PRELIMINARY RESULTS OF ROOT KNOT NEMATODES MONITORING IN CROATIA OVER TWO YEARS (2022-2023)

Tamara REHAK BIONDIĆ[1], Jasna MILANOVIĆ[2], Ivan POJE[3], Barbara GERIČ STARE[4], Karolina VRANDEČIĆ[5], Mirjana BRMEŽ[6]

[1,2,3] Croatian Agency for Agriculture and Food, Centre for Plant Protection, Zagreb, Croatia
[4] Agricultural Institute of Slovenia, Ljubljana, Slovenia
[5,6] University of Josip Juraj Strossmayer in Osijek, Faculty of Agrobiotechnical Sciences, Osijek, Croatia

ABSTRACT

Root-knot nematodes (RKNs, *Meloidogyne* spp.) belong to the economically most important group of plant-parasitic nematodes which consists of approximately 100 species, many of which can be considered dangerous invasive pests in agriculture. In order to prevent the introduction or limit the spread on European territory, certain nematode species have been placed on the quarantine list (EU and EPPO). The EU list of quarantine RKN includes *M. enterolobii* (A1), *M. chitwoodi* and *M. fallax* (A2) and the EPPO list includes *M. ethiopica* (A1) and *M. enterolobii*, *M. luci*, *M. graminicola, M. chitwoodi*, *M. fallax* and *M. mali* (A2). Identification of *Meloidogyne* species based on morphological characteristics is a very difficult task, which is why molecular techniques such as polymerase chain reaction (PCR) are a widely used tool for their identification. In 2022 and 2023, the survey to identify *M. chitwoodi* and *M. fallax* was carried out in potatoes while identification on other vegetables was also carried out as part of an early warning programme for RKN. Visual inspections and sampling were carried out in the indoor and outdoor growing areas on the mainland and in the coastal regions of Croatia. The collected samples were analysed in the Laboratory for Nematology at the Centre for Plant Protection (CAAF, Croatia). During this period, 87 soil samples of potatoes were analysed, 5 of which were positive for the presence of RKNs. As part of an early warning programme, a total of 42 samples were analysed, 31 of which were positive for the presence of RKNs. Molecular identification revealed that the most prevalent species were *M. incognita* and *M. hapla.* The species *M. chitwoodi* and *M. fallax* were not identified in any of the samples. Identification the pests present in the growing area is crucial for the next steps in pest control.

[1] BSc, Gorice 68B, Zagreb, Croatia, e-mail: tamara.rehak.biondic@hapih.hr
[2] PhD, Gorice 68B, Zagreb, Croatia
[3] MSc, Gorice 68B, Zagreb, Croatia
[4] PhD, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[5] prof. PhD, Vladimira Preloga 1, Osijek, Croatia
[6] prof. PhD, Vladimira Preloga 1, Osijek, Croatia

Key words: *Meloidogyne* species, samples analysed, molecular identification, Croatia

1 INTRODUCTION

Root-knot nematodes (RKNs) of the genus *Meloidogyne* belong to the economically most important group of plant-parasitic nematodes and also to the most important plant pests causing major economic losses in crop production (Bebber *et al*. 2014, Gerič Stare *et al*., 2017, Susič, 2020). On the list of the ten most damaging genera of plant-parasitic nematodes, the genus *Meloidogyne* ranks first (Jones *et al*., 2013). They are polyphagous, well-adapted obligate endoparasites of almost all higher plant species, including important agricultural crops. They reproduce in the roots of the host plant, and usually form small to extremely large root-knots. The infested plant is physiologically weakened, and the quantity and quality of yield are reduced (Moens *et al*., 2009). Within the genus *Meloidogyne* (Gőldi, 1892), about 100 species have been described, of which four species: *M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla* are the most important crop pests found in soils worldwide (Karssen, 2002. Karssen *et al*., 2013). Twenty known species from the tropical group of RKNs such as M. *incognita, M. arenaria, M. javanica, M. enterolobii, M. ethiopica, M. luci,* and M. *hispanica* are now already widespread in the warm temperate climates (i.e., in the Mediterranean and in the protected areas) and are expected to become the important pests in the temperate climates due to the newly created climatic and environmental conditions, more favorable for their development (Bebber *et al*., 2014, Širca *et al*., 2021). In order to prevent the introduction or limit the spread on European territory, certain nematode species have been placed on the quarantine list (EU and EPPO). The EU list of quarantine RKN includes *M. enterolobii* (A1), *M. chitwoodi* and *M. fallax* (A2) and the EPPO list includes *M. ethiopica* (A1) and *M. enterolobii*, *M. luci*, *M. graminicola, M. chitwoodi*, *M. fallax* and *M. mali* (A2).

Identification of *Meloidogyne* species based on morphological characteristics is a very difficult task, which is why molecular techniques such as polymerase chain reaction (PCR) are a widely used tool for their identification. An accurate identification of species is necessary for an appropriate pest management, such as crop rotation, resistant cultivars, and regulatory and quarantine programs (Elling, 2013, Gerič Stare *et al*., 2017, EFSA, 2019).

The aim of this monitoring was to molecularly and morphologically identified the isolates of the RKNs found in the national survey in potatoes and in an early warning programme for RKNs from different growing area and host plants across Croatia.

2 MATERIALS AND METHODS

In 2022 and 2023, the national survey for *M. chitwoodi* and *M. fallax* was carried out in potatoes, while inspection on other vegetables was also carried out as part of an early warning program for RKNs. Visual inspections and sampling were carried out in the indoor and outdoor growing areas on the mainland and in the coastal regions of

Croatia. The collected samples were analysed in the Laboratory for Nematology at the Centre for Plant Protection – Croatian Agency for Agriculture and Food, Croatia.
In total of 87 soil samples and 42 root samples of simptomatics host plants with soil were collected for subsequent laboratory analysis. Nematodes were extracted according to the EPPO protocol PM 7/119 (1). The suspension was observed under a stereomicroscope (Olympus SMZ16) and specimens of *Meloidogyne* observed using an optical microscope (Olympus BX51) for confirmation. Roots from sampling were observed under stereomicroscope. The female with egg mass or a single egg mass were handpicked from infected roots and used for molecular and morphological identification. The second-stage juveniles (J2) from positive sample also used for morphological characterization.
Species identification was confirmed by molecular analyses using group specific primers JMV1, JMV2 and JMV hapla for species *M. chitwoodi, M. fallax* and *M. hapla* developed by Wishart *et al*. (2002) and the species-specific SCAR primers Finc/Rinc for *M. incognita* developed by Zijlstra et al. (2000). The PCR product of the reaction with multiplex primers is 440 bp for *M. hapla* and 1200 bp for *M. incognita* with the species-specific SCAR markers (Figure 2).

3 RESULTS AND DISCUSSION

During 2022 and 2023, the national survey of quarantine species *M. chitwoodi* and *M. fallax* was conducted in potatoes on a total of 87 soil samples from 80 locations and 15 counties of Croatia. From the total of samples collected, genus *Meloidogyne* spp. was detected in five, corresponding to 5,75%. In two samples were identified *M. hapla* in Virovitica-Podravina county and one sample in Brod-Posavina county. Species *M. incognita* was identified in one sample in Zadar county and one sample unknown species of *Meloidogyne* spp. in Istria county (Table 1). The quarantine species *M. chitwoodi* and *M. fallax* were not determined in any of the analysed samples in potatoes. A total of 42 root samples of symptomatics host plants with soil from 11 locations and 4 counties were taken within the early warning program for RKNs. Samples were collected in fields and greenhouses. Majority of samples were taken from tomato, carrot, cucumber, melon and pepper which are the most important cultivated host plants of RKNs. From the 42 samples analysed, genus *Meloidogyne* spp. was detected in 31 samples (73,8 %) of them showing a high parasitizing most of the crops surveyed (Table 2, Figure 1).

Table 1: Results of national survey of RKNs on potatoes in Croatia (2022-2023).

Year	N°of counties	N°of locations	N°of soil samples	N°of positive findings	*Meloidogyne* species (County/Locality)
2022	15	36	37	1	*M. hapla* (Brod - Posavina /Donji Lipovac)
2023	14	44	50	4	*M. hapla* - 2 samples (Virovitica - Podravina /Sedlarica); *M. incognita* (Zadar/Tinj); *Meloidogyne* spp. (Istria/Pomer)
TOTAL	**14-15**	**80**	**87**	**5**	

Molecular and morphological identification revealed that the most prevalent species were *M. incognita* and *M. hapla.* The species *M. chitwoodi* and *M. fallax* were not identified in any of the samples (Figure 2).

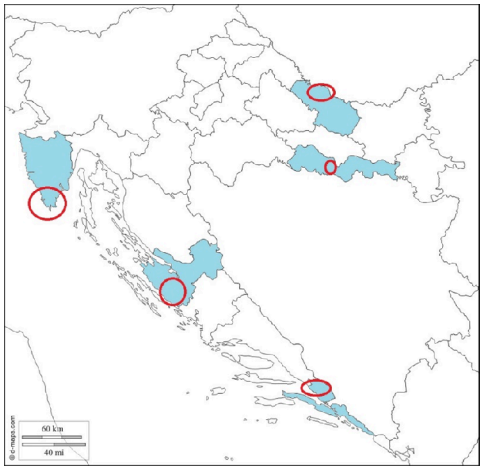

Figure 1. Map of Croatia specifying the locations where *Meloidogyne* spp. positive samples were found.

Table 2: Preliminary results of identification RKNs as part of early warning program in Croatia (2022-2023).

Years	**Counties**	**Locations**	**N°of samples / N° of positive samples**	**Crops /Production type**		***Meloidogyne* species**
				Field	**Greenhouse**	
2022	Zadar	Biograd n/M	1/1	kale (*Brassica oleracea* var. *sabauda*)		*M.incognita*
			1/0	carrot (*Daucus carota* subsp. *sativus*)		ND
		Vrana	1/1	tomato (*Solanum lycopersicum*)		*M.incognita* & *Meloidogyne* spp.
		Pakoštane	1/1	*Buddleja davidii*		*Meloidogyne* spp.
		Kakma	1/1	carrot (*Daucus carota* subsp. *sativus*)		*M. hapla*

		Tinj	1/0	carrot (*Daucus carota* subsp. *sativus*)		ND
	Dubrovnik - Neretva	Metković	1/1		tomato (*Solanum lycopersicum*)	*M. incognita* & *M.hapla*
			1/1		melon (*Cucumis melo*)	*M. incognita* & *M.hapla*
			2/0		lettuce (*Lactuca sativa*)	ND
		Opuzen	1/1	pomegranate "Wonderful"		*M. incognita*
	Istria	Pomer	1/0	pepper (*Capsicum annuum*)		ND
2023	Istria	Pomer	8/8		cucumber (*Cucumis sativus*)	*M. incognita*
			2/0		tomato (*Solanum lycopersicum*)	ND
			4/4		pepper (*Capsicum annuum*)	*M. incognita*
			3/2	potato (*Solanum tuberosum*)		*Meloidogyne* spp.
	Dubrovnik - Neretva	Metković	3/3		cucumber (*Cucumis sativus*)	*M. incognita*
			1/1		tomato (*Solanum lycopersicum*)	*M. incognita*
			1/1	watermelon (*Citrullus lanatus*)		*M. incognita*
			1/1		melon (*Cucumis melo*)	*M. incognita*
			1/1		pepper (*Capsicum annuum*)	*M. incognita*
			1/1		beans (*Phaseolus*	*M. incognita*

					vulgaris)	
		Opuzen	1/1		melon (*Cucumis melo*)	*Meloidogyne* spp.
		Komin	1/0	strawberry (*Fragaria x ananassa*)		ND
			1/1	pepper (*Capsicum annuum*)/ tomato (*Solanum lycopersicum*)		*Meloidogyne* spp.
	Varaždin	Krkanec	1/0	tomato (*Solanum lycopersicum*)		ND
		Papinec	1/0	tomato (*Solanum lycopersicum*)		ND
TOTAL	**4**	**11**	**42/31**	**8**	**7**	**2 species 3 mixed populations 4 unknown species *Meloidogyne* spp.**

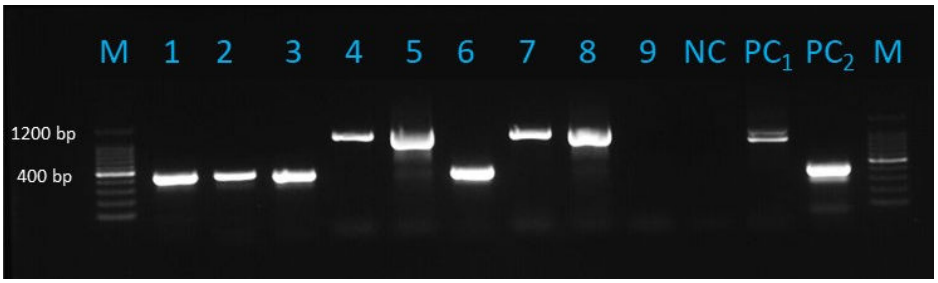

Figure 2. Molecular identification of *Meloidogyne hapla* (440 bp) using species-specific primers (8) and *M. incognita* (1200 bp) using SCAR markers (9);100-bp DNA ladder (M), samples (1-9), negative control (NC), positive controls (PC_1- *M. incognita*; PK_2- *M. hapla*).

4 CONCLUSIONS

The RKNs of the genus *Meloidogyne* are one of the most devastating plant-parasitic nematodes. This preliminary study of the RKNs monitoring revealed their were widely distributed in the growing areas in Croatia. Molecular and morphological identification revealed that the most prevalent species were *M. incognita* and *M. hapla.* The species *M. chitwoodi* and *M. fallax* were not identified in any of the analysed samples. As identification of abundant *Meloidogyne* species is a very challenging task in itself, further efforts in monitoring of *Meloidogyne* in Croatia and access to the latest methods for unambiguous identification, could expand the

knowledge of *Meloidogyne* species distribution in the growing area and to be the crucial for the integrated pest management.

5 REFERENCES

Bebber, D. P., Holmes, T., & Gurr, S. J. 2014. The global spread of crop pests and pathogens. Global Ecology and Biogeography, 23(12), 1398-1407

EFSA (European Food Safety Authority), den Nijs, L., Camilleri, M., Diakaki, M., Schenk, M., & Vos, S. 2019. Pest survey card on *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *EFSA Supporting Publications*, *16*(2), 1572E.

Elling, A.A. 2013. Major emerging problems with minor Meloidogyne species. Phytopathology, 103 (11)

EPPO List of Pest Recommended for Regulation as Quarantine Pests. Version 2023-09. Available online:https://www.eppo.int/ACTIVITIES/plant_quarantine/A1_A2_recent_add (accessed on 5 December 2023)

EPPO 2013. National regulatory control systems PM 9/17 (1) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. EPPO Bulletin 43(3), 527-533. DOI: 10.1111/epp.12079

Gerič Stare, B., Strajnar, P., Susič, N., Urek, G., & Širca, S. 2017. Reported populations of *Meloidogyne ethiopica* in Europe identified as *Meloidogyne luci*. Plant disease, 101(9), 1627-1632.

Hartman, K.M. & Sasser, J.N. 1985. Identification of *Meloidogyne* species by differential host test and perineal pattern morphology. In: Barker, K.R., Carter, C.C.& Sasser, J.N. (Eds.), An advanced treatise on *Meloidogyne* (pp. 69-77). Vol II Methodology. Raleigh N.C. Department of Plant Pathology, North Carolina State University

Jones, J. T., Haegeman, A., Danchin, E.G.J., Gaur, H. S., Helder, J., Jones, M. G.K., Kikuchi T., Manzanilla-Lopez R., Palomares-Rius J.E., Wesemael W.M.L. and Perry, R. N. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. Molecular plant pathology, 14(9), 946-961. DOI: 10.1111/mpp.12057

Karssen, G., Wesemael, W., & Moens, M. 2013. Root-knot nematodes. Plant nematology, (Ed. 2), UK Wallingford, CAB International, 73-108

Karssen, G. 2002. The plant parasitic nematode genus *Meloidogyne* Göldi, 1892 (Tylenchida) in Europe. (pp 92), Leiden, NL: Brill NV

Moens, M., Perry, R. N., & Starr, J. L. 2009. *Meloidogyne* species - a diverse group of novel and important plant parasites. In Perry, R. N., Moens, M and Starr, J.L. (Eds.) *Root-knot nematodes*, *(*pp 488), CABI OEPP/EPPO Bulletin 2016. Diagnostics PM 7/41 (3) *Meloidogyne chitwoodi* and *Meloidogyne fallax*.

OEPP/EPPO Bulletin 46(2), 171–189.

OEPP/ EPPO Bulletin 2013. Diagnostics PM 7/119 (1) Nematode extraction, OEPP /EPPO Bulletin 43(3), 471-495

Susič, N. 2020. The use of advanced technologies for integrated management of root-knot nematodes (*Meloidogyne* spp., Nematoda: Meloidogynidae), Doctoral dissertation, Ljubljana, University of Ljubljana, Biotechnical faculty

Širca, S., Gerič Stare, B., Strajnar, P., Knapič, M., Žibrat, U., Folcher, L., Fabrice O., Buisson A., Chappe A-M., Inacio M. L., Leidy R., Filomena N., de Andrade E., Gerrit K., van Hesse E., Warbroek T., Abrantes I. L.P.M., Conceicao I., Maleita C. and Bacic, J. 2021. Global warming and distribution of root-knot nematode species of the tropical group (MeloTrop), Report of the Euphresco project 2016- A-199, 1-39. URL: https://zenodo.org/record/5171594 (11.5.2022.)

Wishart J. Philips M.S. and Blok V.C. 2002. Ribosomal Intergenic Spacer: a polymerase chain reaction diagnostic for *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla*. Phytopathology 92, 884-892

Zijlstra C., Donkers-Venne, D.T.H.M. and Fargette, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays, Nematology. Vol. 2(8), 847-853