla* and *G. pallida* sequences, and truncated to obtain a common start and end point. The alignment allowed the identification of the additional coding sequence of each sequence and the removal of introns. The amino acid sequences were deduced, from the 206 bp and 396 bp DNA sequences for the partial *vap-1* and *far-1* sequences, respectively. Protein phylogenetic trees were constructed using the Neighbor-Joining (Saitou & Nei, 1987) and Maximum-Likelihood (Jones *et al*., 1992) algorithms, and topology of the trees were generated from evolutionary distances computed using the Poisson correction method (Zuckerkandl & Pauling, 1965), included in MEGA5 (Tamura *et al*., 2011). The topology of the trees generated was evaluated by performing bootstrap analysis (Felsenstein, 1985) of 500 resamplings of the data set. All positions with less than 75% site coverage were eliminated. The protein alignment was used to determine the nucleotide position in the DNA sequences alignment, that was further used to perform DNA sequences phylogenetic analysis as described above, but the evolutionary distances were computed using the Jukes–Cantor correction method included in MEGA5 (Jukes & Cantor, 1969; Tamura *et al*., 2011).

2.4 Results

2.4.1 Bioinformatics analysis and amplification of *vap-1* and *far-1* genes

The protein homology search of VAP-1 (accession Nº ABO38109) and FAR-1 (accession Nº CAA70477.2) in the databank, using tblastn, revealed 76% (Minc17158) and 53% (Minc08986) protein identity, respectively to *M. incognita* and 85% (MhA1_Contig2874) and 88% (MhA1_Contig113) to *M. hapla*. Based in analysis of EST from *M. incognita* and *M. hapla*, a pair of primers was designed for each region which permitted the successful amplification of a fragment of approximately 280 bp for *vap-1* and 700 bp for *far-1*, from all *Meloidogyne* species used in this study (data is only shown for *M. hispanica* J2, Fig. 2.1).

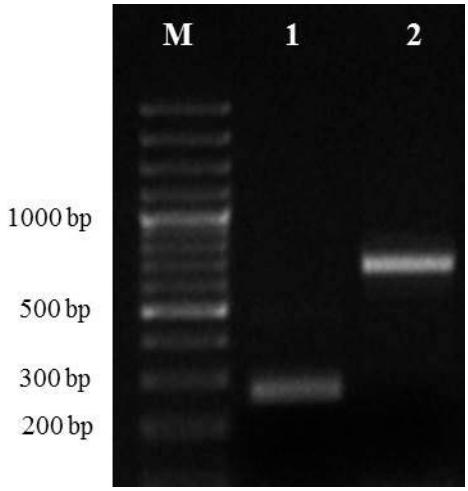


Figure 2.1 DNA amplification products of *Meloidogyne hispanica* second-stage juveniles using MIHA-VAP-1f/MHIHA-VAP-1r (1) and MIHA-FAR-1f/MIHA-FAR-1r (2) primers. Lane M, DNA marker (GeneRuler 1 kb Plus DNA ladder, Fermentas).

2.4.2 Transcription analysis of *Mhi-vap-1* and *Mhi-far-1* genes

Reverse transcription polymerase chain reaction was used to evaluate the expression of the genes *Mhi-vap-1* and *Mhi-far-1* in three *M. hispanica* developmental stages (eggs, J2 and females), and specific bands of approximately 200 bp and 400 bp were, respectively amplified in these developmental stages (Fig. 2.2). The cDNA

fragments were amplified in all of the nematode samples. However, the expression of *Mhi-vap-1* was higher in J2 when compared with those obtained in eggs and females. For *Mhi-far-1*, the expression was equally higher in all the developmental stages (Fig. 2.2). In order to localize the expression of the genes *Mhi-vap- 1* and *Mhi-far-1* in nematodes, *in situ* hybridization experiments were performed. Sense and antisense cDNA probes were used against *M. hispanica* J2. The genes specific antisense cDNA probe specifically hybridized with mRNA accumulated within the subventral oesophageal gland cells of J2 (Fig. 2.3 B, D). No hybridization signal was detected in the nematode when using control sense probes (Fig. 2.3 A, C).

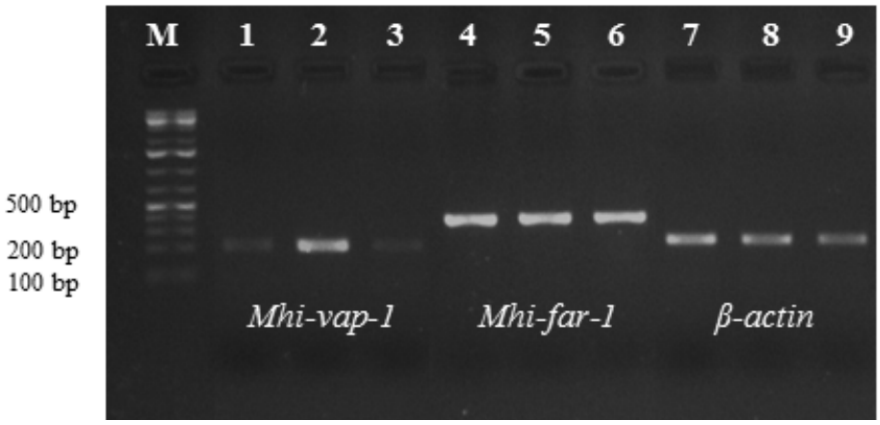


Figure 2.2 Expression of the *Mhi-vap-1* and *Mhi-far-1* genes detected by reverse transcriptase mediated PCR amplification of cDNAs from *Meloidogyne hispanica* eggs, second-stage juveniles (J2) and females. Lanes 1, 4 and 7 cDNA templates from eggs; lanes 2, 5 and 8 J2 DNA; lanes 3, 6 and 9 females cDNA; lane M, DNA marker (GeneRuler 1 kb DNA ladder, Fermentas). As a positive control, cDNA templates were amplified with the primers of *β-actin* gene.

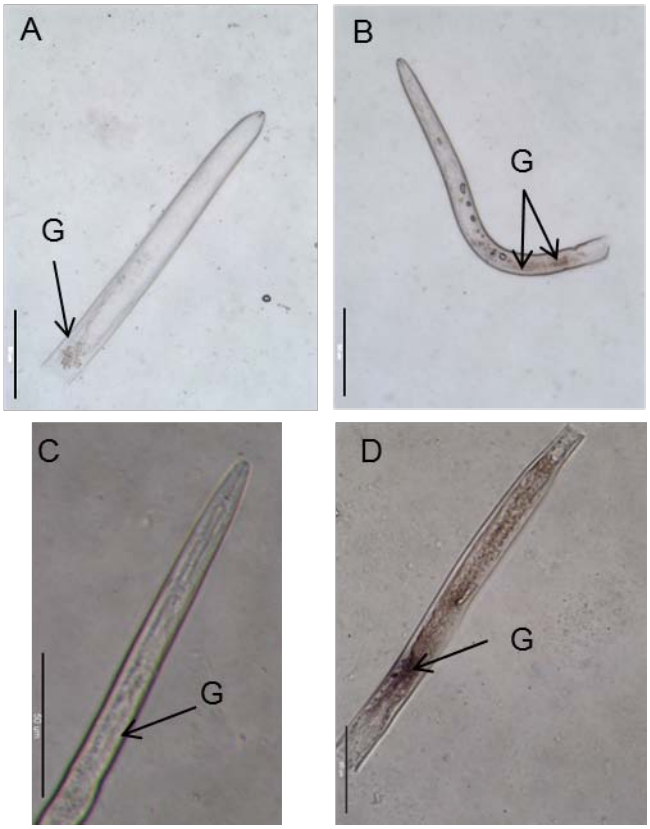


Figure 2.3 *Meloidogyne hispanica* second-stage juveniles (J2) sections hybridized with digoxigenin-labeled sense and antisense cDNA probes derived from the *Mhi-vap-1* (A and B) and *Mhi- far-1* (C and D) genes. A and C - Alkaline phosphatase staining is absent in J2 that have been incubated with the sense probe. B and D – Alkaline phosphatase staining shows specific binding of the antisense probe to the cytoplasm of the subventral oesophageal gland cells (G). Scale bars=50 µm.

2.4.3 Phylogenetic analysis

Using the primers designed in this work (MIHA-VAP-1f/r and MIHA- FAR-1f/r), *vap-1* and *far-1* were identified and successfully amplified in *M. hispanica* and in three additional *Meloidogyne* species. The sequences obtained were used for further phylogenetic analysis of the predicted amino acid and partial gene sequences (Figs. 2.5 and 2.6). Analyzing the multiple alignments obtained to VAP-1 and FAR-1, the MHI-VAP-1 sequence differed by two amino acid positions from *M. incognita* and 11 from *M. hapla* while MHI-FAR-1 differed from *M. hapla* six positions (Fig. 2.4). *Globodera pallida*, GP-FAR-1, showed 43 amino acid differences in alignment with

the other *Meloidogyne* species (Fig. 2.4 B). The MHI-VAP-1 amino acid sequences displayed sequence identities ranging from 77.2 (*M. hapla*) to 100% (*M. arenaria* and *M. javanica*) when compared with the other species and the MHI-FAR-1 from 63.9 (*G. pallida*) to 100% (*M. arenaria*, *M. incognita* and *M. javanica*) (Tables 2.2 and 2.3). The phylogenetic analysis revealed that VAP-1 shared high protein homology and phylogenetic relations with *M. hispanica*, *M. arenaria*, and *M. javanica* (Fig. 2.5 A), whereas FAR-1 was closely related to the four RKN species, *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* (Fig. 2.5 B). In both proteins, *M. hapla* was the most divergent RKN species (Fig. 2.5).

The topology of the VAP-1 tree was identical to the one obtained from DNA sequences (Figs. 2.5 A and 2.6 A). The topology of the FAR-1 protein and DNA trees exhibited some differences, specifically on the cluster formed by *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* (Figs. 2.5 B and 2.6 B). These results are congruent with the differences observed between the identity values determined for the protein and DNA alignments (Tables 2.3 and 2.5). *Mhi-vap-1* sequence exhibited sequence similarity values ranging from 83.7% (*M. hapla*) to 100% (*M. arenaria* and *M. javanica*) (Table 2.4) and formed a closed cluster with *M. arenaria* and *M. javanica* (Fig. 2.6 A). *Mhi-far-1* displayed sequence similarity values of 55.5% towards *G. pallida* and from 87.7 to 100% towards *Meloidogyne* spp. (Table 2.5) and formed a well-supported clade with *M. incognita* with 100% bootstrap (Fig. 2.6 B). *Meloidogyne hapla* was the most divergent *Meloidogyne* spp. (Fig. 2.6 A, B).

In fact, the protein alignment identity values decreased slightly when compared to the values obtained from DNA sequences alignment (Tables 2.3 and 2.5), most probably related with the presence of synonymous mutation, thus explaining the difference of the phylogenetic results.

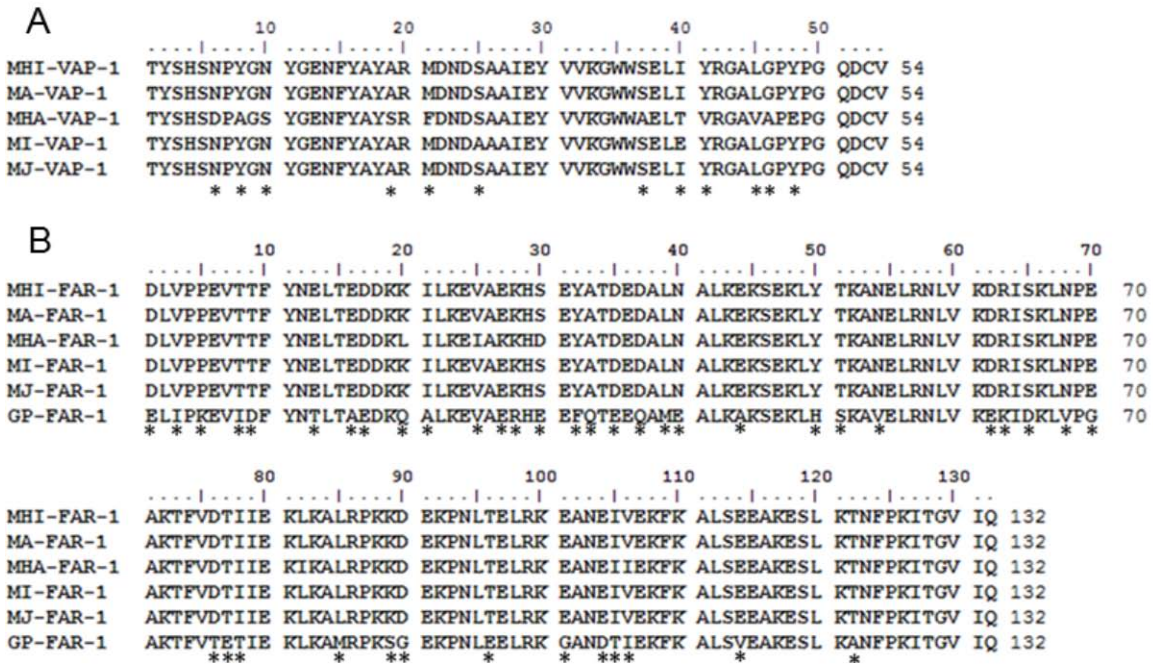


Figure 2.4 Multiple sequence alignment of *Meloidogyne hispanica* venom allergen-like protein, MHI-VAP-1 (KF030969) (A), and fatty acid and retinol binding protein, MHI-FAR-1 (KF030974) (B), amino acid sequences with homologues from other phytoparasitic nematodes. MA-VAP-1 (KF030971) and MA-FAR-1 (KF030973) from *M. arenaria*; MHA-VAP-1 (MhA1_ contig2874) and MHA-FAR–1 (Mh10g200708_contig113) from *M. hapla*; MI-VAP-1 (KF030970) and MI-FAR-1 (KF030975) from *M. incognita*; MJ-VAP-1 (KF030972) and MJ-FAR-1 (KF030976) from *M. javanica* and GP-FAR-1 (CAA70477.2) from *Globodera pallida*. Differences between amino acids are indicated by asterisks.

Table 2.2. Pairwise sequence identities among *Meloidogyne hispanica* (MHI-VAP-1, KF030969), *M. arenaria* (MA-VAP-1, KF030971), *M. hapla* (MHA-VAP-1, MhA1_contig2874), *M. incognita* (MI-VAP-1, KF030970) and *M. javanica* (MJ-VAP-1, KF030972) sequences of VAP-1 protein using the Poisson correction method included in MEGA5.

	MHI-VAP-1	**MI-VAP-1**	**MA-VAP-1**	**MJ-VAP-1**
MI-VAP-1	96.2			
MA-VAP-1	100	96.2		
MJ-VAP-1	100	96.2	100	
MHA-VAP-1	77.2	74.9	77.2	77.2

Table 2.3. Pairwise sequence identities among *Meloidogyne hispanica* (MHI-FAR-1, KF030974), *M. arenaria* (MA-FAR-1, KF030973), *M. hapla* (MHA-FAR-1, Mh10g200708_contig113), *M. incognita* (MI-FAR-1, KF030975), *M. javanica* (MJ-FAR-1, KF030976) and *Globodera pallida* (GP-FAR-1, CAA70477.2) sequences of FAR-1 protein using the Poisson correction method included in MEGA5.

	MHI-FAR-1	**MI-FAR-1**	**MA-FAR-1**	**MJ-FAR-1**	**MHA-FAR-1**
MI-FAR-1	100				
MA-FAR-1	100	100			
MJ-FAR-1	100	100	100		
MHA-FAR-1	95.3	95.3	95.3	95.3	
GP-FAR-1	63.9	63.9	63.9	63.9	61.7

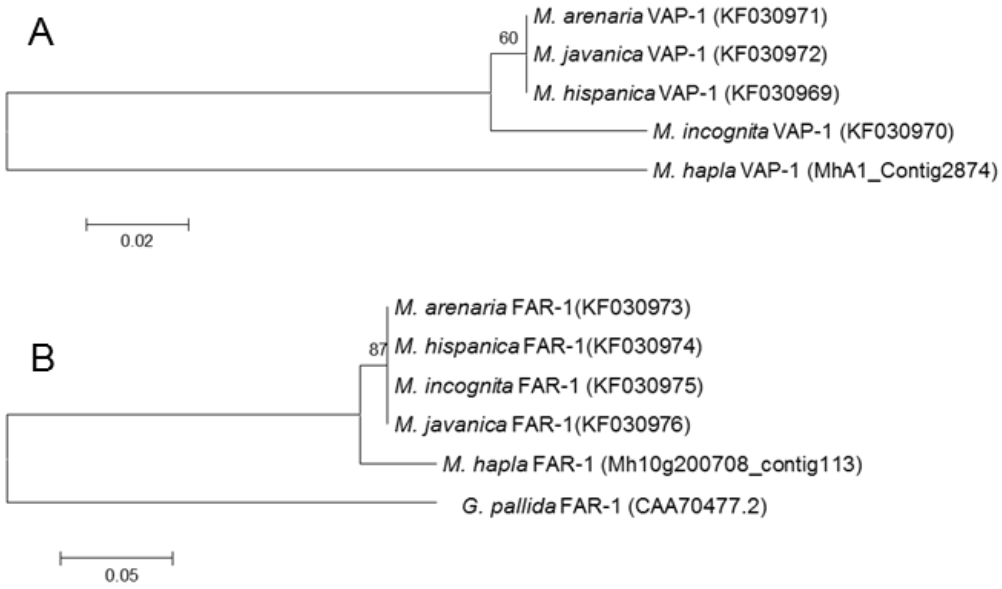


Figure 2.5 Phylogenetic trees constructed on the basis of the predicted venom allergen-like protein (VAP-1) sequences from *Meloidogyne hispanica*, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* (A) and fatty acid and retinol binding protein (FAR-1) sequences from *M. hispanica*, *M. arenaria*, *M. hapla*, *M. incognita*, *M. javanica* and *Globodera pallida* (B). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

Table 2.4. Pairwise sequence similarities between *Meloidogyne hispanica* (*Mhi-vap-1*, KF030969), *M. arenaria* (*Ma-vap-1*, KF030971), *M. hapla* (*Mha-vap-1*, MhA1_contig2874), *M. incognita* (*Mi-vap-1*, KF030970) and *M. javanica* (*Mj-vap-1*, KF030972) sequences of *vap-1* gene using MEGA5*.

	Mhi-vap-1	***Mi-vap-1***	***Ma-vap-1***	***Mj-vap-1***
Mi-vap-1	97.5			
Ma-vap-1	100	97.5		
Mj-vap-1	100	97.5	100	
Mha-vap-1	83.7	81.4	83.7	83.7

* Analyses were conducted using the Maximum Likelihood model.

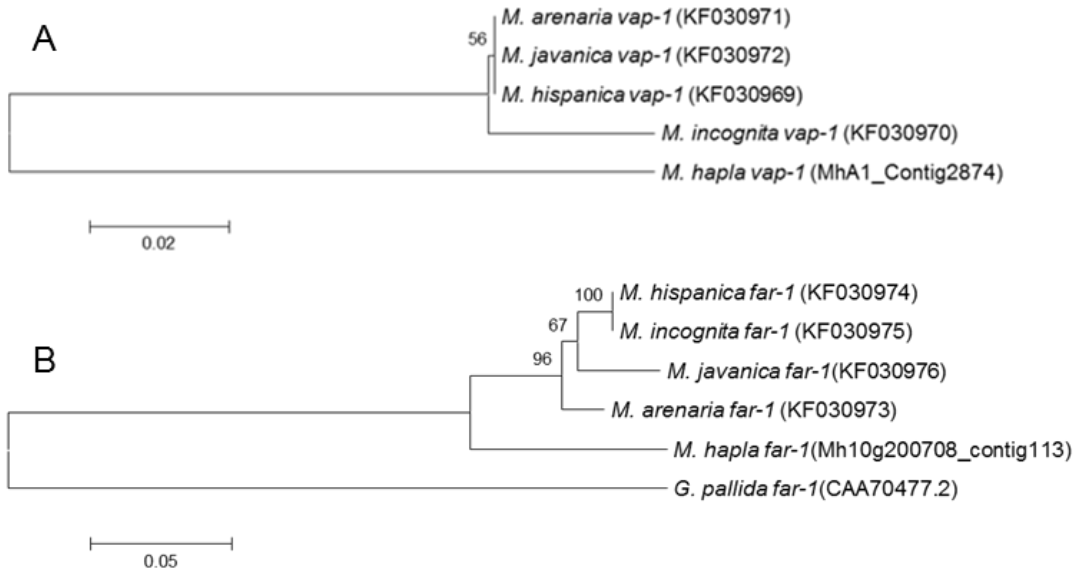


Figure 2.6 Phylogenetic analysis of the venom allergen-like protein (*vap-1*; A) and fatty acid and retinol binding protein (*far-1*; B) gene sequences. *Meloidogyne hapla* and *Globodera pallida* were included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

Table 2.5. Pairwise sequence similarities between *Meloidogyne hispanica* (*Mhi-far-1*, KF030974), *M. arenaria* (*Ma-far-1*, KF030973), *M. hapla* (*Mha-far-1*, Mh10g200708_contig113), *M. incognita* (*Mi-far-1*, KF030975), *M. javanica* (*Mj-far-1*, KF030976) and *Globodera pallida* (*Gp-far-1*, CAA70477.2) sequences of *far-1* gene using MEGA5*.

	Mhi-far-1	***Mi-far-1***	***Mj-far-1***	***Ma-far-1***	***Mha-far-***
Mi-far-1	100				
Mj-far-1	95.8	95.8			
Ma-far-1	96.9	96.9	94.8		
Mha-far-1	87.7	87.7	87.1	88.3	
Gp-far-1	55.5	55.5	53.6	55.9	53.6

* Analyses were conducted using the Maximum Likelihood model.

2.5 Discussion

The effector genes *vap-1* and *far-1* (encoding proteins VAP-1 and FAR-1, respectively) have been identified for the first time in *M. hispanica* and the partial sequences of these genes were successfully amplified. The phylogenetic analysis, conducted on VAP-1 and FAR-1 and on partial genes sequences, determined that *M. hispanica* was most closely related with *M. arenaria, M. incognita* and *M. javanica*, and *M. hapla* was the most divergent of the *Meloidogyne* species. The presence of synonymous mutation was also observed indicating a conservation of the protein sequences within the *Meloidogyne* species, most probably due to its functional specificity. Two types of VAPs have been identified in nematodes: a short single domain type, of approximately 220 amino acids, and a longer double domain type of approximately 425 amino acids (Bin *et al*., 1999; Hawdon *et al*., 1999; Gao *et al*., 2001). *Meloidogyne hispanica* MHI-VAP-1 is representative of the single domain venom allergen-like protein, the most common type found in nematodes (Gao *et al*., 2001). Multiple sequence alignment showed that MHA-VAP-1 contains the most variant amino acids residues compared with *M. hispanica*, *M. arenaria* and *M. javanica*. MHI-VAP-1

partial predicted amino acid sequence has 100% homology with MA-VAP-1 and MJ-VAP-1, indicating a strong conservation of these proteins. Previous phylogenetic studies demonstrated that FAR homologues from the animal parasitic nematodes *Onchocerca, Brugia*, *Wuchereria*, *Loa*, *Acanthocheilonema*, *Ascarissuum*, *Toxocara canis*, and *Litomosoides*, and the plant-parasitic nematodes *G. pallida*, *G. rostochiensis*, *M. javanica, M. arenaria*, *H. schachtii*, *P. vulnus* and *R. similis*, and the free-living nematode *Caenorhabditis elegans* were closely related and they all share conserved amino acid sequences in its primary and secondary structures (Prior *et al*., 2001; Garofalo *et al*., 2002; Iberkleid *et al.,* 2013). Mj-FAR-1 grouped closely with FARs belonging to other parasitic nematodes (animal, sedentary, and migratory plant-parasitic nematodes). However, FAR proteins clearly distinguished and grouped different nematode species according to their trophic group and nematode parasitism strategy. The highest predicted amino acid identity of Mj-FAR-1 was found amongst *M. incognita*, *M. arenaria* and *M. hapla* and the lowest observed between *M. javanica* and *M. chitwoodi* (Iberkleid *et al.,* 2013). Although, there is a strong conservation for this group of proteins, *M. hapla* in this study showed to be the most divergent when compared with *M. hispanica*, which might account for a particular mode of parasitism adaptation and/or reproduction. *Meloidogyne hapla* reproduce by facultative meiotic or mitotic parthenogenesis while *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* reproduce by obligatory mitotic parthenogenesis (Chitwoodi & Perry, 2009). The high degree of conservation in the lipid-binding characteristics of FAR proteins and their presence at the host parasite interface, across multiple families of parasitic nematodes, support the hypothesis that this nematode restricted family of proteins play a crucial role in the life cycle, and in the parasitism of their host (Bath *et al*., 2009). Only six differences were

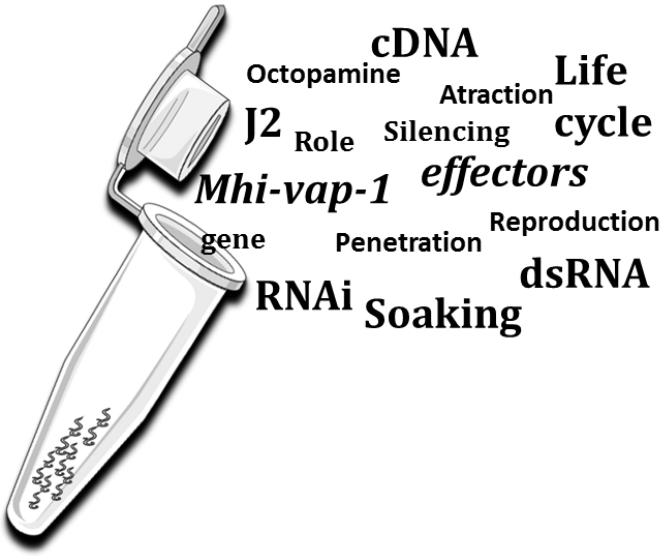
observed in the predicted amino acid sequences between MHA-FAR-1 and MHI-FAR-1, MA-FAR-1, MI-FAR-1 and MJ-FAR-1. The cDNA transcription analysis demonstrated that *Mhi-vap-1* and *Mhi-far-1* genes were transcribed in eggs, J2 and females. Moreover, a high expression of MHI-VAP-1 was evidenced in *M. hispanica* J2 and a very low level of expression was detected in eggs. In contrast, almost equal expression of MHI-FAR-1 was detected in eggs, J2 and females, which suggest a potential role during the different developmental stages. The localization of the *Mhi-vap-1* and *Mhi-far-1* transcripts in J2, by *in situ* hybridization, revealed that there is a specific binding of the antisense probe to the subventral oesophageal glands. These results suggest that, in *M. hispanica* VAP-1 and FAR-1 might be natural components of the nematode secretions which are released through the stylet by the J2. Remarkable similarities of VAP proteins to allergens from hymenopteran insect venoms were found and the *in situ* hybridization showed that homologues of this gene are present in the gland cells of *H. glycines* and *Ditylenchus destructor* (Gao *et al*., 2001; Peng *et al.,* 2013). Furthermore, the venom allergen AG5-like protein and the *Mi-vap-2* gene were expressed exclusively in the oesophageal glands of pre-parasitic and parasitic J2 of *M. incognita* (Ding *et al.,* 2000; Wang *et al*., 2007) while the *Mhi-vap-1* was detected in oesophageal glands of pre-parasitic J2 being weakly transcribed in eggs and females. Animal parasitic allergen genes have been associated with the induction of a host immune response (Chen *et al.,* 2010) and the effector Gr-VAP-1, localized in the subventral oesophageal glands of *G. rostochiensis* pre-parasitic J2, has also been implicated in resistance (Lozano-Torres *et al*., 2012). Using RT-PCR, this study shows that equal expression of MHI-FAR-1 was detected in eggs, J2 and females however, quantitative real time PCR indicates that for *M. javanica* the lowest level of expression for *mj-far-1* transcripts was detected within

eggs and the highest in J2 within the first hours after root infection, which suggest a potential role of MJ-FAR during the different parasitic stages (Iberkleid *et al*., 2013). Using *in situ* hybridization, this work localized the *Mhi-far-1* in the subventral oesophageal glands of J2 and the same localization, using different approaches, was identified for a protein similar to the Gp-FAR in the oesophageal glands of *M. incognita*, another FAR protein of the plant parasitic nematode *D. africanus* was also identified in the oesophageal glands (Bellafiore *et al*., 2008; Haegeman *et al*., 2009). However, in contrast with this work, *G. pallida* and *M. javanica* FAR-1 proteins have been shown to be highly expressed in the migratory and parasitic J2 with transcription in the nematode surface and also within the adult female body, using immunolocalization studies with the same antiserum (Prior *et al*., 2001; Iberkleid *et al*., 2013). Although *M. hispanica far-1* formed a well supported clade (100%) with *M. incognita* it showed only 67% bootstrap with *M. javanica* which could account for a particular mode of parasitism adaptation of this gene in *M. hispanica* and *M. incognita*. We also showed in this paper that the localization of *Mhi-far-1* is similar to *M. incognita* FAR protein but differs in *M. hispanica* in comparison with *M. javanica* (Bellafiore *et al*., 2008; Iberkleid *et al*., 2013). The FAR-1 protein binds to linolenic and linoleic acid, which are precursors of plant defence compounds in the jasmonic acid signaling pathway (Prior *et al*., 2001; Curtis, 2007). It was recently detected in the *M. javanica* cuticle surface and along the adjacent host root tissues and a continuous secretion of this protein into the intercellular space between the nematode body and the host cells was proposed (Iberkleid *et al*., 2013). The authors also reported that tomato plants over expressing of *Mj-far-1* are highly susceptible to nematode infection indicating that the FAR protein might be involved in the manipulation of host lipid-based defences playing an important role in the

parasitism of RKN (Iberkleid *et al.,* 2013). The localization of the expression of the genes *vap-1* and *far-1* in the subventral oesophageal glands suggest a potential parasitic function for these genes in *M. hispanica*. Further work is being conducted in order to assess whether the silencing of these genes affects *M. hispanica* behaviour and whether they are essential for successful infection.

Chapter 3

RNAi silencing of the venom allergen-like protein (*Mhi-vap-1*) gene in the root-knot nematode *Meloidogyne hispanica*



Written as a Journal article:

Duarte, A., Maleita, C., Egas, C., Abrantes, I. & Curtis, R. (2014). RNAi silencing of the venom allergen-like protein (*Mhi-vap-1*) gene in the root-knot nematode *Meloidogyne hispanica.* Plant Plathology (*in preparation*).

3.1 Abstract

Gene silencing using RNAi is a powerful tool for functional analysis of nematode genes and can provide a new strategy for the management of root-knot nematodes (RKN). The transcript of the *Meloidogyne hispanica* venom allergen-like protein gene (*Mhi-vap-1*) was localised in the second-stage juveniles (J2) subventral oesophageal gland cells and shown to be highly transcribed in this developmental nematode stage. The purpose of this study was to assess whether the silencing of the *Mhi-vap-1 gene* could affect nematode attraction and or penetration of tomato roots. The optimum soaking time to generate silencing of this gene was evaluated at 24, 48 and 32 h, J2s were incubated in a soaking solution containing double stranded RNA (dsRNA) and the relative expression of *Mhi-vap-1* gene determined at each time point by qRT-PCR. At 48 h, the relative expression of *Mhi-vap-1* decreased, which indicates that this gene is susceptible to the RNAi method and 48 h is the optimum incubation time. Results confirmed the silencing of *Mhi-vap-1* gene, and a reduction in nematode attraction and penetration to tomato roots was observed. The use of host-delivered RNAi targeting *Meloidogyne* effector genes such as *Mhi-vap-1*, either when nematodes are moving in the rhizosphere or during migration inside the root tissue could achieve resistance as this gene is important for the host-parasite interaction and has shown to be involved in suppression of host immune defences.

Keywords: effectors, phenotype, quantitative real-time PCR (qRT-PCR), RNAi, root-knot nematodes, soaking, venom allergen-like protein.

3.2 Introduction

Nematodes of the genus *Meloidogyne*, commonly known as root-knot nematodes (RKN), belong to a group of plant-parasitic nematodes (PPN) that is widely dispersed around the world. These nematodes virtually can parasitize all crops affecting their production and quality. *Meloidogyne hispanica* was described for the first time in Seville, Spain, from a peach rootstock (*Prunus persica silvestris* Batsch) and has been found in Africa, Asia, Australia, Europe, and North, Central and South America associated with economically important crops (Hirschmann, 1986; Maleita *et al*., 2012a). Cultural control is widely practiced and several strategies have been used: crop rotation, use of allelopathic plants that release nematicidal compounds into the rhizosphere, trap crops, green manure, soil amendments, removal or destruction of infected host plants and growing of resistant cultivars (Halbrendt & La Mondia, 2004). However, chemical nematicides, including soil fumigants, are the most reliable means for the management of RKN, but most of them are non-specific and notoriously toxic, which poses a threat to the soil ecosystem, ground water, and human health. As the use of agrochemicals is restricted and will probably be more drastically reduced in the future, the search for new alternatives of RKN control are of great importance. A new strategy based on RNA interference (RNAi) has recently been proven to be successful to control nematode infection of plants (Gheysen & Vanholme, 2007; Matsunaga *et al*., 2012). RNAi is a reverse genetics technique which ablates mRNA by introduction of complementary double-stranded RNA (dsRNA) (Hammond *et al*., 2001). It was first used in *Caenorhabditis elegans* (Fire *et al*., 1998) and has become an established experimental technique to investigate the function of different genes involved in nematode parasitism. This strategy has allowed firstly, to understand the function of some essential nematode genes and secondly has been

used to engineer resistance to host plants that express dsRNA and small interfering RNAs (siRNA) to silence specific nematode genes (Rosso *et al*., 2009). The efficacy of RNAi depends of the turnover of the target gene, susceptibility of the organism to the delivered RNAi, base composition and position of dsRNA construct in a target gene, length of dsRNA sequence, mode of delivery and type of target tissue (Fire *et al*., 1998; Orii *et al*., 2003; Rosso *et al*., 2005).

The successful application of RNAi as a tool for functional genomics and has been demonstrated in several PPN, such as *Bursaphelenchus xylophilus, Globodera pallida*, *Heterodera glycines*, *M. incognita*, *M. javanica*, *Pratylenchus thornei, P. zeae* and *Radopholus similis* (Urwin *et al*., 2002; Bakhetia *et al*., 2005; Rosso *et al*., 2005; Adam *et al*., 2008; Park *et al*., 2008; Haegeman *et al.*, 2009a; Niu *et al*., 2012; Tan *et al*., 2013). Recently, Papolu *et al.* (2013) through host derived resistance RNAi demonstrated that two FMRFamide like peptide genes (*flp-14* and *flp-18*) are important for infection and development of resistance to *M. incognita* in transgenic tobacco plants.

The secretions from sedentary endoparasites are particularly intriguing because of the complex changes in phenotype, function and gene expression that they modulate in the parasitized plant cells (Hussey *et al*., 2002). A large number of effectors are synthesized in the oesophageal glands and play a crucial role in the relationship with host promoting the infection process, inducing the differentiation of root cells and the formation of the feeding site, but their exact role is a complex subject (Baum *et al*., 2007; Mitchum *et al*., 2013). Therefore, nematode genes highly expressed in the subventral oesophageal gland cells of pre-parasitic second-stage juveniles (J2), are good candidates to gene silencing by RNAi (Sukno *et al*., 2007).

The venom allergen-like proteins (VAPs), characterized by the highly conserved spacing of cysteine residues mainly in the carboxy-terminal region (Hawdon *et al*., 1996), are transcribed during plant nematode parasitism. The *vap* genes were isolated in *B. mucronatus, B. xylophilus, Ditylenchus africanus, H. glycines*, *M. hispanica and M. incognita* (Gao *et al*., 2001; Wang *et al*., 2007; Haegeman *et al*., 2009; Kang *et al*., 2012; Yan *et al*., 2012; Duarte *et al*., 2014). In *B. xylophilus* and *B. mucronatus,* six new VAPs were found, which were not homologous to known VAPs, and their functions still remain to be elucidated (Yan *et al*., 2012). Recently, *vap-1* gene was localized, by *in situ* hybridization, in the subventral oesophageal gland cells of *M. hispanica* J2 (Duarte *et al*., 2014). Because such transcripts accumulated exclusively within the subventral oesophageal gland cells of nematodes (Lin *et al*., 2011), this gene may play an important role in the *M. hispanica* infection of host plants.

The purpose of this study was to assess the effect of *Mhi-vap-1* silencing on J2 attraction and penetration of tomato plants

3.3 Materials and methods

3.3.1 Nematode isolate

The *M. hispanica* isolate obtained from infected fig tree roots, *Ficus carica* L., in Odeceixe, Faro, Portugal, was reared on tomato, *Solanum lycopersicum* L., cv. Easypeel. Two months after the inoculation, with 10 egg masses (EM), freshly J2 were obtained from EM placed on a 25 µm mesh sieve. Second-stage juveniles collected during the first 24 h were discarded and only the subsequent 48 h *M. hispanica* J2 were used in the experiments. The identification of the RKN isolate was

based on the isoesterase phenotype (Hi4) (Abrantes *et al*., 2008; Maleita *et al*., 2012b).

3.3.2 Gene silencing strategy

Total RNA was extracted from *M. hispanica* J2. The specimens were placed in liquid nitrogen and homogenized using Trizol reagent (Invitrogen, UK). Afterwards, the RNA was isolated using the RNeasy Mini Kit and the DNA digested by DNase I, using RNase-Free DNase Set (QIAGEN, UK). The concentration and purity of the RNA was determined in a Nanodrop ND-1000 Spectrophotometer (Labtech International, UK). Total RNA (50 ng) was reverse transcribed into cDNA using the Omniscript RT Kit (QIAGEN, UK) and Oligo-dT primers, according to the manufacturer's instructions. After the reverse transcriptase reaction, a standard PCR was performed with the *M. hispanica* specific primers MHI-VAP-1f/r (Table 3.1) (Duarte *et al*., 2014).

The PCR amplifications, performed in a mixture containing 25 ng of synthesized cDNA template and 5 units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's and 10 μM of each primer, were carried out using the following conditions: 3 min at 95°C, 39 cycles at 95°C for 30 s, 40°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 5 min, in a MJ Mini TM Personal Thermal Cycler (Bio-Rad Laboratories Inc., USA). Quality and yield of the reactions were checked on a 1% agarose gel prepared with 1X TBE and the PCR products purified using the QIAquick PCR Purification Kit (QIAGEN, UK).

Table 3.1. Primers used for reverse transcription, qRT-PCR, and for generating templates for dsRNA synthesis.

Primer name	Primer sequence 5´→3'
MHI-VAP-1f	TTATGGAGAGATTTCTATGC
MHI-VAP-1r	GTGTCCAATGTCCAATACCT
MHI-VAP-1T7f	TAATACGACTCACTATAGGGATGCTTATGCAAGAATGGACAA
MHI-VAP-1T7r	TAATACGACTCACTATAGGGTTTGAGGTGCATCAAAAGCA
MHIq-ACTINf	TGTATCCAGGCATTGTGATCGT
MHIq-ACTINr	CATTGTTGATGGTGCCAAAGC
MHIq-VAP-1f	CCTTATCCTGGCCAAGACTGC
MHIq-VAP-1r	TTGTGTCCAATGTCCAATACCTCT

3.3.3 Synthesis of double stranded RNA

The *M. hispanica* cDNA was used as a template for double stranded RNA (dsRNA) synthesis with two primers non-overlapping dsRNA within the coding region of the gene T7-labeled gene-specific primers, designed in the E-RNAi web service (http://www.e-rnai.org/) from GenBank sequence KF030969 and used to amplify a region of approximately 180 bp of the *Mhi-vap-1* gene (Table 3.1, MHI-VAP-1T7f/r). The PCR reaction was the same as referred before and the amplification carried out with the following conditions: 95°C for 3 min, followed by 39 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1.5 min with a final extension at 72°C for 5 min. The PCR products were analyzed on 1% agarose gel electrophoresis in 1X TBE, and the remainder purified using the QIAquick PCR Purification Kit (QIAGEN, UK). The reaction of transcription of dsRNA was performed using 2 µg of DNA according to the MEGAscriptRNAi Kit (Ambion, USA). DNA was incubated with the T7 enzyme mix and 75 mM each of ribonucleotides for 16 h at 37°C to increase RNA yield, and a

extra annealing step at 75°C for 5 min was done to ensure the formation of dsRNA before nuclease digestion treatment. The dsRNA was purified with a solid-phase adsorption system to remove proteins and mono and oligonucleotides. The dsRNA was quantified and qualified in Nanodrop and in a 1% agarose gel prepared with 1X TBE, respectively.

3.3.4 Nematode soaking and dsRNA treatment

Freshly hatched *M. hispanica* J2 (≈10,000) were soaked in 0.25X M9 buffer (43.6 mM Na_2HPO_4, 22 mM KH_2PO_4, 2.1 mM NaCl and 4.7 mM NH_4Cl) with 3.8 mg/mL dsRNA and 50 mM octopamine (neurostimulant) for 24, 32 or 48 h in the dark at room temperature on a rotator. Soaking solutions with or without octopamine and dsRNA were used as controls.

3.3.5 Effect of RNAi treatment in *M. hispanica* attraction and penetration

3.3.5.1 qRT-PCR of *Mhi-vap-1* gene

The optimisation of the nematode incubation period with dsRNA was carried out to determine the best soaking period of time for complete silencing of the *Mhi-vap-1* gene. For that J2 were incubated with dsRNA or controls and 500 J2/nematode sample were removed at 24, 32 and 48 h, washed three times in RNase-free water and RNA extracted with RNeasy Mini Kit as described before. After treatment with DNase I, the RNA was used to synthesize first strand cDNA and performed a qRT-PCR, as previously described, in order to ascertain the *Mhi-vap-1* expression.

For qRT-PCR, 20 µL of reaction mix contained 3 µL of cDNA, 1X Fast SYBR Green Master Mix and 200 nM each of the gene specific primer pair (MHIq-VAP-1_f/r, Table 3.1), which amplify 140 bp. qRT-PCR primers for *vap-1* and *β-actin* genes

(Table 3.1) were designed from the sequences KF030969 of *M. hispanica* and BE225475.1 of *M. incognita*, respectively, obtained from Genbank, using the software Primer Express v3.0 (Applied Biossystems). Primers MHIq-ACTIN_f/r (Table 3.1), from *β-actin* gene that amplified 58 bp, were used as reference for gene expression normalization. This gene was previously identified as showing constant expression in similar experiments and has been reported to be a useful qRT-PCR endogenous baseline control in PPN (Painter & Lambert, 2003). The qRT-PCR primers sets were optimized for working concentration, annealing temperature and analyzed by dissociation curve for contamination or non-specific amplification by primer dimer. Efficiencies of the PCR reactions were determined according to Pfaffl (2001) (Applied Biosystem, version 2.0.4). Two independent qRT-PCR reactions were carried out, the PCR done in triplicate and the mean Ct values determined. qRT-PCR was performed using the following conditions: 95°C for 2 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. Reactions were performed in the 96 well Fast Thermal Cycling Plates (Applied Biosystems) and capped with Optical Adhesive Covers (Applied Biosystems). Relative expression of the *Mhi-vap-1* gene transcripts was determined using the ΔΔCT method according to 7500 Fast Real -Time PCR System (Applied Biosystem, version 2.0.4).

3.3.5.2 Attraction and penetration bioassay of dsRNA treated J2 of *M. hispanica*

Meloidogyne hispanica J2 (≈10,000) were soaked for 48 h with dsRNA or controls and after incubation washed to remove the soaking solution. Part of the J2 were used to performed a qRT-PCR and confirm the reduction of *Mhi-vap-1* expression, as referred above, and the remaining used in the attraction and penetration bioassays. Attraction and penetration assays were performed according to Dutta *et al*. (2011).

Two hundred J2s were added to 24 mL pluronic gel F-127 (23%) and mixed uniformly at 15°C. Three mL of suspension/treatment were transferred to Petri dishes (35x10 mm), four day-old tomato cv. Easypeel seedlings placed in the center and incubated at room temperature in the dark. Each treatment was replicated three times. Nematodes in defined zone of attraction (DZA, 1 cm^2 around the root tip) and touching root tip (TRT) were counted at 2, 8 and 24 h after inoculation (HAI). At 36 HAI, roots were washed gently to remove all J2 outside the roots, stained with acid fuchsin (Byrd *et al*., 1983) and the number of J2 that penetrated the roots recorded.

3.3.6 Data analysis

Gene expression was calculated in relation to expression levels of control groups (M9+octopamine and M9) and normalized against the reference *β-actin* gene, using REST© 2009 software (Pfaffl, 2001; Pfaffl *et al*., 2002). Up-and down-regulated genes and respective differences were considered significant at $P < 0.05$.

In order to compare the effect of the treatments on attraction and penetration, the results were analyzed using Statsoft Statistica version 7 for Windows. Data were checked for evidence of a normal distribution and homogeneity of variances using Kolmogorov–Smirnov test and Hartley, Cochran, and Bartlett's tests, respectively. All data were normally distributed, and One-way ANOVA Fischer LSD post-hoc test was performed to evaluate the differences among treatments, at 5% level of significance.

3.4 Results

3.4.1 qRT-PCR of *Mhi-vap-1* gene

An initial experiment amplifying *Mhi-vap-1*, from a dilution series over a 10^4 range of cDNA, established an expected increase in threshold cycle. The slope values for

vap-1 gene was -3.3 and -3.5 for *β-actin* gene, equivalent to 98 and 93% efficiency, respectively. Data of *Mhi-vap-1* gene expression was obtained in three different time points (24, 32 and 48 h) from the dsRNA soaked J2s (Fig. 3.1). At 24 and 48 h, *Mhi-vap-1* was significantly down-regulated when J2 were soaked with dsRNA+M9+octopamine and the gene expression, calculated in relation to expression levels of control M9+octopamine ($P < 0.05$, Fig. 3.1 A). At 32 h, non significant down-regulated expression was obtained, $P \geq 0.05$ (Fig. 3.1 A).

The analyses performed with the control M9 showed a non significant down-regulated expression at the three different time points, $P > 0.05$ (Fig. 3.1 B).

When the J2 were soaked with dsRNA+M9 and compared with the respective control M9, the gene *Mhi-vap-1* was significantly up-regulated at 24 h with an expression level of 3.150, $P < 0.05$ (Fig. 3.1 C). At 32 and 48 h, the *Mhi-vap-1* gene was not significantly up/down-regulated, $P > 0.05$ (Fig. 3.1 C).

The variation of the expression the *Mhi-vap-1* gene was evaluated at different time points and at 48 h the down-regulation of the *Mhi-vap-1* gene was confirmed (Fig. 3.1).

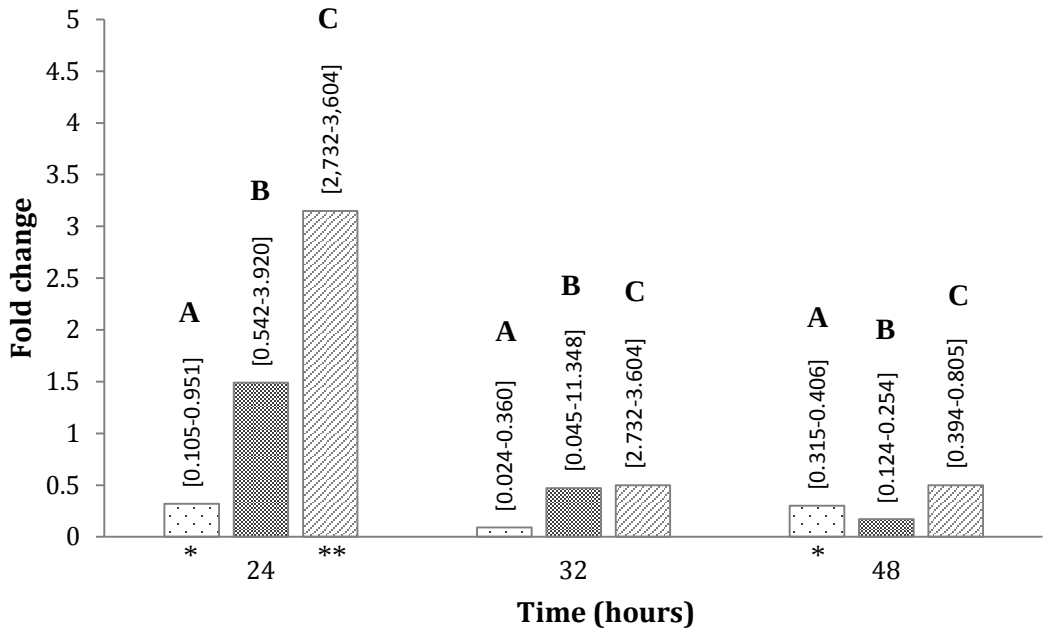


Figure 3.1 Relative *Mhi-vap-1* gene expression at 24, 32 and 48 h, after soaking of 500 *Meloidogyne hispanica* second-stage juveniles, obtained in relation to the expression levels in control treatments: A- dsRNA+M9+octopamine vs M9+octopamine (control); B- dsRNA+M9+octopamine vs M9 (control); C- dsRNA+M9 vs M9 (control). 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* down-regulation and ** up-regulation).

3.4.2 Effect of RNAi treatment in *M. hispanica* attraction and penetration

A negative impact of *vap-1* dsRNA on *M. hispanica* J2 attraction and penetration to tomato roots was observed and at 48 h of soaking, in all treatments, a statistically significant reduction in the expression of *Mhi-vap-1* gene was confirmed by qRT-PCR (Figs. 3.2 and 3.3).

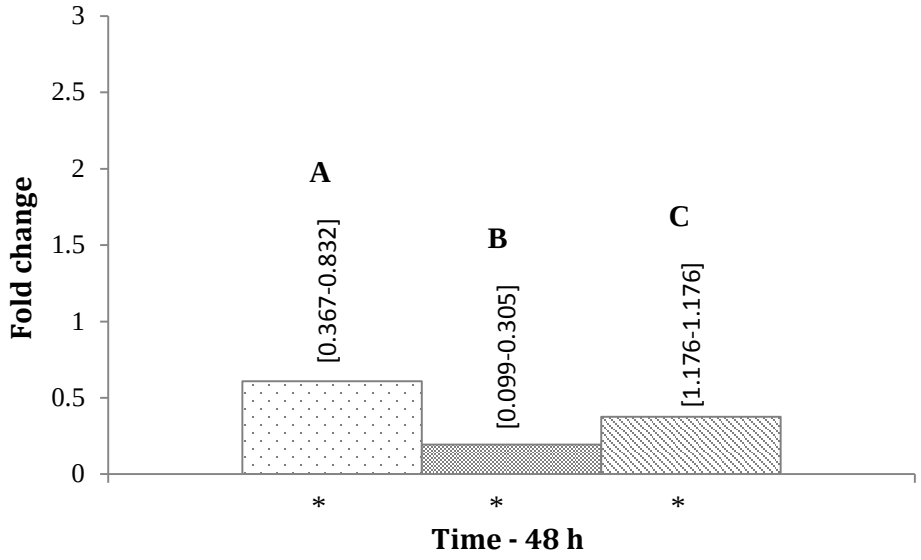


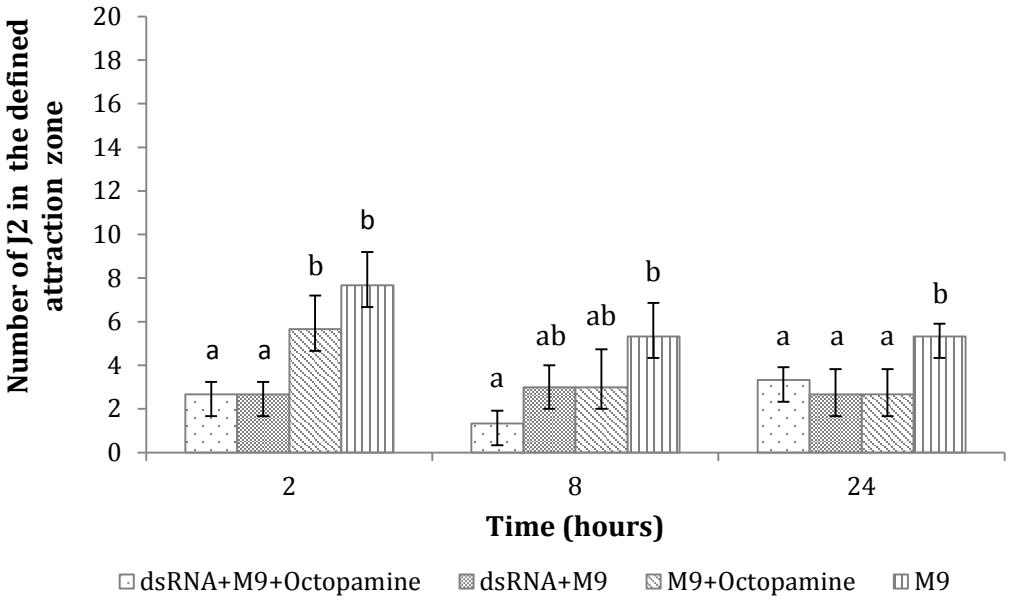
Figure 3.2 Relative *Mhi-vap-1* gene expression at 48 h, after soaking of 500 *Meloidogyne hispanica* second-stage juveniles, obtained in relation to expression levels in control treatments: A- dsRNA+M9+octopamine vs M9+octopamine (control); B- dsRNA+M9+octopamine vs M9 (control); C- dsRNA+M9 vs M9 (control). 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* down-regulation) as determined by REST.

The effect of the different treatments in the number of J2 in DZA and TRT were analyzed at 2, 8 and 24 HAI. No statistical significant differences were observed in treatments over time ($P > 0.05$, data not show). For DZA and TRT, significant differences were detected at 2 HAI in J2 treated with dsRNA+M9+octopamine and dsRNA+M9 and their controls, M9+octopamine and M9, respectively. The number of J2 in DZA and TRT was significantly less when treated with *Mhi-vap-1* dsRNA (Fig. 3.3). At 8 and 24 HAI, the number of J2 in DZA treated with dsRNA+M9+octopamine tended to decrease when compared with the respective control (M9+octopamine), however, no statistical significantly differences were detected, except between the treatments dsRNA+M9+octopamine and M9 (Fig. 3.3 A). At 24 HAI, significant

differences were only found between the treatments dsRNA+M9+octopamine and dsRNA+M9 with the control M9 (Fig. 3.3 A).

Significant differences ($P < 0.05$) were detected, at 2 and 8 HAI, between the number of J2 TRT treated with dsRNA+M9+octopamine and dsRNA+M9 when compared with their respective controls (M9+octopamine and M9). Finally, at 24 HAI, the results obtained for the number of J2 TRT were similar with the results obtained withf J2 in DZA at 8 HAI (Fig. 3.3). The number of J2 TRT, at 2 and 8 HAI, was significantly reduced when treated with dsRNA+M9 with/without octopamine than when with M9+octopamineor M9 ($P < 0.05$, Fig. 3.3 B). Results in the number of J2 in DZA and TRT revealed that the differences between treatments have a tendency to decrease over time (Fig. 3.3 A, B).

A



B

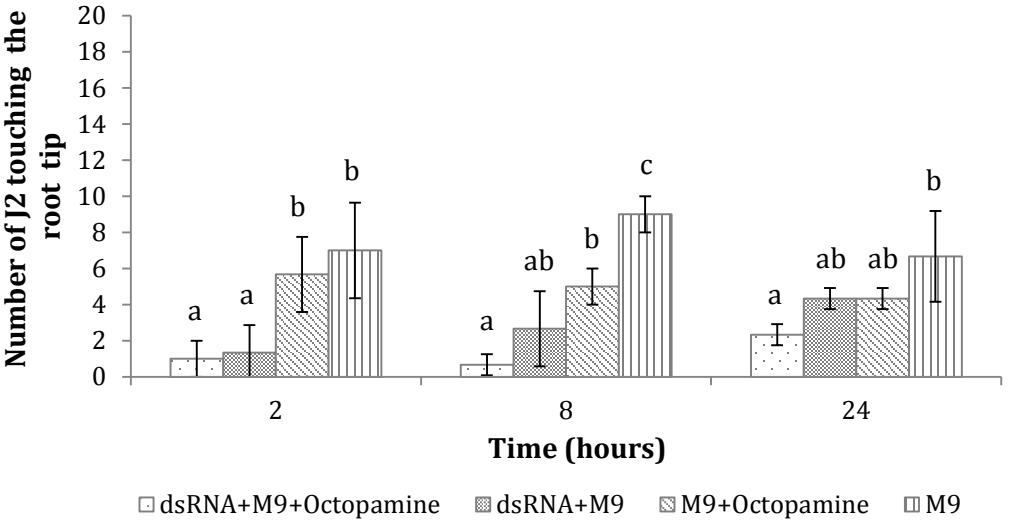


Figure 3.3 Number of *Meloidogyne hispanica* second-stage juveniles (J2) after soaking in dsRNA+M9+octopamine, dsRNA+M9, M9+octopamine (control) and M9 (control) in the defined zone of attraction (A, 1 cm^2 around the root tip) and touching root tip (B) at 2, 8 and 24 h after inoculation. Initial population density of approximately 25 J2. The value of each bar represents the mean ± SE of three replicates; the same letter within each observation time point indicates that values are not different ($P > 0.05$) according to One-way ANOVA Fischer LSD post-hoc test.

A decline in the number of J2 inside the roots was observed when J2 were treated with *vap-1* dsRNA ($P \leq 0.05$, Fig. 3.4). Significant differences were also observed between controls (J2 treated with or without octopamine) where a decrease in nematode penetration after soaking in M9+octopamine was found (Fig. 3.4). Overall, the number of J2 in DZA, TRT and inside the roots tended to decrease when J2 were treated with dsRNA+M9+octopamine vs dsRNA+M9 and M9+octopamine vs M9 (Figs 3.3 A, B and 3.4).

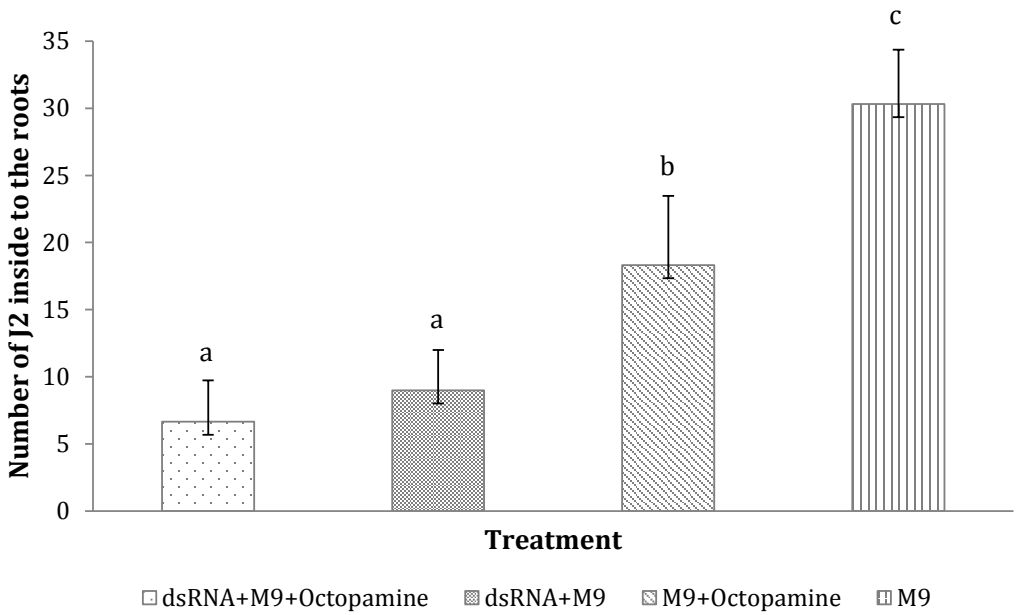


Figure 3.4 Number of *Meloidogyne hispanica* second-stage juveniles (J2) inside the tomato cv. Easypeel roots, after 36 h of inoculation. Soaking solutions with or without octopamine and dsRNA were used as controls. The value of each bar represents the mean ± SE of three replicates, and bars with different letters denote a significant difference at $P \leq 0.05$, according to One-way ANOVA Fischer LDS post-hoc test.

3.5 Discussion

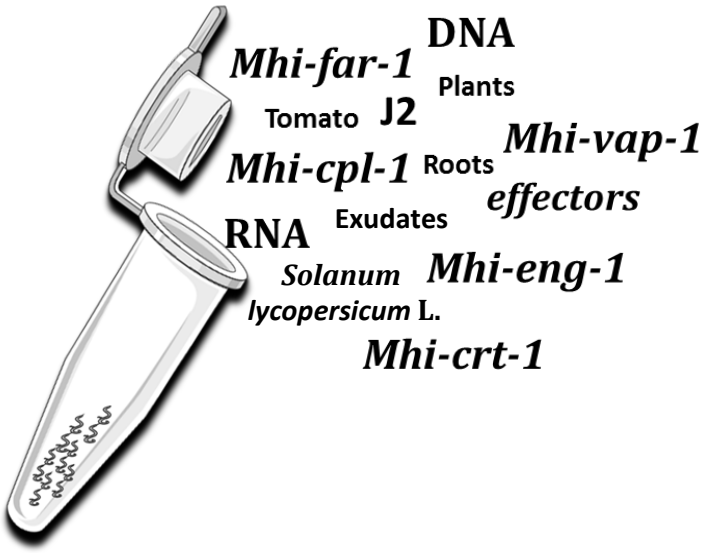
The exact function of the effector gene *vap-1* in parasitism is unknown and this study provide, for the first time, information about the effect of mediated gene silencing of *Mhi-vap-1* in J2 attraction and penetration. The *Mhi-vap-1* gene was only significantly down-regulated after 48 h of incubation of J2 with dsRNA+M9 with or without octopamine. These results suggest that the RNAi effect in *Mhi-vap-1* is not instantaneous and several hours of soaking are necessary. Successful *in vitro* RNAi have been observed in cyst nematodes and RKN treated with dsRNA, with incubation periods that ranging from 4 h to 7 days (Rosso *et al*., 2005). Neurostimulants, as octopamine, may not be absolutely required to stimulate solute ingestion by RKN (Bakhetia *et al*., 2007; Rosso *et al*., 2009). Our study indicates that the octopamine slightly affect the uptake of dsRNA. Some authors did not observe silencing after incubation in octopamine (Urwin *et al*., 2002; Rosso *et al*., 2005). However, a toxic effect in J2 can be considered, because there is a tendency to the expression level of *Mhi-vap-1* to decrease when the J2 were treated with this neurostimulant (dsRNA+M9+octopamine and M9+octopamine). In *G. pallida* and *M. incognita* infective juveniles, the dsRNA of neuropeptide targets also induce phenotype modification including an inhibitory effect on motility (Dalzell *et al*., 2009). Our results suggest that the reduction of the transcript *vap-1* gene is associated with a decrease in *M. hispanica* attraction and penetration. However, in a recent study, where the effect of RNAi and levels of transcripts were evaluated in 12 novel genes, the results revealed that in two of these genes there was significant and reproducible diminution of the infestation, but not at transcript levels (Danchin *et al*., 2013). The effect of dsRNA *Mhi-vap-1* in J2 attraction seems to decrease over time, because the differences between treatments with dsRNA and controls are likely to

decrease. Nonetheless, statistical differences were obtained in the number of J2 that penetrated roots between treatments, at 36 HAI. The duration of time that the *Mhi-vap-1* gene is down-regulated was not investigated; but in most cases the depletion of transcripts after soaking is transient (Rosso *et al*., 2009). The transcript level of the targeted genes would return to normal once nematodes are removed from exposure to dsRNA (Niu *et al*., 2012). Overall, the *vap-1* gene in *M. hispanica* is expressed and secreted from the infective stage stimulated by root exudates of the host plant. *Mhi-vap-1* gene was up-regulated after exposition of *M. hispanica* J2 to tomato root exudates for 24 h (unpublished results). According to Gao *et al.* (2001) and Wang *et al*. (2007), various allergen proteins were described as being highly transcribed during plant nematode parasitism. In the attraction assay, a significant reduction in the number of J2 treated with dsRNA+M9 and with or without octopamine was detected in DZA at 2 HAI, when compared with the controls. These results are partially consistent with the findings of Niu *et al*. (2012) that reported an interruption of the attraction and, consequently, a reduction of the nematode infection, ranging from 55.2 to 66.5%, when the *Rpn7* gene in *M. incognita* was silenced. In *M. hispanica*, the *vap-1* gene silencing was also supported by the J2 penetration results. At 36 HAI, the number of J2 inside the tomato roots decrease after soaking in dsRNA+M9 with or without octopamine. This result sustains the potential role of the VAP-1 in the early parasitic stages of the infection process (Duarte *et al*., 2014). The *Mhi-vap-1* was differentially expressed during *M. hispanica* development and its amplification products were observed from cDNA of eggs, J2 and adult females with the highest level of expression in J2 (Duarte *et al*., 2014). Our study clarifies the importance of the effector *Mhi-vap-1* gene in the nematode parasitism and its role in the infection process, more specifically in the attraction

and penetration of tomato roots. This data also demonstrates the efficacy of the RNAi in the *Mhi-vap-1* gene silencing. Nevertheless, the silencing of the *Mhi-vap-1* gene is not immediate and required 48 hs of incubation time with dsRNA. *Vap-1* can be a potential target for RNAi mediated nematode control by delaying the damage of important crops. *Meloidogyne hispanica* J2 can reach the tomato roots even after silencing, but the number of J2 was significantly less. Some proof of concept studies have shown that partial resistance against plant-parasitic nematodes can be achieved by expressing hairpin RNAs corresponding to nematode-specific genes. The use of host-delivered RNAi targeting *Meloidogyne* effector genes such as *Mhi-vap-1*, either when nematodes are moving in the rhizosphere or during migration inside the root tissue could achieve resistance as this gene is important for the host-parasite interaction and has been shown to be involved in suppression of host immune defences.

Chapter 4

Tomato root exudates induce transcriptional changes of *Meloidogyne hispanica* genes



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Duarte, A., Maleita, C., Abrantes, I. & Curtis, R (2014). Tomato root exudates induce transcriptional changes of *Meloidogyne hispanica* genes. Phytopathologia Mediterranea (submitted).

4.1 Abstract

Meloidogyne hispanica is a polyphagous root-knot nematode (RKN) recognized as a species of emerging importance able to infect a broad range of plants and tomato (*Solanum lycopersicum* L.) crops can be severely damaged due to the presence of this RKN species. The objective of this research is to investigate whether tomato root exudates regulate the expression of five parasitism candidate genes previously identified and sequenced in *M. hispanica* (*Mhi*): calreticulin (*crt-1*), cathepsin L cysteine protease (*cpl-1*), *β*-1,4 endoglucanase-1 (*eng-1*), fatty acid retinol binding protein (*far-1*) and venom allergen-like protein (*vap-1*). In particular the transcripts of the *Mhi-far-1* and *Mhi-vap-1* which were localized in the oesophageal subventral gland cells of the second-stage juveniles (J2). This paper reports for the first time that tomato root exudates induce changes in the gene expression of candidate parasitism genes in the *M. hispanica* pre-parasitic J2. The *M. hispanica cpl-1, crt-1*, *far-1*, and *vap-1* genes were differentially up-regulated ($P < 0.05$) in the pre-parasitic J2 after exposure to tomato root exudates. The *crt-1* and *vap-1* genes showed approximately 6 fold increase when compared with control and *cpl-1* and *far-1* approximately 2 fold increase, whilst the expression of gene *eng-1* was only slightly affected ($P = 0.05$) by the treatment. It is possible that these candidate parasitism genes have a strategic function during the plant-nematode interaction in the early events of infection and that their up-regulation prior to root infection possibly contribute to successful parasitism of plants.

Key words: effectors**,** plant-nematode interactions, root-knot nematodes, secretions.

4.2 Introduction

The sedentary endoparasitic root-knot nematodes (RKN), *Meloidogyne* spp., are among the most economically damaging plant-parasitic nematodes (PPN). They can affect an entire crop, reducing the yield and quality of the product, and tomato (*Solanum lycopersicum* L.) plants are particularly highly susceptible to RKN infestation (Moens *et al*., 2009). The RKN *M. hispanica* Hirschmann, 1986, detected for the first time in Spain from peach rootstock (*Prunus persica silvestris* Batsch) has a worldwide distribution being found in all continents, associated with a wide range of plant hosts including tomato cultivars (Hirschmann, 1986; Maleita *et al*., 2012a).

Sedentary PPN, such as *Meloidogyne* spp., have co-evolved with their hosts to develop mechanisms that optimize the chances of successful root invasion. Infective nematode stages rely on responses to plant signals originating from root exudates or sites of previous nematode penetration to find a host in the soil and when a root is encountered, its surface is explored for a suitable penetration site (reviewed in Curtis, 2008). The infective second-stage juveniles (J2) of RKN do not feed during their migration in soil and roots. Therefore, plant behavioural cues are essential for nematodes to localize the hosts and establish the feeding sites before their lipid reserves have been too depleted; nematodes with > 60% of their lipid reserves depleted are no longer able of directed movement (Curtis *et al*., 2009). Plant chemicals originating from root exudates have been shown either to attract nematodes to the roots or to result in repellence, motility inhibition, or even death (Curtis *et al*., 2009). A combination of signals emanating from different areas of the roots affects nematode behaviour in a given plant-nematode interaction (Prot, 1980). Pre-parasitic J2 of *Meloidogyne* spp. are attracted to the zone of elongation in growing root tips and display characteristic nematode exploratory behaviour at the

root surface, including stylet thrusting, release of secretions in preparation for root penetration, aggregation and an increase in mobility (Von Mende, 1997). This exploratory behaviour was induced *in vitro* by compounds present in root exudates, and a number of plant compounds, such as catechol and caffeic acid, induced nematode stylet thrusting and production of secretions (reviewed in Curtis, 2007). Root exudates components such as tannic acid, flavonoids, glycosides and fatty acids may regulate the pre-parasitic J2 chemotaxis by repulsion or attraction (Chitwood, 2002; Bais *et al*., 2006). Indeed many crops naturally release nematotoxic compounds into the environment either from their roots or directly from plant tissue to suppress RKN (Bais *et al*., 2006; Dutta *et al*., 2011). Dutta *et al*., 2012 showed that semiochemicals such as small lipophilic molecules emitted by root exudates of tomato and rice (*Oryza sativa* L.) affected stylet thrusting and motility of RKN J2 and might exert a repellent or allelopathic effect on these nematodes. It was shown that *in vitro*, plant signals present in root exudates, trigger a rapid alteration of the surface cuticle of sedentary PPN and that the same changes were also induced by phytohormones, in particular auxin (Curtis *et al*., 2006; Curtis, 2007). Therefore, molecules present in root exudates may act as environmental signals to induce behavioural changes and consequently play a vital role in the host-recognition processes and might help these nematodes to adapt and survive to the plant defence processes. Until recently, little was known about RKN gene expression and the signaling mechanisms occurring in the early stages of infection before nematodes penetrate the roots. For the first time, Teillet *et al*., (2013) and Dong *et al*., (2014) showed that PPN are able to perceive root signals before root penetration and respond by changing their behaviour and gene expression. The complex molecular communication during the early stages of plant-nematode interactions was

associated with a number of nematode genes being up or down-regulated in response to signals present in the root exudates of *Arabidopsis thaliana* (Teillet *et al*., 2013). Lauric acid present in the root exudate of *Chrysanthemum coronarium* L. was shown to down-regulated the expression of the *M. incogni*ta *Mi-flp-18* gene and affect nematode motility (Dong *et al*., 2014).

The potential impact of *M. hispanica* in agriculture strengthens the urgent need for the development of new control strategies. The objective of this research was to investigate whether tomato root exudates regulate the expression of five parasitism candidate genes identified in *M. hispanica* (*Mhi*): calreticulin (*crt-1*), cathepsin L cysteine protease (*cpl-1*), β-1,4 endoglucanase-1 (*eng-1*), fatty acid retinol binding protein (*far-1*) and venom allergen-like protein (*vap-1*) (unpublished data). These genes have been considered to play important roles in the plant-nematode interactions and they are strongly associated with a potential digestive role (*cpl-1*), plant cell-wall degradation (*eng-1*) and suppression or manipulation of plant basal defences (*crt-1*, *far-1* and *vap-1*) (Jaouannet & Rosso, 2013). The transcripts of the *Mhi-far-1* and *Mhi-vap-1* genes were localized in the J2 oesophageal subventral gland cells (Duarte *et al*., 2014). We show for the first time that, *in vitro*, tomato root exudates induce changes in gene expression of candidate parasitism genes in the pre-parasitic J2 of *M. hispanica*.

4.3 Materials and methods

4.3.1 Nematode isolate

The Portuguese *M. hispanica* isolate, obtained from fig-tree (*Ficus carica* L.) roots, was reared on tomato cv. Easypeel. The species identification was confirmed by

esterase phenotype analysis (Abrantes *et al.*, 2008; Maleita *et al.*, 2012b). The freshly hatched J2 were obtained from egg masses placed on a 25 µm mesh sieve.

4.3.2 Root exudates and J2 incubation

Root exudates of three tomato plants cv. Easypeel were obtained from the root system of four week-old plants. The roots were washed gently and transferred to an Erlenmeyer with 250 mL of sterilized distillated water, with agitation during 4 h (Shepherd, 1986; Bellafiore *et al.*, 2008). The roots exudates were filtered using Whatman filter paper grade 1. Afterwards, 1000 *M. hispanica* J2 were exposed overnight to tomato root exudates. Water was used as control.

4.3.3 RNA extraction and reverse transcriptase

After exposure to tomato root exudates or water, the *M. hispanica* J2 were centrifuged at 8000 x g for 2 min, washed tree times with RNAase free water and stored at -80°C until RNA extraction. *Meloidogyne hispanica* J2 were homogenized in 400 µL Trizol reagent (Invitrogen, UK) and vortexed for 2 min. Three cycles of freezing, in liquid nitrogen, and thawing, at 37°C, were performed. Then, 200 µL of Trizol were added, the samples incubated for 5 min, at room temperature, and subsequently 140 µL of chloroform were added and the samples incubated for 2 min, at room temperature. The samples were centrifuged for 15 min at 12000 x g and 4°C, and the aqueous phase transferred to a new tube. Afterwards, the RNA was isolated using the RNeasy Mini Kit including RNase-Free DNase Set (QIAGEN, UK). Total RNA (50 ng) was reverse transcribed into cDNA using the Omniscript RT Kit (QIAGEN, UK), according to the manufacturer's instructions. The concentration of the cDNA

was determined in a Nanodrop ND-1000 Spectrophotometer (Labtech In ternational, UK).

4.3.4 Gene expression analysis by quantitative RT-PCR

In order to ascertain the effect of tomato root exudates on the expression of the *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-eng-1*, *Mhi-far-1* and *Mhi-vap-1* genes, a quantitative real time PCR (qRT-PCR) was carried out with the SYBR Green PCR Master Mix (Applied Biosystems 7500 Fast Real-Time PCR System). qRT-PCR primers for *cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1 M. hispanica* genes and *β-actin* gene were designed from the *M. hispanica* sequences KF030974, KF679110, KF679121, KF679116 and KF030969 and *M. incognita* BE225475.1 sequence, respectively, obtained from Genbank, using the software Primer Express v3.0 (Applied Biossystems) (Table 4.1). *β-actin* gene was used as reference gene for normalization. The qRT-PCR primers sets were optimized for working concentration, annealing temperature and analyzed by dissociation curve for contamination or non-specific amplification by primer dimer. Efficiencies of the PCR reactions were determined according to Pfaffl (2001) using the Applied Biosystems software to guarantee that only a single PCR product was obtained. All primers pairs had efficiencies over 86%. PCR negative control, without cDNA template, confirmed that there were no non-specific PCR products. The qRT-PCR reaction mix (20 µL) contained 3 µL of cDNA in 1× Fast SYBR Green Master Mix and 200 nM of primers for *Mhi-vap-1* and *β-actin*, 100 nM for *Mhi-cpl-1*, *Mhi-crt-1* and *Mhi-eng-1* or 80 nM for *Mhi-far-1* (Table 4.1). The qRT-PCR was performed with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. PCR was done in triplicate and the mean Ct values determined. The relative expression of the genes was calculated by the ΔΔCT method according to

Applied Biosystems 7500 Fast Real-Time PCR System, (software version 2.0.4). The entire experiment was performed three times and the statistical analysis of the results was performed according to the iteration test (REST© 2009 Software).

Table 4.1. – Genes and primers used in the qRT-PCR.

Gene name	**Primer name**	**Primer sequence 5'→3'**
Calreticulin	MHIq-CRT-1f	AGACTTGAGCTGATTGGGAGTTG
(crt-1)	MHIq-CRT-1r	TCTTTGCGTCAGGGTCCTTAA
Cathepsin L cysteine protease	MHIq-CPL-1f	TTGGATACGGCACAGATGACA
(*cpl-1*)	MHIq-CPL-1r	TTCCCCCCAACTATTTTTAACAAG
β-1,4-endoglucanase-1	MHIq-ENG-1f	CGTTCTCGGTACAACAACATGGT
(*eng-1*)	MHIq-ENG-1r	TGTGCCGCTTACAGGATTGTTA
Fatty acid and retinol binding	MHIq-FAR-1f	ATTGACCGAGGACGACAAGA
protein (*far-1)*	MHIq-FAR-1r	TCAGTTGCATATTCACTGTGCTTCT
Venom allergen-like protein-1	MHIq-VAP-1f	CCTTATCCTGGCCAAGACTGC
(*vap-1*)	MHIq-VAP-1r	TTGTGTCCAATGTCCAATACCTCT
β-actin (control)	MHIq-ACTINf	TGTATCCAGGCATTGTGATCGT
	MHIq-ACTINr	CATTGTTGATGGTGCCAAAGC

4.4 Results and discussion

The results revealed that tomato root exudates induce changes in gene expression of candidate parasitism genes in *M. hispanica* pre-parasitic J2, suggesting that this effect takes place before root penetration. The *M. hispanica cpl-1*, *crt-1*, *far-1* and *vap-1* genes were differentially up-regulated ($P < 0.05$) after exposition of pre-parasitic J2 to tomato root exudates, whilst the expression of gene *eng-1* was only slightly affected by the treatment (P = 0.05, Fig. 4.1). Gene expression was validated and confirmed in all cases. The *crt-1* and *vap-1* genes showed approximately 6 fold increase when compared with control and *cpl-1* and *far-1* approximately 2 fold (Fig. 4.1).

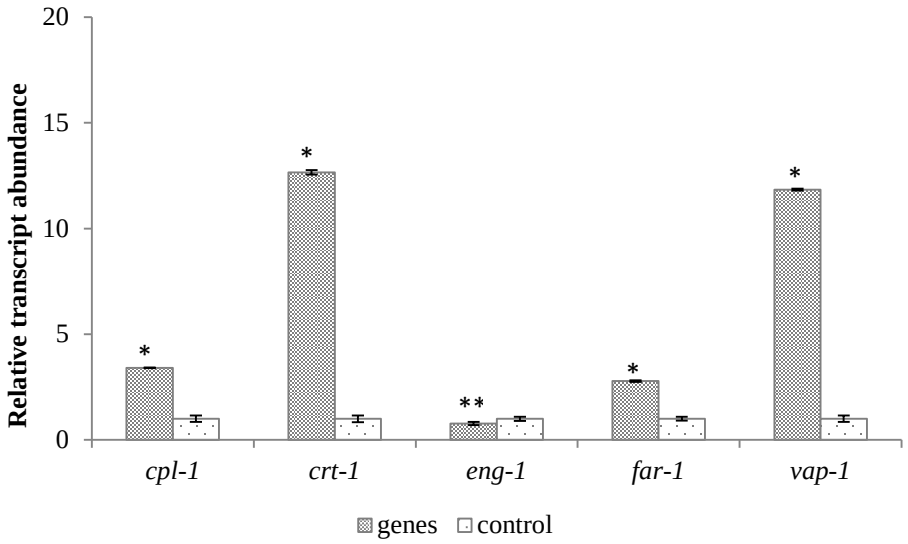


Figure 4.1 Relative transcript abundance of *Meloidogyne hispanica cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1* genes determined by qRT-PCR after exposure of second-stage juveniles to tomato root exudates. Values are means of three replicates. Significant differences (* $P < 0.05$ and ** $P = 0.05$) were assessed by iteration test (REST© 2009 Software).

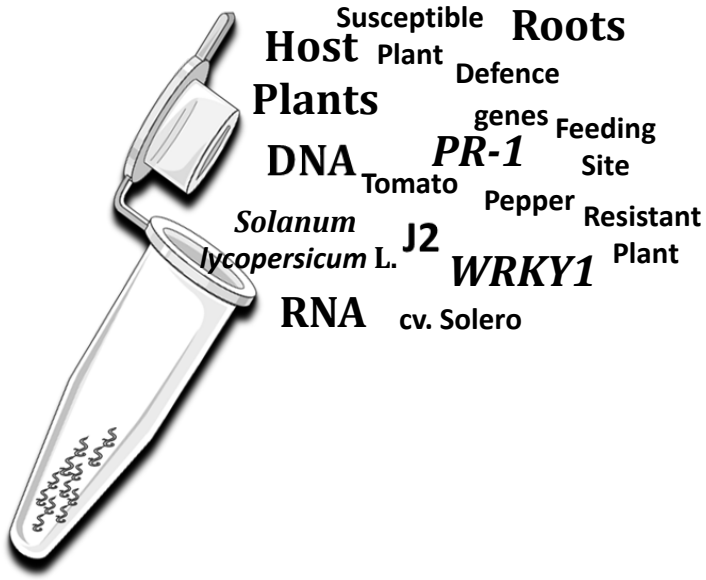
It has been extensively reported that plant signals present in root exudates induced nematodes behaviour changes and the regulation of nematode gene expression by root signals has been studied specifically in relation to the hatching of cyst nematodes (reviewed in Curtis *et al*., 2008). However, the works of Teillet *et al*., (2013) and Dong *et al*., (2014) were the first to show that a number of nematode genes of *M. incognita* were differentially expressed in response to signals present in the root exudates, *in vitro* and in planta studies. Teillet *et al*., (2013) also showed that the genes continued to be up-regulated post nematode penetration, during migration and feeding site initiation. The genes studied here have been shown to be expressed during migration and feeding site formation (unpublished data; Duarte *et al*., 2014), however, this is the first report showing that RKN genes potentially involved in parasitism are up-regulated by tomato root exudates prior to nematode

infection. Understanding the complexity of the molecular signal exchange and response during the early stages of the host parasite interactions is important to identify vulnerable points in the parasite life cycle that can be used to target disruption of nematode-host recognition. Dong *et al.* (2014) demonstrated the importance of such studies and showed that a possible novel control strategy can be devised by implementing a tomato–crown daisy intercropping system. The crown daisy root exudates in this system are responsible for the down-regulation of the expression of the *M. incognita Mi-flp-18* gene, which negatively affected nematode motility and led to a decrease in nematode infection of the tomato plants. Our data shows that *M. hispanica* pre-parasitic J2 are able to recognize signals present in root exudates that trigger a change in gene expression in juveniles. The *Mhi-crt-1* and *Mhi-vap-1* genes were the most expressed, with a 6 fold increase after incubation with root exudates (Fig. 4.1) suggesting that these two genes might be involved in the early events of recognition between the plant and the nematode. In previous studies, the *crt-1* gene was shown to be abundantly secreted into the apoplasm by *M. incognita* sedentary stages during induction and maintenance of the giant cells (Jaubert *et al*., 2005) and the function of the Mi-CRT-1 as a suppressor of host innate immunity was recently demonstrated (Jaouannet & Rosso, 2013). The venom allergen-like protein effector was also associated with the suppression of host defence (Peng *et al*., 2013). The expression of the *Mhi-cpl-1* and *Mhi-far-1* genes was up-regulated with a 2 fold increase in the pre-parasitic J2, after exposure to tomato root exudates (Fig. 4.1). The *cpl-1*, in *M. incognita*, is a digestive enzyme expressed in the nematode intestine whilst the *far-1* is as a fatty acid retinol binding protein that has an important function in the infection process, through the manipulation of jasmonate-dependent defence response that induces susceptibility to RKN

(Iberkleid *et al*., 2013). These candidate parasitism genes might have a strategic function during the plant-nematode interaction in the early events of infection and their up-regulation prior to root infection contributes to a successful parasitism. Gene silencing should provide some information to whether these genes are vital for nematode penetration and survival inside the roots. The identification of the plant signals present in the tomato root exudates, responsible for up-regulation of these parasitism genes, may lead to the devise of novel approaches to PPN control. It is possible to modify the rhizosphere and interfere with host recognition process.

Chapter 5

Expression of the defence genes (*PR-1* and *WRKY1*) in tomato and pepper after *Meloidogyne hispanica* infection



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5.1 Abstract

Plants employ defensive strategies to protect themselves from a variety of pathogens including root-knot nematodes, *Meloidogyne* spp. These plant defence mechanisms are regulated by a large number of genes which expression is differentially regulated during nematode infection. The purpose of this study was to evaluate the expression of the plant defence genes, *PR-1* and *WRKY1,* after *M. hispanica* infection of susceptible tomato, *Solanum lycopersicum*, cv. Easypeel and resistant pepper, *Capsicum annuum*, cv. Solero. Tomato and pepper four-week-old seedlings were inoculated with 2000 freshly hatched *M. hispanica* second-stage juveniles and the expression of the *PR-1* and *WRKY1* genes were evaluated by quantitative real time (qRT-PCR) at 12 h after inoculation (HAI) and 2, 6 and 12 days after inoculation (DAI). In the susceptible tomato cv. Easypeel, the *PR-1* and *WRKY1* genes were significantly high up-regulated upon the first signals of *M. hispanica* infection, at 12 HAI and 2 DAI, which reinforces the possible involvement of these genes in the regulation of primary host plant defence pathways and in the early events of the compatible plant-nematode interaction. At 6 and 12 DAI, the expression of *WRKY1* genes in tomato plants was significantly down-regulated. In contrast, in the resistant pepper cv. Solero, the expression of the *PR-1* and *WRKY1* genes was significantly down-regulated upon *M. hispanica* infection at all the time points tested, suggesting that these defence genes might not play a vital role in the defence mechanism of pepper against *M. hispanica*.

Key words: effectors genes, host plants, parasitism, qRT-PCR, root-knot nematodes.

5.2 Introduction

Plants are hosts of a wide range of pathogens that exploit them as source of nutrients to support their development and reproduction. On the other hand, plants employ several defence strategies against pathogens, such as physical and chemical barriers and induce immune responses that are regulated by complex signalling networks (Rushton & Somssich, 1998; Singh *et al*., 2002). Plants react to pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs), which are detected through leucine-rich repeat receptors, promoting the innate immunity in plants in a process called pathogen triggered immunity (PTI). These mechanisms of defence are regulated by a large number of genes, including *PR-1* and *WRKY*, which encode regulatory proteins (Glazebrook, 2001; Singh *et al*., 2002; Huang *et al*., 2012). In contrast, plant-parasitic nematodes have co-evolved innovative strategies and use molecules collectively known as effectors to manipulate and suppress host defences to protect them and their feeding sites from direct attack by the host (Maule & Curtis, 2010; Smant & Jones, 2011).

The plant defence response is a complex process induced by several hormones, such as salicylic acid (SA), indole acetic-acid, jasmonic acid and ethylene (Wubben *et al*., 2008; Matthews *et al*., 2013). The SA in general activates the defence response to biotrophic and hemi-biotrophic pathogens, triggers the systemic acquired resistance (SAR) inducing the expression of SAR associated pathogenesis-related (*PR*) genes (Glazebrook, 2001; Jones & Dang, 2006; Matthews *et al*., 2013). The PR proteins implicated in active plant defence can be induced by various types of pathogens during plant-pathogen interaction such as oomycetes, fungi, bacteria, viruses, plant-parasitic nematodes and phytophagous insects and in some cases these proteins can

restrict the pathogen development and spread (Antoniw *et al*., 1980; Van Loon *et al*., 1994, 2006; Van Loon & Van Strien, 1999; Hamamouch *et al*., 2011).

PR proteins have been classified in at least seventeen families, on the basis of their sequence or predicted sequence of amino acids, and grouped into different classes based on serological relationship and enzymatic or biological activity (Van Loon & Van Strien, 1994; Van Loon *et al*., 2006). The PR-1 class of proteins, the most abundant in plants reaching to about 1-2% of total leaf protein content, has been detected in tobacco, tomato, barley, maize, parsley and other plants (Sudisha *et al*., 2012). The expression of *PR-1* gene has been used as a molecular marker of SAR in plants in response to biotic stress, to indicate plant defence response, but the exact activity of the gene remain unclear (Mitsuhara *et al*., 2008; Sudisha *et al*., 2012; Matthews *et al*., 2014). The SA-induced *PR1b* gene is also generally used as a marker of SAR, induced in rice leaves during fungal, bacterial and pathogen attack (Mitsuhara *et al*. 2008). To date few studies have investigated the expression of *PR* genes in the incompatible plant-nematode interaction (Mazarei *et al*., 2011; Tirumalaraju *et al.,* 2011; Molinari *et al.,* 2014). Molinari *et al*. (2014) analysed the *PR-1* gene expression in roots and shoots of susceptible and resistant tomato plants uninfected and infected with the RKN *M. incognita.* The *PR-1* gene expression was up-regulated in roots and shoots of resistant tomato plants. In susceptible plants an inhibition of the expression of *PR* genes was detected in roots after infection with second stage juveniles (J2).

The WRKY family of transcription factors, originally believed to be unique to plants, represents an important class of transcriptional regulators in higher plants (Ulker & Somssich, 2004; Pan *et al*., 2009). This family of proteins, each with approximately 60 amino acids long, contains a conserved amino acid sequence at the N-terminus,

which interact with its cognate DNA binding site, known as the W-box (TTGACY) (Rushton *et al*., 1996; Ciolkowski *et al*., 2008; Yan *et al*., 2013). WRKY factors were classified into three major groups: group I, characterized by two WRKY domains, containing a C_2H_2 zinc-finger motif; group II, *WRKY* genes with only one domain, characterized by a C_2H_2 zinc-finger motif; and group III with a single WRKY domain containing a C_2H_2C zinc-finger motif (Eulgem *et al*., 2000). *WRKY* genes have been identified in: *Arabidopsis thaliana*; rice, *Oryza sativa*; soybean, *Glycine max*; pine, *Pinus monticola*; barley, *Hordeum vulgare*; tobacco, *Nicotiana attenuata*; and the green alga *Chlamydomonas reinhardtii* (Wu *et al*., 2005; Shen *et al.*, 2007; Skibbe *et al*., 2008; Zhou *et al.*, 2008; Liu & Ekramoddoullah, 2009; Tao *et al*., 2009; Bhattarai *et al*., 2010; Rushton *et al*., 2010). More recently, the *WRKY* transcription factors were identified in tomato, *Solanum lycopersicum*, and grapevine, *Vitis vinifera* (Huang *et al*., 2012; Wang *et al*., 2014). WRKY proteins were associated with several development processes in plants, such as seed development, senescence, dormancy, germination and also in the regulation of the defence against biotic and abiotic stresses (Huang *et al*., 2012; Wang *et al*., 2014). In *Arabidopsis* and tomato plants, the *SlWRKY72a* and *b* genes were studied and the results indicate the participation of these genes in plant defence response against *M. incognita* (Bhattarai *et al*., 2010).

Meloidogyne hispanica Hirschmann, 1986, detected for the first time in Spain from peach rootstock, *Prunus persica silvestris* Batsch, has a wide range of plant hosts (Hirschmann, 1986; Maleita *et al*., 2012a). Seventy six, out of 82 commercial plants, comprising 18 plant species and representing 10 botanical families, were susceptible to *M. hispanica*, five hypersusceptible or poor hosts and only pepper, *Capsicum annuum*, cvs. Aurelio and Solero were considered resistant (Maleita *et al*., 2011, 2012a). The aim of the present study was to evaluate the response of *PR-1* and

WRKY1 plant defence genes in susceptible and resistant plants to *M. hispanica*. *Meloidogyne hispanica* is able to reproduce in cultivars with the *Mi-1.2* gene and to date no tomato cultivar was identified as resistant to this RKN. Therefore, the gene expression of these defence genes was evaluated during a time course of infection using the susceptible tomato cv. Easypeel and the resistant pepper cv. Solero. The understanding of the molecular mechanisms of the plant defences against RKN is essential to develop environmentally friendly nematode control strategies.

5.3 Materials and methods

5.3.1 Nematode isolate

Portuguese *M. hispanica* isolate (PtHi3) obtained from infected fig-tree, *Ficus carica*, roots was maintained on tomato cv. Easypeel in a growth chamber at 25±2°C, with a 12 h photoperiod and ±75% relative humidity. The species identification was confirmed by isoesterase phenotype (Abrantes *et al*., 2008; Maleita *et al*., 2012b).

5.3.2 Plant material

The response of plant defence genes, *PR-1* and *WRKY1*, was evaluated in the roots of tomato cv. Easypeel and pepper cv. Solero after *M. hispanica* inoculation. Twelve tomato or pepper four-week-old seedlings, grown in a 250 cm^3 pot filled with autoclaved sand, were inoculated with 2000 freshly hatched J2, obtained from egg masses placed on a 25 µm mesh in water, and maintained in the growth chamber. Uninfected tomato and pepper plants were used as controls.

Gene expression was monitored in roots of tomato and pepper at 12 h after inoculation (HAI) and 2, 6 and 12 days after inoculation (DAI) with *M. hispanica* J2. The plants were harvested, the roots washed free of soil and grounded to a fine

power in liquid nitrogen, using a mortar and pestle. The powder was kept at -80°C until needed for RNA extraction.

5.3.3 RNA extraction and reverse transcription

Total RNA was extracted from root tissues with the RNeasy Plant Mini Kit including RNase-Free DNase Set (QIAGEN, UK) and the concentration and purity determined in the Nanodrop ND-1000 Spectrophotometer (Labtech International, UK). Total RNA (25 ng/µL) was reverse transcribed into cDNA, in a volume of 20 µL, using the Omniscript RT Kit (QIAGEN, UK), according to the manufacturer's instructions and the concentration determined.

5.3.4 Plant defence gene expression analysis by quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was used to ascertain the effect of nematode infection on the expression of two plant defence genes in susceptible tomato and resistant pepper plants at various time points. The experiment was carried out with the SYBR Green PCR Master Mix in a 7500 Fast Real-Time PCR System (Applied Biosystems, version 2.0.4). qRT-PCR primers (Table 5.1) were designed from sequences searched in the National Center for Biotechnology Information: AAB40095 and ABHO3630 for *β-actin* gene; NP_001234128 and AAK30143 for *PR-1* gene and NM_001247372 and ACT80136 for *WRKY1* gene in tomato and pepper, respectively, using the software Primer Express v3.0 (Applied Biossystems).

For qRT-PCR, the reaction mix (20 µL) contained 3 µL of cDNA, 1× Fast SYBR Green Master Mix and 100, 80 or 100 nM of the *β-actin*, *PR-1* and *WRKY1* gene specific primers, respectively (Table 5.1). Quantitative PCR was performed with the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C

for 1 min. The efficiency of PCR for each primer pair was assessed using a serial dilution over a 10^4 range of cDNA (50 ng/µL). Efficiencies of the PCR reactions and the relative expression of the gene transcripts were determined using the $\Delta\Delta$CT method according to 7500 Fast Real-Time PCR System. Relative *PR-1* and *WRKY1* genes expression was calculated in relation to expression levels in control group (uninfected plants) and normalized against the reference *β-actin* gene, using REST© 2009 software (Pfaffl, 2001; Pfaffl *et al.*, 2002). Up- and down-regulated genes and respective differences were considered significant at $P < 0.05$. The experiment was repeated twice and qRT-PCR assays performed in triplicate.

Table 5.1. Primers used in this study.

Genes	**Primer name**	**Primer sequence 5'→3'**
β-actin	SOLq-ACTINf	TTCAACACCCCTGCCATGTA
	SOLq-ACTINr	GTCCACTGGCATAGAGGGAAAG
	CAPSq-ACTINf	TTCCCGGGTATTGCTGATAGA
	CAPSq-ACTINr	TGCTGCTAGGAGCCAATGC
PR-1	SOLq-PR-1f	GCCCTTACGGCGAAAACCT
	SOLq-PR-1r	AGCACCAGCAGCGTTTAGCT
	CAPSq-PR-1f	GCTCACAATGCAGCTCGTAGAC
	CAPSq-PR-1-r	GCTAGCCTATTGTCCCATGTCAT
WRKY1	SOLq-WRKY1-f	CATCCACGAACCTCAAAACCA
	SOLq-WRKY1-r	AATCGGCTGGCTGTGGAA
	CAPSq-WRKY1f	TGCACGATGGCTGTTGGT
	CAPSq-WRKY1r	TCCTCCGCACACCTTTGC

5.4 Results

The relative transcript abundance of *PR-1* and *WRKY1* genes in susceptible tomato and resistant pepper plants inoculated with *M. hispanica* and respective uninfected controls were evaluated by qRT-PCR, using *β-actin* gene as reference for normalization, at various time points (12 HAI and 2, 6 and 12 DAI). The slope values obtained for *PR-1* and *WRKY1* genes were -3.4 and -3.5, respectively, with 96 and 93% efficiency. For the reference gene *β-actin*, the slope value was -3.14 with 107% efficiency. In pepper plants, the slope values obtained were -3.2, -3.7 and -3.5 for *PR-1*, *WRKY1* and *β-actin* genes, respectively, with 105, 86 and 93% efficiency. In the susceptible tomato cv. Easypeel plants, the *PR-1* and *WRKY1* genes were significantly ($P < 0.05$) up-regulated at 12 HAI and 2 DAI. At 6 DAI, both genes were significantly down-regulated. The expression level of *PR-1* gene increased at 12 DAI, however *WRKY1* gene remained significantly down-regulated ($P < 0.05$, Fig. 5.1).

In resistant pepper plants, at all observation times, the *PR-1* and *WRKY1* genes were significantly down-regulated ($P < 0.05$, Fig. 5. 1).

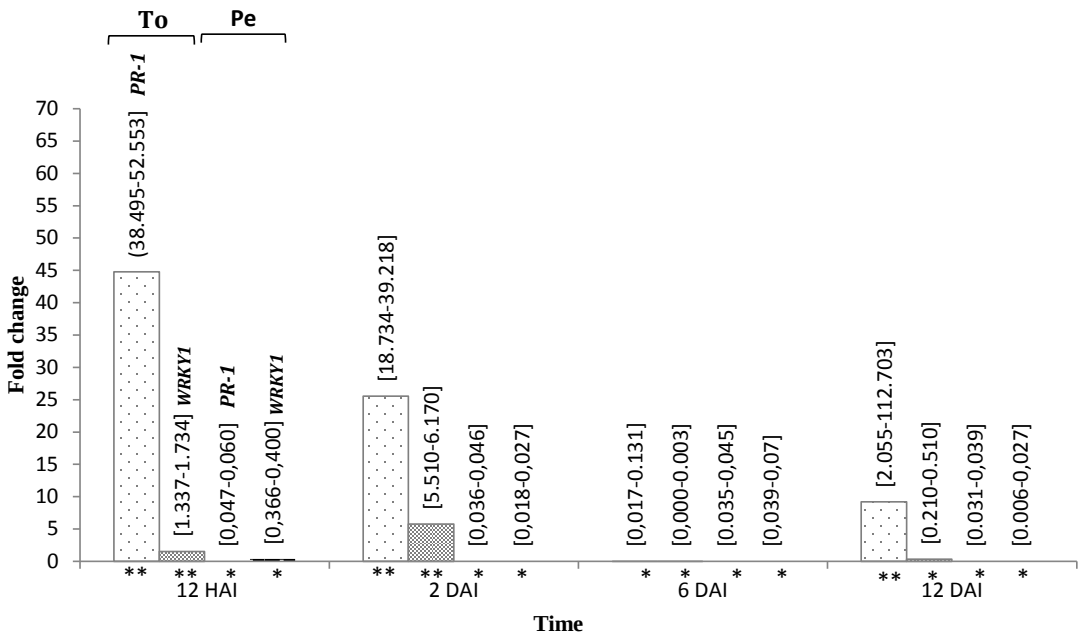


Figure 5.1 Relative expression of *PR-1* and *WRKY1* genes in susceptible tomato cv. Easypeel (To) and resistant pepper cv. Solero (Pe), inoculated with 2000 *Meloidogyne hispanica* second-stage juveniles. HAI - hours after inoculation; DAI - days after inoculation. 95% confidence limits are shown in square brackets. Asterisks indicate significant gene expression (* down-regulation and ** up-regulation).

5.5 Discussion

Nematode infection differentially changes the gene expression of numerous plant genes and RKN infection not only triggers but also suppresses the defence response of the plants (Ibrahim *et al*., 2011). These changes in gene expression are pivotal to the success of parasitism in susceptible plants which culminates in the formation of permanent nematode feeding sites that allows nematode development and reproduction (Bird, 1996). Temporal expression of the *PR-1* and *WRKY1* genes was validated and qRT-PCR results showed significant changes in expression of these defence genes upon *M. hispanica* inoculation in susceptible and resistant plants. In tomato roots, the expression of the *PR-1* and *WRKY1* genes was early up-regulated (12 HAI and 2 DAI) and decreased over time, suggesting that during the early events

of infection (penetration and formation of the feeding site), nematodes trigger a strong PTI culminating with an increase of the *PR-1* and *WRKY1* genes expression. However, the expression of *WRKY1* gene was subsequently suppressed as the nematode infection progresses. The *PR-1* gene was down-regulated at 6 DAI possibly by the action of nematode effector proteins released from J3 and/or J4 developmental stages which do not feed during this period of time as the stylet and the medium bulb degenerate (Triantaphyllou & Hirschmann, 1960). This was followed by an increase in the expression level of this gene at 12 DAI, which was comparatively lower (10x change fold) than the change fold induced by the J2 at 12 HAI (40x change fold). This suggests that induction of the *PR-1* gene by MAMPS released by adult females were less effective in triggering PTI or less concentrated.

The down-regulation of *PR-1* and *PR-2* genes at 10 DAI following infection with *M. javanica* has also been reported previously in susceptible tomato plants (Sanz-Alferez *et al*., 2008). Other studies showed that the transcript levels of *PR-1* genes increased at 3, 6 and 9 DAI in *A. thaliana* infected with *Heterodera schachtii* or *M. incognita* (Hamamouch *et al*., 2011) and an increase in the expression of this gene was also observed in soybean 3 DAI with *H. glycines* (Matthews *et al*., 2011). The intensification of the *PR-1* expression suggests an increase in the level of SA known as a signal molecule for defence against plant-parasitic nematodes (Branch *et al*., 2004; Chen *et al*. 2009).

Our experiments showed that expression of the *WRKY1* gene was induced early in the susceptible tomato cv. Easypeel and decreased at 6 and 12 DAI, having a weak response of *WRKY1* defence gene during *M. hispanica* infection. According to Eulgem (2005), 17 of the 21 *WRKY* genes identified are down-regulated after nematode infection in susceptible plants. However, *WRKY23* gene expression increased rapidly

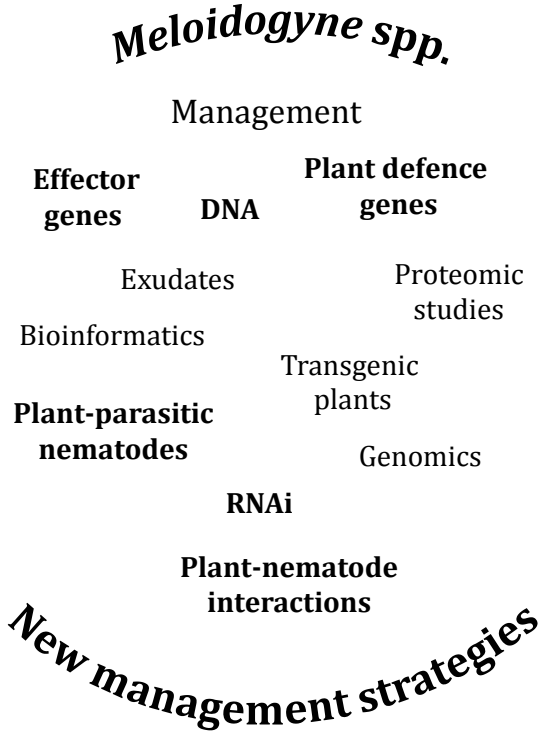
and was highly induced upon infection of *A. thaliana* roots with *M. incognita* and *H. schachtii* and at 10 DAI the expression of this gene started to decrease and faded away (Grunewald *et al.*, 2008). Nonetheless, Atamian *et al*. (2012) showed the transcript level of *SlWRKY70* gene at 12 HAI was induced with *M. javanica* inoculation only in resistant tomato roots when compared with susceptible roots and at 36 HAI, the *SlWRKY70* gene was induced in both genotypes in response to RKN. Furthermore, this study suggested that *SlWRKY70* is required for *Mi-1* function, because when the *SlWRKY70* was silenced, the resistance against RKN was attenuated (Atamian *et al*., 2012).

In pepper, the *PR-1* and *WRKY1* genes were weakly expressed in all time points tested, indicating that these genes may not have a direct involvement in the defence during the interaction of *M. hispanica* with the resistant pepper cv. Solero.

The strong expression of the *PR-1* and *WRKY1* genes after 12 HAI and 2 DAI in tomato plants reinforces the possible involvement of these genes in the regulation of the primary host plant defence pathways in the early events of the compatible plant-parasitic interaction. *PR-1* gene potentially can be considered a marker for the early and also later events of infection by *M. hispanica* in susceptible tomato plants.

The different responses of the defence genes expression against pathogens can provide new insights to understand the mechanism in plant defence responses into host-parasite interactions.

General discussion and future perspectives



General discussion

It is generally accepted that nematode parasitism genes encode for secreted molecules known as nematode effectors, play a key role in the plant-nematode interactions. These effectors are secreted into the host tissue and able to change plant physiology and assist the infection process. Some effector are able to manipulate and supress host defences to protect them and their feeding sites from direct attack by the host which allows nematode development inside the roots and completion of their life-cycle (Jaouannet & Rosso, 2013). The identification and characterization of the nematode effector genes is important to provide a better understanding of the plant-nematode interaction and may lead to the identification of vulnerable points in the nematode life-cycle which can be used for the development of new approaches for the control and management of plant-parasitic nematodes. In this study, 13 effector genes and homologous predicted proteins were searched in the National Center for Biotechnology Information (NCBI) and using information available from the genomes of *M. incognita* and *M. hapla*. Cathepsin L cysteine protease (*cpl-1*), β-1,4–endoglucanase-1 (*eng-1*), β-1,4–endoglucanase-2 (*eng-2*), pectase lyase 3 (*pel-3*), polygalacturonase (*gp-1*), manganese superoxide dismutase (*mnsod*), glutathione-S-transferase (*gsts-1*), fatty acid binding retinol (*far-1*), annexin-2 (*nex-2*), calreticulin (*crt-1*), 14-3-3a (*14-3-3a*), venom allergen-like protein-1 (*vap-1*) and venom allergen–like protein-2 (*vap-2*) were selected for the research conducted in this study to accomplish the following objectives:

1. To identify *M. hispanica* effector genes potentially important for the plant-nematode interaction and to determine whether these genes were differentially

expressed during *M. hispanica* development and their phylogenetic relationship with other *Meloidogyne* spp.;

2. To identify and isolate the effector genes venom allergen-like protein (*vap-1*) and fatty acid and retinol binding protein (*far-1*) in *M. hispanica* and determine their temporal and spatial expression patterns in the nematode eggs, second-stage juveniles (J2) and females. Also, to study the degree of the conservation of these effector genes between *M. hispanica* and other plant-parasitic nematodes;

3. To evaluate the putative role of the effector gene venom allergen-like protein (*Mhi-vap-1*) during nematode infection of tomato plants by silencing this nematode gene using RNAi;

4. To assess the effect of tomato root exudates in *M. hispanica* genes *cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1* expression;

5. To evaluate the effects of the defence genes *PR-1* and *WRKY1* in tomato and pepper plants after *M. hispanica* infection.

In **chapter 1,** the annexin-2 (*nex-2*), β-1,4-endoglucanase-1 and 2 (*eng-1* and *eng-2*), cathepsin L cysteine protease (*cpl-1*), calreticulin (*crt-1*), glutathione-S-transferase (*gsts-1*), manganese superoxide dismutase (*mnsod*) pectase lyase 3 (*pel-3*), polygalacturonase (*gp-1*), venom allergen-like protein -2 (*vap-2*) and 14-3-3a (*14-3-3a*) effector genes were identified for the first time in *M. hispanica*. The partial *cpl-1*,

crt-1, *eng-1* and *mnsod* gene sequences were obtained for *M. hispanica*, *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica.* The expression of these genes in eggs, J2 and females provided new information about their putative roles in the early events of nematode infection and during nematode development (**objective 1**). The expression of *Mhi-cpl-1* gene was similar in the eggs, J2 and females, suggesting that this gene has a significant role in all the steps of *M. hispanica* life cycle. These results are not in agreement with the findings of Shingles *et al*. (2007) who reported a weaker intensity for the expression of *cpl-1* gene in mature females from *M. incognita*. Studies conducted with the CPL-1 protease showed an involvement in the J2 nutrition and digestion processes and therefore may influence the host-parasite relationship (Neveu *et al.,* 2003; Ultaigh *et al*., 2009; Haegeman *et al*., 2012).

The *Mhi-crt-1* gene is present in all developmental stages, showing a strong expression in *M. hispanica* females, suggesting an important role in the later events of the infection (possibly during eggs deposition in the gelatinous matrix). During feeding, cell induction and maintenance, this protein accumulates along the wall of the giant cells and might have an important role in the suppression of plant basal defences and in targeting plant signaling pathways (Jaubert *et al*., 2002, 2005; Hassan *et al*., 2010; Haegeman *et al*., 2012; Jaouannet *et al*., 2012, 2013). The presence of the expression the *Mhi-crt-1* gene in J2 has already been detected by Ithal *et al.* (2007), Opperman *et al*. (2008) in *M. hapla* and in *Heterodera glycines*. According to Dubreil *et al*. (2009), the silencing of *Mi-CRT* in the pre-parasitic J2 reduced the ability of the nematodes to induce galls on tomato, highlighting the potential importance of this effector protein in the initiation of the feeding site and consequently for the life cycle completion.

The expression of the *eng-1* gene was found in *M. hispanica* eggs and J2 and may have a function in the early events of infection. The expression of this gene was already demonstrated in *Rotylenchulus reniformis* J2 and females (Wubben *et al*., 2010).

The MNSOD enzyme has been localized in the intestine of *M. incognita* J2, which suggest a putative function as a detoxification enzyme and could play an important role in protecting the nematode from plant host defences inside the host (Rosso, 2009). Nonetheless, the *Mhi-mnsod* gene was only detected in *M. hispanica* eggs, which indicate that this gene could have an additional function in the life-cycle (such as embryogenesis and or egg hatching) of this RKN. The differential expression of *cpl-1*, *crt-1*, *eng-1* and *mnsod* genes in different development stages (eggs, J2 and females) highlighted the importance of these genes in the parasitism.

The *cpl-1*, *crt-1, eng-1* and *mnsod* genes were identified and sequenced not only in *M. hispanica* but also in *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. A close phylogenetic relationship was observed among predicted amino acid sequences obtained for CPL-1, CRT-1, ENG-1 and MNSOD in *M. arenaria*, *M. hispanica*, *M. incognita* and *M. javanica*, however, *M. hapla* appeared as an out group. *Meloidogyne hispanica* displayed a predicted amino acid sequence identity ranging from 88 to 100% when compared with CPL-1, CRT-1, ENG-1 and MNSOD sequences of the other *Meloidogyne* species, being the lowest values always obtained for *M. hapla*. Similar results were obtained for the gene sequences similarity values. The results of the topology of the MHI-CPL-1, MHI-CRT-1, MHI-MNSOD and MHI-ENG-1 trees revealed slight differences compared to the ones obtained from DNA sequences, but the phylogenetic relations were essentially the same. In general, high level of identity

values among DNA and protein sequences was shown for the RKN nematode species analysed.

In **chapter 2,** the effector genes venom allergen-like protein (*vap-1*) and fatty acid retinol binding protein (*far-1*) were identified and sequenced in *M. hispanica* and shown to be differentially expressed in the *M. hispanica* developmental stages. The *Mhi-vap-1* and *Mhi-far-1* genes were present in eggs, J2 and in females. Nevertheless, the *Mhi-vap-1* showed the highest level of expression in J2 and a low level in eggs, while the expression of *Mhi-far-1* was detected in equal intensity in eggs, J2 and females, suggesting a potential function in these developmental stages. The *in situ* hybridization analysis revealed that the two genes are present in the subventral oesophageal glands, therefore may play an important role in the early stages of the infection process (**objective 2**). Previous *in situ* hybridization studies revealed that homologues for the VAP proteins were localized in the gland cells of *H. glycines* and *Ditylenchus destructor* (Gao *et al*., 2001; Peng *et al*., 2013). The *vap-2* gene in *M. incognita* was also expressed in the oesophageal glands of pre-parasitic and parasitic J2 and also transcribed in eggs and in females (Ding *et al*., 2000; Wang *et al*., 2007). Venon allergen proteins have not only been associated with the induction of a host immune response but have also been implicated in disease resistance by triggering programmed cell death in tomato plants infected with *Globodera rostochiensis* (Chen *et al*., 2010; Lozano-Torres *et al*., 2012). Multiple sequence alignments between MHI-VAP-1, MA-VAP-1 and MJ-VAP-1 showed 100% homology and therefore, a strong conservation between these proteins. In MHA-VAP-1, a high variability in the amino acids residues was found, when alignment of *M. hispanica*, *M. arenaria* and *M. javanica* were compared. The topology of the VAP-1 tree was identical to the one obtained from DNA sequences. According to Gao *et al*. (2001), a short single domain

type and a longer double domain type have been identified in nematodes and MHI-VAP-1 is a representative of the single domain, the most usual type found in nematodes.

The predicted amino acid sequence for FAR-1 proteins showed a strong conservation when compared with other *Meloidogyne* species but few differences were found in the predicted amino acids sequence of *M. hapla.* Prior *et al*. (2001), Garofalo *et al*. (2002), and Iberkleid *et al*. (2013) found conserved amino acid sequences for FAR-1 homologue proteins in different animal parasitic nematodes (*Onchocerca*, *Brugia*, *Wucheria*, *Loa, Acanthocheilonema*, *Ascaris suum*, *Toxocara canis* and *Litomosoides* and the plant-parasitic nematodes *G. pallida*, *G. rostochiensis*, *M. javanica*, *M. arenaria*, *H. schachtii*, *Pratylenchus. vulnus* and *Radopholus. similis*, and in the free-living nematode *Caenorhabditis elegans*). The high degree of conservation detected in the lipid-binding feature of FAR proteins and their presence at the host parasite interface, suggest that this restricted family of proteins play an important role in diferent life-cycles and in the parasitism (Bath *et al*., 2009). Overall, the results of the phylogenetic analysis, for VAP-1 and FAR-1 proteins, revealed that *M. hapla* was the most divergent species when compared with *M. hispanica*, which can be associated to the mode of parasitism and/or reproduction. *Meloidogyne hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* reproduce by obligatory mitotic parthenogenesis while *M. hapla* reproduce by facultative meiotic or mitotic parthenogenesis. These results provide insights into the important role of the VAP-1 and FAR-1 proteins in the parasitism.

In chapter 3, studies with VAP 1 supplied information for its potential to be used in the development of nematode control strategies. The transcript of the *Mhi-vap-1* was

localized in the subventral oesophageal gland cells with highest transcriptional levels in J2 (**chapter 2**). Detailed information about the putative role of the *Mhi-vap-1* gene in the parasitism was obtained by silencing this gene using the RNAi method and by observing nematode behaviour towards tomato roots using attraction and penetration bioassays (**objective 3**). The expression of the *Mhi-vap-1* gene is susceptible by silencing induced by RNAi but this effect is not immediate, as several hours of incubation with the RNAi probes were needed for silencing to take place and this is in agreement with Rosso *et al*. (2005). The results suggest that the neuro-stimulant octopamine slightly affect the uptake of dsRNA. According to Bakhetia *et al*. (2007) and Rosso *et al*. (2009), this neuro-stimulant may not be absolutely required to stimulate solute ingestion by RKN. In previous studies, no effects in silencing were observed after incubation in octopamine (Urwin *et al*., 2002; Rosso *et al*., 2005). The expression level of the *Mhi-vap-1* gene in J2 soaked with dsRNA, M9 buffer and octopamine, was down-regulated at 48 h, indicating a silencing effect of the *Mhi-vap-1* gene while in J2 without treatment, the transcript level at 48 h was highest, indicating that this gene is highly active in the infective stage. The silencing effect induced differences in the attraction and penetration of the J2. These studies support the results concerning the importance of *Mhi-vap-1* gene in the early stages of infection and also prior to infection (**chapters 2 and 4**). These results suggest that *vap-1* gene could be a potential target for novel nematode control strategies by silencing this nematode gene.

In **chapter 4**, the expression of *M. hispanica cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1* genes, previously identified (**chapters 1 and 2**), were quantified in the pre-parasitic J2 in order to determine whether tomato root exudates induced changes in gene expression of these genes. Plant signals, such as allelochemicals, present in root

exudates are known to induce changes in nematode behaviour and cause an effect on the regulation of nematode gene expression (Curtis, 2008).

Meloidogyne hispanica pre-parasitic J2 were able to sense and respond to signals present in root exudates by generating a change in the expression of several genes. This confirms the hypothesis of this study that signals from root exudates play an important role in preparing the nematode for infection of host roots by up-regulating genes which play important roles at the early stages of infection. The highest expression was observed in *Mhi-crt-1* and *Mhi-vap-1* genes followed by the *Mhi-cpl-1* and *Mhi-far-1*, which were also up-regulated while the expression of the *Mhi-eng-1* gene was less affected by the molecules present in the root exudates. This novel information highlighted the importance of these genes in the parasitism of plants and can contribute to a better understanding of the putative role of some *M. hispanica* genes in the infection process (**objectives 3 and 4**). The RKN effectors *Mhi-crt-1* and *Mhi-vap-1,* have been shown to be involved in the recognition process between the nematode and the host plant, specifically in early parasitic stage of the infection process where they are able to suppress host immune defences (Jaounnet & Rosso, 2013; Peng *et al*., 2013). These genes were also shown to be expressed during migration and feeding site formation (**chapters 1 and 2),** confirming their important role in parasitism of plants. Furthermore, the expression of *cpl-1* was also up-regulated after exposure to tomato root exudates, demonstrating that this gene has a significant role in all steps of the *M. hispanica* life cycle. The up-regulation of *far-1* gene was also observed in J2, which is another evidence of its putative role in the early events of infection.

In chapter 5, the expression of *PR-1* and *WRKY1* plant defence genes was evaluated in a susceptible tomato, *Solanum lycopersicum*, cv. Easypeel and in a resistant pepper,

Capsicum annuum, cv. Solero after *M. hispanica* inoculation (**objective 5**). *Meloidogyne hispanica* infection induced differential expression of *PR-1* and *WRKY1* genes, which is in agreement with observation made by Ibrahim *et al*. (2011) with *M. incognita*. The expression of the *PR-1* and *WRKY1* genes in the susceptible tomato was up-regulated at 12 h and 2 days after inoculation (DAI), indicating that during the early events of infection (penetration and formation of the feeding site), nematodes induce a strong response of these defence genes. This up-regulation supports the possible involvement of the *PR-1* and *WRKY1* genes in the regulation of the primary host plant defence pathways in the early events of the compatible plant-nematode interaction. The *PR-1* gene can be considered a marker for the early events of infection by *M. hispanica* in susceptible tomato plants. However, at 6 DAI, the *PR-1* gene was down-regulated and this can be associated with secreted effectors from the J3 and J4 developmental stages. In these stages, the stylet and the medium bulb degenerate and, consequently, these juveniles do not feed during this period (Triantaphyllou & Hirschmann, 1960) but secretions from other nematode organs could have triggered the down-regulation of these genes. At 12 DAI when the nematode have developed into adult females, a small increase in the expression level of *PR-1* was observed, suggesting the susceptible tomato plants at this time point were able to overcome the effect of nematode effectors. The expression of the *WRKY1* gene was up-regulated in the susceptible tomato plants at 2 DAI and was down-regulated at 6 and 12 DAI, showing that this defence gene might be only active when *M. hispanica* J2 become parasitic and have initiated feeding site formation. In a previous study, the expression of several *WRKY* genes has also been shown to be down-regulated in susceptible plants after nematode infection (Eulgem, 2005).

In pepper, the expression of *PR-1* and *WRKY1* gene were progressively suppressed as the nematode infection progress, suggesting that these genes may not have a direct involvement in the defence during the interaction of *M. hispanica* with the resistant pepper cv. Solero.

Future Perspectives

Meloidogyne hispanica is one of the lesser known RKN and a species of emerging importance as it has the ability to spread towards northern Europe and is not controlled by cultivars with *Mi* gene. This work reports the identification and subsequent molecular and biological studies of several *Meloidogyne hispanica* effector genes. The results showed novel data regarding the putative roles of several nematode effector genes which enhanced the scientific knowledge and contributed to further understanding of plant-nematode interactions. There has been an enormous development in the field of nematode genes research since the first report in 1998 (Smant *et al*., 1998). Many of these developments have derived from genomics studies, such as analysis of expressed sequence tags of various nematode species (Furlanetto *et al*., 2005; Jacob *et al*., 2008; Haegeman *et al*., 2009, 2011). Bioinformatics and proteomic studies, from *M. incognita* and *M. hapla* secretions (Bellafiore *et al*., 2008; Bellafiore & Briggs, 2010; Mbeunkui *et al*., 2010), have also contributed to the advances in the knowledge of important RKN effector genes and proteins. The whole sequence of the genome of *M. incognita* and *M. hapla* (Abad *et al*., 2008; Opperman *et al*., 2008) provided the first genome sequences for phytoparasitic nematodes (Jones *et al*., 2013). The recent advances in RNAi methodology have contributed to the functional analysis of nematode parasitism genes and helped to determine the potential functions of some plant effectors genes

(Mitchum *et al.*, 2013). The identification of the nematode parasitism genes and the knowledge of their function can lead to the identification of potential nematode targets for the development of new strategies for nematode control.

Novel strategies for nematode control could be devised based on the knowledge of which molecules present in root exudates are responsible for up-regulation of the parasitism genes described in this work. These candidate parasitism genes have a strategic function during the interaction of the nematode in the early events of infection and their early up-regulation prior to root infection possibly contribute to their successful parasitism of plants. Gene silencing should provide some information to whether these genes are vital for nematode penetration and survival inside the roots.

Another alternative is to modify the rhizosphere and interfere with host recognition process by blocking or inactivating the host molecules present in the root exudates which are acting as signals to up-regulated parasitism genes or by adding compounds which are repellent and therefore could make the plants invisible to nematodes.

Further work to understand the regulatory role of hormones and plant defence genes during nematode infection of plants and specially during feeding site formation will be crucial to understand how these genes act in a concerted action during the infection process. They can operate as agonists or antagonists resulting in enhanced infection or the opposite effect as for example, some defence genes have been shown to act as susceptibility genes by enhancing nematode infection. Monitoring the host proteins targeted by plant nematode or even multiple pathogens can lead to the understanding of disease resistance mediated by single plant immune receptors.

Some proof of concept studies have shown that partial resistance against plant-parasitic nematodes can be achieved by expressing small hairpin RNA corresponding to nematode specific genes. The identification of nematode specific genes essential for nematode development based on lethal RNAi phenotypes could enhance this area of research on producing transgenic resistant plants.

The use of host-delivered RNAi targeting *Meloidogyne* effector genes such as *Mhi-vap-1*, either when nematodes are moving in the rhizosphere or during migration inside the root tissue, could induce enhance host resistance as these genes are important for the host-parasite interaction and are responsible for suppressing host immune defences.

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"No fundo, todos temos necessidade de dizer quem somos e o que é que estamos a fazer e a necessidade de deixar algo feito, porque esta vida não é eterna e deixar coisas feitas pode ser uma forma de eternidade."

José de Sousa Saramago

La Provincia, Las Palmas de Gran Canaria, 20 de Julho de 1997