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EFFECT OF NAPHTHOQUINONES IN *MELOIDOGYNE LUCI* LIFE CYCLE AND EVALUATION OF THEIR RECOVERY THROUGH GENE EXPRESSION ANALYSIS

Dissertação no âmbito do Mestrado em Ecologia, orientada pela Doutora Carla Maria Nobre Maleita e coorientada pela Professora Doutora Isabel Maria de Oliveira Abrantes, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Doutora Carla Maria Nobre Maleita e coorientação da Professora Doutora Isabel Maria de Oliveira Abrantes.

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Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

Thomas A. Edison (1847-1931)

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List of publications and scientific communications related to this thesis

Publications

Santos, D., Correia, A., Abrantes, I., & Maleita, C. (2019). New hosts and records in Portugal for the root-knot nematode *Meloidogyne luci*. *Journal of Nematology*, *51*, 1-4.

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Communication

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Abstract

Root knot nematodes (RKN), *Meloidogyne* spp., are a highly polyphagous group of nematodes distributed worldwide, causing great economic losses. *Meloidogyne luci*, an emerging species, first described in 2014, and reported in Portugal in 2018 and 2019, was added to the EPPO alert list in 2017. The main objectives of this study were to develop standard molecular protocols for *M. luci* identification and to investigate the response of *M. luci* life cycle and gene expression to 1,4-naphthoquinone (1,4-NTQ) in order to develop a potential sustainable management strategy. A Portuguese *M. luci* isolate was studied by biochemical (esterase phenotype) and molecular characteristics. Accurate identification and differentiation of *M. luci* was achieved by esterase phenotype (L3). The analysis of mitochondrial DNA region, from COII and 16S rRNA genes, resulted in amplification products of 1,800 bp for the *M. ethiopica*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. luci* isolates, being digested with *Hinf*I, *Alu*I and *Dra*III restriction enzymes, allowing the differentiation of *M. luci* from the other isolates, except *M. ethiopica*. Available sequences from *M. luci* were analysed, revealing huge similarities between *M. luci* and *M. ethiopica* sequences, without new restriction sites available. RAPD markers were, subsequently, used to differentiate these two species, producing different band patterns. The accurate identification of RKN is crucial for the development of sustainable strategies for the management of these nematodes. RKN management is mostly performed using chemical fumigants, which are particularly effective, but awareness towards human and environmental health has led to the development of alternative "eco-friendly" strategies. The effect of different concentrations of 1,4-NTQ on root attraction and infectivity, and the gene expression profile of *gst* and *ache* genes from *M. luci* was assessed. The bioassays revealed a slight impact on the second-stage juveniles (J2) root attraction, but showed a negative effect on the J2 infectivity at 2.5, 5 and 10 ppm concentrations. After J2 exposure to 10 ppm 1,4-NTQ concentration, the *ache* gene lacked expression and *gst* gene was less expressed. This study depicts a contribution for the RKN accurate differentiation and identification, and also highlights the potential of 1,4-NTQ as a low concentration natural-origin nematicide for the sustainable management of *M. luci*.

Keywords: 1,4-naphthoquinone; bioassay; gene expression; identification; infectivity; integrated management; *Meloidogyne*; nematicides; plant-nematode interactions; sustainability.

Resumo

Os nemátodes das galhas radiculares (NGR), *Meloidogyne* spp., são um grupo de nemátodes polífagos, distribuídos mundialmente, com grande impacto económico. *Meloidogyne luci*, uma espécie emergente, descrita pela primeira vez em 2014, e reportada em Portugal em 2018 e 2019, foi adicionada à lista de alerta da EPPO em 2017. Os objetivos deste estudo foram desenvolver uma metodologia molecular para a identificação de *M. luci*, e avaliar o efeito de 1,4-naftoquinona (1,4-NTQ) no seu ciclo de vida e expressão de genes, para o desenvolvimento de uma potencial estratégia de controlo sustentável. Um isolado português de *M. luci* foi caracterizado bioquimicamente (análise do padrão de esterases) e molecularmente. A análise do padrão de esterases permitiu a identificação e diferenciação de *M. luci* (L3). A análise da região do DNA mitocondrial entre os genes COII e 16S rRNA resultou em produtos de amplificação de 1.800 pb para os isolados de *M. ethiopica*, *M. hispanica*, *M. incognita*, *M. javanica* e *M. luci*, sendo posteriormente digeridos com as enzimas de restrição *Hinf*I, *Alu*I e *Dra*III, permitindo a diferenciação de *M. luci* dos demais isolados, exceto de *M. ethiopica*. Sequências de *M. luci* foram analisadas, revelando semelhanças com *M. ethiopica*, não tendo sido possível identificar novos locais de restrição. Marcadores RAPD foram, posteriormente, usados para diferenciar estas duas espécies, produzindo padrões de bandas diferentes. A identificação de NGR é crucial para o desenvolvimento de estratégias sustentáveis para a gestão destes nemátodes. A gestão de NGR é realizada principalmente através do uso de fumigantes químicos, particularmente eficazes, mas a consciencialização relativa à saúde humana e ambiental potenciou o desenvolvimento de estratégias ecológicas alternativas. O efeito de diferentes concentrações de 1,4-NTQ na atração e infetividade ao hospedeiro, e o perfil de expressão dos genes *gst* e *ache* de *M. luci* foram avaliados. Os ensaios revelaram um ligeiro impacto na atração dos jovens do 2º estádio (J2) à raiz, mas mostraram um efeito negativo na infetividade após exposição a concentrações de 2,5, 5 e 10 ppm. Após a exposição de J2 a 10 ppm de 1,4-NTQ, o gene *ache* não foi expresso e a expressão do gene *gst* diminuiu. Este estudo contribui para a diferenciação e identificação precisas de NGR, e demonstra o potencial da 1,4-NTQ como um nematodicida natural de baixa concentração para a gestão sustentável de *M. luci*.

Palavras-chave: 1,4-naftoquinona; bioensaio; expressão de genes; identificação; infetividade; gestão integrada; *Meloidogyne;* nematodicidas; interação nemátode-planta; sustentabilidade.

General introduction

Nematodes, phylum Nematoda, are the most abundant group of multicellular animals on Earth (van den Hoogen et al., 2019). Nematodes can be found in, virtually, all habitats – terrestrial, freshwater or marine – mostly due to their great adaptability to adverse conditions and their condition as polyphagous species (Abad and Williamson, 2010), representing an important part in the soil food web. Although the majority are free-living species, part of them parasitise plants and animals, including mammals and insects.

Plant-parasitic nematodes (PPN) comprise a large number of species, with more than 4,100 species described (Decraemer and Hunt, 2006). These parasites severely impact agricultural production, with extensive yield losses estimated to be around 100 billion dollars/year, which is probably an underestimate since low infestation cause almost imperceptible symptoms on crops (Oka et al., 2000; Nicol et al., 2011).

This group of nematodes can be split into ectoparasites and endoparasites, depending on how they interact with the host (Williamson and Hussey, 1996). Ectoparasites never enter the host, using the stylet to feed on the plant cells. Endoparasites enter the host and migrate inside the root system, in order to feed and complete their life cycle (Jones et al., 2013). Moreover, endoparasites can be classified into migratory, which migrate into and out of plant roots, causing numerous lesions, such as root lesion nematodes, *Pratylenchus* spp., or sedentary, as root knot nematodes (RKN), *Meloidogyne* spp., establishing a permanent feeding site, on which they feed on throughout their life cycle (Manzanilla-López and Starr, 2009; Quist et al., 2015).

Root knot nematodes

Meloidogyne Göldi, 1892, are obligate sedentary endoparasites with a worldwide distribution, wider than any group of PPN (Sasser, 1977). The origin of their common name, RKN, comes from a structure induced by these nematodes in their host plants called root knots or galls (Wesemael et al., 2011). Other visible symptoms, but not specific, include stunting, yellowing and wilting (Khalil, 2013).

Until now, more than a hundred species have been described (Seid et al., 2015), with four being considered the most prevalent – the tropical species *M. arenaria, M. incognita* and *M. javanica* and the temperate *M. hapla*. Each one of this species has a wide host range from agricultural crops, to ornamental plants and weeds (Eisenback and Triantaphyllou, 1991; Hunt and Handoo, 2009).

In Portugal, some RKN species have been found in various regions, namely *M. arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. hapla*, *M. hispanica*, *M. incognita*, *M.*

javanica, *M. luci* and *M. lusitanica*. (Pais and Abrantes, 1989; Abrantes and Santos, 1991; Abrantes et al., 2008; Conceição et al., 2009; Maleita et al., 2012, 2018; Esteves et al., 2015; EPPO, 2018; Santos et al., 2019).

As sedentary endoparasites, RKN spend a large portion of their life span inside their host (Fig. 1), leading to the growth of deeply changed plant cells to feed (Caillaud et al., 2008). While inside the host, females lay eggs into a protective gelatinous matrix, normally, on the surface of galled roots. After the embryonic development, the first-stage juvenile (J1) remains inside the egg, suffers the first moult after which, if the conditions are favourable, the infective second-stage juvenile (J2) hatches. J2 migrates through the soil to find a host, usually in direction of a susceptible root system when resistant and susceptible hosts are present (Curtis et al., 2009). After the parasitic J2 penetrates, near or behind the root tip, it migrates intercellularly until it finds a suitable feeding site in the zone of differentiation of the vascular cylinder (Moens et al., 2009). During the feeding site formation, secretions are released from the nematodes' oesophageal glands into the root cells, using the stylet, giving rise to powerful physiological modifications in the parasitised cells (Davis et al., 2008). These modifications, and the feeding of the J2 on protoxylem and protophloem cells, leads to the formation of multinucleate cells called giant cells, that grant the fundamental nutrients, cortical cells proliferation and hypertrophy, in association with the enlargement of the nematode body causing the formation of typical galls. After the nematode turns sedentary, it endures three moults into third- (J3) and fourth-stage (J4) juveniles and adult male or female (Fig. 1). The J3 and J4 stages are much shorter than the previous one and they do not feed, due to the lack of a functional stylet (Moens et al., 2009; Favery et al., 2016).

Regarding the adult stage, RKN have uneven sex ratios, since females are much more common than males. The males are vermiform and leave the roots, moving through the soil. Most of the times, males do not take part in reproduction, since most of the *Meloidogyne* spp. are parthenogenic (Castagnone-Sereno, 2006). Pear-shaped females remain sedentary and deposit 300-500 eggs in a gelatinous matrix known as egg mass (Fig.1) (Moens et al., 2009).

The completion of the RKN life cycle largely relies on temperature. For instance, *M. chitwoodi* and *M. hapla* species are known cryophils, being able to survive soil temperatures close to and below 0ºC, contrary to the thermophils *M. javanica* and *M. arenaria* that need soil temperature over 10ºC to survive (Evans and Perry, 2009).

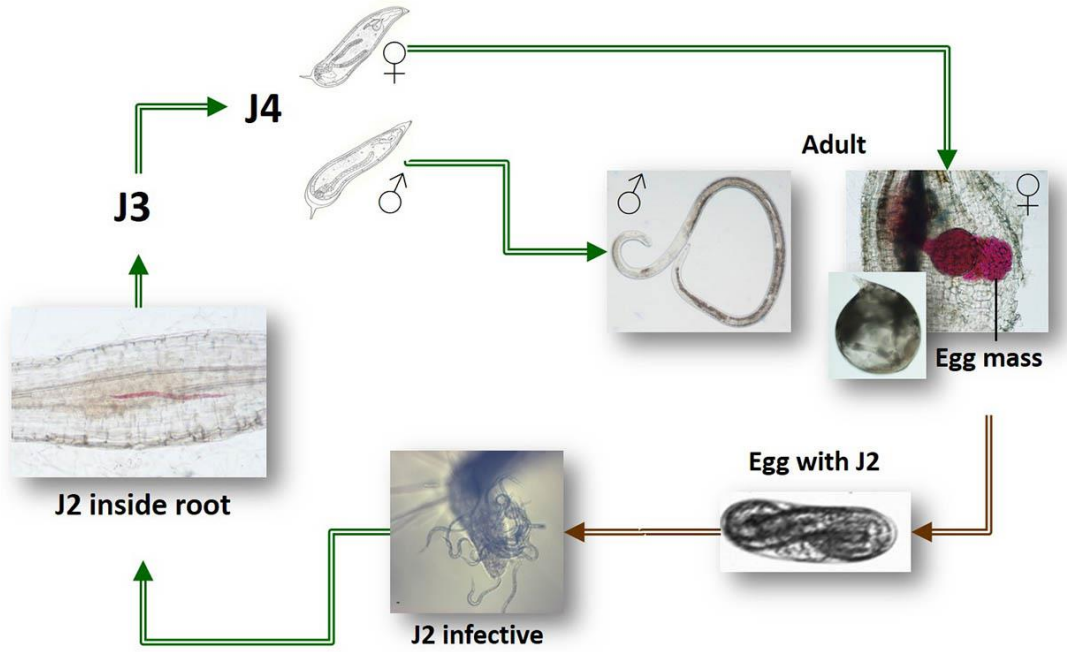


Figure 1. Root knot nematode, *Meloidogyne* spp., life cycle. J2, J3, J4 - Second-, third and fourth-stage juveniles (Adapted from Inácio et al., 2018).

The life cycle completion of the RKN species requires a strict and durable interaction with their host as it influences the host cellular processes, taking advantage of them, due to distinct interactions with specific host proteins. By the time of infection, genes that encode host cell wall degrading enzymes, like endoglucanase, are increasingly expressed (Duarte et al., 2014). Some other genes related to key plant functions, such as water transport in the xylem, have been reported to be also up-regulated after J2 infection (Gheysen and Fenoll, 2002). Contrarily, various genes related to host defence response, such as calreticulin and glutathione-S-transferase, are down-regulated following nematode infection, demonstrating the influence of the nematode feeding site on the suppression of plants protection (Gheysen and Fenoll, 2002; Quentin et al., 2013).

Several biological and chemical methods have been applied towards RKN management. In the last half century, chemical nematicides, namely fumigants, have been the most meaningful strategies to nematode management, which operates by killing the nematodes or preventing the completion of their life cycle (Nyczepir and Thomas, 2009).

Meanwhile, awareness towards environmental and human matters has been rising, stimulating the search and development of alternative methods to reduce the negative impact of chemical nematicides on human and environment health. In this sense, the European Commission (EC) has been encouraging the improvement and search of new,

competent and, above all, eco-friendly strategies (European Commission, 2009a,b). For instance, crop rotation, development and growth of resistant cultivars, or even destruction of the infected root systems are some of the procedures known to be effective on managing RKN populations, although these strategies require experience and knowledge of nematode species composition and characteristics (Barker and Koenning, 1998; Nyczepir and Thomas, 2009). In fact, plant genes conferring resistance to RKN have been recognised (Williamson and Roberts, 2009). The tomato gene *Mi*-1, for example, has been the most extensively used source of resistance against the most prominent RKN species – *M. incognita*, *M. javanica* and *M. arenaria* (Williamson, 1998). However, the *Mi*-1 gene breaks down the resistance when soil temperatures exceed 28°C and its resistance can be surpassed by virulent nematode populations (Dropkin, 1969; Ammati et al., 1986; Maleita et al., 2011; Tzortzakakis et al., 2014).

Furthermore, a considerable amount of secondary metabolites from plants have been identified and reported to have great potential as natural-origin nematicides, being an alternative to the hazardous chemical fumigants (Ntalli and Caboni, 2012). These bionematicides are generally safer to humans and to the environment than conventional synthetic chemical pesticides, have limited field persistence, present no residual threats, and have a highly-specific mode of action and narrow target ranges (Dubey, 2011). Nematicides with botanical origin like thiocyanates, glucosides, alkaloids, phenolics, fatty acids, among others, should be effective against nematodes at low doses, with low toxicity to non-target soil organisms, and degradable (Chitwood, 2002). Recently, naphthoquinones, 1,4-naphthoquinone (1,4-NTQ) and juglone, present in extracts from walnut, *Juglans regia* L., fruit hulls residues, have been found to have high nematicidal activity against RKN and a potential alternative to synthetic nematicides (Maleita et al., 2017).

Meloidogyne luci

Meloidogyne luci was described in 2014, in Brazil, from an isolate obtained from lavender (*Lavandula spica* L.) (Carneiro et al., 2014). Nevertheless, this nematode has been identified in various countries around the world, namely Argentina, Bolivia, Chile, Ecuador, Greece, Guatemala, Iran, Italy, Slovenia and Turkey (Širca et al., 2004; Conceição et al., 2012; Maleita et al., 2012, 2018; Aydinli et al., 2013; Aydinli and Mennan, 2016; Bellé et al., 2016; Machado et al., 2016; Stare et al., 2017a,b; Santos et al., 2019).

In Portugal, *M. luci* was first reported parasitising potato, *Solanum tuberosum* L., roots from Coimbra region (Maleita et al., 2018). Later on, this nematode species was reported infecting roots of tomato (*S. lycopersicum* L.), of the ornamental plant *Cordyline australis* (Forst f.) Hook. F and of the weed *Oxalis corniculata* L., in distinct locations within the Coimbra region (Figueira da Foz and Montemor-o-Velho) (Santos et al., 2019).

This species has a close relationship with *M. ethiopica*, a tropical RKN that have been included in the EPPO alert list (EPPO Standards, 2016), with which it shares some identical biochemical and morphological characteristics. Populations from Europe and Turkey formerly identified as *M. ethiopica* were reclassified, through molecular and biochemical procedures, as *M. luci* (Stare et al., 2017b). Routinely, the most appropriate method to distinguish these two species is the esterase (EST) phenotype analyses (Dickson et al., 1970; Esbenshade and Triantaphyllou, 1985) and the EST phenotype of *M. luci* (L3) and *M. ethiopica* (E3) is fairly similar. However, there is a difference, since the first band of *M. luci* is positioned at the same level as the first band of *M. javanica*, the reference isolate (Carneiro et al., 2014). On the other hand, the first band of the *M. ethiopica* phenotype is slightly above, when compared to the first band of *M. javanica*, allowing the differentiation of the two species (Maleita et al., 2018).

Additionally, molecular-based analyses can also be a good tool for the species identification and differentiation, with different regions already described as effective, namely: ITS1 rRNA region and D2-D3 fragment of 28S rRNA region (Carneiro et al., 2014; Machado et al., 2016); mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) region (Maleita et al., 2018); and mtDNA cytochrome oxidase subunit II (COII) region (Stare et al., 2017b). However, ITS, SSU, and LSU of the rDNA regions were, later, considered inappropriate for studying relationships among the tropical group of RKN (Maleita et al., 2018; Stare et al., 2017b). Stare et al. (2017b) selected the mtDNA COII region as the most useful for the identification and differentiation of *M. luci* from closely related species, whereas Maleita et al. (2018) designated mtDNA COI region as the most effective. Considering the great diversity of RKN species, and the close relationship between *M. luci* and *M. ethiopica*, the use of biochemical and molecular procedures, selecting multiple DNA regions, should be considered when attempting to properly identify this group of nematodes.

The pathogenicity of *M. luci* on potato and tomato genotypes was already reported to be fairly high, since all of the potato (GI = 5 and Rf > 1) and the tomato (GI>2 and Rf>1) genotypes with the *Mi* gene absent were deemed susceptible to *M. luci* (Maleita et

al., 2018; Santos et al., 2019), although tomato genotypes carrying the *Mi* gene were effective in suppressing *M. luci* reproduction (Santos et al., 2019). The potential repercussions of the presence of *M. luci* in agricultural fields supports the demand for the development of novel control strategies.

Objectives

By studying the expression of RKN candidate genes known to be involved in key functions - such as protection against plant defences or nerve impulse transmission - it is possible to elucidate about the regulation mechanisms of the RKN *M. luci* in response to the presence of 1,4-NTQ, leading to the development of potential, new, effective and integrated pest management programs. Nonetheless, to implement these programs and prevent *M. luci* dispersion and suppress reproduction, an accurate diagnosis of this species is also required. The main objectives of this study were: 1) to evaluate different molecular tools in order to develop standard molecular protocols for *M. luci* identification; and 2) to investigate the effect of 1,4-NTQ on *M. luci* life cycle and gene expression.

The specific objectives were:

1. To provide a tool for the correct identification and differentiation of *M. luci* from *M. ethiopica* based on sequence analysis of different DNA regions and different molecular techniques;

2. To assess the effect of 1,4-NTQ on *M. luci* life cycle (attraction and infectivity assays) and gene expression;

3. To select the lowest 1,4-NTQ concentration with effect on *M. luci* life cycle.

Chapter 1

Meloidogyne luci identification and differentiation

Introduction

Root knot nematodes (RKN), *Meloidogyne* spp., are obligate sedentary endoparasites distributed worldwide (Jones et al., 2013). This group of plant pathogens parasitises a large number of plant species ranging from the major food crops, vegetables and fruits to ornamental plants, and are responsible for economic losses of several billion dollars (Eisenback and Triantaphyllou, 1991; Elling, 2013).

The *Meloidogyne* genus encompasses around 100 described species, but research on RKN is mostly focused on the four major species - *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* – due to their wide host range, broad distribution and high economic impact (Moens et al., 2009; Karssen et al., 2013). However, several other *Meloidogyne* spp. are being recognised as emerging species. For instance, *M. ethiopica*, *M. graminicola* and *M. luci* were added to the EPPO alert list (EPPO, 2017) and *M. chitwoodi*, *M. enterolobii*, *M. fallax* and *M. mali* were added to the European list of quarantine organisms, due to their potential to cause serious damage to agriculture (Elling, 2013; Hunt and Handoo, 2009).

Meloidogyne luci is a recently described RKN species that highly damages plants, inducing the formation of medium to large galls on the plant root system (Carneiro et al., 2014; Maleita et al., 2018). This nematode species has been associated with some economically important plant species, such as broccoli (*Brassica oleracea* var. italic L.), carrot (*Daucus carota* L.), common bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), grapevine (*Vitis vinifera* L.), kiwi (*Actinidia deliciosa* Liang and Ferguson), lavender (*Lavandula angustifolia* Mill.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), potato (*Solanum tuberosum* L.), rose (*Rosa* sp.), sedum (*Hylotelephium spectabile* L.), snapdragon (*Antirrhinum majus* L.), soybean (*Glycine max* L.), tomato (*S. lycopersicum* L.) and yakon (*Polymnia sonchifolia* (Poepp.) H. Rob.) (Carneiro et al., 2014; Bellé et al., 2016; Janssen et al., 2016; Machado et al., 2016; Maleita et al., 2018; Santos et al., 2019).

This RKN species shares some morphological and biochemical similarities with *M. ethiopica* that led to the misidentification of several *M. luci* populations in Europe (Stare et al., 2017b). Currently, the strongest method to differentiate these two species is the esterase (EST) isozyme phenotype analysis, despite the similarity between *M. luci* (L3) and *M. ethiopica* (E3) phenotypes (Janssen et al., 2016; Stare et al., 2017b; Maleita et al., 2018; Santos et al., 2019).

Proper identification of RKN is essential to successfully control emergent group of parasites. Morphology based identification of RKN to the species level, frequently centred in female perineal pattern analysis, is hard and often unreliable and requires high expertise to perform (Carneiro et al., 2000; Blok and Powers, 2009).

Analyses of EST electrophoretic profiles are widely used to differentiate *Meloidogyne* spp., with a large number of species-specific isozyme patterns already published (Blok and Powers, 2009). However, there are some limitations: the use of egg-laying females is mandatory, although galled root tissue has also been used (Ibrahim and Perry, 1992); intraspecific variability may occur; and there is a great diversity of RKN species, some of them with no EST phenotype described (Blok and Powers, 2009).

Molecular techniques can be an alternative tool when assessing RKN differentiation. Contrarily to biochemical procedures, molecular methods are much more reliable, exhibit higher sensitivity and are independent of the nematode life cycle (Powers et al., 2005); but can be expensive and more complex, leading to increase difficulty when tested in extensive analysis (Molinari et al., 2005). The ribosomal DNA (rDNA), including 18S, 28S and internal transcribed spacer (ITS) regions have been extensively used for both phylogenetic studies and diagnostic purposes (Blok and Powers, 2009). Mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI), mtDNA cytochrome oxidase II (COII) and 16S rRNA regions have also been used for phylogenetic analysis, due to its uniparental inheritance and low level of recombination (Powers and Harris, 1993; Blok and Powers, 2009).

Other molecular methods are, presently, being used to a certain degree of success: random amplified polymorphic DNA (RAPD); restriction fragment length polymorphisms (RFLP) and sequence characterised amplified regions (SCAR).

RFLP is a PCR technique based on DNA digestion by one or more restriction enzymes, followed by electrophoresis in agarose gel (Blok and Powers, 2009). This method is moderately complex and requires considerable amounts of DNA. RAPD is also a type of PCR technique resulting in randomly amplified DNA segments. Normally, short primers (8-12 nucleotides) are used on a large DNA template resulting in a potently unique profile (Blok and Powers, 2009). Finally, the SCAR technique is based on the development of specific primers to amplify, via PCR, a specific and known sequence (Blok and Powers, 2009). This technique is frequently applied after a RAPD analysis: the differential bands are isolated, sequenced and specific primers are designed.

Accurate *M. luci* identification is of crucial importance when attempting to monitor the distribution and spread of this RKN species and implement integrated pest management programs. The main objective of this study was to provide a tool for the correct identification and differentiation of *M. luci* from other RKN species based on the analysis of different regions of DNA and techniques.

Materials and Methods

1. Nematode isolates

Thirteen *Meloidogyne* spp. isolates, corresponding to nine *Meloidogyne* spp., were included in this study: *M. arenaria*, *M. chitwoodi*, *M. enterolobii* (=*M. mayaguensis*), *M. ethiopica*, *M. hapla*, *M. hispanica*, *M. incognita, M. javanica* and *M. luci* (Table 1.1). Isolates were maintained in the NEMATO-lab at CFE, on tomato plants, genotype Coração-de-Boi, in a temperature-controlled growth chamber (25±2°C) with a daily 12 h light period. For long-term maintenance, 10 egg masses were transferred every two months, to new seedlings.

Table 1.1. *Meloidogyne* isolates, hosts and geographic origin included in this study.

***Meloidogyne* species (Code)**		**Host plant species**	**Geographic origin**
M. arenaria (PtA)		*Oxalis corniculata*	Coimbra, Portugal
M. chitwoodi (PtCh)		*Solanum tuberosum*	Porto, Portugal
M. enterolobii (VnEn)		*Malpighia glabra*	Cabudare, Venezuela
M. ethiopica (BrE)		*Actinidia deliciosa*	Rio Grande do Sul, Brasil
M. hapla (PtH)		*S. lycopersicum*	Coimbra, Portugal
M. hispanica (PtHi)		*Ficus carica*	Setúbal, Portugal
M. incognita (PtI)		*Cucumis melo*	Santa Maria (Açores), Portugal
M. javanica (PtJ)		*S. tuberosum*	Guarda, Portugal
M. luci	PtL	*S. tuberosum*	Coimbra, Portugal
	GrL	*Zea mays*∗	Greece
	ItL	*S. lycopersicum*∗	Italy
	SlL	*S. lycopersicum*∗	Slovenia
	TrL	*Cucumis sativus*∗	Turkey

∗*M. luci* isolates only included in genetic analysis diversity by RAPD.

2. Biochemical identification

Five egg-laying females of *M. luci* were handpicked from infected tomato roots and transferred, individually, to micro-haematocrit tubes with 5 µL of extraction buffer (20% sucrose and 1% Triton X-100). The females were macerated with a pestle, frozen and stored at −20°C. Before electrophoresis, performed according to Pais and Abrantes (1989), the samples were centrifuged at 8905 g, at -5°C for 15 minutes. Electrophoresis was carried out at 6 mA/gel during the first 15 minutes and then at 20 mA/gel for about 45 minutes using the Mini-Protean Tetra Cell System (Bio-Rad Laboratories, Hercules, CA, USA). The polyacrylamide gels were stained for EST activity with the substrate α-naphthyl acetate, in the dark at 37°C, for ±30 minutes. Protein extracts from five females of *M. javanica* were included in each gel as reference. Protein extracts from five females of *M. ethiopica* were also included in the gel for comparison (Table 1.1).

3. Molecular identification

3.1. DNA extraction

Egg masses from each *Meloidogyne* species isolate (Table 1.1) were handpicked from tomato infected roots, placed on a mesh sieve, and incubated in moist chambers at 25°C. Hatched J2 were collected, centrifuged (336 g; 3 minutes) and stored at -20ºC until DNA extraction. DNA was extracted and purified using the DNeasy Blood & Tissue Kit for purification of total DNA from animal tissues (QIAGEN, Germany). Genomic DNA concentration was determined in a Nanodrop 2000c spectrophotometer (ThermoScientific), and the samples stored at -20°C until PCR.

3.2. Mitochondrial DNA COII gene region amplification

The mtDNA COII gene region located between COII and 16S rRNA genes was selected to conduct the molecular characterisation of *M. luci* isolates. This region was amplified with the primer set C2F3 (5′-GGT CAA TGT TCA GAA ATT TGT GG-3′) and MRH106 (5′-AAT TTC TAA AGA CTT TTC TTA GT-3′) (Powers and Harris, 1993; Stanton et al., 1997). PCR reactions were performed in a 25 µL volume containing 1× Taq reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM each primer, 2.5 U Taq DNA polymerase and 50 ng of nematode DNA as a template. The amplifications were carried out using the following conditions: an initial denaturation at 94°C for 4 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 2 minutes; and a final extension for 10 minutes at 72°C (Maleita et al., 2012). The PCR reaction was analysed on a 1.0% agarose gel electrophoresis in 1× Tris-borate-EDTA (TBE) buffer stained with GreenSafe (NZYTech, Portugal).

3.3. Restriction Fragment Length Polymorphism (RFLP)

Amplification products with approximately 1,800 bp were digested with 5 U of *Hinf*I. If no digestion occurred, the amplified products were digested with 5 U of *Alu*I. If, again, no digestion occurred the amplified products were digested with 5 U of *Dra*III. All restriction enzymes were applied in 7 µL of PCR products. The digestion was conducted at 37°C for 5 h to *Dra*III and for 18 h to *Hinf*I and *Alu*I restriction enzymes (Maleita et

al., 2012). Restriction fragments were separated on a 2% agarose gel electrophoresis in 1×TBE buffer stained with GreenSafe.

3.4. *Meloidogyne* species genome analysis

As no differences were found between *M. luci* and *M. ethiopica* for *Hinf*I, *Alu*I and *Dra*III restriction enzymes, sequences from *M. luci* mtDNA COI, mtDNA COII and D2-D3 fragment of the 28S rRNA gene regions in the GenBank nucleotide database (National Center of Bio-technology Information, www.ncbi.nlm.nih.gov) were aligned with ClustalW within BioEdit software with sequences of *M. ethiopica* (Table 1.2).

The online software Web-Cutter 2.0 was also used to determine restriction enzyme maps based on altered nucleotides between the sequences to create new restriction sites.

Table 1.2. List of GenBank accession numbers used in this study.

***Meloidogyne* species**	**mtDNA COI**	**mtDNA COII**	**D2-D3***
M. ethiopica	KU372162	AY942848 KU852490 LN626953 LN626960	KF482372 KF482373
M. luci	KY563093 MF280976 KU372173	AY942858 JN673275 KM042848 LN626956 LN626958 LN626954	KF482369 KF482370 KF482371

* D2-D3 fragment of 28S rDNA.

3.5. Rapid Amplified Polymorphic DNA (RAPD)

The genetic diversity of 13 *Meloidogyne* spp. isolates was assessed using RAPD markers: five *M. luci* isolates and one isolate from each of the RKN species *M. arenaria*, *M. chitwoodi*, *M. enterolobii, M. ethiopica*, *M. hapla, M. hispanica, M. incognita* and *M. javanica* (Table 1.1). The DNA was amplified using 16 random 6-mer primers obtained from STAB VIDA (Lisbon, Portugal): OPA-06, OPA-08, OPA-09, OPA-17, OPB-01,

OPB-14, OPC-06, OPC-08, OPD-01, OPE-06, OPE-07, OPG-04, OPK-02, OPM-01, OPO-06 and OPR-09. PCR assays were performed in a final volume of 13 µL containing 1× Taq reaction buffer, 1.8 mM of $MgCl_2$, 0.2 mM of dNTP's, 0.3 µM of primer, 2 U of Taq DNA polymerase and 25 ng of nematode DNA as a template. PCR amplifications were carried out using the following parameters: an initial denaturation at 94°C for 5 minutes; followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 36°C for 45 seconds, and extension at 72°C for 2 minutes; and a final extension at 72°C for 10 minutes. The amplified products were analysed on a 1.5% agarose gel electrophoresis in 1×TBE buffer stained with GreenSafe.

Results

1. Biochemical identification

Three EST bands were observed in the *M. javanica* isolate used as a reference to determine the relative position of *M. luci* and *M. ethiopica* EST bands (Fig. 1.1).

Three bands of EST activity were detected in the Portuguese *M. luci* isolate (Rm: 0.38; 0.43; 0.49), identical to the EST L3, attributed to *M. luci* from Brazil. The *M. ethiopica* isolate included for comparison exhibited also three EST activity bands (Rm: 0.36; 0.43; 0.49), corresponding to the phenotype E3.

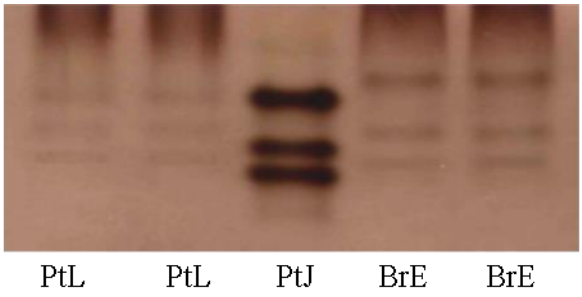


Figure 1.1. Esterase phenotypes of protein homogenates from five egg-laying females of *Meloidogyne* spp. isolates. PtL - *M. luci;* BrE - *M. ethiopica*; PtJ - *M. javanica* (reference isolate). For isolate codes, see Table 1.1.

2. Molecular identification

The mtDNA region between COII and 16S rRNA genes was picked to perform the molecular characterisation of *M. luci* isolates. The amplification of this region, using

the primer set C2F3/MRH106, resulted in four different sizes of bands (approximately 600, 800, 1,200, and 1,800 bp; Fig. 1.2). The isolates of *M. hapla* and *M. chitwoodi* had a PCR product of ±600 bp; the *M. enterolobii* isolate displayed a fragment of 800 bp; the *M. arenaria* isolate a fragment of 1,200 bp; and the isolates of *M. luci*, *M. ethiopica*, *M. javanica*, *M. hispanica* and *M. incognita* a fragment of ±1,800 bp (Fig. 1.2).

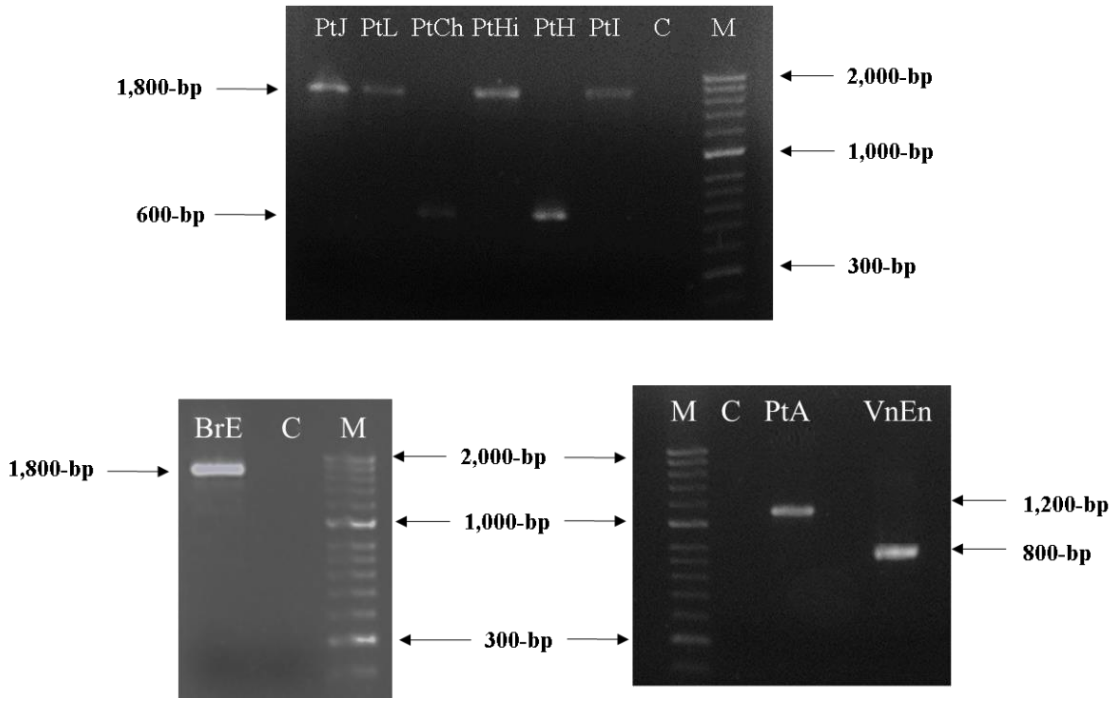


Figure 1.2. DNA amplification products obtained from nine isolates of *Meloidogyne* spp. using C2F3 and MRH106 primers. PtJ - *M. javanica*; PtL - *M. luci*; PtCh - M. *chitwoodi*; PtHi - *M. hispanica*; PtH - *M. hapla*; PtI - *M. incognita*; BrE - *M. ethiopica*; PtA - *M. arenaria*; VnEn - *M. enterolobii*; C - negative control; M - DNA Marker (HyperLadder II; Bioline). For isolate codes, see Table 1.1.

Amplified products of approximately 1,800 bp were subsequently digested with the restriction enzyme *Hinf*I, resulting in two patterns of digestion, even though only *M. incognita* was distinctively differentiated (Fig. 1.3). Two fragments of approximately 1,200 and 350 bp were observed in the *M. incognita* isolate and one fragment of approximately 1,600 bp was produced in *M. luci*, *M. ethiopica*, *M. javanica* and *M. hispanica* isolates.

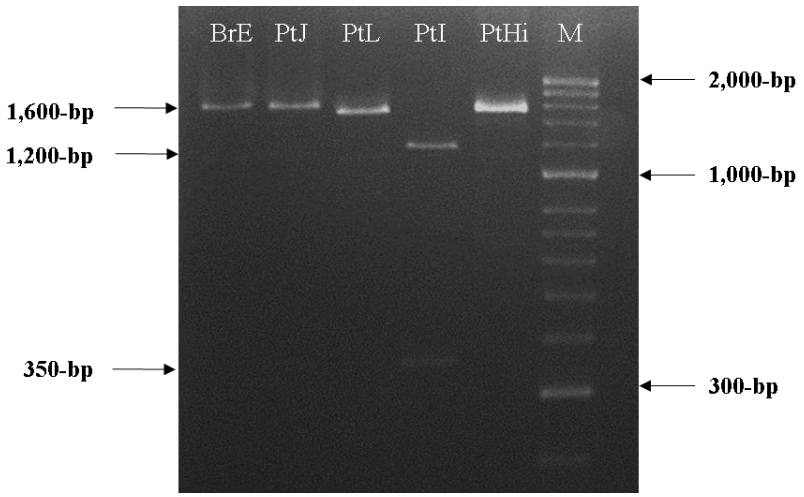


Figure 1.3. *Hinf*I digestion patterns of the approximately 1,800 bp amplification products from *Meloidogyne* spp. after 18 h of digestion. BrE - *M. ethiopica*; PtJ - *M. javanica*; PtL - *M. luci*; PtI - *M. incognita*; PtHi - *M. hispanica*; M - DNA Marker (HyperLadder II; Bioline). For isolate codes, see Table 1.1.

Isolate amplification products not discriminated after *Hinf*I PCR-RFLP were digested with the restriction enzymes *Alu*I and *Dra*III. After the digestion with the restriction enzyme *Alu*I, the differentiation of *M. hispanica* and *M. javanica* (two fragments of approximately 1,000 and 550 bp) from *M. luci* and *M. ethiopica* (one fragment of approximately 1,200 bp) (Fig. 1.4) was achieved and after the digestion with the restriction enzyme *Dra*III, the discrimination between *M. javanica* (two fragments of approximately 950 and 850 bp) and *M. hispanica* (one fragment of approximately 1,700 bp) was attained (Fig. 1.5).

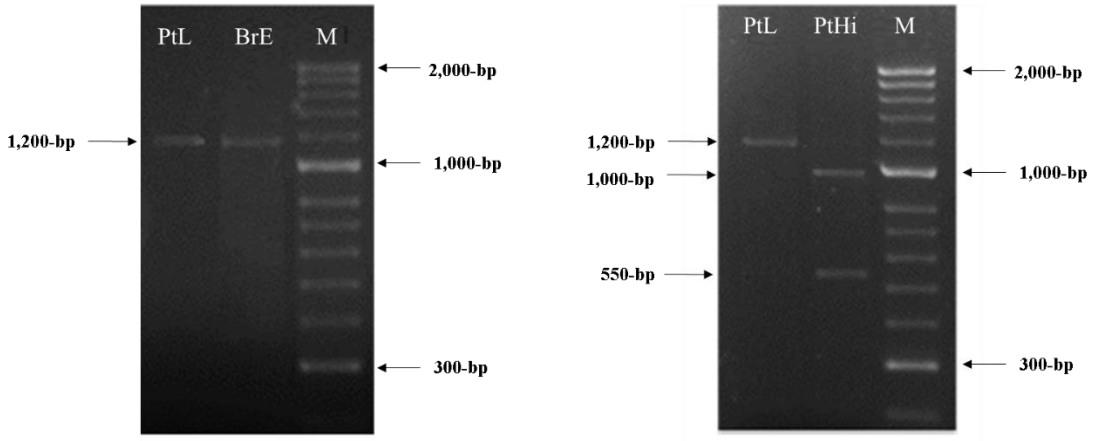


Figure 1.4. *Alu*I digestion patterns of the approximately 1,800 bp amplification products from *Meloidogyne* spp. after 18 h of digestion. PtL - *M. luci*; BrE - *M. ethiopica*; PtHi - *M. hispanica*; M - DNA Marker (HyperLadder II; Bioline). For isolate codes, see Table 1.1.

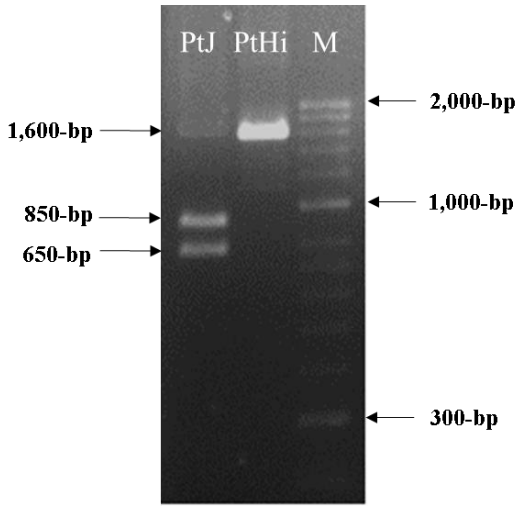


Figure 1.5. *Dra*III digestion patterns of the approximately 1,800 bp amplification products from *Meloidogyne* spp. after 5 h of digestion. PtJ - *M. javanica*; PtHi - *M. hispanica*; M - DNA Marker (HyperLadder II; Bioline). For isolate codes, see Table 1.1.

Restriction of the mtDNA COII amplified products with *Alu*I or *Dra*III did not differentiated *M. luci* from *M. ethiopica*. Sequences of mtDNA COII from *M. luci* and *M. ethiopica* isolates (Table 1.2) were retrieved from GenBank nucleotide database (National Center of Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov) and aligned with ClustalW within BioEdit software, to search for altered nucleotides which create new restriction sites. Several nucleotides were removed from the *M. luci* and *M. ethiopica*

sequences to obtain a common start and end point and when compared (1,580 bp), three nucleotide substitutions and two nucleotide insertions were detected in the *M. luci* sequences in comparison with *M. ethiopica*. The *M. luci* sequences differed by nine nucleotide positions: one insertion; seven substitutions; and one deletion (*data not shown*). Restriction enzyme maps were built, using the on-line WebCutter 2.0 software, and no different restriction sites for this two species on mtDNA COII gene region were perceived (Fig. 1.6). Additionally, sequences from mtDNA COI and D2-D3 fragment of the 28S rRNA gene from both isolates (Table 1.2) were retrieved from GenBank nucleotide database, aligned with ClustalW within BioEdit software and compared. *Meloidogyne luci* mtDNA COI region sequences (551 bp) revealed one nucleotide substitution in comparison with *M. ethiopica* sequences, which is not recognised by any available restriction enzyme (*data not shown*). *Meloidogyne luci* D2-D3 fragment analysis revealed 20 nucleotide substitutions when compared with *M. ethiopica* sequences. Despite this difference the restriction enzyme maps revealed no new restrictions sites for the *M. luci* sequences when placed side by side with *M. ethiopica* sequences (*data not shown*).

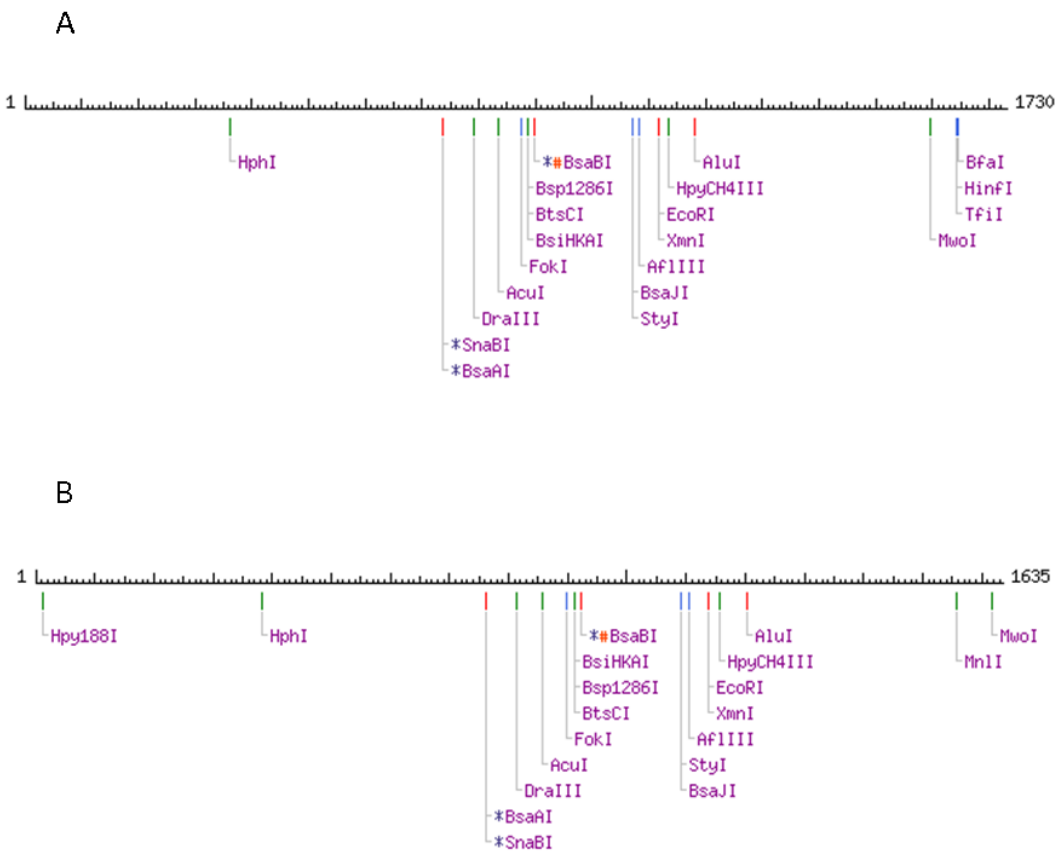


Figure 1.6. (A) *Meloidogyne luci* (KM042848) and (B) *M. ethiopica* (KU852490) restriction enzyme maps retrieved from the online software Web-Cutter 2.0.

Inability to differentiate *M. luci* from *M. ethiopica* through PCR-RFLP, using different DNA regions, stimulated the use of a different technique. In order to design a potential species-specific primer for *M. luci* identification and differentiation, 16 random 6-mer primers were tested. Most primers did not produce amplifications (OPA-17, OPE-06, OPE-07, OPM-01) or were unable to differentiate *M. luci* from *M. ethiopica* and the RKN species included for comparison (OPA-08, OPA-06, OPA-09, OPB-14, OPC-06, OPC-08, OPD-01, OPG-04, OPO-06, OPR-09), and were discarded.

On the other hand, the primer OPK-02 has distinguished *M. luci* from the other isolates, but the results were not reproducible and intraspecific variability among *M. luci* isolates was found (*data not shown*). Finally, the primer OPB-01 resulted in different band patterns for the *M. luci* and *M. ethiopica* isolates, specifically a band of ±450 bp was present in all *M. luci* isolates and absent in the *M. ethiopica* isolate. However, a band of ±450 bp was also observed for the *M. arenaria* and *M. javanica* isolates (Fig. 1.7).

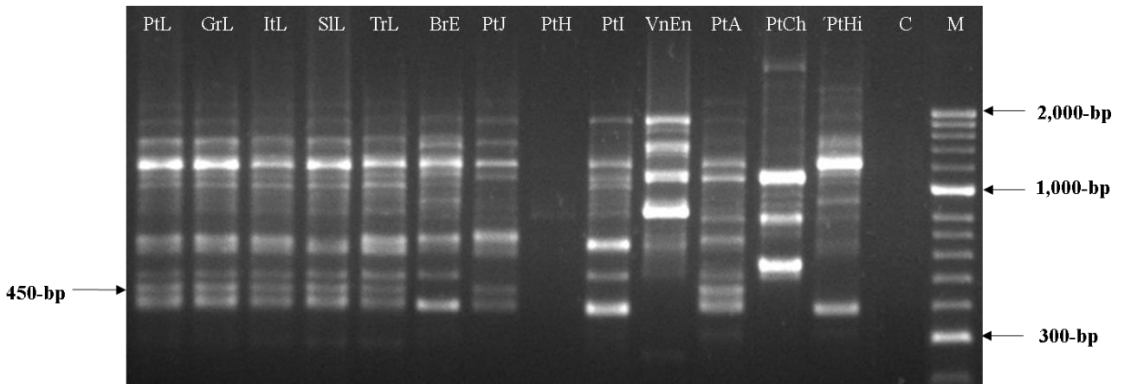


Figure 1.7. RAPD-PCR amplification products obtained from 13 isolates of *Meloidogyne* spp. using the OPB-01 primer. PtL - *M. luci*; GrL - *M. luci*; ItL - *M. luci*, SlL - *M. luci*; TrL - *M. luci*; BrE - *M. ethiopica*; PtJ - *M. javanica*; PtH - *M. hapla*; PtI - *M. incognita*; VnEn - *M. enterolobii*; PtA - *M. arenaria*; PtCh - *M. chitwoodi*; PtHi - *M. hispanica*; C - negative control; M - DNA Marker (HyperLadder II; Bioline). For isolate codes, see Table 1.1.

Discussion

Effective *M. luci* integrated pest management approaches demand accurate and rapid identification tools for this RKN species. However, proper identification of *Meloidogyne* spp. is difficult due to morphological similarity between species and intraspecific variability (Hunt and Handoo, 2009). Some of the characters, such as morphology of the perineal patterns, are highly variable and can be found in isolates from other species: *M. ethiopica* exhibits a mix of *M. arenaria* and *M. incognita* patterns, and *M. inornata* patterns are similar to *M. incognita* (Carneiro et al., 2014).

The EST phenotypes of *M. luci* and *M. ethiopica* corresponded, respectively, to the previously described species-specific L3 and E3 EST phenotypes (Carneiro et al., 2014; Maleita et al., 2018). Both EST activity patterns are very alike, displaying three activity bands, with a slight difference in the migration rate of the upper band of *M. luci*. Such small difference can be misleading when analysing the phenotypes alone, but is, still, an accurate way to identify and differentiate these two species, especially when included on the same gel.

The mtDNA COII amplified products of *M. arenaria* (ca. 1,200 bp), *M. chitwoodi* (ca. 600 bp), *M. enterolobii* (ca. 800 bp), *M. ethiopica* (ca. 1,800 bp), *M. hapla* (ca. 600 bp), *M. hispanica* (ca. 1,800 bp), *M. incognita* (ca. 1,800 bp), *M. javanica* (ca. 1,800 bp) and *M. luci* (ca. 1,800 bp) are in agreement with previous reports (Powers and Harris, 1993; Blok et al., 2002; Handoo et al., 2005; Maleita et al., 2012). The PCR-RFLP

analysis performed to distinguish *M. luci* from the other RKN species was effective to a certain extent. *Meloidogyne luci* was distinguished from *M. arenaria*, *M. chitwoodi*, *M. hapla* and *M. enterolobii* by the size of the amplification products; from *M. incognita* through *Hinf*I digestion patterns, and from *M. hispanica* and *M. javanica* by the size of amplified products after digestion with *Dra*III and *Alu*I. The detection of the tropical RKN *M. luci* in European countries has been raising awareness towards this highly impactful species, therefore accurate identification may be the key to successfully manage this RKN species. The results demonstrate the effectiveness of this technique when differentiating *M. luci* from the four 'major' RKN species and emerging quarantine species, such as *M. chitwoodi* and *M. enterolobii* (Moens et al., 2009; EPPO Standards, 2016). Nonetheless, differentiation of *M. luci* from *M. ethiopica* using the PCR-RFLP method was unsuccessful due to close molecular similarities between these two species.

In order to obtain different restriction sites for the two species, the mtDNA COI and COII regions and the D2-D3 fragment of the 28S rRNA gene, widely used for phylogenetic studies and diagnostic purposes, were analysed and compared to find nucleotide differences between *M. luci* and *M. ethiopica* (Hugall et al., 1999; Blok and Powers, 2009; Carneiro et al., 2014; Janssen et al., 2016; Machado et al., 2016; Stare et al., 2017b; Maleita et al., 2018).

The D2-D3 fragment revealed slight differences between the *M. luci* and *M. ethiopica* sequences, however no new restriction sites for *M. luci* were found. High variability in 28S rRNA gene has been previously reported (Hugall et al., 1999; Stare et al., 2017b), possibly due to the hybrid origin of apomictic RKN species, such as *M. luci* (Lunt et al., 2014).

On the other hand, mtDNA in obligate mitotic parthenogenic species was previously used for RKN species identification (Janssen et al., 2016; Maleita et al., 2018), but after analysis of the mtDNA COI and COII regions, respectively, one and five nucleotide differences were observed between sequences from *M. luci* and *M. ethiopica*, rendering the identification of new restriction sites unsuccessful. Although the mtDNA COI and COII regions were reported as suitable for *M. luci* species identification (Stare et al., 2017b; Maleita et al., 2018), the close proximity between *M. luci* and *M. ethiopica* requires a joint use of different molecular techniques in addition to EST phenotype analysis.

RAPD-PCR analysis usefulness for diagnostic purposes depends on the reproducibility of the results (Cenis, 1993). In an attempt to differentiate *M. luci* from *M.*

ethiopica, the amplification with primer OPB-01 resulted in a distinct fragment of ±450 bp for all five *M. luci* isolates, when compared with the *M. ethiopica* isolate. This difference on band pattern suggests a possible path to distinguish these closely related species, leading to proper identification of *M. luci*. This fragment could be excised from the gel, cloned, sequenced, and converted into a *M. luci* species-specific primer, to be applied when adult females are not available. Nevertheless, a similar size fragment was also observed on the *M. arenaria* and *M. javanica* isolates. To unambiguously discriminate *M. luci* from *M. arenaria* and *M. javanica* and validate the results, *M. arenaria* and *M. javanica* fragment should be also sequenced and compared with *M. luci* sequence and a broader range of *M. luci* isolates, from different hosts and geographical origins, included in further studies.

The relationship of *M. luci* and *M. ethiopica* has been subject of many studies ever since the *M. luci* description, in 2014 (Carneiro et al., 2014). In fact, populations of *M. ethiopica* from Europe and Turkey were reclassified as *M. luci* and there is still no consensus about the true significance of this close relationship, being recently considered as part of the *M. ethiopica* group (Stare et al., 2017b, 2019). The identification of *M. ethiopica* group-specific primers, due to the close relationship among *M. ethiopica*, *M. inornata* and *M. luci* species, instead of species-specific primers for each species, was already developed. The similarity between *M. ethiopica*, *M. inornata* and *M. luci* can be seen at various levels: common hosts, overlapping morphological characteristics and at the molecular level (Stare et al., 2019). In fact, to the best of our knowledge, reliable differential hosts were not found between *M. ethiopica* and *M. luci*, being apparent that these two species parasitise similar plant species (Stare et al., 2017b). Regarding *M. inornata*, a narrow host range is known, but all reported hosts for this species can also be parasitised by *M. ethiopica* and *M. luci* (Carneiro et al., 2008; Machado et al., 2013). The efficient and fast identification of *M. ethiopica*, *M. inornata* and *M. luci*, economically important PPN, are of extreme importance to monitor its distribution and implement effective and environmentally friendly management strategies.

Chapter 2

Effect of naphthoquinones in *Meloidogyne luci* life cycle and evaluation of their recovery through gene expression analysis

Introduction

Plant-parasitic nematodes (PPN) impact on crop losses is estimated to be around 100 billion dollars/year (Oka et al., 2000). Root knot nematodes (RKN), *Meloidogyne* spp., are considered the most damaging PPN worldwide (Jones et al., 2013). The majority of the species are polyphagous with a wide host range and global distribution, leading to ineffective control of this group of nematodes (Wesemael et al., 2011).

RKN management is mostly based on three different approaches: chemical, including fumigants and non-fumigants; cultural, such as crop and fallow rotation, plant resistance, trap crops or amendments; and biological control, using other pathogens like bacteria and fungi (Hallmann et al., 2009; Nyczepir and Thomas, 2009). Although fumigant nematicides are considered the most efficient, their use has been reduced, due to severe impacts on human health and on environment, requiring the development of sustainable management strategies (Martin, 2003).

The increasing concern of producers and consumers about the risks posed by these chemicals has stimulated research to the development of "natural" nematicides, mostly extracted from plants (Elbadri et al., 2008; Caboni et al., 2013; Esteves et al., 2017; Maleita et al., 2017), which are generally safer to humans and the environment than their chemical homologous. Botanical origin nematicides include aldehydes, alkaloids, flavonoids, glucosides, ketones, limonoids, phenolics, quinones, thiocyanates, among others (Ntalli and Caboni, 2012). Such nematicides should be degradable and effective at low doses and display low toxicity to non-target soil organisms (Chitwood, 2002). Recently, naphthoquinones (NTQ) present in walnut (*Juglans regia* L.) husks and leaves were demonstrated to have significant nematicidal potential against RKN (Esteves et al., 2017; Maleita et al., 2017).

In Brazil, Iran, Chile, Guatemala, and Turkey, *M. luci* has been found associated with several important vegetable plants and fruit tree species (Aydınlı et al., 2013; Carneiro et al., 2014; Bellé et al., 2016; Janssen et al., 2016; Machado et al., 2016) and in Europe, was found parasitising maize (*Zea mays* L.) and kiwi (*Actinidia* spp.) in Greece; and tomato (*Solanum lycopersicum* L.) in Italy and Slovenia (Širca et al., 2004; Conceição et al., 2012; Maleita et al., 2012). In Portugal, *M. luci* was detected in a potato field near Coimbra, in 2013, and more recently on the ornamental plant *Cordyline australis* (Forst f.) Hook. F in Figueira da Foz, and the weed, *Oxalis corniculata* L., and

tomato in Montemor-o-Velho, all in Coimbra district (Maleita et al., 2018; Santos et al., 2019). The recent detection of *M. luci,* draw attention to the importance of understand the *M. luci*-plant interaction to develop new, effective, eco-friendly and integrated pest management programs and decrease the risk of spread and the potential economic impact.

In order to *Meloidogyne* spp. complete their life cycle, these nematodes develop a close relationship with the host plant. For instance, the activity of the RKN oesophageal gland cells varies throughout the different stages of parasitism, a strong activity in the subventral gland cells can be observed during nematode root penetration, and the activity of the dorsal gland cells is higher as feeding cells are formed and maintained (Davis et al., 2004).

Nematode effector genes, also called parasitism genes, are expressed in the nematode oesophageal gland cells and are considered the genetic determinants of nematode parasitism, having key roles, like cell-wall degrading or giant cell formation and maintenance (Hussey, 1989; Davis et al., 2004; Rosso et al., 2011; Quentin et al., 2013; Xie et al., 2016). Effector genes codify enzymes, such as β-1,4 endoglucanase and pectate lyase that modify the plant cell wall in order to facilitate intercellular migration (Rosso et al., 1999; Huang et al., 2004). Other effector genes, such as glutathione-S-transferase (*gst*) or venom allergen-like protein, are involved in the suppression of host defences during the infection stages, or even manganese superoxide dismutase that codifies anti-oxidant enzymes to protect nematodes from host-induced oxidative damage (Rosso, 2009; Abad and Williamson, 2010). Another effector, acetylcholinesterase (AChE), is involved in the termination of impulse transmission by rapid hydrolysis of ACh and effectors like *MiMsp40* and *MeTCTP* can suppress programmed host cell death (Niu et al., 2016; Zhuo et al., 2017; Chatonnet et al., 2017).

Using several procedures, a list of genes encoding candidate effector proteins has been produced, although some of the specific roles in parasitism are unknown, meaning that many effectors remain to be assessed (Haegemen et al., 2012; Xie et al., 2016).

The objectives of this study were: 1) to assess the effect of 1,4-naphthoquinone (1,4-NTQ) on *M. luci* second-stage juveniles (J2) infectivity and attraction to tomato roots and gene expression; and 2) to select the lowest compound concentration with effect on *M. luci*.

Materials and Methods

1. Nematode inoculum

Egg masses were handpicked from infected tomato roots with the *M. luci* isolate PtL obtained from a potato field in Coimbra (Maleita et al., 2018; Table 2.1), placed in a mesh sieve and incubated in a moist chamber at 25°C. Hatched J2 from the first 24 h were discarded. The subsequent J2, from the second 24 h and thereafter were collected and stored at 4°C until inoculation, for a maximum of five days.

2. Exposure to 1,4-naphthoquinone

Pure bioactive compound 1,4-NTQ was solubilised in Triton X-100 (laboratory grade) aqueous solutions (100 ppm) to obtain concentrations of 5.0, 10 and 20 ppm. Solutions were stirred for three days, at 37ºC, in the dark. J2 were incubated in these solutions to obtain the final concentrations of 2.5, 5.0 and 10 ppm (J2 suspension:1,4-NTQ; 1:1 v/v), at 25ºC also in the dark. Water and Triton X-100 were used as controls. After 24 h, J2 were concentrated by centrifugation for 2 minutes at 336 g and washed two times with RNAse-free water. Then, 2800 J2 were used in the attraction and infectivity bioassays and the remaining (± 5000 individuals) stored at -80°C in 2 mL Eppendorf tubes, until RNA extraction.

3. Attraction and infectivity bioassays

3.1. Attraction bioassays

Tomato seeds were placed in a moist chamber for two days at 25°C to germinate. Plates (5 cm Ø) were filled with 1% water-agar (5 mL/dish) and one seedling (0.5-1 cm long) was placed in one side of the plate. After 6 h, 20 *M. luci* J2, previously exposed to the different concentrations of 1,4-NTQ (2.5, 5.0 and 10 ppm), Triton X-100 and water, were inoculated 3 cm away from the tomato root tip. Each treatment was replicated three times and assayed twice. The plates were kept in the dark at 25°C. Following inoculation, a circular area (2 cm Ø) centred on the tomato root tip was placed beneath the plate. The number of nematodes inside that area was recorded at 3 and 18 h after inoculation.

3.2. Infectivity bioassays

Twenty-five tomato plants, genotype Coração-de-Boi (two-weeks old), were transferred to pots containing autoclaved sandy loam soil and sand. Plants were divided into five groups (2.5, 5.0 and 10 ppm 1,4-NTQ; Triton X-100; and water) with five replicates each. Each plant was inoculated with 100 *M. luci* J2 (initial nematode population density, Pi), previously exposed to the different concentrations of 1,4-NTQ or controls, and left at ±25°C. Three days after inoculation, all the roots were removed, washed, stained with acid fuchsin (Byrd et al., 1983), and the number of nematodes inside the roots quantified. The assay was repeated three times.

4. Gene expression analysis

4.1. RNA extraction, purification and reverse transcription

Total RNA was extracted from J2 of *M. luci* and isolated according to the Affymetrix standard protocol with Trizol reagent (Invitrogen). Nematodes were homogenised in Trizol reagent through six freeze-thawing-TyssueLyser cycles (liquid nitrogen; 37ºC; and 30 seconds at 50 Hz, respectively). Afterwards, the RNA was purified using the Direct-zol RNA kit (Zymo research) and any remaining DNA was digested by the TURBO DNA-free kit (Ambion). The concentration and purity of the RNA was determined in a Nanodrop 2000c spectrophotometer (NanoDrop; Labtech International), and the samples stored at -80°C.

RNA was converted into cDNA by reverse transcription (RT) using the iScript™ Reverse Transcription Supermix Kit (Bio-Rad Laboratories, USA) in a volume of 20 µL, according to the instructions, and the samples stored at -20°C, until PCR analysis.

4.2. Expression analysis

The expression of two genes was evaluated: *ache* that encodes an enzyme responsible for the primary termination of cholinergic nerve impulse transmission; and *gst* encodes an enzyme related to protection against the plant defences. Primer sets used are described in Table 2.1 PCR reactions were performed in a 25 µL volume containing 1× Taq reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 µM each primer, 2.5 U of Taq DNA polymerase and 25 ng of nematode cDNA as a template. A positive control – *β*-actin - was included. The amplifications were carried out using the following

conditions: an initial denaturation at 94°C for 3 minutes; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds; and a final extension for 10 minutes at 72°C. PCR reactions were analysed on a 1.5% agarose gel electrophoresis in 1× Tris-borate EDTA buffer stained with GreenSafe (NZYTech, Portugal)

Table 2.1. Primer sets used in the PCR reactions, as described in Duarte et al. (2014) and Cui et al. (2017).

Protein name	Primer name	Primer sequence 5' → 3'
Acetylcholinesterase	AChE_F	AACCGCAATCCAGACAATTCTTAT
	AChE_R	TCTTCTTGGCCCAGTTCCTATTCG
Glutathione S-transferase	GST_F	GAAAAATGGCCAGCCGAGAA
	GST_R	TCCTTTCCAGCCAAACCTTT
β*-actin	Actin_F	GATGGCTACAGCTGCTTCGT
	Actin_R	GGACAGTGTTGGCGTAAAGG

* Positive control.

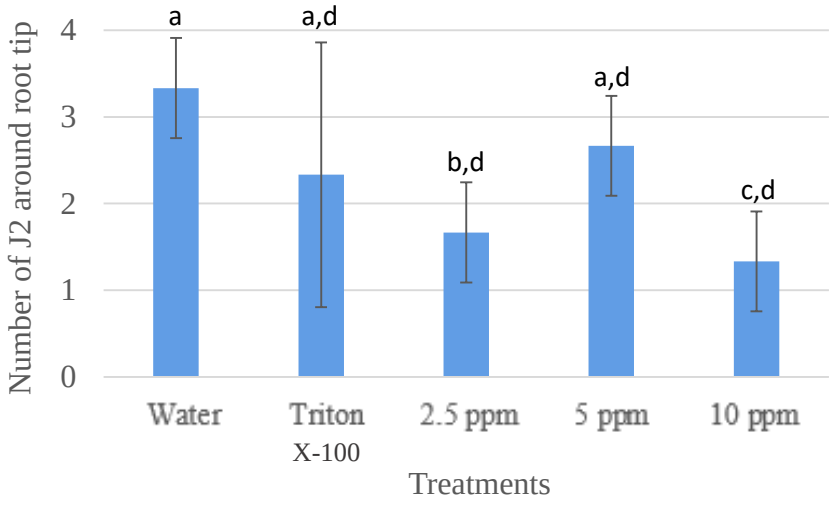
Results

1. Attraction and infectivity bioassays

1.1 Attraction bioassays

The impact of 1,4-NTQ on the attraction behavior of *M. luci* J2 was recorded at 3 h and 18 h after inoculation (Fig. 2.1). After 3 h of inoculation, significant differences ($p>0.05$) between water and 1,4-NTQ at 2.5 and 10 ppm were observed (Fig. 2.1A). The number of J2 recorded around the root tip was very low and when the experiment was repeated no significant differences were found between treatments, as observed at 18 h after inoculation (Fig. 2.1B). High heterogeneity among replicates was also observed (Fig. 2.1).

A



B

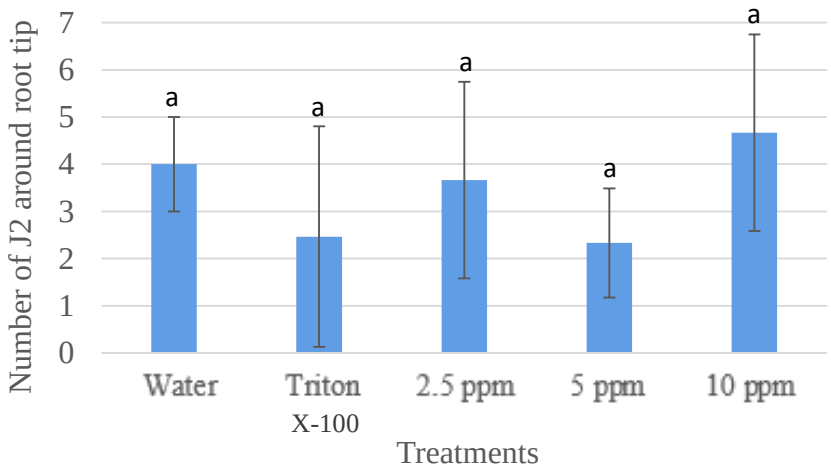


Figure 2.1. Attraction of *Meloidogyne luci* second-stage juveniles (J2) to tomato roots genotype Coração-de-Boi root tip after exposure for 24 h to 1,4-naphthoquinone 2.5, 5 and 10 ppm. Water and Triton X-100 (100 ppm) were used as controls. (A, B) Number of J2 on the designated attraction area at 3 h (A) and 18 h (B) after nematode inoculation. Each bar represents the average ± standard deviation of three replicates and bars denoted by different letters differ significantly at $p>0.05$ (according to the Fisher's LSD test).

1.2. Infectivity bioassays

Meloidogyne luci J2 were found inside the tomato roots in all treatments, but their relative numbers were significantly different (Fig. 2.2). A negative effect on *M. luci* J2 root penetration was observed after J2 incubation in 1,4-NTQ 2.5, 5 and 10 ppm. The

number of J2 inside the roots, when exposed to 1,4-NTQ, decreased approximately in about 50% when compared to the controls. The number of J2 found in tomato roots was not significantly different among all 1,4-NTQ treatments (Fig. 2.2).

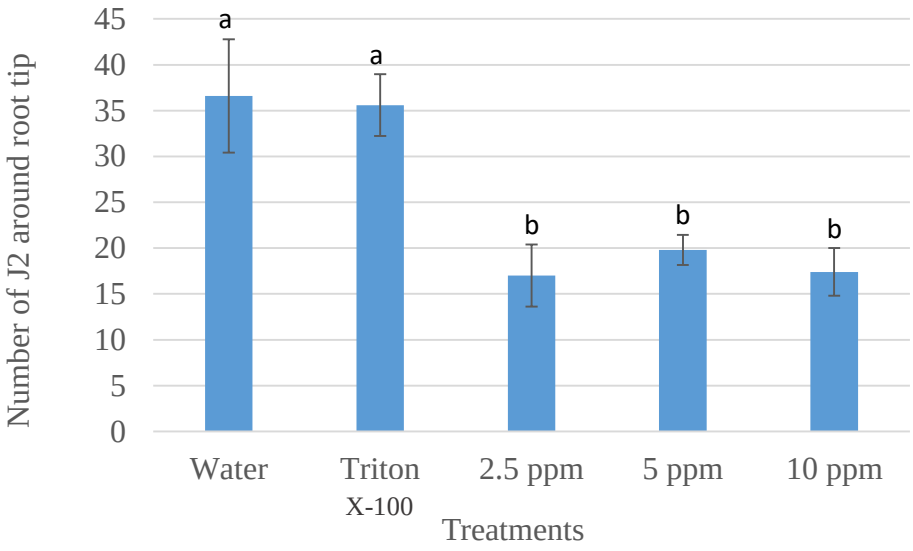


Figure 2.2. Number of *Meloidogyne luci* second-stage juveniles (J2) found inside tomato gemotype Coração-de-Boi roots three days after inoculation. Before inoculation, J2 were soaked for 24 h in 1,4- naphthoquinone 2.5, 5 and 10 ppm, in a 100 ppm Triton X-100 solution, and in water. Each bar represents the average ± standard deviation of five replicates and bars denoted by different letters differ significantly at p>0.05 (according to the Fisher's LSD test).

2. Gene expression analysis

Reverse transcription polymerase chain reaction, using the primers for *ache* and *gst* genes, showed the expression of these genes in *M. luci* J2. Amplification of the *β-actin* gene was used as a positive control (Fig. 2.3). The specific band for the *ache* gene has a molecular weight of approximately 150 bp and for the *gst* gene of 95 bp (Fig. 2.3). The cDNA fragments of *ache* and *gst* genes have a clear difference in band intensity. For both genes, the bands were equally intense in water, Triton X-100 and 1,4-NTQ 2.5 ppm treatments, and less intense in 1.4-NTQ 5 ppm when compared with those obtained in water and Triton X-100. However, for 1.4-NTQ 10 ppm no expression was detected for *ache* gene, while for *gst* gene the band was less intense when compared with those obtained in water, Triton X-100 and 1.4-NTQ 5 ppm (Fig. 2.3). Regarding the *β-actin* gene, the cDNA fragments were equally amplified in all treatments.

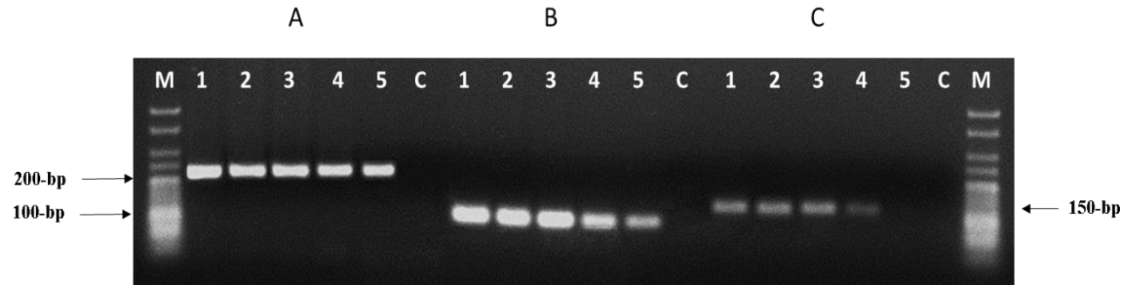


Figure 2.3. Expression of the genes *β-actin* (A), *gst* (B), and *ache* (C) by PCR amplification of cDNA from *Meloidogyne luci* second-stage juveniles, after exposure for 24 h to 1,4-naphthoquinone 2.5, 5 and 10 ppm. Water and Triton X-100 (100 ppm) were used as controls. 1 - water; 2- Triton X-100; 3- 1,4-NTQ 2.5 ppm; 4- 1,4-NTQ 5 ppm; 5 – 1,4-NTQ 10 ppm; C - negative control; M - DNA Marker (HyperLadder V; Bioline).

Discussion

The effect of 1,4-NTQ on the expression of *ache* and *gst* genes, with key roles in parasitism, was evaluated on *M. luci* J2. The potential use of 1,4-NTQ as a nematicidal agent against *Meloidogyne* species was previously demonstrated (Dama, 2002; Esteves et al., 2017; Maleita et al., 2017), resulting in high nematode mortality for concentrations above 50 ppm. Despite that, an approach on a supposed effect of low concentrations of 1,4-NTQ on nematode life cycle completion was not yet performed.

The expression of two genes with important roles in plant-nematode interaction was assessed. Acetylcholinesterase (AChE) is responsible for nematode nerve impulse transmission and it is the predominant target of chemical nematicides used in agriculture against PPN (Combes et al., 2001; Cui et al., 2017). Several *ache* genes have been reported in RKN, such as *M. incognita* and *M. javanica* (Piotte et al., 1999; Laffaire et al., 2003). No *ache* gene expression was found at 10 ppm 1,4-NTQ. Previous studies reported that inhibition of the *ache* gene could reduce the parasitic ability of PPN, including RKN (Opperman and Chang, 1990; Kang et al., 2013; Cui et al., 2017), demonstrating the key importance of this gene in the plant-nematode interaction, and a possible impairment of the nematode life cycle completion.

Glutathione-S-transferase (GST) is an effector involved in the suppression of host defences during the infection stages (Xie et al., 2016). Knockout of the transcripts

encoding this enzyme has led to a decrease in parasitism (Favery et al., 2016). The analysis of the results showed that J2 *gst* expression after exposure to 10 ppm 1,4-NTQ concentration decreased. The use of phytocompounds to inhibit GST activity in RKN was previously demonstrated (Babu et al., 2012) due to the antioxidant and detoxification properties of this enzyme, playing a major role in the survivability of the nematode inside the host. The negative effect of 1,4-NTQ on the *gst* gene expression may conduct to the impairment of the nematode ability to develop a permanent feeding site, preventing the completion of the life cycle.

The absent expression of the *ache* gene and the decreased expression of the *gst* gene, when exposed to a low concentration of 1,4-NTQ, suggests a decrease in the nematode ability to infect its host, revealing 1,4-NTQ as a potential disruptor of the RKN life cycle completion, and its potential use as a low concentration eco-friendly nematicide.

In order to find a host, RKN possess chemosensory structures which guide their movements after sensing chemical gradients originating from root diffusates (Eisenback and Hunt, 2009). A number of different chemicals was already proven effect on the nematode chemotactic behavior (Yang et al., 2016). The effect of 1,4-NTQ on *M. luci* J2 attraction to tomato root system was assessed, revealing, in a first experiment, significant differences between the water and 2.5 ppm and 10 ppm 1,4-NTQ treatments, 3 h after inoculation. Nonetheless, no significant differences were observed in a second experiment and at 18 h after inoculation. The poor consistency of the results revealed the lack of support to the existence of an effect of 1,4-NTQ on the chemotactic behavior of *M. luci* J2.

Instead, nematode root penetration was significantly affected by all 1,4-NTQ concentrations (2.5, 5 and 10 ppm), which implies a possible delay, or even impairment, of *M. luci* J2 root penetration and host infectivity, leading to a reduction of the nematode population density in the soil, preventing yield losses without the use of chemical control. Maleita et al. (2017) reported a negative impact on *M. hispanica* J2 penetration after exposure, for three days, to a *J. nigra* extract (50:47 ppm, 1,4-NTQ:juglone), revealing a reduction of approximately 50% in the number of J2 inside the roots. This negative impact is in agreement with the present study, however similar results were obtained with a lower time of exposure (24 h) and 1,4-NTQ concentration (10 ppm).

Meloidogyne luci was included in the EPPO alert list in 2017 (EPPO, 2017) proving the high impact of this emerging nematode species. Our findings demonstrate that low concentrations of 1,4-NTQ (10 ppm) have a strong nematicidal activity against

M. luci J2, and negatively influence the expression of key genes involved in parasitism. The toxicity of 1,4-NTQ to plants and soil invertebrates, including non-target soil nematode communities was already evaluated revealing a negative impact for 1,4-NTQ concentrations above 20 ppm. Our results indicate that a 1,4-NTQ concentration of 10 ppm might be effective for RKN management and could be environmentally safe to plant and soil invertebrates (Chelinho et al., 2017).

According to our results, the use of 1,4-NTQ as an alternative to fumigant nematicides is very encouraging, but further studies are needed before this natural-origin nematicide could be integrated in a new pest management strategy. More studies assessing the effect of low concentration 1,4-NTQ, when applied on the soil, should be performed, to understand if its effect on nematode life cycle completion persists.

Conclusions

Meloidogyne luci, a tropical RKN first described in Brazil, has a wide host range, known to parasitise highly important crops, and a large geographical distribution, being reported throughout Europe, including in Portugal. The close similarities between *M. luci* and *M.ethiopica* has led to the misidentification of these species in the past. *Meloidogyne luci* was added to the EPPO alert list in 2017, and sustainable management of this nematode has been the subject of growing concern in the last few years.

The main aims of this study were 1) to evaluate different molecular tools in order to develop standard molecular protocols for *M. luci* identification and differentiation, and 2) to investigate the effect of 1,4-NTQ on *M. luci* life cycle and gene expression.

In Chapter I, *M. luci* identification and differentiation from other RKN species was assessed and the main conclusions were:

1. Esterase phenotype differentiates *M. luci* from *M. ethiopica*, and is a practical tool to identify these two species;
2. RFLP-PCR analysis distinguish *M. luci* from *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. enterolobii*;
3. RFLP-PCR analysis was unable to discriminate *M. luci* from *M. ethiopica*;
4. The alignment of sequences from *M. luci* and *M.ethiopica* revealed a close similarity;
5. RAPD primer OPB-01 gave different band patterns for *M. ethiopica* and *M. luci*, displaying an additional band of ± 450 bp for *M. luci*.

In Chapter II, the effect of 1,4-NTQ in *M. luci* life cycle and the expression analysis of two genes with key roles in plant-nematode interaction were studied and the main findings were:

1. 10 ppm 1,4-NTQ is the lowest compound concentration with effect on *M. luci* gene expression and life cycle completion;
2. 2.5 ppm 1,4-NTQ is the lowest compound concentration with effect on *M. luci* root penetration;
3. All 1,4-NTQ concentrations tested negatively impacted *M. luci* root penetration.

The use of proper identification techniques to monitor the distribution and impact of *M. luci* and the implementation of effective and environmentally friendly management strategies is highly desirable considering the potentially great economic impact of this species. This study depicts a contribution for the RKN accurate differentiation and identification, and also highlights the potential of 1,4-NTQ as a low concentration natural-origin nematicide for the sustainable management of *M. luci*.

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