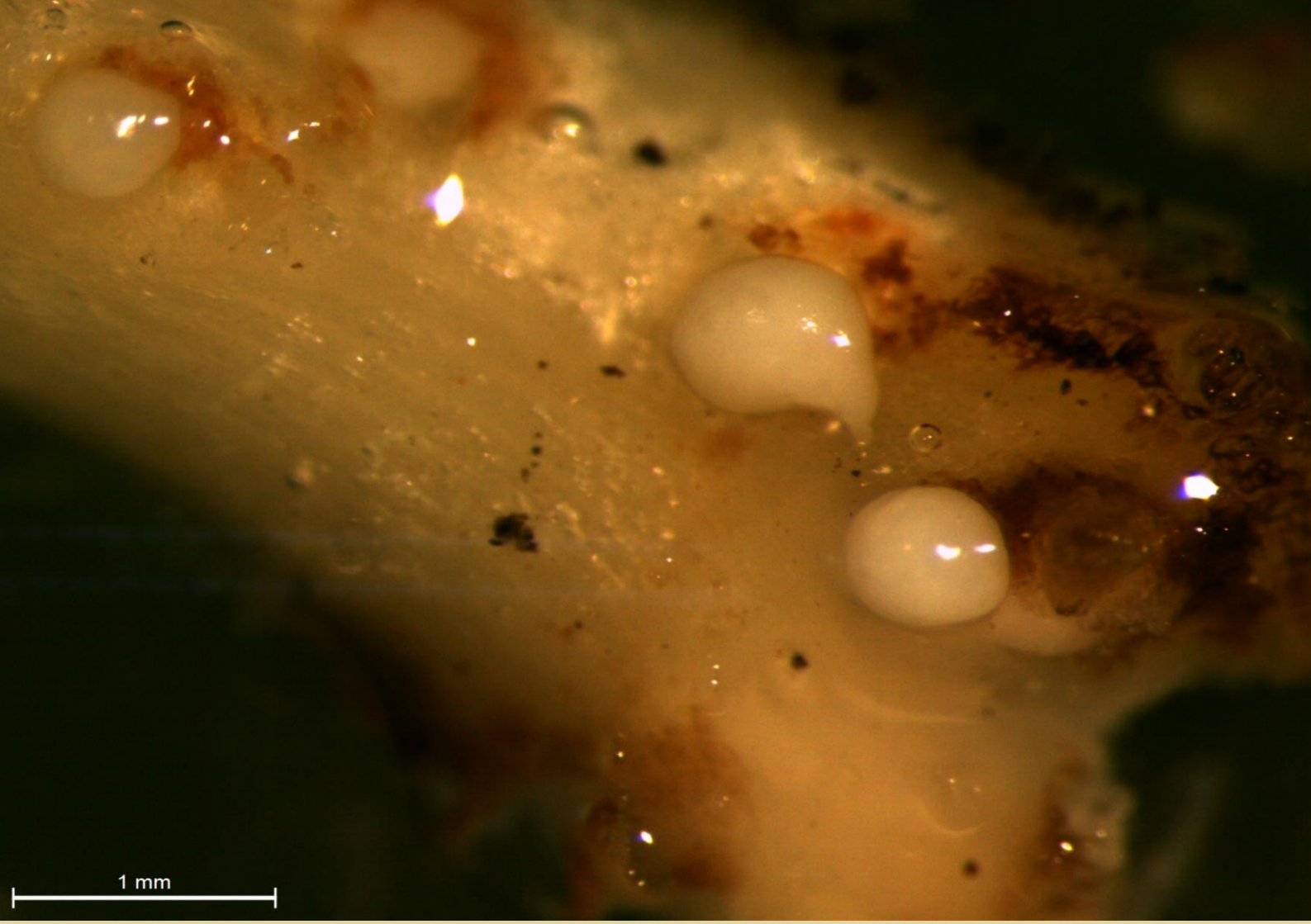

Duarte António Fernandes Santos

Meloidogyne luci: characterisation of a tropical root-knot nematode species in Portugal

Dissertação de Mestrado em Biodiversidade e Biotecnologia Vegetal, 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 da Universidade de Coimbra

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Cover figure: *Cordyline australis* infected root with *Meloidogyne luci* females.

Meloidogyne luci: characterisation of a tropical root-knot nematode species in Portugal

FCTUC FACULDADE DE CIÊNCIAS E TECNOLOGIA UNIVERSIDADE DE COIMBRA

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

Thesis submitted to University of Coimbra for the degree of Masterin Biodiversity and Plant Biotechnology, supervised by Dr. Carla Maria Nobre Maleita and co-supervised by Professor Dr. Isabel Maria de Oliveira Abrantes

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Beautiful is what we see, more Beautiful is what we know,
most Beautiful by far is what we don't know.

Nicolas Steno (1638–1686)

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Publications

Santos, D., Correia, A., Abrantes, I. & Maleita, C. (2018). New record of the root knot nematode *Meloidogyne luci* in Portugal. Plant Disease (To be submitted).

Santos, D., Abrantes, I. & Maleita, C. (2018). *Meloidogyne enterolobii*, a new quarantine root knot nematode - a potential threat to Portugal and Europe. Plant Disease (To be submitted).

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Communications

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Santos, D., Correia, A., Abrantes, I. & Maleita, C. New record of the root-knot nematode *Meloidogyne luci* in Portugal. 33rd Symposium of the European Society of Nematologists, 9-13th September 2018, Ghent, Belgium (Poster).

Santos, D., Abrantes, I. & Maleita, C. Effect of the *Mi* gene on the reproduction of *Meloidogyne luci* on tomato genotypes. 33rd Symposium of the European Society of Nematologists, 9-13th September 2018, Ghent, Belgium (Poster).

Abstract

The tropical root-knot nematode (RKN) *Meloidogyne luci* is a highly polyphagous and damaging agricultural pest, which has been reported, recently, from several European countries. In 2013, *M. luci* was first detected in Portugal in a potato field from Coimbra region, but the extent of its geographic distribution, and of other RKN species, in Portugal is unknown. Incidence and diversity of RKN on subsistence farms and public and private gardens, in Coimbra region, were evaluated. In total, forty eight RKN isolates were identified, based on esterase phenotype, corresponding to six *Meloidogyne* species: *M. arenaria, M. enterolobii, M. hapla, M. hispanica, M. incognita* and *M. luci*. *Meloidogyne luci* was recorded in two new locations, which indicates that this RKN species is already established in Portugal, and was found parasitising two new host plants (*Cordyline australis* and *Oxalis corniculata)*. The quarantine nematode *Meloidogyne enterolobii* was also reported for the first time in Portugal and three new host plants were identified (*Physalis peruviana*, *Cereus hildmannianus* and *Lampranthus*). The effect of the *Mi-1.2* gene in the reproduction of *M. luci* was also evaluated and compared with *M. ethiopica*, in order to increase the knowledge of its biology and to define sustainable management strategies in tomato production areas. Twenty seven tomato genotypes were screened for the RKN resistance *Mi-1.2* gene, by amplification of Mi23 marker. Thirteen heterozygous (Mimi) tomato genotypes at the *Mi* locus, two homozygous (MiMi) and twelve lacking the *Mi* gene for resistance to RKN were identified. The pathogenicity assays revealed that this *R*-gene is effective in supressing *M. luci* and *M. ethiopica* reproduction and can be used in integrated pest management programmes in tomato production areas, particularly the genotypes Reconquista (*Mi*-homozygous) and Vimeiro F1, Paipai, Sahel, Agora F1, Amaral, Valoasis RZ F1, and SV1917 (heterozygous), classified as resistant to *M. luci* and *M. ethiopica*. This study represents an important contribution for the RKN knowledge in Portugal and also highlights the potential of the tomato genotypes with the *Mi-1.2* gene for the management of the RKN *M. luci* and *M. ethiopica* as an alternative to the use of nematicides.

Keywords: diversity; esterase phenotype; management; *Meloidogyne*; *Mi-1.2* gene; Mi23 marker; plant resistance; quarantine nematode; resistance genes; *Solanum lycopersicum*; tomato genotypes.

Resumo

O nemátode das galhas radiculares (NGR) *Meloidogyne luci*, recentemente reportado em vários países Europeus, é uma espécie polífaga capaz de causar prejuízos em várias culturas economicamente importantes. Em 2013, *M. luci* foi descoberta pela primeira vez em Portugal num campo de batata da região de Coimbra, mas até agora a sua distribuição geográfica assim como a de outras espécies de NGR, em Portugal, ainda é pouco conhecida. Neste estudo, foi avaliada a incidência e a diversidade de NGR, em explorações de agricultura de subsistência e jardins públicos e privados, na região de Coimbra. No total, quarenta e oito isolados de NGR foram identificados, com base nos fenótipos de esterases, correspondendo a seis espécies de NGR: *M. arenaria*, *M. enterolobii*, *M. hapla*, *M. hispanica*, *M. incognita* e *M. luci*. *Meloidogyne luci* foi registada em duas novas localidades e em duas novas plantas hospedeiras (*Cordyline australis* e *Oxalis corniculata*), o que indica que esta espécie de NGR já está estabelecida em Portugal. O nemátode de quarentena *Meloidogyne enterolobii* foi detetado pela primeira vez em Portugal e três novas plantas hospedeiras foram identificadas (*Physalis peruviana*, *Cereus hildmannianus* e *Lampranthus*). O efeito do gene *Mi-1.2* na reprodução de *M. luci* foi avaliado e comparado com *M. ethiopica*, a fim de ampliar o conhecimento da sua biologia e definir estratégias de controlo mais sustentáveis em áreas de produção de tomate. A presença do gene *Mi-1.2* em vinte e sete genótipos de tomateiro foi determinada, através da amplificação do marcador Mi23. Treze genótipos de tomate foram identificados como heterozigóticos (Mimi), dois como homozigóticos (MiMi) para o locus *Mi* e doze não apresentavam este gene. Os ensaios de patogenicidade revelaram que o gene *Mi-1.2* é eficaz na supressão da reprodução de *M. luci* e *M. ethiopica*, podendo, os genótipos Reconquista (*Mi*-homozigótico) e Vimeiro F1, Paipai, Sahel, Agora F1, Amaral, Valoasis RZ F1, e SV1917 (heterozigóticos), que foram classificados como resistentes a *M. luci* e *M. ethiopica*, ser utilizados em programas de proteção integrada. Este estudo representa uma contribuição importante para o conhecimento dos NGR em Portugal e evidencia o potencial dos genótipos de tomate com o gene *Mi-1.2* para o controlo dos NGR *M. luci* e *M. ethiopica* como uma alternativa ao uso de nematodicidas.

Palavras-chave: controlo; diversidade; fenótipos de esterases; gene *Mi-1.2*; genes de resistência; genótipos de tomateiro; marcador Mi23; *Meloidogyne;* nemátode de quarentena; resistência de plantas; *Solanum lycopersicum*.

General Introduction

Nematodes

The Nematoda phylum is one of the most diverse taxa in the animal kingdom, being the most abundant multicellular group of animals on the Earth, accounting for about 80% of all individual animals, with densities often exceeding 1 million individuals/m^2 in the soil (Lorenzen, 1994). These small round worms, usually with microscopic size (<1 mm in length), live in nearly every habitat on Earth. Most of them are free-living species inhabiting both aquatic and terrestrial environments feeding on algae, bacteria, fungi, organic debris and other small animals (Yeates, 2004; Quist et al., 2015). However, some nematodes are parasites of plants and animals, including humans and insects.

Over 4,100 plant-parasitic nematodes (PPN) species have been identified (Decraemer & Hunt, 2013) and can be further divided according to their parasitic habits as: ectoparasites (uses its stylet to feed on plant root cells without entering the plant tissue) and endoparasites (enter plant tissue to feed and complete at least part of their life cycle inside the root system).

Root-knot nematodes

The most notorious group of PPN is the sedentary endoparasitic root-knot nematodes (RKN) of the genus *Meloidogyne* Göldi, 1892. These nematodes form complex feeding relationships with their hosts by establishing permanent feeding sites in the vascular cylinder of infected roots from which they draw off nutrients to complete their life cycle (Abad et al., 2003).

RKN are considered worldwide one of the most important group of nematodes and the most widely distributed (Jones et al., 2013). Damage caused by these pathogens to agriculture has been estimated at US$100 billion (Oka et al., 2000). So far, about 100 *Meloidogyne* species have been described, and each of these species typically has a wide range of hosts, including many crop plants, ornamental plants, and common weed species (Eisenback & Triantaphyllou, 1991; Hunt & Handoo, 2009). It was hypothesised that the wide host range of most important RKN species is a consequence of their reproduction strategy (Trudgill, 1997). Mitotic parthenogenesis is thought to drive and maintain a wide host range, because it slows host–parasite coevolution and genetic drift. However a handful of species present a narrow host range, for instance *M. baetica* and *M. lusitanica* are only know to infect olive trees (Abrantes & Santos, 1991; Castillo et al., 2003).

Historically, the majority and most important research have been focused on the four ‘major’ RKN species: *M. incognita*, *M. arenaria*, *M. javanica* and the temperate *M. hapla*. But recent studies have been raising awareness about the so-called ‘minor’ *Meloidogyne* spp. demonstrating their importance and impact in sustainability of farming systems (Castagnone-Sereno, 2012; Elling, 2013).

Life cycle

RKN maintain a durable relationship with their host plants for 3 to 8 weeks depending on several factors, such as availability of a suitable host, temperature and moisture (Taylor & Sasser, 1978). The first-stage juvenile (J1) remain inside the egg, suffer a moult and hatch as a second-stage juvenile (J2) (Fig. 1). This free-living stage migrates through the soil to a new host, usually penetrating near or behind the root cap. After breaching the root epidermis, the J2 move through the plant tissue until it finds a suitable feeding site in the zone of differentiation of the vascular cylinder (Wyss et al., 1992). Plant cell walls around the head of the nematode are pierced with the stylet, and secretions from the esophageal glands are released, inducing the formation of giant cells. Giant cells (2 to 12 cells, each with several nuclei) result from nuclear divisions, without cytokinesis. Giant cell formation and cortical cells proliferation and hypertrophy in association with the enlargement of the nematode body is accompanied by enlargement of the root giving rise to typical galls, the main visible symptom of infection (Hussey & Mims, 1991; Vanholme et al., 2004). During post-embryonic development J2 becomes sedentary and flash-shaped and moults three times into the third (J3) and fourth-stage (J4) and adult (Williamson & Hussey, 1996). Males are vermiform and leave the root moving freely through the soil; females are globose and sedentary and deposit 300-500 eggs on the root surface or inside the galls in a protective gelatinous matrix (Abad et al., 2003; Ornat & Sorribas, 2008) (Fig. 1).

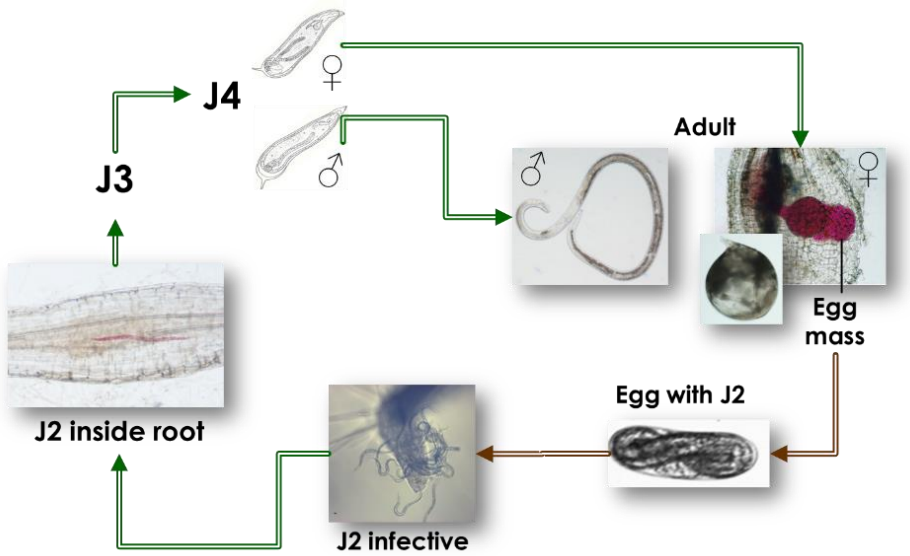

Figure 1. Root-knot nematode life cycle, *Meloidogyne* spp. J2, J3, J4 - Second-, third and fourth-stage juveniles (From Inácio et al., 2018).

Heavily infected roots can show large galls hampering the main root functions: water and nutrients uptake and translocation are substantially reduced resulting into poor growth and low yield (Williamson & Hussey, 1996). Additionally, mechanical wounding of the root favours the entry of other pathogens, such as fungi, bacteria and/or virus leading to disease complexes. *Fusarium* species are frequently found associated with RKN (Jeffers & Roberts, 1993; Bertrand et al., 2000).

Management and Control

Several strategies have been employed in RKN management, including cultural, chemical and biological approaches. For the last 50 years, the RKN control largely relied on treatments with chemical nematicides, mainly fumigants, which act by killing or interfering with the reproduction cycle of nematodes. However, increasing awareness about the negative impact of these chemicals on the environment and human health led the European Commission (EC) to change its policy and eliminate or reassess many synthetic pesticides (Directive 91/414/EEC; Regulation 2009/1107/EC; Directive 2009/128/EC), so that only a handful of nematicides are available for farmers (European Commission, 1991, 2009a,b); and has stimulated the search for alternative safe, effective and eco-friendly strategies for *Meloidogyne* species management (Zuckerman & Esnard,

1994). Furthermore, it is known that repeated applications of the same nematicide results in an effectiveness decrease (Viglierchio, 1990).

Alternative non-chemical strategies, such as destruction of infected root systems after harvest, rotation with non-hosts or fallow periods, growth of resistant plants and adjust planting and harvest date, have been reported as effective on RKN control (Barker & Koenning, 1998). In most cases, these strategies are accessible and inexpensive, since most of them do not require special skills for application. Nevertheless, they rely on the knowledge of the taxonomy and biology of *Meloidogyne* species present in the soil, in order to define efficient and taxa-specific control measures, as a consequence of pathogenic variability between and within species (Sasser et al., 1983).

Implementation of some of these strategies, namely crop rotation fallow periods, in intensive crop production systems can be difficult and costly due to the fallow periods where no income is obtained. Moreover, weeds can be alternative hosts and serve as RKN reservoirs, which restrict the use of this strategy (Rich et al., 2009).

Sustainable use of nematicides, prioritising the use of non-chemical strategies, in compliance with the principles of integrated pest management, is imperative in order to reduce the risks that this chemicals pose on human health and environment, and increase the productivity of agrosystems.

Identification

An accurate identification of nematodes of the genus *Meloidogyne* is essential to devise integrated nematode management strategies and is mandatory for research. In the past, *Meloidogyne* species were identified on the basis of morphological and biometric characters of females, males and J2, and host preferences (Eisenback et al., 1980). However, diagnosis based on morphology is not always easy, even for qualified taxonomists, due to the great inter and intra-specific variability and to the frequent occurrence of more than one species in the same sample (Jepson, 1987; Carneiro et al., 2000; Blok & Powers, 2009). This prompted the researchers to search for alternative methodologies to confirm and complement RKN species identification.

The use of biochemical and molecular methods for RKN identification became very common, as these methods are fast, reliable and efficient and not require nematological expertise in comparison with morphological analyses (Esbenshade & Triantaphyllou, 1990). The biochemical electrophoretic analysis of non-specific esterases

(EST) and malate dehydrogenases (MDH) (Dickson et al., 1970; Esbenshade & Triantaphyllou, 1985), remains, actually, one of the first steps in the identification of the most common *Meloidogyne* spp. Nonetheless, it can only be done with egg-laying females. Emphasis has also been given on developing DNA-based methodologies, particularly those based on polymerase chain reaction (PCR), as these do not rely on the genome expression, and are independent of the environment and nematode life stage (Floyd et al., 2002; De Ley et al., 2005).

Meloidogyne luci

Meloidogyne luci was described, in 2014, from a Brazilian isolate originated from lavender (*Lavandula spica* L.) (Carneiro et al., 2014). However, since 1985 this nematode species have also been detected in Argentine, Bolivia, Brazil, Chile, Ecuador, Greece, Guatemala, Iran, Italy, Slovenia and Turkey (Širca et al., 2004; Conceição et al., 2012; Maleita et al., 2012a; Aydinli et al., 2013, 2016; Carneiro et al., 2014; Bellé et al., 2016; Machado et al., 2016; Stare et al., 2017a). More recently, *M. luci* was first reported in Portugal parasitising potato (*Solanum tuberosum* L.) roots, from Coimbra region, but the full extent of its geographic distribution is unknown (Maleita et al., 2018).

This RKN species shares some morphological and biochemical similarities with *M. ethiopica* so that several populations has been misidentified. All populations previously identified as *M. ethiopica* in Europe and Turkey being, recently, reclassified as *M. luci* using biochemical and molecular analysis (Stare et al., 2017b). Identification based on morphological characters, such as perineal pattern, which is often used as a diagnostic characteristic, is hard and often unreliable. *Meloidogyne luci* and *M. ethiopica* share an ovoid to squarish perineal pattern with a low and rounded to moderately high dorsal arch. In addition, most of the biometric characters of J2, females and males are highly variable and can be found in populations from other species (Carneiro et al., 2014; Stare et al., 2017b; Maleita et al., 2018).

Analyses of EST phenotypes in combination with phylogenetic analysis of the mitochondrial DNA (mtDNA) region, located in the 3' portion of the gene that codes for cytochrome oxidase subunit II (COII) through a portion of the 16S rRNA gene, proved to be very useful to distinguish *M. luci* from *M. ethiopica*. The easiest method to differentiate these two species is through biochemical analysis. Although the EST phenotype detected in *M. luci* (L3) was similar to *M. ethiopica* (E3), the first band of *M.*

luci was located at the same level of the first band of the reference isolate, *M. javanica*. *Meloidogyne ethiopica* phenotype is clearly different and the first band located above the first band of *M. javanica. Meloidogyne luci* L3 EST phenotype is unique and useful to differentiate this species from other RKN species (Maleita et al., 2018). Nevertheless, there is a great diversity of *Meloidogyne* species, some of their described many years ago when isozyme characterisation was not yet a practice, so there are many species with no EST phenotype described.

Molecular analysis can also be used for an accurate species identification, D2-D3 sequences (Carneiro et al., 2014; Machado et al., 2016), mtDNA COII (Stare et al., 2017b) and mtDNA cytochrome oxidase subunit I (COI) region (Maleita et al., 2018) were shown to be good tools to differentiate these two closely related species. These techniques have been used so far with varying degrees of success, allowing for a reliable differentiation of *M. luci* populations. Stare et al. (2017b) have considered the mtDNA COII region very useful for the identification and phylogenetic relationship of *M. luci* closely related species, while Maleita et al. (2018) results depicted mtDNA COI region as effective.

Meloidogyne luci has a wide host range, and has been associated with several economically important crops, including 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 & Ferguson), lavender (*Lavandula angustifolia* Mill.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), okra (*Abelmoschus esculentus* 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. Endl.) (Carneiro et al., 2014; Bellé et al., 2016; Machado et al., 2016; Janssen et al., 2016; Maleita et al., 2018).

Furthermore, it has been shown that *M. luci* has the potential to survive under a sub-Mediterranean and continental climate conditions, even in locations where soil temperatures drop below zero, like Slovenia (Strajnar et al., 2011). Therefore, *M. luci* may constitutes a threat to southern Europe agrosystems, which justify the need for further research to understand its distribution and host range, for the development of new management strategies.

Objectives

The main objectives of this study were: a) to determine the incidence of RKN in subsistence farms and gardens, in Coimbra region; and b) to increase the knowledge of *M. luci* biology, in order to define sustainable management strategies in tomato production areas.

The specific objectives were:

1. To determine RKN incidence and diversity on subsistence and public/private gardens, in Coimbra region;

2. To identify RKN isolates by biochemical electrophoretic analysis of non-specific esterases;

3. To confirm the identification of new RKN species found in Portugal by molecular characters;

4. To screen the RKN resistance *Mi-1.2* gene in 27 tomato genotypes by DNA amplification using the Mi23 marker;

5. To evaluate the ability of a Portuguese isolate of *M. luci* and a Brazilian isolate of *M. ethiopica* to reproduce on tomato genotypes.

Chapter 1

Root-knot nematodes, *Meloidogyne* spp., on subsistence farms and gardens

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are considered the most damaging phytoparasites, as a result of their wide geographic distribution and polyphagous nature, affecting the production and quality of a number of plants of economic importance (Sasser, 1977; Trudgill & Blok, 2001). Their potential host range encompasses more than 3000 plant species including monocotyledons, dicotyledons and herbaceous and wood plants (Rich et al., 2009).

During post-embryonic development, RKN, sedentary endoparasites, alter the root physiology, resulting in the formation of typical galls, and disturb the normal uptake and transport of water and nutrients. Above-ground symptoms exhibited by infected plants are unspecific and generally involve, depending of the severity of the infection, severe growth retardation, lack of vigour, wilting, particularly in periods of water stress and high temperatures, and leaf nutritional deficiencies, such as chlorosis (Netscher & Sikora, 1990).

Currently, *Meloidogyne* genus comprises more than 100 described species (Karssen et al., 2013), so far, and 23 have been found in Europe (Wesemael et al., 2011). *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla* are considered the most abundant and damaging, often classified as “major" RKN species (Moens et al., 2009). However, some species previously considered as “minor” agricultural pests, such as *M. enterolobii*, *M. chitwoodi* and *M. fallax*, are regarded as emerging species with potential to cause significant damage to crops and to overcome known resistance genes (Elling, 2013). These three emerging species have received increase attention in the past years, and are present in the A2 List of pests of the European and Mediterranean Plant Protection Organization (EPPO), recommended for regulation as quarantine pests (EPPO/PQR, 2017).

In Portugal, the RKN species *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. hispanica*, *M. incognita*, *M. javanica*, *M. luci* and *M. lusitanica* have been found in different regions, associated with several economic important crops (Pais & Abrantes, 1989; Abrantes & Santos, 1991; Abrantes et al., 2008; Conceição et al., 2009; Esteves et al., 2015; Maleita et al., 2018). In 2013, the tropical RKN *M. luci* was found in a potato, *Solanum tuberosum* L., field near Coimbra, Portugal. This was the first record of this species parasitising potato worldwide. In 2017, *M. luci* was added to the EPPO Alert List (EPPO, 2017) and all *M. ethiopica* isolates identified in Europe (Slovenia, Italy and Greece) and Turkey

were reclassified, using biochemical and molecular analyses as *M. luci* (Stare et al., 2017b). In Slovenia, this species was detected in tomato roots first in 2003 and then in 2015 (Širca et al., 2004; Stare et al., 2017a). *Meloidogyne luci* was also found in Brazil, Chile, Guatemala and Iran (Carneiro et al., 2014).

Accurate identification of *Meloidogyne* spp. is a prerequisite for the implementation of efficient management strategies, such as crop rotation and resistant cultivars, and regulatory and quarantine programmes (Elling, 2013). Despite numerous studies, RKN identification to the species level based on morphology represents a huge challenge for many researchers, even for qualified taxonomists due to the occurrence of intra- and inter-specific variability (Eisenback, 1985; Jepson, 1987; Carneiro et al., 2000; Blok & Powers, 2009). Furthermore, the classic identification techniques based on light microscopy has proven complex and time-consuming, requiring a detailed analysis of several morphological characters of females, particularly perineal pattern morphology, males and second-stage juveniles (J2) (Eisenback et al., 1980). The use of morphological characters, as the only criterion for RKN diagnosis, can lead to misidentifications (Carneiro & Cofcewicz 2008). Therefore, there is a need to embrace other methodologies in combination with classical methods to carry out the identification of RKN populations.

Electrophoretic analysis of isozymes, mainly malate dehydrogenase (MDH) and esterase (EST), resolved in polyacrylamide gel electrophoresis, are used routinely for RKN identification (Xu et al., 2004; Brito et al., 2008; Kolombia et al., 2017). The EST profile analysis is reliable, widely used and very useful in the detection of population mixtures that can be easily separated to obtain pure isolates (Pais & Abrantes, 1989; Cofcewicz et al., 2005; Brito et al., 2008, 2010). It remains relevant over the years despite the emergence of new modern techniques based on polymerase chain reaction (PCR), since EST analysis are effective and requires less expensive equipment (Janssen et al., 2016). Nevertheless, isozyme analysis is life stage dependent, only egg-laying females can be used.

Molecular techniques based on DNA are independent of the nematode life cycle, fast and reliable (Zijlstra et al., 2004; Powers et al., 2005). These methods are very sensitivity and have recently become widely used not only in *Meloidogyne* spp. diagnosis, but also to provide important knowledge on nematode phylogeny (De Ley et al., 2002; McClure et al., 2012). Despite the reliability of molecular methods, time-consuming, complexity and expense are important constraints when applied in large surveys (Molinari et al., 2005).

Different molecular techniques are available for *Meloidogyne* species identification including: amplified fragment length polymorphism (AFLP), random amplified polymorphism DNA (RAPD), restriction fragment length polymorphism variation (RFLP) and sequence characterised amplified region markers (SCAR) (Blok et al., 1997, 2002; Adam et al., 2007). These approaches depend on the occurrence of polymorphisms in DNA sequences between nematode species, especially in nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) (Janssen et al., 2016).

The SCAR is a PCR technique that is based on the sequence information from a specific DNA fragment of a target organism genome, e.g., species-specific DNA bands from RAPD or AFLP methods are cloned, sequenced and converted to SCAR markers. DNA can be extracted from single nematodes, eggs or juveniles; and, through a simple PCR or real-time PCR, the species identity is or not confirmed by the presence/absence of an amplification product, respectively (Zijlstra et al., 2000; Randig et al., 2002; Tigano et al., 2010). Despite offering many advantages, SCAR markers show some limitations: primers are not available for all RKN species, like *M. luci*; and for a given *Meloidogyne* sample it is often difficult to define the appropriate primers to use. Consequently, random selection of primers or combination of primers in a multiplex PCR assay is required for the identification of unknown RKN isolates (Powers et al., 2005; Baidoo et al., 2016).

Congruence between two or more taxonomic characters is important to reach an accurate *Meloidogyne* spp. identification (Padial et al., 2010). A combination of morphological, biochemical and molecular methods is crucial and required (Carneiro et al., 2014; Castillo et al., 2009; Tao et al., 2017). A limited number of surveys have been conducted in Portugal to evaluate the incidence and diversity of RKN, particularly in ornamental plants. The objectives of this study were to find out the RKN incidence and diversity on subsistence farms and gardens, in Coimbra region.

Materials and methods

SAMPLING AND NEMATODE CULTURES

Forty one root samples were collected, from October 2017 to April 2018, on subsistence farms and private and public gardens from three municipalities (Figueira da Foz, Montemor-o-Velho and Coimbra) of Coimbra region (Fig. 1; Table 1), comprising a wide range of host plants including vegetables, fruit trees, weeds and ornamental plants.

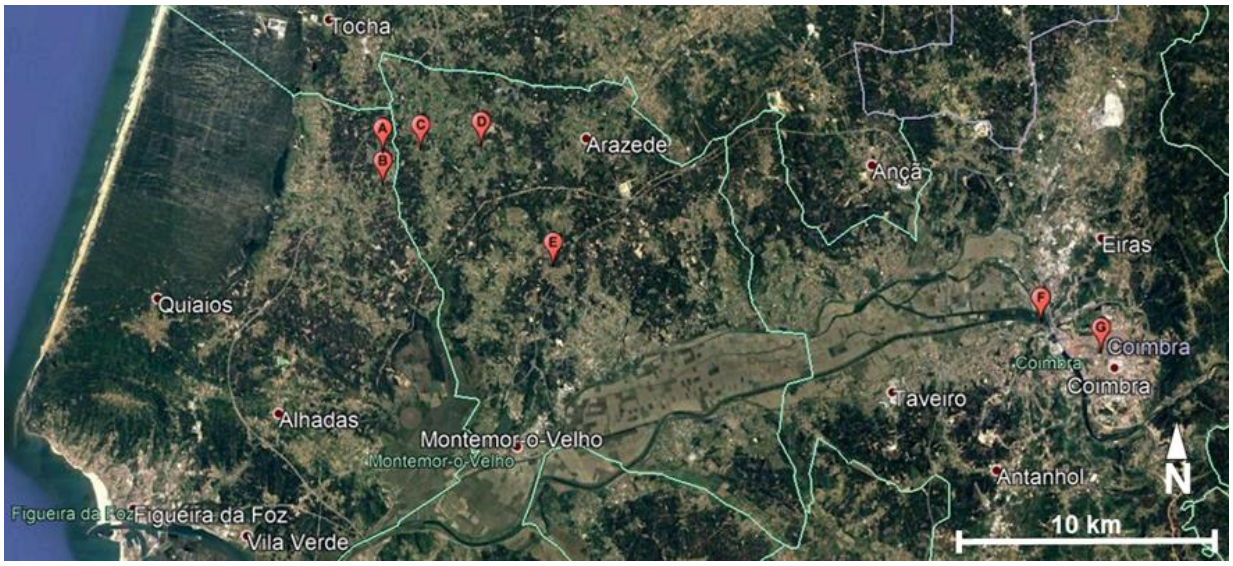

Figure 1. Localisation of the sampling sites (villages and municipalities) in Coimbra region. A - Coentros, Figueira da Foz; B - Tromelgo, Figueira da Foz; C - Tojeiro, Montemor-o-Velho; D - Catarruchos, Montemor-o-Velho; E - Carapetos, Montemor-o-Velho; F - Santa Cruz (Mata do Choupal), Coimbra; G - Santa Cruz (Jardim da Sereia), Coimbra (Image created using Google Earth Pro 7.3.1).

Roots were gently rinsed with tap water and examined under a stereomicroscope to look for RKN symptoms (galls and/or egg masses). From each root sample, at least 8 young egg-laying females were, individually and randomly, handpicked with the respective egg mass to glass blocks with NaCl 0.9%. In order to obtain pure isolates, individual young egg-laying females were characterised biochemically by electrophoretic analysis of EST, before egg mass inoculation on tomato, *S. lycopercicum* L., cv. Coração-de-Boi plants. Egg masses from females with similar EST phenotype were grouped and inoculated in tomato plants, into 10 cm-diameter pots filled with a mixture (1:1:1) of sterilised soil, sand and substrate .Tomato plants were maintained in a growth chamber at 25±2°C, with a 12 h photoperiod, in the NEMATO-lab, and the isolates maintained

through periodically sub-culturing. When young egg-laying females were not available on the root samples, ten egg masses were randomly handpicked and inoculated in tomato plants. After 60 days, tomato roots were washed, young egg-laying females and egg masses teased out of galls/roots and followed the procedure described before.

Meloidogyne enterolobii, *M. ethiopica*, *M. hispanica*, *M. javanica* and *M. luci* isolates, selected from NEMATO-lab *Meloidogyne* spp. collection, were included in the biochemical and/or molecular studies for comparison.

BIOCHEMICAL CHARACTERISATION

Esterase electrophoresis was performed following the method described by Pais and Abrantes (1989). Females were transferred, individually, to micro-hematocrit tubes containing 5 µL of extraction buffer (20% sucrose and 1%Triton X-100), macerated with a pestle, and stored at -20°C. Before electrophoresis, the samples were centrifuged at 9969 rpm, at -5°C for 15 minutes. Electrophoresis was performed at 6 mA/gel during the first 15 min and then at 20 mA/gel for about 45 min using the Mini-Protean Tetra System (Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained for EST activity with the substrate α-naphthyl acetate, in the dark at 37°C. Protein extracts from five females of *M. javanica* was included in each gel as a reference. Protein extracts from females of *M. ethiopica* and *M. luci* isolates, selected from NEMATO-lab, were also included in gels for comparison (isolates B3, C1, C2, C11, C12, C13 and C14; Table 1). Esterase phenotypes were designated with a letter corresponding to nematode species followed by a number indicating the number of bands of EST activity (Esbenshade & Triantaphyllou, 1985; Pais & Abrantes, 1989; Carneiro et al., 2000).

MOLECULAR CHARACTERISATION

Biochemical characterisation of isolates with unusual EST phenotype (isolates C11, C12, C13 and C14; Table 1) was complemented using molecular methods. Egg masses from each isolate were handpicked from infected roots and maintained in moist chambers at 25°C to obtain freshly J2 that were concentrated by centrifugation for 2 min at 2000 rpm, and stored at –20°C in Eppendorf tubes, until DNA extraction. Nematodes were mechanically disrupted with a pestle before DNA extraction and purification with the DNeasy Blood and Tissue Kit (QIAGEN, Manchester, UK) according to manufacturer's instructions.

Molecular characterisation of *M. enterolobii* isolates was conducted by DNA amplification with the species-specific primers MK7-F (5′-GAT CAG AGG CGG GCG CAT TGC GA-3′) and MK7-R (5′-CGA ACT CGC TCG AAC TCG AC-3′) (Tigano et al., 2010). Each PCR reaction (25 µL) contained: 1X PCR buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5 U Taq DNA polymerase (Bioline, London), and 50 ng DNA. Amplification was carried out in a GeneAmp PCR System 2700 Thermal cycler (Applied BioSystems, Carlsbad, CA, USA) using the following conditions: initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, and a final extension for 10 min at 72°C. PCR products were analysed by electrophoresis in 1% agarose gel stained with Greensafe premium® (Nzytech, Portugal). DNA from selected *M. enterolobii* and *M. hispanica* isolates were also included as *M. enterolobii* DNA positive and negative control, respectively.

Results and discussion

In all the 41 root samples collected were detected RKN, infecting 27 plant species from 17 botanical families (Table 1; Fig. 2). These endoparasitic nematodes were frequently found in *Solanaceae* plants, with 27% of the samples belonging to this botanical family.

Nematode species identification was first based on EST phenotypes and the isolates with unusual EST phenotypes were submitted to an additional molecular characterisation, to confirm the identification.

Forty eight isolates were identified corresponding to six *Meloidogyne* species (Table 1; Figs. 3 and 4). However, small differences in the rate of migration (Rm) of the bands were observed, between gels and previous studies (Esbenshade & Triantaphyllou, 1985; Xu et al., 2004; Maleita et al., 2012a; Carneiro et al., 2014) as a consequence of variations in laboratory equipment and electrophoretic conditions. For that reason protein extracts of *M. javanica* were always included in each gel as reference.

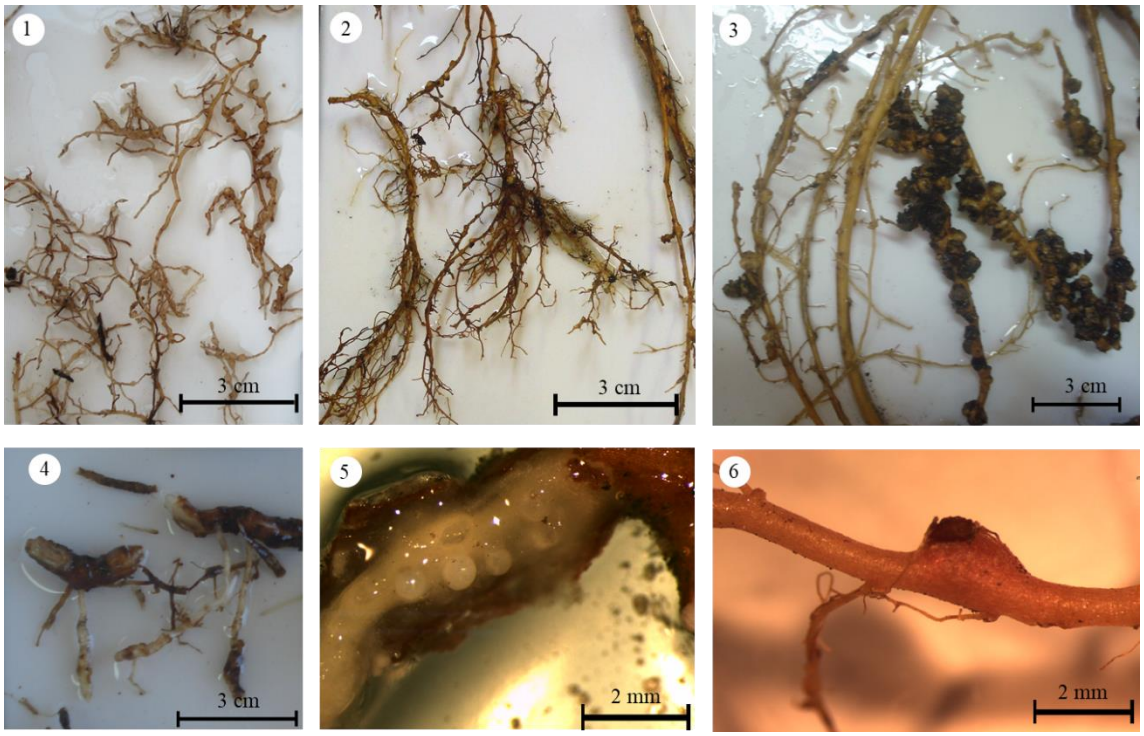

Figure 2. Root-knot nematode infected roots in Coimbra region: 1 – *Actinidia deliciosa* (Isolate A16); 2 – *Prunus persica* (Isolate A5); 3 – *Callistemon* sp. (Isolate C11); 4 – *Cordyline australis* (Isolate B3); 5 – *Yucca gigantea* (Isolate A3); 6 – *Amaranthus powellii* (Isolate C5). For isolate code, see Table 1.

Eleven EST phenotypes were detected. Usually each *Meloidogyne* species is characterised by major bands, which are always present; although EST phenotypes can display extra minor bands. The EST patterns of *M. enterolobii*, *M. hispanica* and *M. incognita* isolates displayed differences in the number of minor bands between samples. These fainted bands can be related either to intraspecific variability or to the low EST activity, requiring a large amount of young egg-laying females for their detection (Esbenshade & Triantaphyllou, 1985; Carneiro et al., 1996) (Figs. 3 and 4).

Meloidogyne incognita was the most prevalent species (33.3%; 16 isolates out of 48), which is in agreement with the findings of previous studies (Conceição et al., 2009; Esteves et al., 2015), followed by *M. hapla* (25.0%), *M. hispanica* (16.7%), *M. arenaria* (10.4%), *M. enterolobii* (8.3%), and finally *M. luci* (6.4%) (Table 1). In 82.9% of the root samples only one *Meloidogyne* species was identified/sample, but mixtures of RKN species were detected in 7 out of 41: *M. hispanica* and *M. incognita* (3 samples); *M. arenaria* and *M. enterolobii* (2 samples); *M. arenaria* and *M. incognita* (1 sample); and *M. hapla* and *M. incognita* (1 sample).

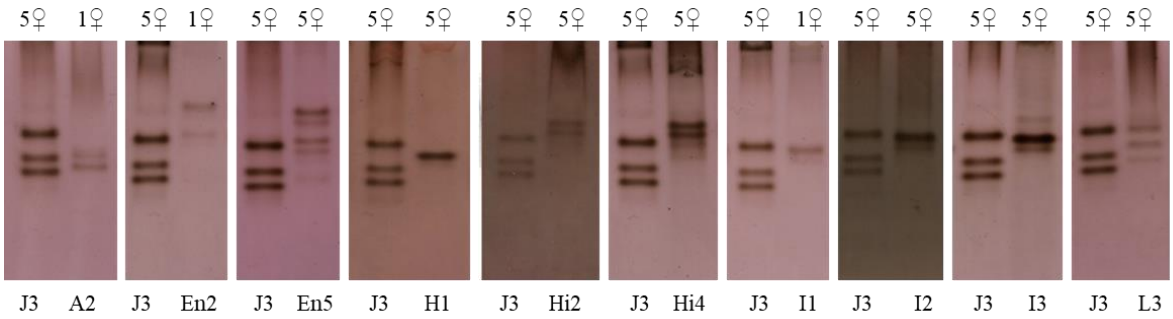

Figure 3. Esterase phenotypes of Portuguese *Meloidogyne* spp. isolates identified in Coimbra region. A2 - *M. arenaria*; En2 and En5 - *M. enterolobii*; H1 - *M. hapla*; Hi2 and Hi4 - *M. hispanica*; I1, I2 and I3 - *M. incognita*; J3 - *M. javanica* (reference isolate); L3 - *M. luci*. Number of females used in each protein homogenate is indicated above.

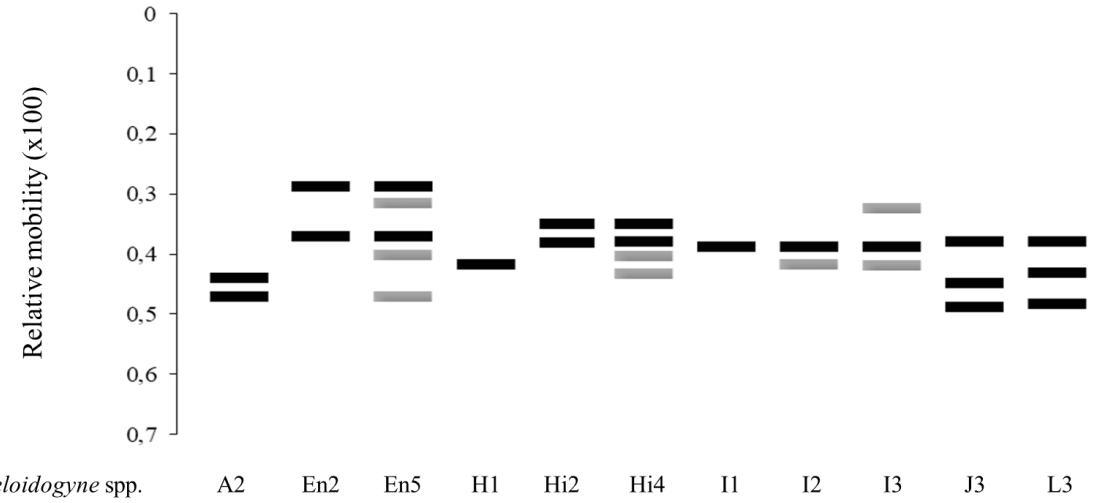

Figure 4. Schematic representation of esterase phenotypes of *Meloidogyne* spp. isolates from Coimbra region. A2 - *M. arenaria*; En2 and En5 - *M. enterolobii*; H1 - *M. hapla*; Hi2 and Hi4 - *M. hispanica*; I1, I2 and I3 - *M. incognita*; J3 - *M. javanica* (reference isolate); L3 - *M. luci*. Black lines correspond to major bands, used to characterise the isolates, and grey lines indicate fainted bands, which can vary with the amount of protein and staining conditions (Carneiro et al., 1996).

Table 1. Root-knot nematodes, *Meloidogyne* spp., isolate code, host plant (species/botanical family), esterase phenotypes and species identification.

Isolate code[1)]	Host plant		Esterase phenotype[2)]	*Meloidogyne* species
	Species	Botanical family		
A1	*Solanum lycopersicum*	*Solanaceae*	I1	*M. incognita*
A2	*Datura stramonium*	*Solanaceae*	I2	*M. incognita*
A3	*Yucca gigantea*	*Asparagaceae*	I1	*M. incognita*
A4	*Passiflora caerulea*	*Passifloraceae*	I1	*M. incognita*
A5	*Prunus persica*	*Rosaceae*	I2	*M. incognita*
A6	*Ficus carica*	*Moraceae*	I2	*M. incognita*
A7	*Rumex crispus*	*Polygonaceae*	I3	*M. incognita*
A8	*Vitis vinifera*	*Vitaceae*	A2	*M. arenaria*
A9	*Brugmansia suaveolens*	*Solanaceae*	H1 + I1	*M. hapla* + *M. incognita*
A10	*Aucuba japonica*	*Garryaceae*	A2 + I1	*M. arenaria* + *M. incognita*
A11	*Chenopodium album*	*Amaranthaceae*	Hi2 + I2	*M. hispanica* + *M. incognita*
A12	*Y. gigantea*	*Asparagaceae*	Hi2 + I1	*M. hispanica* + *M. incognita*
A13	*Lycianthes rantonnei*	*Solanaceae*	Hi4 + I3	*M. hispanica* + *M. incognita*
A14	*B. suaveolens*	*Solanaceae*	H1	*M. hapla*

[1)] Each letter of the sample code corresponds to specific locations (villages and municipalities). A - Coentros, Figueira da Foz; B -Tromelgo, Figueira da Foz; C - Tojeiro, Montemor-o-Velho; D - Catarruchos, Montemor-o-Velho; E - Carapetos, Montemor-o-Velho; F - Santa Cruz (Mata do Choupal), Coimbra; G - Santa Cruz (Jardim da Sereia), Coimbra.

[2)] Esterase phenotypes are designated by a letter suggestive of the nematode species followed by a number corresponding to the number of bands.

Table 1. (Continued) Root-knot nematodes, *Meloidogyne* spp., isolate code, host plant (species/botanical family), esterase phenotypes and species identification.

Isolate code[1)]	Host plant		Esterase phenotype[2)]	*Meloidogyne* species
	Species	Botanical family		
A15	*Melissa officinalis*	*Lamiaceae*	H1	*M. hapla*
A16	*Actinidia deliciosa*	*Actinidiaceae*	H1	*M. hapla*
A17	*S. chenopodioides*	*Solanaceae*	H1	*M. hapla*
A18	*Malva multiflora*	*Malvaceae*	Hi4	*M. hispanica*
A19	*Amaranthus powellii*	*Amaranthaceae*	Hi4	*M. hispanica*
A20	*F. carica*	*Moraceae*	Hi2	*M. hispanica*
B1	*Callistemon* sp.	*Myrtaceae*	A2	*M. arenaria*
B2	*Cordyline australis*	*Asparagaceae*	I2	*M. incognita*
B3	*C. australis*	*Asparagaceae*	L3	*M. luci*
C1	*S. lycopersicum*	*Solanaceae*	L3	*M. luci*
C2	*Oxalis corniculata*	*Oxalidaceae*	L3	*M. luci*
C3	*S. chenopodioides*	*Solanaceae*	Hi4	*M. hispanica*
C4	*B. suaveolens*	*Solanaceae*	Hi2	*M. hispanica*
C5	*A. powellii*	*Amaranthaceae*	I3	*M. incognita*

[1)] Each letter of the sample code corresponds to specific locations (villages and municipalities). A - Coentros, Figueira da Foz; B - Tromelgo, Figueira da Foz; C - Tojeiro, Montemor-o-Velho; D - Catarruchos, Montemor-o-Velho; E - Carapetos, Montemor-o-Velho; F - Santa Cruz (Mata do Choupal), Coimbra; G - Santa Cruz (Jardim da Sereia), Coimbra.

[2)] Esterase phenotypes are designated by a letter suggestive of the nematode species followed by a number corresponding to the number of bands.

Table 1. (Continued) Root-knot nematodes, *Meloidogyne* spp., isolate code, host plant (species/botanical family), esterase phenotypes and species identification.

Isolate code[1)]	Host plant		Esterase phenotype[2)]	*Meloidogyne* species
	Species	Botanical family		
C6	*V. vinifera*	*Vitaceae*	I2	*M. incognita*
C7	*A. deliciosa*	*Actinidiaceae*	H1	*M. hapla*
C8	*B. suaveolens*	*Solanaceae*	H1	*M. hapla*
C9	*A. deliciosa*	*Actinidiaceae*	H1	*M. hapla*
C10	*Salvia involucrata*	*Lamiaceae*	H1	*M. hapla*
C11	*Callistemon* sp.	*Myrtaceae*	A2 + En5	*M. arenaria* + *M. enterolobii*
C12	*Cereus hildmannianus*	*Cactaceae*	A2 + En5/En2	*M. arenaria* + *M. enterolobii*
C13	*Lampranthus* sp.	*Aizoaceaes*	En5/En2	*M. enterolobii*
C14	*Physalis peruviana*	*Solanaceae*	En5/En2	*M. enterolobii*
D1	*A. deliciosa*	*Actinidiaceae*	H1	*M. hapla*
E1	*A. deliciosa*	*Actinidiaceae*	H1	*M. hapla*
F1	*Myriophyllum* sp.	*Haloragaceae*	H1	*M. hapla*
G1	*Solanum mauritianum*	*Solanaceae*	I3	*M. incognita*

[1)] Each letter of the sample code corresponds to specific locations (villages and municipalities). A - Coentros, Figueira da Foz; B - Tromelgo, Figueira da Foz; C - Tojeiro, Montemor-o-Velho; D - Catarruchos, Montemor-o-Velho; E - Carapetos, Montemor-o-Velho; F - Santa Cruz (Mata do Choupal), Coimbra; G - Santa Cruz (Jardim da Sereia), Coimbra.

[2)] Esterase phenotypes are designated by a letter suggestive of the nematode species followed by a number corresponding to the number of bands.

Meloidogyne incognita isolates exhibited three different EST phenotypes: I1 (Rm: 0.39), I2 (Rm: 0.39; 0.42) and I3 (Rm: 0.33; 0.39; 0.42) (Figs. 3 and 4; Table 1) that can be associated with intraspecific variability among the isolates. EST I1 and EST I2 phenotypes were already reported by Pais &Abrantes (1989) and Carneiro et al. (1996, 2000). EST I3 phenotype was never record for *M. incognita*, but it seems to be not stable depending on the host plants, since several attempts to purify this phenotype in tomato have resulted in an I2 EST phenotype. *Meloidogyne incognita* is a major pest of vegetables, particularly solanaceous crops, such as tomato, pepper (*Capsicum annum* L.) and potato, *S. tuberosum* L., due to its high level of pathogenicity (Netscher & Sikora, 1990; Conceição et al., 2009).

Phenotype EST H1 (Rm: 0.42), corresponding to *M. hapla*, was found in all kiwi, *Actinidia deliciosa* Liang et Ferguson, 1984, root samples, being the second most abundant phenotype detected (Figs. 3 and 4; Table 1). This species is the most common RKN species in temperate regions (Moens et al., 2009). Kiwi is considered highly susceptible to RKN and *M. hapla* is one of the most frequent RKN species parasitising kiwi plants (Knight, 2001; Tao et al., 2017).

All the isolates of *M. arenaria* identified in this study showed the single EST phenotype A2 (Rm: 0.44; 0.47) and were found parasitising *Vitis vinifera* and four ornamental plants, *Aucuba japonica, Cereus hildmannianus* and 2 different plants of the genus *Callistemon* (Figs. 3 and 4; Table 1). This RKN species is one of the four most important *Meloidogyne* spp. being responsible for significant yield losses in horticulture in tropical and subtropical regions (Hunt & Handoo, 2009).

Phenotypes EST Hi2 (Rm: 0.35; 0.38) and Hi4 (Rm: 0.35; 0.38; 0.41; 0.44) (Figs. 3 and 4; Table 1) occurred in nine isolates associated with a wide range of host plants and were attributed to *M. hispanica*. In Portugal, *M. hispanica* was previously found parasitising carnation (*Dianthus caryophyllus* L.), corn (*Zea mays* L.), fig-tree (*Ficus carica* L.), potato and tomato (Santos et al., 1992; Abrantes et al., 2008; Landa et al., 2008; Conceição et al., 2009). This nematode species considered an emerging RKN species with a wide range of host plants and a great aggressiveness to tomato cultivars, can overcome tomato *Mi*-mediated resistance, which limit the use of either crop rotation or tomato resistant cultivars, and can spread northwards in Europe, which emphasizes its importance (Maleita et al., 2011, 2012b,c).

The EST L3 (Rm: 0.38; 0.43; 0.49), attributed to *M. luci*, was observed in three isolates (Figs. 3-5; Table 1) and is the most useful characteristic to differentiate this

species from other closely related, such as *M. ethiopica* (Rm: 0.36; 0.43; 0.49) (Carneiro et al., 2014; Maleita et al., 2018) (Fig. 5). This tropical RKN species was already detected in several European countries and is causing increasing concern, since it may enter and become established in more countries, with high negative impact in several crops (EPPO, 2017). *Meloidogyne luci* was reported for the first time in Portugal, in 2018, parasitising potato roots in Coimbra region (Maleita et al., 2018). In the present study, this RKN species was recorded in two new locations (Tromelgo, Figueira da Foz; and Tojeiro, Montemor-o-Velho), which can be an indication that this nematode species could already be established and widespread in Portugal. Furthermore, it is reported for the first time as parasites of plants of the genera *Cordyline* and *Oxalis*.

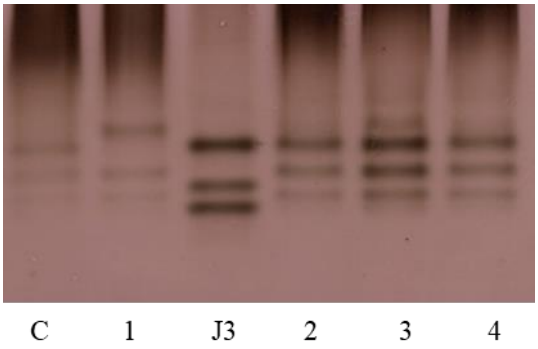

Figure 5. Esterase phenotypes of protein homogenates from five egg-laying females of *Meloidogyne* species isolates. C – *M. luci* (positive control); 1 – *M. ethiopica* isolate from Brazil; J3 – *M. javanica* (reference isolate); 2, 3 and 4 – *M. luci* isolates (Isolates B3, C1 and C2, respectively); For isolate code, see Table 1.

The unusual EST En2 (Rm: 0.29; 0.37) and En5 (Rm: 0.29; 0.32; 0.37; 0.40; 0.47) phenotypes, detected for the first time in Portugal, were identified as belonging to *M. enterolobii* (Figs. 3, 4 and 6). In some samples an unusual much fainted band (Rm: 0.47) was detected, being present even in homogenates from single females (Fig. 3). These phenotypes were detected in isolates parasitising four nearby plants (*Callistemon* sp., *Cereus hildmannianus*, *Lampranthus* sp. and *Physalis peruviana*), either alone or in mixed populations with *M. arenaria* (Table 1; Fig. 6). This nematode has been previously found associated with plants of the genus *Callistemon* in Florida, USA (Brito et al., 2010). Nonetheless, this is the first report of *Physalis peruviana*, *Cereus hildmannianus* and *Lampranthus* as hosts of *M. enterolobii*. Typically, all roots parasitised by this RKN species presented large and irregular galls (Fig. 2.3). Species-specific primers set MK7-

F/MK7-R were used to validate the identification of these four isolates. A unique size fragment of 520 bp was obtained from all *M. enterolobii* isolates, as expected (Fig. 7) (Tigano et al., 2010).

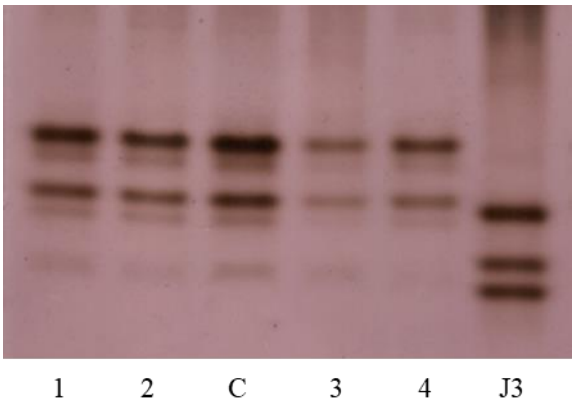

Figure 6. Esterase phenotypes of protein homogenates from five young egg-laying females of *Meloidogyne enterolobii.* 1 – Isolate C13; 2 – Isolate C11; C – *M. enterolobii* (positive control); 3 – Isolate C14; 4 – Isolate C12; J3 – *M. javanica* (reference isolate). For isolate code, see Table 1.

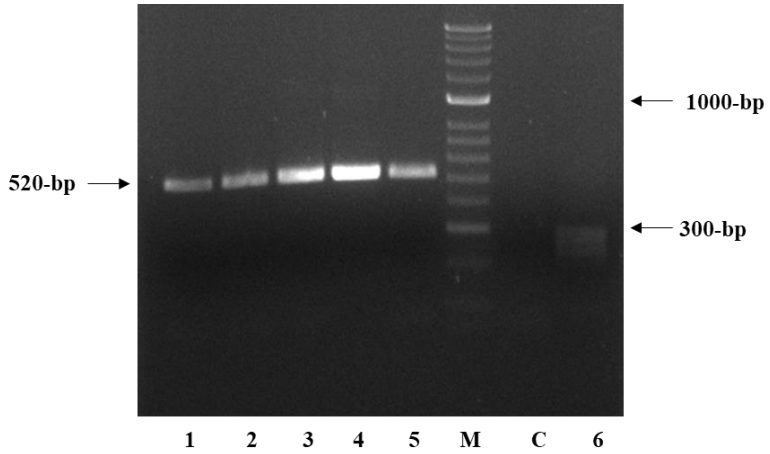

Figure 7. PCR products of *Meloidogyne enterolobii* isolates (≈520bp) amplified with the species-specific primers set MK7-F/MK7-R (Tigano et al., 2010). 1 – Isolate C14; 2 – Isolate C11; 3 – Isolate C12; 4 – Isolate C13; 5 – *M. enterolobii* (positive control); 6 – *M. hispanica* (DNA negative control)*;* M – DNA marker (HyperLadder II; Bioline); C – negative control (without DNA). For isolate code, see Table 1.

Meloidogyne enterolobii is a highly pathogenic parasite able to overcome several sources of resistance against RKN, and represent a global threat to food production systems (Castagnone-Sereno, 2012). This pest is considered to be one of the most important RKN species regulated as a quarantine species in the EPPO region (EPPO/PQR, 2017). This nematode species has increasingly been reported on a wide range of host plants from different parts of the world, including several African countries, Central and South America, China, Vietnam, USA (Florida), France and Switzerland (CABI, 2017).

Meloidogyne spp. J2 can move only a few centimetres in the soil (Prot & Van Gundy, 1981), however they can be disseminated for long distances through transport of infected plant material or infested soil adhering to machinery, tools or even footwear. The first detection of *M. enterolobii* in Portugal from ornamental plants, lead us to believe that this species may have been accidentally introduced from imported plant material. In the Netherlands, this pathogen has intercepted several times in many different imported plant material from different parts of the world, where this species was already detected (EPPO, 2008). Introduction of a non-native plant-parasitic nematode into a new habitat may have a profound effect on agricultural productivity and natural ecosystem function (Vovlas et al., 2013). Generally, once RKN are established, it is very hard to control or eradicate. Thus, more research is needed in order to understand the full extent of the RKN distribution in Portugal, not only of *M. enterolobii*, but also of *M. luci.*

In Portugal, RKN surveys have been more focused on specific crops, such as potato, fig-trees and olives (Pais & Abrantes, 1989; Abrantes & Santos, 1991; Abrantes et al., 2008; Conceição et al., 2009; Esteves et al., 2015). According to Manzanilla-Lopez & Starr (2009), these systems are quite homogeneous resulting in the dominance of just one RKN species. This is the first study, in Portugal, on ornamental and weeds, which may serve as a source of RKN inoculum for plants of economic importance (Castillo et al., 2008). Despite the small number of sampling sites, a great diversity of *Meloidogyne* species were identified as result of the wide range of host plants collected.

Subsistence farms and gardens, unlike intensive agriculture where monoculture is the model, present a great diversity of suitable host plants surrounded by non-host plants. These systems are comparable with insular environment, where any area of habitat suitable for a specific species is surrounded by an area of unsuitable habitat, which tends to restrict the dispersal range of species. Cyclic disturbance like crop rotation or transplantation of rooted plantlets promotes heterogeneity of patches and maintenance of

this diversity, preventing a strong competitor species from dominating the entire system. On the contrary, in industrial farming species initially accumulate through succession (by dispersal and establishment) are later lost through competition (White & Jentsch, 2001; Sheil & Burslem, 2003).

Root-knot nematodes are poikilothermic animals, and therefore soil temperature affects its population dynamics. In field conditions, the populations generally reach a maximum in the fall and are at a minimum in the spring (Starr & Jegger, 1985). These nematodes endure the cold winter temperature in order to survive until spring, when soil temperatures are more suitable and nematode populations start to build-up. However, tolerance to cold conditions differs among *Meloidogyne* species (Madulu & Trudgill, 1994; Trudgill et al., 2005). For instance, *M. hapla* eggs survived at -2°C for 12 days, whereas *M. javanica* eggs, a RKN species limited to areas where the soil does not freeze during winter, does not survive when exposed to the same conditions (Daulton & Nusbaum, 1961). Dávila-Negrón & Dickson (2013) sort *Meloidogyne* spp. in descending order of cold tolerance as follows: *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. arenaria* and *M. javanica*. This data fits with our results, *M. javanica* was not found and *M. arenaria* was detected in a low number probably due to their low resilience to cold temperatures, regularly recorded in Portugal. *Meloidogyne hapla* was found mostly in perennial plants, like kiwi and *Brugmansia suaveolens*, where it can outcompetes the most common species during winter periods. On the other hand, among the RKN, *M. incognita* has the largest range of known hosts that, combined with the Portuguese environmental conditions, explain the high prevalence of this nematode species. This RKN species is able to infect a large range of host plants, being probably the most common and the most damaging RKN species, causing serious problems on several economically important agricultural crops (Trudgill & Blok, 2001).

Chapter 2

Detection of *Mi* gene and reproduction of *Meloidogyne luci* on tomato genotypes

Introduction

Tomato, *Solanum lycopersicum* L., is the second most important vegetable crop next to potato, and is by far the most popular home garden vegetable in the world. According to Food and Agriculture Organization of the United Nations, the worldwide production of tomato, in 2016, was estimated around 177 million ton (FAO, 2016). The top 5 largest producers are China, European Union, India, United States of America and Turkey, accounting for 70% of global production. In the European Union, Portugal is the third biggest tomato producer, with 1.69 million tonnes, accounting for 9.4% of total production behind Italy (33.3%) and Spain (29.1%) (Eurostat, 2016).

Solanum lycopersicum is susceptible to many pests and diseases, responsible for significant quality and quantity losses on tomato production. Root-knot nematodes (RKN), *Meloidogyne* Göldi 1892, are considered the most important and widely distributed group of plant-parasitic nematodes (PPN), causing serious yield losses in tomato crop worldwide (Taylor & Sasser, 1978; Jones et al., 2013). Their feeding activity inside the roots induce the formation of giant cells and cell hypertrophy, resulting in the formation of root galls, which hampered the main functions of roots. These nematodes drain the plant's photosynthates and nutrients to support its development and reproduction. RKN damage results in poor growth and reduced resistance to water stress and high temperatures and plants become more susceptible to attack by other pathogens (Williamson & Hussey, 1996).

In 2014, a new RKN species, *M. luci* was described from lavender roots (*Lavandula spica* L.) collected in Rio Grande do Sul, Brazil (Carneiro et al., 2014). This *Meloidogyne* species was also reported in Argentine, Bolivia, Chile, Ecuador, Guatemala, and Iran (reviewed in Carneiro et al., 2014; Janssen et al., 2016). Because of its morphological resemblance to *M. ethiopica* and similar esterase phenotype, *M. luci* might have been misidentified as *M. ethiopica* in a number of surveys. In 2017, all populations previously identified as *M. ethiopica* in Europe (Greece, Italy and Slovenia) and Turkey were reclassified as *M. luci*, using biochemical and molecular analyses (Stare et al., 2017b). *Meloidogyne luci* was initially detected in Portugal parasitising potato, *S. tuberosum* L., roots from Coimbra region (Maleita et al., 2018). More recently, this pathogen was detected again in Portugal, associated with *Oxalis corniculata* L., *Cordyline* sp. and *S. lycopersicum* roots indicating that might be already established, representing a potential threat to tomato production in Portugal and other parts of the world (Chapter 1).

Traditionally, RKN management have relied on the application of chemical nematicides, mainly fumigants. However, the negative impact of these chemical compounds on the environment and human health has stimulated the search for new harmless alternatives of RKN management (Martin, 2003; McSorley 2011). Plant resistance is an economically and environment-friendly strategy able to suppress or delay invasion by a potential pathogen (Roberts, 2002). Moreover, plant resistance is compatible with organic farming production methods and the demand for these products has been increasing in recent years. In Solanaceous species, like tomato, resistance is achieved by introgression of several *R*-genes which improve their defences against RKN. Nevertheless, its efficacy depends on the temperature, nematode isolate and inoculum level (Williamson & Kumar, 2006; Barbary et al. 2015).

RKN resistance in tomato is conferred by a multigene locus designated *Mi-1* (Roberts, 1992), containing several genes and pseudogenes. Among them only *Mi-1.2* is responsible for resistance to the three most common warm-climate *Meloidogyne* species (*M. arenaria, M. incognita,* and *M. javanica*) (Milligan et al., 1998), but not immunity since a handful of juveniles are able to infect roots and reproduce (Talavera et al., 2009). Several studies also demonstrated that *Mi-1.2* gene is more effective on homozygous (MiMi) genotypes than on heterozygous (Mimi) genotypes, indicating a dosage effect of the gene (Tzortzakakis et al., 1998; Jacquet et al., 2005; Maleita et al., 2011). Therefore, the *Mi-1.2* gene is widely used to RKN management in tomato production areas, allowing farmers to grow resistant cultivars in infected fields with minimum yield and quality losses (Rich & Olson, 1999; Sorribas et al. 2005).

Despite its effectiveness, *Mi*-mediated resistance has some constraints, breaking down when soil temperatures exceed 28°C (Dropkin, 1969; Ammati et al. 1986). In addition, the use of single major resistance genes combined with the possibility of emergence of virulent nematode populations, capable of overcoming these *R*-genes, may constitute an important limitation to this control strategy (Ornat et al., 2001; Jacquet et al., 2005; Maleita et al., 2011; Tzortzakakis et al., 2014). Sustainable and integrated management of *R*-genes is vital to maintain their effectiveness and durability preventing the selection of virulent RKN populations. Incorporation of different resistant genes in rotation practices and combination of different resistance genes in one genotype has already proved to be effective strategies (Djian-Caporalino et al., 2014).

The main goal of the current study was to evaluate whether the *Mi-1.2* gene confers resistance to *M. luci* and compare with *M. ethiopica*, in order to define nematode

management strategies to be used in infested tomato production areas. The specific objectives were: i) to screen for the presence of RKN resistance *Mi*-1.2 gene (referred from now as *Mi* gene) in 27 tomato genotypes by DNA amplification, using Mi23 markers; and ii) to evaluate the ability of *M. luci* and *M. ethiopica* to reproduce on tomato genotypes.

Materials and methods

NEMATODE ISOLATES

Two *Meloidogyne* spp. isolates were included in this study: a Portuguese *M. luci* isolate obtained from a potato field in Coimbra (Maleita et al., 2018), and a Brazilian *M. ethiopica* isolate obtained from infected kiwi roots collected in Rio Grande do Sul (Carneiro et al., 2004). *Meloidogyne ethiopica* isolate was included for comparison of *Meloidogyne* spp. ability to reproduce on tomato genotypes. *Meloidogyne* isolates identification was confirmed by biochemical electrophoretic analysis of non-specific esterase phenotype (Chapter 1, Fig. 5). The RKN isolates were maintained on tomato plants genotype Coração-de-Boi, in a temperature-controlled growth chamber (25±2°C) with daily 12 h light period, in the NEMATO-lab at CFE.

ANALYSIS OF TOMATO DNA FOR THE MI23 ALLELES

Plant material

To assess the presence and/or influence of the homozygous or heterozygous state of *Mi* gene on nematode reproduction, 27 commercially available tomato genotypes (Table 1) were grown from seeds, in a growth chamber at 23-25°C in Petri dishes with filter paper soaked in distilled water. After three days, seedlings were individually transplanted to 5 cm diam. plastic pots filled with a mixture of sterilised sandy loam soil, sand and substrate (1:1:2).

Table 1. Origin and resistances of tomato genotypes used in this study.

Genotype	Seed company	Resistance
Addalyn F1	Hazera	Fol:1-2, ToMV, TSWV, TYLCV, Vd
Agora F1	Vilmorim	Fol:0-1, ToMV, S, V:0, N(MaMiMj)
Amaral	Enza Zaden	Ff:A-E, Fol:0-1, For, Lt, N(MaMiMj) ToMV:0-2, TSWV, Va:0, Vd:0,
Anairis F1	Seminis	Fol:0-1, N(MaMiMj), ToMV, TSWV, Va, Vd,
Basileia	Seminis	Fol:0-1, N(MaMiMj), TSWV, TYLCV, Va:0, Vd:0
Belle	Enza Zaden	Fol:0-1, ToMV:0-2, Va:0, Vd:0
Bermello RZ F1	Rijk Zwaan	Ff:A-E, Fol:0-1, N(MaMiMj), Sbl, ToMV, TYLCV:0-2, TSWV, Va:0, Vd:0
Clemente	Semillas Fitó	Ff:A-E, Fol:0-1, ToMV, TSWV, TYLCV, Va, Vd
Coração-de-Boi	Casa César Santos	-
Eshkol	Seminis	Ff:A-C, Fol:0-1, For, N(MaMiMj), ToMV:0-2, TSWV, TYLCV, Va:0, Vd:0
Matias	Seminis	Ff:A-E, Fol:0-1, N(MaMiMj), ToMV:0-2, Va:0, Vd:0
Matissimo	Seminis	Cf, N(MaMiMj), TSWV, TYLCV
Monita	TGSC	-
Montfavet 63/5 F1	Vilmorim	F2, ToMV

Resistance information withdrawn from product catalogues provided by the seed companies: *Cladosporium* sp. (Cf); *Fulvia fulva* (Ff) ; *F. fulva* races A, B and C (Ff:A-C); *F. fulva* races B, C, D (Ff:B-D); *F. fulva*races A, B, C, D and E (Ff:A-E); *Fusariu*m *oxysporum* f. sp. *lycopersici* races 0 and 1 (Fol:0-1); *F. oxysporum* f. sp. *lycopersici* races 1 and 2 (Fol 1–2); *F. oxysporum* f. sp. *radicis-lycopersici* (For); *F. oxysporum* race 2 (F2); Nematodes, *Meloidogyne arenaria*, *M. incognita*, *M. javanica* [N(MaMiMj)]; Powdery mildew (Lt); *Pseudomonas syringae* pv. (Pst); *Stemphylium botryosum* f. sp. *lycopersici* (Sbl); *Stemphylium* spp. (S); *S. solani* (Ss); Tomato mosaic virus (ToMV); Tomato spotted wilt (TotV); Tomato spotted wilt virus (TSWV); Tomato yellow leaf curl virus (TYLCV); *Verticillium albo-atrum* (Va); *V. albo-atrum* race 0 (Va:0); *V. dahliae* (Vd); *V. dahliae* race 0 (Vd:0); *Verticillium* sp. (V); TGSC (Tomato Genetics Stock Center, University of California, Davis, CA, USA)

Table 1. (Continued) Origin and resistances of tomato genotypes used in this study.

Genotype	Seed company	Resistance
Paipai	Enza Zaden	Fol:0-1, N(MaMiMj), ToMV, TSWV, Va, Vd
Reconquista	De Ruiter Seeds	Fol:0-1, Lt, N(MaMiMj), Pst, S, ToMV, TSWV, Va
Roma VF	Vilmorim	V:0, Fol:0
Sahel	Syngenta	Fol:1-2,For, N(MaMiMj), S, Ss, ToMV:0-2,V
San Marzano 2	Vilmorim	-
San Pedro	Vilmorim	-
SV7886TH	Seminis	Ff:B-D, Fol: 0-1, ToMV:0-2, TYLCV, TSWV, Va:0, Vd:0
SV1917	Seminis	Fol:0-1, Lt, N(MaMiMj), ToMV:0-2, TSWV, TYLCV, Va:0, Vd:0
Tisey F1	Seminis	Fol:0-1, N(MaMiMj), ToMV, ToTV, TYLCV, Va, Vd
Valoasis RZ F1	Rijk Zwaan	Fol:0-1, N(MaMiMj), ToMV:0-2, TSWV, Va:0, Vd:0
Vimeiro F1	Semillas Fitó	Fol:0-1, N(MaMiMj), ToMV:0-2, TYLCV, Va, Vd
Visconti	Clause	Fol:0-1, ToMV, TSWV, TYLCV, Va, Vd,
Zinac	Seminis	Fol:0-1, N(MaMiMj), ToMV, Va, Vd

Resistance information withdrawn from product catalogues provided by the seed companies: *Cladosporium* sp. (Cf); *Fulvia fulva* (Ff) ; *F. fulva* races A, B and C (Ff:A-C); *F. fulva* races B, C, D (Ff:B-D); *F. fulva*races A, B, C, D and E (Ff:A-E); *Fusariu*m *oxysporum* f. sp. *lycopersici* races 0 and 1 (Fol:0-1);.*F. oxysporum* f. sp. *lycopersici* races 1 and 2 (Fol 1–2); *F. oxysporum* f. sp. *radicis-lycopersici* (For); *F. oxysporum* race 2 (F2); Nematodes, *Meloidogyne arenaria*, *M. incognita*, *M. javanica* [N(MaMiMj)]; Powdery mildew (Lt); *Pseudomonas syringae* pv. (Pst); *Stemphylium botryosum* f. sp. *lycopersici* (Sbl); *Stemphylium* spp. (S); *S. solani* (Ss); Tomato mosaic virus (ToMV); Tomato spotted wilt (TotV); Tomato spotted wilt virus (TSWV); Tomato yellow leaf curl virus (TYLCV); *Verticillium albo-atrum* (Va); *V. albo-atrum* race 0 (Va:0); *V. dahliae* (Vd); *V. dahliae* race 0 (Vd:0); *Verticillium* sp. (V); TGSC (Tomato Genetics Stock Center, University of California, Davis, CA, USA)

DNA extraction

Plant DNA extraction was performed using a classical method as described by Edwards et al. (1991). However, due to the difficulty of amplification of some samples, a NucleoSpin® Plant II (Macherey-Nagel) kit was used for six samples (Agora F1; San Marzano 2; Matias; Valoasis RZ F1; Roma VF; and SV7886TH), with some modifications: young leaves were smash in liquid nitrogen, and the DNA was resuspended in distilled water at 65˚C. The genomic DNA was quantified by using Nanodrop 2000 C spectrophotometer (Thermo Scientific) and stored at -20ºC until PCR.

Detection of *Mi* gene presence

DNA amplification was done using Mi23 marker to assess the present/absence of the *Mi* gene on each genotype. The primers used were: Mi23F (5'-TGG AAA AAT GTT GAA TTT CTT TTG-3') and Mi23R (5'-GCA TAC TAT ATG GCT TGT TTA CCC-3'). DNA amplification was performed as described by Seah et al. (2007). DNA concentration was adjusted to approximately 25 ng/µL. PCR was carried out in 25µL reactions containing 2.5 µL 10X Taq buffer, 1 µL 2 mM $MgCl_2$, 1.25 µL 5 mM dNTPs, 0.1 µL Taq DNA polymerase (Bioline, London), 2.5 µL of each primer at 10 µM, and 5 µL of DNA. Amplifications were conducted in a GeneAmp PCR System 2700 Thermalcycler (Applied BioSystems, Carlsbad, CA, USA) as described in Maleita et al. (2011). Amplified products were visualised under UV illumination after electrophoresis on 1.5% agarose gels stained with Greensafe premium® (Nzytech, Portugal).

PATHOGENICITY ASSAYS

Reproduction of *M. luci* isolate was assessed on 27 commercial genotypes (Table 1) and compared with *M. ethiopica*. Three-week old tomato seedlings were transplanted, one/pot, to 10 cm diam. pots (500 cm^3) filled with sterilised soil, sand and substrate (1:1:1). The inoculum was obtained by extraction of eggs from infected tomato roots with 0.5% sodium hypochlorite (NaOCL) solution (Hussey & Barker, 1973). Five plants from each tomato genotype were inoculated with 5000 eggs (initial population density, Pi) of *M. luci* or *M. ethiopica* isolates. The pots were arranged in a completely randomised design, maintained in a temperature controlled growth chamber at 25±2°C, with a 12 h photoperiod, and ±60% relative humidity, and watered at each two days. Tomato

genotype Coração-de-Boi was used as a positive control. Non-inoculated plants of each genotype were included to assess plant development and soil sterility.

Sixty days after inoculation, the plants were uprooted and the root systems washed carefully. The number of galls/plant was recorded, under a stereoscopic microscope, and categorised using a 0–5 scale (0=no galls, 1=1-2, 2=3-10, 3=11-30, 4=31-100, 5≥100 galls) (Taylor & Sasser, 1978). Eggs were extracted from each plant with 0.75-1% NaOCl solution (Hussey & Barker, 1973), counted to determine the final population density (Pf) and the reproduction factor (Rf=Pf/Pi) was calculated. Host suitability was assessed on the basis of root gall index (GI), an indicator of plant damage, and the reproduction factor (Rf), an indicator of nematode host efficiency. Plants with GI≤2 and Rf≤1 were considered resistant, GI>2 and Rf≤1 resistant/hypersensitive, GI>2 and Rf>1 susceptible and with GI≤2 and Rf>1 classified as tolerant (Sasser et al., 1984; Huang, 1985).

DATA ANALYSIS

Data (Rf) on host status of tomato genotypes were checked for evidence of a normal distribution and variance homogeneity using the Kolmogorov-Smirnov and Levene's tests, respectively. These two assumptions of analysis of variance (ANOVA) were violated and the non-parametric Kruskal-Wallis test was therefore performed. Statistically significant differences between *M. luci* and *M. ethiopica* Rf, for each tomato genotype, were also determined by *t*-test ($P<0.05$). Statistical analysis of the data was performed using Statsoft Statistica version 7 for Windows.

Results

ANALYSIS OF TOMATO DNA FOR THE MI23 ALLELES

The Mi23 amplified products, using DNA from all tomato genotypes, resulted in one band of approximately 380 bp for homozygous resistant genotypes (MiMi), or 430 bp for tomato genotypes without the *Mi* gene (mimi). The heterozygous genotypes (Mimi) displayed two bands of 430 bp and 380 bp (Fig. 1). Our results indicate that two out of 27 tomato genotypes were found homozygous for *Mi* gene, 13 heterozygous and in the remaining 12 genotypes the *Mi* gene was absent (Fig. 1).

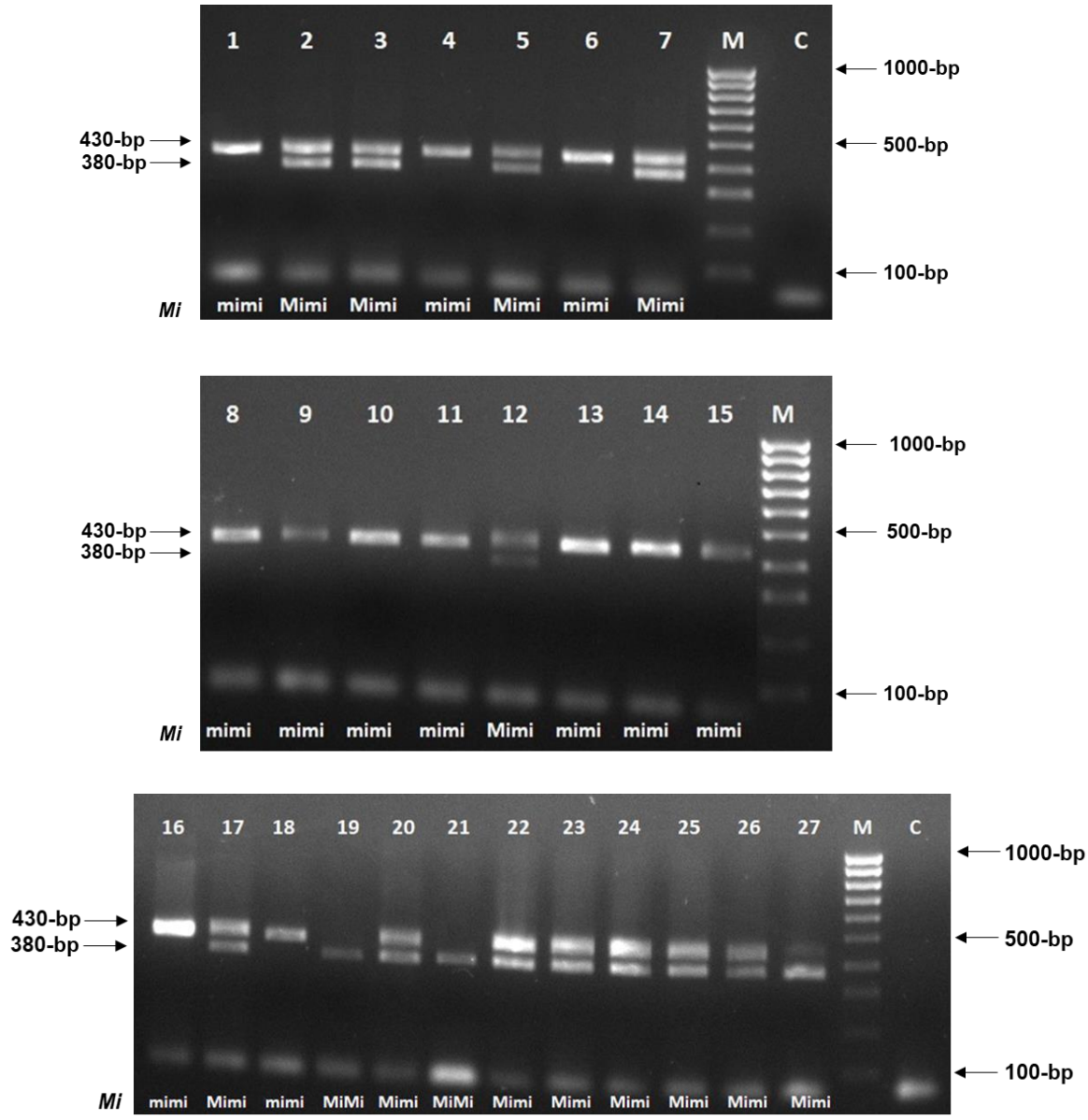

Figure 1. PCR products of tomato material amplified using the Mi23 marker. 1 - San Marzano 2; 2 - SV1917; 3 - Matias; 4 - SV7886TH; 5 - Valoasis RZ F1; 6 - Roma VF; 7 - Agora F1; 8 - San Pedro; 9 - Basileia; 10 - Clemente; 11 - Addalyn F1; 12 - Bermello RZ F1; 13 - Montfavet 63/5 F1; 14 - Coração-de-Boi; 15 - Tisey F1; 16 - Visconti; 17 - Sahel; 18 - Belle ; 19 - Monita; 20 - Eshkol ; 21 - Reconquista; 22 - Matissimo; 23 - Zinac; 24 - Vimeiro F1; 25 - Paipai; 26 - Anairis F1; 27 - Amaral; M - DNA marker (HyperLadder IV; Bioline); C - negative control (without DNA).

PATHOGENICITY ASSAYS

A total of 54 nematode isolate-tomato genotype combinations were evaluated. For each tomato genotype–nematode interaction, five plants were analysed to assess the ability of *M. luci* and *M. ethiopica* to reproduce on tomato genotypes and to evaluate the impact of allelic condition on reproduction of the *Meloidogyne* spp. isolates. For the genotype Monita, tested with *M. ethiopica*, only four plants were analysed (Table 2).

As expected, the tomato genotype Coração-de-Boi, used to maintain the *Meloidogyne* species in the lab and included in the assay as positive control, was highly susceptible with Rf=46.96 and 35.35, respectively, for *M. luci* and *M. ethiopica*, confirming the viability of the inoculum used in the experiment (Table 2).

Tomato genotypes varied in their response either when inoculated with *M. luci* or *M. ethiopica*. *Meloidogyne luci* reproduced (Rf>1) on 16 out of the 27 genotypes, being 15 classified as susceptible (2.38≤Rf≤99.73; GI≥3) and one as tolerant (Monita; Rf=1.16; GI=2) (Table 2). On the other hand, *M. ethiopica* reproduced on 12 genotypes (1.05≤Rf≤63.64). San Marzano 2 and San Pedro were the most susceptible genotypes to *M. luci* (Rf=99.73) and *M. ethiopica* (Rf=63.64), respectively. Genotypes Matissimo, Basileia and Visconti, classified as susceptible to *M. luci* (2.43≤Rf≤4.22; GI≥4) were considered resistant/hypersensitive to *M. ethiopica* (0.49≤Rf≤0.94; GI≥4). Genotypes Bermello RZ F1 and Tisey F1 were classified as resistant/hypersensitive, to both *Meloidogyne* species; nematode induced gall formation (GI≥3) but a small number of eggs were recovered (Rf=0.45 and 0.61 for *M. luci* and *M. ethiopica*, respectively, for Tisey F1; Rf=0.66 and 0.03, respectively, for Bermello Rz F1). Nine tomato genotypes were resistant to *M. luci* with Rf values varying from 0.00 (SV1917 and Reconquista) to 0.14 (Vimeiro F1) (Table 2). From these, eight were also resistant to *M. ethiopica* and one (Eshkol) classified as resistant/hypersensitive (Rf=0.36; GI=3). Genotype Monita was resistant to *M. ethiopica* (Rf=0.07; GI=1) (Table 2).

Overall, *M. luci* showed higher Rf values in comparison with *M. ethiopica*, becoming significant for tomato genotypes San Marzano 2, Coração-de-Boi, Addalyn F1, Montfavet 63/5 F1, Belle, Clemente, Matias, Matissimo, Basileia and Visconti (Table 2). Although *M. ethiopica* tended to present lower Rf values, GI was generally greater in genotypes carrying the *Mi* gene (Table 2).

Table 2. Gall index (GI), reproduction factor (Rf) and host status of 27 tomato, *Solanum lycopersicum* L., genotypes to *Meloidogyne luci* and *M. ethiopica*,60 days after inoculation with 5000 eggs/plant and respective Mi23 profile.

Genotype	Mi23 profile	*M. luci*			*M. ethiopica*		
		GI[1)]	Rf[2)]	Host status[3)]	GI[1)]	Rf[2)]	Host status[3)]
San Marzano 2	mimi	5	99.73 a-e∗	S	5	52.90a-c	S
San Pedro	mimi	5	57.42 a-f	S	5	63.64 a-c	S
Coração-de-Boi[4)]	mimi	5	46.96 a-g∗	S	5	35.35 a-d	S
Addalyn F1	mimi	5	45.47 a-h∗	S	5	28.99 a-f	S
Montfavet 63/5 F1	mimi	5	44.91 a-j∗	S	5	28.25 a-f	S
Belle	mimi	5	43.97 a-i∗	S	5	22.98 a-f	S
Clemente	mimi	5	42.15 a-j∗	S	5	23.24 a-f	S
Roma VF	mimi	5	38.42 a-j	S	5	37.87 a-e	S
Zinac	Mimi	4	7.2 a-j	S	4	8.36 a-f	S
Matias	Mimi	3	5.13 a-j∗	S	4	1.32 a-f	S
Matissimo	Mimi	4	4.22 a-j∗	S	4	0.94 a-f	RH
SV7886TH	mimi	4	2.67 a-j	S	5	1.88 a-f	S
Basileia	mimi	5	2.51 a-j∗	S	5	0.81 a-f	RH

[1)] GI (0-5): 0=no galls, 1=1-2, 2=3-10, 3=11-30, 4=31-100, 5≥100 galls/root system.

[2)] Rf=final population density/initial population density. Data are means of five replicates, except genotype Monita (four replicates) tested with *M. ethiopica*. Means in this column followed by the same combination of letters do not differ significantly at $P>0.05$, according to the Kruskal-Wallis test. ∗ Rf of *M. luci* differs significantly at $P>0.05$, from *M. ethiopica*, according to the *t*-test.

[3)] Host status categories: S=susceptible (GI>2 and Rf>1), RH=resistant/hypersensitive (GI>2 and Rf≤1), T=tolerant (GI≤2 and Rf>1), R=resistant (GI≤2 and Rf≤1) (Sasser et al., 1984).

[4)] Tomato genotype used as a control.

Table 2.(Continued) Gall index (GI), reproduction factor (Rf) and host status of 27 tomato, *Solanum lycopersicum* L., genotypes to *Meloidogyne luci* and *M. ethiopica*,60 days after inoculation with 5000 eggs/plant and respective Mi23 profile.

Genotype	Mi23 profile	*M. luci*			*M. ethiopica*		
		GI[1)]	Rf[2)]	Host status[3)]	GI[1)]	Rf[2)]	Host status[3)]
Visconti	mimi	4	2.43 a-j∗	S	5	0.49 a-f	RH
Anairis F1	Mimi	4	2.38 a-j	S	3	1.05 a-f	S
Monita	MiMi	2	1.16 a-j	T	1	0.07 a-f	R
Bermello RZ F1	Mimi	4	0.66 a-j	SH	4	0.03 c-f	RH
Tisey F1	mimi	3	0.45 a-j	SH	4	0.61 a-f	RH
Vimeiro F1	Mimi	2	0.14 a-j	R	2	0.07 a-f	R
Paipai	Mimi	1	0.1 b-j	R	2	0.02 d-f	R
Eshkol	Mimi	1	0.03 d-j	R	3	0.36 a-f	RH
Sahel	Mimi	1	0.03 c-j	R	1	0.04 b-f	R
Agora F1	Mimi	1	0.03 d-j	R	2	0.01 d-f	R
Amaral	Mimi	1	0.02 d-j	R	1	0.02 c-f	R
Valoasis RZ F1	Mimi	1	0.01 e-j	R	2	0.00 d-f	R
SV1917	Mimi	0	0.00 f-j	R	0	0.01 d-f	R
Reconquista	MiMi	0	0.00 f-j	R	0	0.00 d-f	R

[1)] GI (0-5): 0=no galls, 1=1-2, 2=3-10, 3=11-30, 4=31-100, 5≥100 galls/root system.

[2)] Rf=final population density/initial population density. Data are means of five replicates, except genotype Monita (four replicates) tested with *M. ethiopica*. Means in this column followed by the same combination of letters do not differ significantly at $P>0.05$, according to the Kruskal-Wallis test. ∗ Rf of *M. luci* differs significantly at $P>0.05$, from *M. ethiopica*, according to the *t*-test.

[3)] Host status categories: S=susceptible (GI>2 and Rf>1), RH=resistant/hypersensitive (GI>2 and Rf≤1), T=tolerant (GI≤2 and Rf>1), R=resistant (GI≤2 and Rf≤1) (Sasser et al., 1984).

[4)] Tomato genotype used as a control.

Evaluating the influence of *Mi* gene status (MiMi, Mimi and mimi) on *M. luci* and *M. ethiopica* reproduction, tomato genotypes with mimi profile showed, in general, higher numbers of Rf and GI being classified as susceptible, with exception of Tisey F1 for both RKN species, and Basileia and Visconti for *M. ethiopica*, with Rf≤1 and GI≥3 (Fig. 1; Table 2). Reproduction factor of RKN species on genotypes Monita and Reconquista, homozygous at the *Mi* locus (MiMi), was similar to that obtained for heterozygous genotypes (Mimi), which indicates that not only the status of the *Mi* gene, but also the genetic background of the genotypes influence nematode reproduction (Fig. 1; Table 2). Significant differences in *Meloidogyne* species reproduction were detected between genotypes within *Mi* allelic conditions (Fig 1; Table 2).

Discussion

Twenty seven tomato genotypes were screen for the presence of *Mi* gene using the Mi23 marker. This marker is tightly linked to *Mi* gene and allows the differentiation of homozygous from heterozygous genotypes, with reduced likelihood of false positives (Seah et al. 2007; Devran et al., 2013). Our results indicate that all RKN resistant tomato genotypes carried the *Mi* resistance gene, either in heterozygous or homozygous state. However, some *Mi*-heterozygous tomato genotypes have been found susceptible, although they present lower RKN reproduction compared with genotypes without *Mi* gene (mimi). The genotypes Basileia, Visconti and Tisey F1, where the *Mi* gene was not detected (mimi), displayed low values of reproduction and were classified as resistant/hypersensitive. Presence/absence of *Mi* gene may not be the only factor determining *M. luci* and *M. ethiopica* reproduction. Root-knot nematode Rf variability possibly will reflect an influence of tomato genetic background. Tomato genotypes included in this study do not share the same progenitors and, consequently, there is a genetic variability that is difficult to control and should be considered (Jacquet et al. 2005; Cortada et al., 2008). This genetic diversity can be demonstrated by the number and quality of disease resistance genes present in each tomato genotype (Table 1). The effect of these plant resistance genes on nematode reproduction is unknown, most of them are specific, but they can be active against *Meloidogyne* species. *Mi* gene, for example, is also a plant resistance gene active against some biotypes of the potato aphid *Macrosiphum euphorbiae* and of the whitefly, *Bemisia tabaci*, biotypes B and Q (Rossi et al., 1998; Nombela et al., 2003). The accumulation of these genes may reduce significantly the

reproductive success of RKN, making these tomato genotypes a valuable resource of resistance to be included in integrated pest management programmes. For example, the genotype SV7886TH has resistance to *Fulvia fulva*, *Fusarium oxysporum* f. sp. *lycopersici*, tomato mosaic virus, tomato yellow leaf curl virus, tomato spotted wilt virus, *Verticillium albo-atrum*, and *V. dahlia*, but not against RKN (mimi). Nonetheless, this genotype showed a moderate level of reproduction (Rf=2.67 and 1.88, respectively, for *M. luci* and *M. ethiopica*) similar to heterozygous genotypes, such as Anairis F1. The higher levels of *M. luci* and *M. ethiopica* reproduction were observed in San Marzano 2, San Pedro and Coração-de-Boi genotypes, which information about resistance genes is not displayed in the product catalogue.

The results of this study confirmed that *Mi* gene is effective to supress *M. luci* and *M. ethiopica* reproduction and can be used as an alternative to the use of chemical nematicides, in integrated nematode management programmes. However, the *Mi* gene did not confer total immunity, since a proportion of nematodes were able to develop and reproduce. Nematodes extracted from these genotypes (Paipai, Amaral, Sahel), for both *Meloidogyne* species, were re-inoculated in the *Mi* homozygous genotype Reconquista, to evaluate the potential of these isolates to overcome the *Mi* gene under laboratory artificial conditions. *Meloidogyne ethiopica* and, as previously suggested, *M. luci* were not able to overcome the resistance conferred by the *Mi* gene in tomato (Strajnar & Širca et al., 2011; Conceição et al., 2012). Resistant genotypes appear to be suitable for the management of the RKN *M. luci* and *M. ethiopica*, helping to reduce nematode density, to prevent yield losses and to diminish the application of chemical nematicides. However, the repeated exposure of tomato genotypes carrying the *Mi* resistance gene to *Meloidogyne* species could lead to the selection of virulent isolates. Virulent populations have been already reported for *M. incognita* and *M. javanica* (Tzortzakakis et al., 1998, 2014; Ornat et al., 2001; Huang et al. 2004; Iberkleid et al., 2014).

Crop rotations with *Mi* gene resistant and susceptible tomato genotypes can be an option to prevent virulence selection of RKN, in intensive tomato production systems (Tavalera et al., 2009). Tomato homozygous (Reconquista) and heterozygous (Vimeiro F1, Paipai, Sahel, Agora F1, Amaral, Valoasis RZ F1 and SV1917) genotypes at the *Mi* locus, available commercially, can be included by farmers in integrated pest management programmes, in combination with other strategies, such as nematicides, fallow periods or solarisation beyond crop rotation, to control, not only the three most common RKN species, but also *M. luci* and *M. ethiopica*.

Meloidogyne ethiopica and *M. luci*, considered as ‘minor’ RKN species, were included in the EPPO Alert List in 2011 and 2017, respectively, which confirm the potential economic impact of these species (EPPO, 2017). Our results showed a high potential impact of *M. luci* compared with *M. ethiopica*, making it a species of emerging importance for tomato crops. Greater tomato losses can be expected from *M. luci* than *M. ethiopica*, but the real impact of both RKN species is unknown. Thus, it is desirable to avoid the introduction and spread of *M. luci* and *M. ethiopica* to regions where they do not exist.

Conclusions

The RKN *Meloidogyne luci* originally described from Brazil, Chile and Iran is distributed in several southern European countries including Portugal, where it was found in a single potato field, so far the full extent of its geographic distribution in Portugal was unknown. This RKN species can be morphologically confused with *M. ethiopica* and, therefore, may have been misidentified as *M. ethiopica* in a number of surveys. This species have received a great interest in the last years, since it is able to parasitise several economical important crops and its full distribution is still unknown, having been added, in 2017, to the EPPO Alert List.

The main aims of this study were to acquire knowledge on RKN diversity in Portugal and to evaluate the suitability of the *Mi* gene to be used as a management tool for *M. luci* infected fields.

In **Chapter 1**, the incidence and diversity of RKN on subsistence farms and public and private gardens, in Coimbra region, were studied, using biochemical and molecular markers, and the principal findings were:

1. Forty eight isolates were identified and 11 esterase (EST) phenotypes detected corresponding to six *Meloidogyne* species (*M. arenaria*, *M. enterolobii*, *M. hapla*, *M. hispanica*, *M. incognita* and *M. luci*);

2. *Meloidogyne enterolobii* was reported for the first in Portugal and for the third time in Europe, and three new host plants were identified (*Physalis peruviana*, *Cereus hildmannianus* and *Lampranthus* sp.);

3. The presence of *M. luci* in Portugal was confirmed and two new host plants were recorded (*Cordyline australis* and *Oxalis corniculata*);

4. Esterase phenotype is a valuable tool for *Meloidogyne* spp. identification when young-egg laying females are available and very useful in the detection of populations with more than one species;

5. This study represents a relevant contribution to the RKN knowledge, in Portugal;

6. Further research is needed in order to understand the full extent of *M. enterolobii* and *M. luci* distribution, in Portugal;

7. Sanitation of infected plants should also be implemented to prevent the spread of these pathogens to new locations.

In **Chapter 2**, the effect of the *Mi* locus gene in the reproduction of *M. luci* and *M. ethiopica* was analysed in pot assays, conducted in a controlled environment growth chamber, and the major conclusions were:

1. The commercial tomato genotypes carrying *Mi* gene were effective in supressing *M. luci* and *M. ethiopica* reproduction and can be used in management programmes for the control of these two RKN species, particularly Reconquista (*Mi*-homozygous) and Vimeiro F1, Paipai, Sahel, Agora F1, Amaral, Valoasis RZ F1, and SV1917 (heterozygous), that exhibit a consistent response in reducing nematode reproduction;

2. Variability in Rf values of the nematode species within the same allele combination was attributed to the tomato genetic background, which varies widely, due to the fact that the these tomato genotypes do not share the same progenitors;

3. The genotypes, where the *Mi* gene was not detected (mimi), surprisingly displayed low values of reproduction and were classified as resistant/hypersensitive, which raise the question related with the influence of the tomato genetic background on nematode reproduction, particularly other resistance genes specific for other diseases;

4. The presence of the *Mi* gene does not necessarily result in RKN resistance, but less damage can be expected in comparison to genotypes where *Mi* gene was not detected;

5. Several attempts to establish virulent isolates in the tomato cv. Reconquista have failed, which suggested that *M. luci* and *M. ethiopica* are not able to overcome the *Mi* resistance gene in tomato.

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