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Root-Knot Nematode Species Associated with Horticultural Crops in the Island of Azores, Portugal

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Abstract: Plant-parasitic nematodes (PPN) are an economically important group of plant pests present throughout the world. In particular, root-knot nematodes (RKN), sedentary endoparasites of a wide variety of economically important hosts with the ability to survive in temperate regions. During 2020–2021 an extensive survey of *Meloidogyne* spp. was undertaken on the island of São Miguel Azores, Portugal. A total of 80 samples comprising 23 species of plants were collected from 13 localities in 4 districts of the island. Samples included field and greenhouse vegetable crops. Bioassays were carried out to obtain females and juveniles for morphological and biochemical characterization of the isolates. The observed morphological features showed high similarity and consistency with previous descriptions of the genus. Concerning the biochemical characterization, the esterase (EST) phenotype displayed the patterns described for *M. incognita*, *M. arenaria*, and *M. javanica*. *Meloidogyne incognita* was found to be the most prevalent species with respect to both natural host range and geographical distribution, followed by *M. arenaria* and *M. javanica*. This is the first field survey that assesses the species of root-knot nematodes associated with horticultural crops in the Azores Island, contributing additional information on the distribution of this genus.

Keywords: identification; EST phenotypes; *Meloidogyne*

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1. Introduction

Agriculture is one of the most important economic sectors and a significant component of the island of Azores, responsible for 46% of the regional economy [1]. According to the latest Land Occupation Report [2], approximately half of the Azores territory is occupied by agriculture (48.8%), followed by forestry and natural and semi-natural vegetation, representing (42.6%) [3]. Plant-parasitic nematodes (PPN) represent a significant constrain to agricultural production, as they cause serious losses in quantity and quality worldwide. Among the PPN, root-knot nematodes (RKN), *Meloidogyne* spp., are one of the oldest known plant parasitic nematodes and most devastating pests of economically important crops [4]. *Meloidogyne* spp. have an extensive host range that includes nearly every horticultural, fruit and ornamental crop comprising more than 100 species [2]. The species *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. hapla* Chitwood, 1949, *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, and *M. javanica* (Trub, 1885) Chitwood, 1949 are regarded as the most important, due to their worldwide distribution and polyphagia [5,6]. In view of the impact this nematode can cause in agricultural areas, the identification of the species is of primary importance. In the past, RKN identification was based on microscopic examination of second-stage juveniles (J2) and female perineal patterns. However, these methods are often unreliable and require expertise due to the inter- and intraspecific variability [7]. Currently, the biochemical electrophoretic analysis of nonspecific esterases (EST) is a widely used method employed to differentiate *Meloidogyne* species, with many species-specific isozyme patterns already published. In Portugal, many species have been reported: *Meloidogyne arenaria*; *M. chitwoodi* Golden et al., 1980; *M. hapla*; *M. hispanica* Hirschman, 1986; *M. incognita*; *M. javanica*; *M. lusitanica* Abrantes and Santos 1991; *M. luci* Carneiro et al., 2014; *M. enterolobii* Yang et al., 1983, and *M. naasi* Franklin, 1965 [8–14]. Due to the risk that various *Meloidogyne* species pose to agricultural production, a national field survey programme that includes the islands of Azores and Madeira was established in 2019 and led to the detection of *M. luci* associated with potato in the island of Pico, Azores [14]. Although this programme is in place, only the species included in the A2 and Alert EPPO lists are surveyed in the samples (*M. chitwoodi, M. fallax* Karsen, 1996, *M. enterolobii*, and *M. luci*) [15,16]. Currently, on the island of Azores, there is a lack of detailed information about the presence of other *Meloidogyne* species. So, the aim of the present study was to determine the frequency of occurrence of the RKN and identify biochemically, the species present in the fields.

2. Materials and Methods

2.1. Sampling and Nematode Isolates

During 2020 and 2021 on the Island of São Miguel, Azores, 80 soil samples were collected from 13 localities in 4 districts. Sampling was carried out from the soil rhizosphere at about 15 cm depth. Each sample consisted of 5 to 8 cores, sampled in zigzag at roughly equal intervals. Each composite soil sample was placed in a polyethylene bag and brought for analysis. A 400 mL subsample was taken from each composite sample and the nematodes extracted according to the protocol PM 7/119 (1) [17]. The suspension was observed under a stereomicroscope (Nikon SMZ1500, Tokyo, Japan) and suspected specimens of *Meloidogyne* were observed using a brightfield light microscope (Olympus BX-51, Hamburg, Germany) for confirmation. Bioassays were performed to obtain material for biochemical identification (mature females) when second-stage juveniles (J2) of *Meloidogyne* were detected in the soil suspensions. Bioassays were carried out by planting tomato plants cv. Oxheart in the remaining soil from the sample and maintained in a quarantine greenhouse for two months. Females were handpicked from the infected tomato roots and second-stage juveniles were extracted from soil. To be used as a reference in the biochemical studies an isolate of *M. javanica* was maintained as described above. Relative migration rates (Rm) were calculated as a ratio of migration distance of EST bands to the upper *M. javanica* EST band.

2.2. Morphological Characterisation

Ten second-stage juveniles (J2) were placed in a drop of water on a glass slide and gently heat-killed for morphological characterization using a bright-field light microscope (Olympus BX-51, Hamburg, Germany) and photographed with a digital camera (Leica MC190 HD, Wetzlar, Germany).

2.3. Biochemical Characterisation

Young egg-laying females were handpicked from infected tomato roots and transferred to micro-hematocrit capillary tubes (one female per tube) with 5 µL of extraction buffer (20% sucrose v/v and 1% Triton X-100 v/v). The females were macerated with a pestle, frozen, and stored a −20 °C until use. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) on thin-slab 7% separating polyacrylamide gels in a Mini-Protean II (BioRad Laboratories, Hercules, CA, USA), according to [7]. The gels were stained for EST activity with the substrate α-naphthyl acetate.

3. Results and Discussion

3.1. Distribution

A total of 80 samples comprising 23 species of plants from 13 localities in 4 municipalities of the island were collected. Samples included field and greenhouse vegetable crops (Table 1).

From the 80 samples analysed, *Meloidogyne* spp. was detected in 60 (75%) of them, showing a high prevalence of this plant-parasitic nematode in the Island of São Miguel, Azores. Positive detections were identified in all the districts prospected. From the total of positive samples, the district of Ponta Delgada accounted for 55%, followed by Ribeira Grande with 31.66%. The district of Lagoa contributed 11.66% and Vila Franca 1.66% of the total. Root-knot nematodes were found parasitizing most of the crops surveyed, both in greenhouses and open fields. From the 60 positive samples, 40% corresponded to greenhouses and 60% to open field production. Nematodes of the following genera were also present in the samples analyzed: *Pratylenchus, Helicotylenchus, Hemicycliophora, Scutellonema*, and *Globodera.*

3.2. Morphological Characterisation

Morphological characterization from the recovered second-stage juveniles was performed and was in agreement with the previous description of the genus [18,19]. The second-stage juveniles were vermiform, slender, and clearly annulated. The head region was slightly set off from the body. The stylet was delicate, narrow, and sharply pointed; the knobs were small and oval-shaped. The excretory pore was distinct. The tail was conoid with a rounded tip and hyaline terminus distinctive (Figure 1). Morphology has been a valuable tool for RKN identification due to its low cost. However, its accuracy depends on the number of specimens and characteristics assessed, which represents a challenge due to the similarity among species of the characters evaluated.

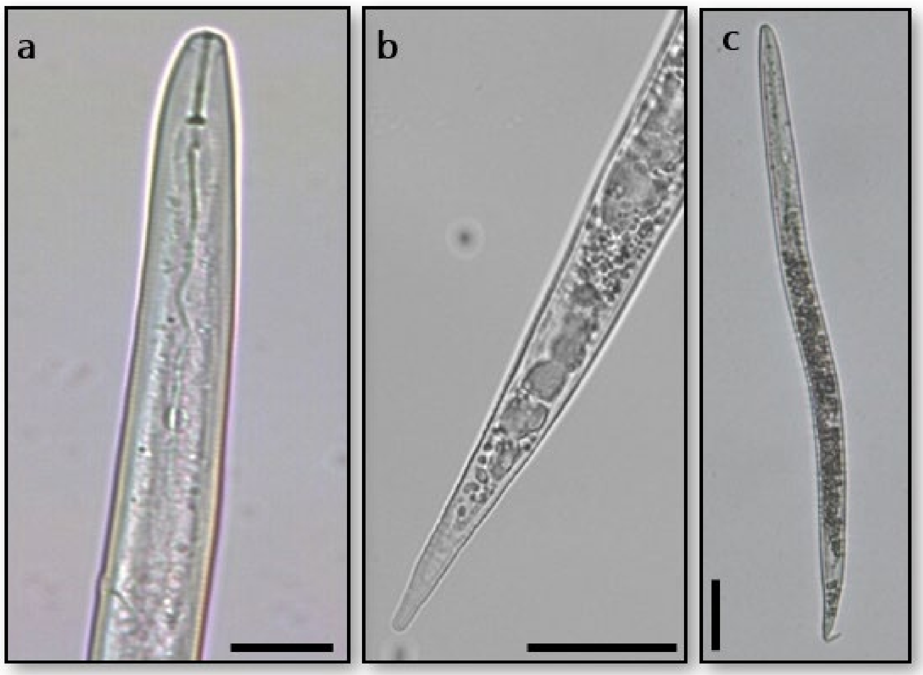

Figure 1. *Meloidogyne* spp. light microscope observations. Second-stage juvenile: (**a**) anterior region; (**b**) tail region, (**c**) whole specimen (bar = 20 µm).

Table 1. Locations and crops surveyed in the Island of São Miguel, Azores, for the detection of root-knot nematode.

District	Locality	Coordinates	Production Type			
			Field	*Meloidogyne* Species	Greenhouse	*Meloidogyne* Species
Ponta Delgada (39 samples = 48.75%)	**São Roque** (14 Samples)	37°45′17.906″ N 25°37′43.507″ W	Leek (*Allium porrum*) Chard (*Beta vulgaris* subsp. vulgaris) Lettuce (*Lactuca sativa*)	ND *M. incognita* *M. incognita*	Broccoli (*Brassica oleraceae* cv. italica) Pepper (*Capsicum annuum*) Pea (*Pisum sativum*)	*M. inognita* *M. incognita* *M. incognita*
		37°45′23.306″ N 25°37′58.962″ W	Carrot (*Daucus carota* subsp. sativus) Potato (*Solanum tuberosum*)	*M. incognita* *M. incognita*	Cucumber (*Cucumis sativus*) Tomato (*Solanum lycopersicum*)	*M. incognita* *M. javanica*
		37°46′21.846″ N 25°37′25.018″ W	Sweet-potato (*Ipomoea batatas*) Carrot (*Daucus carota* subsp. NDsativus)	ND ND	Green beans (*Phaseolus vulgaris*) Lettuce (*Lactuca sativa*)	*M. incognita* *M. incognita*
	Livramento (6 Samples)	37°45′53.100″ N 25°35′27.100″ W	Lettuce (*Lactuca sativa*) Cabbage (*Brassica oleracea* cv. capitata) Spinach (*Spinacia oleracea*) Courgette (*Cucurbita pepo*)	*M. incognita* *M. javanica* *M. incognita* *M. incognita*	Lettuce (*Lactuca sativa*) Tomato (*Solanum lycopersicum*) (2)	*M. incognita* *M. incognita*
	Arrifes (6 Samples)	37°45′26.647″ N 25°41′47.965″ W	Parsley (*Petroselnum cripsum*)	ND	Courgette (*Cucurbita pepo*)	*M. incognita*
		37°45′42.534″ N 25°40′45.588″ W	Lettuce (*Lactuca sativa*)	*M. incognita*	Lettuce (*Lactuca sativa*)	*M.incognita*
		37°45′31.730″ N 25°40′34.838″ W	Watercress (*Nasturtium officinalis*)	ND	Spinach (*Spinacia oleracea*)	*M. javanica*
	São Pedro (2 Samples)	37°45′12.658″ N 25°39′54.464″ W	Onion (*Allium cepa*)	ND	Tomato (*Solanum lycopersicum*)	*M. javanica*
	Ginetes (10 Samples)	37°50′24.565″ N 25°49′58.649″ W	Cabbage (*Brassica oleracea* cv. capitata)	*M. incognita*	Cucumber (*Cucumis sativus*) Lettuce (*Lactuca sativa*) Green beans (*Phaseolus vulgaris*)	*M. incognita* *M. incognita* *M. incognita*
		37°50′38.130″ N 25°50′45.420″ W	Potato (*Solanum tuberosum*) (6)	*M. incognita*		
	Mosteiros (1 Sample)	37°53′18.427″ N 25°48′49.597″ W	Potato (*Solanum tuberosum* cv. Rudolph)	*M. incognita*		

Table 1. *Cont.*

District	Locality	Coordinates	Production Type			
			Field	***Meloidogyne* Species**	**Greenhouse**	***Meloidogyne* Species**
Ribeira Grande (30 samples = 37.5%)	**Rabo de peixe** (12 Samples)	37°47′45.038″ N 25°35′44.624″ W	Cabbage (*Brassica oleracea* cv. capitata) Onion (*Allium cepa*)	*M. arenaria* ND		
		37°48′16.643″ N 25°35′31.848″ W	Potato (*Solanum tuberosum*) (4) Rocket (*Eruca sativa*)	*M. incognita* ND	Passion fruit (*Passiflora edulis*) Tomato (*Solanum lycopersicum*) Cucumber (*Cucumis sativus*) Green beans (*Phaseolus vulgaris*) Courgette (*Cucurbita pepo*)	ND *M. incognita* *M. incognita* *M. arenaria* ND
	Pico da pedra (4 Samples)	37°48′28.440″ N 25°37′26.627″ W	Strawberry (*Fragaria X Ananassa*) Potato (*Solanum tuberosum*) Broccoli (*Brassica oleraceae* cv. italica) Cabbage (*Brassica oleracea* cv. capitata)	ND *M. incognita* *M. incognita* ND		
	Calhetas (4 Samples)	37°49′1.074″ N 25°35′57.127″ W	Strawberry (*Fragaria X Ananassa*) Parsley (*Petroselnum cripsum*)	ND ND	Tomato (*Solanum lycopersicum*) Cucumber (*Cucumis sativus*)	*M. incognita* ND
	Ribeira seca (10 Samples)	37°48′27.552″ N 25°32′43.628″ W	Potato (*Solanum tuberosum* cv. Rudolph) (3)	*M. incognita*		
		37°48′26.508″ N 25°33′9.157″ W	cv. Tonmedo	ND		
		37°48′43.950″ N 25°33′6.360″ W	cv. Red Scarlett	*M. incognita*		
		37°48′13.677″ N 25°32′23.734″ W	cv. Yona	*M. incognita*		
		37°48′19.435″ N 25°32′11.068″ W	cv. Picasso (2)	*M. incognita*		
		37°48′30.272″ N 25°33′1.325″ W	cv. Perdiz	*M. incognita*		
		37°48′43.950″ N 25°33′6.360″ W	cv. Agria	*M. incognita*		

Table 1. *Cont.*

District	**Locality**	**Coordinates**	**Production Type**			
			Field	***Meloidogyne* Species**	**Greenhouse**	***Meloidogyne* Species**
Lagoa **(9 samples = 11.25%)**	**Água de Pau** (2 Samples)	37°43′4.717″ N 25°29′35.569″ W	Potato (*Solanum tuberosum* cv. Agria) cv. Rudolph	*M. incognita* ND		
	Santa Cruz (7 Samples)	37°44′34.365″ N 25°33′17.830″ W	Cabbage (*Brassica oleracea* cv. capitata) Parsley (*Petroselnum cripsum*)	*M. incognita* ND	Lettuce (*Lactuca sativa*) Tomato (*Solanum lycopersicum*)	ND *M. incognita*
		37°44′15.399″ N 25°33′13.514″ W	Potato (*Solanum tuberosum* cv. picasso) (3)	*M. incognita*		
Vila Franca **(2 samples = 2.5%)**	**Ribeira seca** (2 samples)	37°43′25.704″ N 25°25′11.182″ W	Potato (*Solanum tuberosum* cv. picasso) cv. Red scarlett	*M. incognita* ND		

3.3. Biochemical Characterisation

From young egg-laying females three different phenotypes were observed in the samples collected from the Island of São Miguel, Azores. *Meloidogyne incognita* I2 phenotype (relative mobility, Rm: 1.04; Rm; 1.11) exhibited two bands slightly below the first band of the reference isolate; *M. arenaria* A2 phenotype (Rm: 1.16; Rm: 1.22), presented two bands between the first and second band of the reference isolate and *M. javanica* J3 phenotype (Rm: 1; Rm: 1.17; Rm: 1.26). *Meloidogyne javanica* J3 phenotype was used as a reference in all the gels to determine the relative position of other species [17,20,21] (Figure 2a–d).

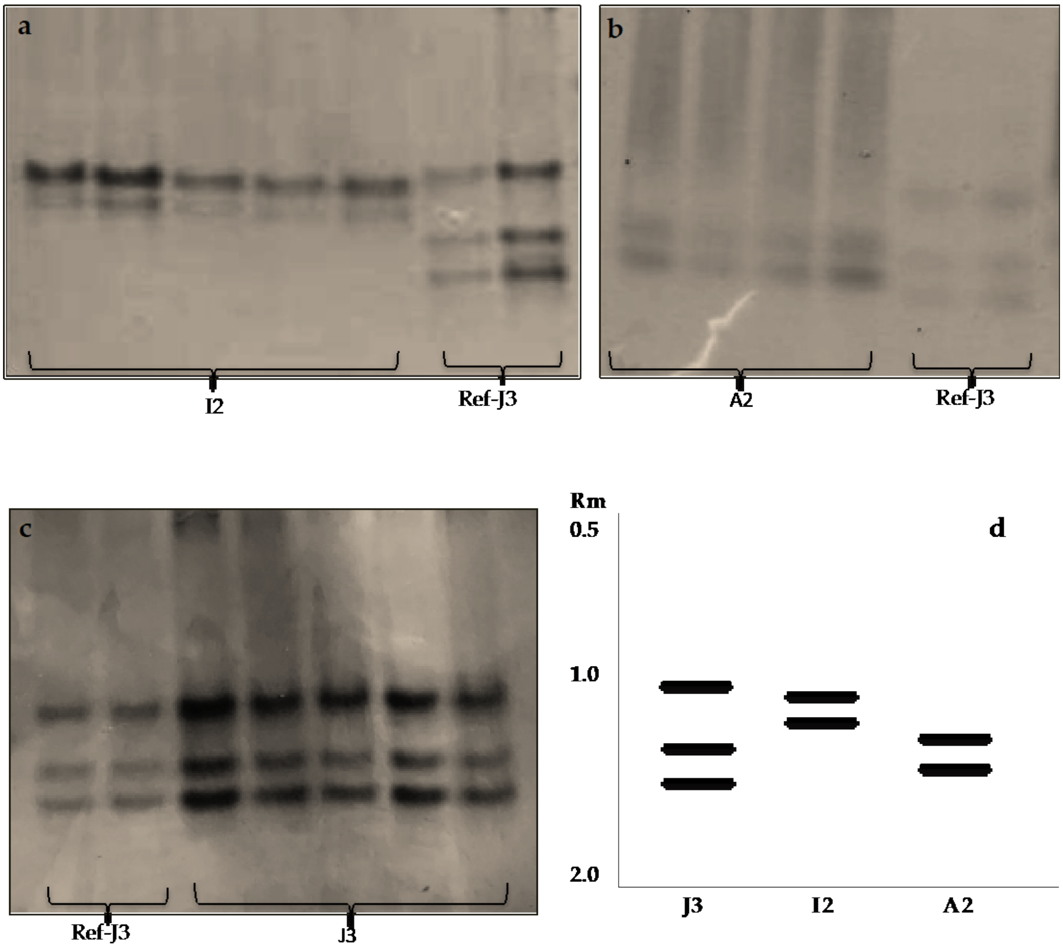

Figure 2. Esterase phenotypes of protein homogenates from one egg-laying female of *Meloidogyne* species; (**a**)—*M. incognita* (I2); (**b**)—*M. arenaria* (A2); (**c**)—*M. javanica* (J3); (**d**)—Relative mobility J3: *M. javanica*; I2: *M. incognita*; A2: *M. arenaria*.

The functionality of the nonspecific EST phenotype has been shown in many studies as a quicker, reliable, and stable method for *Meloidogyne* identification, with the main drawback being the use of a specific developmental stage of the nematode [17,20,21]. In this study, the EST activity helped us to determine the species that are associated with horticultural crops grown on the island. Based on this analysis, the most prevalent esterase phenotype detected was *M. incognita* I2, which was found in 91.6% of the samples (55 samples) and was present in all the districts surveyed. The presence of *M. javanica* was detected in 3 samples and *M. arenaria* in 2 samples corresponding to 5 and 3.4% of the total, respectively. Its presence was limited to one district (Figure 3). The population density of *M. incognita* ranged between 35–45 nematodes/400 mL, *M. arenaria* and *M. javanica* had a span of 20–25 nematodes/400 mL.

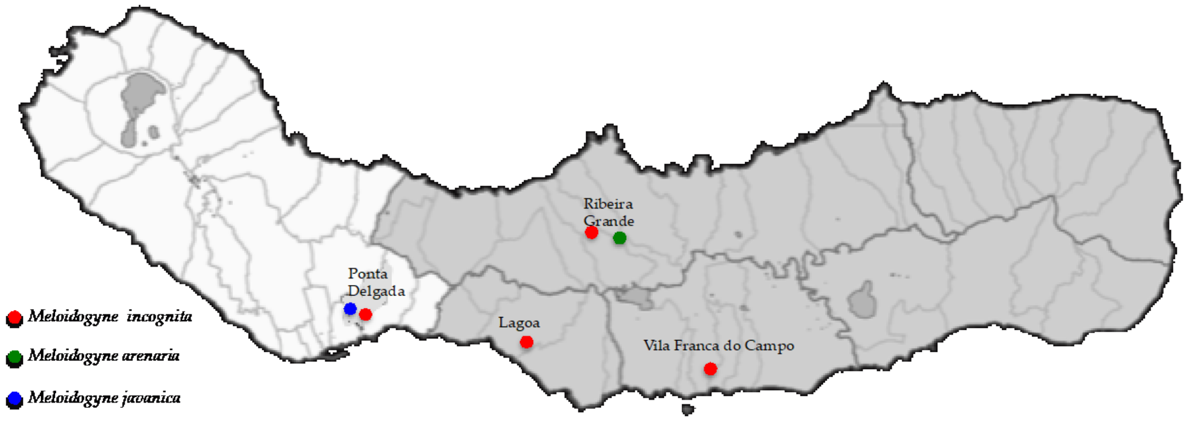

Figure 3. Species of *Meloidogyne* present in the districts surveyed in São Miguel Island, Azores.

4. Conclusions

The root-knot nematodes are one of the most widely distributed pests causing economically important damages in a large number of crops. In particular, three species, *M. incognita*, *M. arenaria,* and *M. javanica*, which due to their reproductive rate, short generation time, and wide host range, are very difficult to control. Due to this fact, the identification of *Meloidogyne* species is of great importance for the development of effective nematode management practices as well as for quarantine purposes. To our knowledge, this is the first survey carried out in horticultural crops in the island of São Miguel, Azores, as well as the first report of *Meloidogyne incognita* associated with broccoli, pea, chard, cucumber, and courgette. It is also the first report of *M. arenaria* parasitizing green beans and cabbage and *M. javanica* parasitizing spinach.

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