Scientific Papers. Series A. Agronomy, Vol. LXVII, No. 1, 2024
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

MELOIDOGYNE SPECIES THAT POSE A THREAT TO POTATO CROP IN ROMANIA

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Abstract

The most important root-knot nematodes specific for potatoes crops in Europe are Meloidogyne chitwoodi and M. fallax which are regulated as Union quarantine pests, according to Commission Implementing Regulation Annex II B of EU Reg. 2019/2072). A monitoring program is ongoing for detection and identification of quarantine and regulated non quarantine pests with economic importance for Solanum tuberosum in Romania. One of the goals of this program is to have a clear situation of nematodes which can occur in order to establish the status of them in Romania and their distribution. In the literature there is mentioned also another root-knot nematode species which can be present on potato, namely Meloidogyne hapla. Official surveys are based on taking sample consists of soil and potatoes tubers. For extraction of juvenile nematodes (J_2) from soil samples were used Oostenbrink elutriator and Baermann funnel and for females from potato tubers samples by enzymatic digestion of potato peels. The identification of Meloidogyne species was done by morphological and molecular methods. It was the first record of M. hapla on Solanum tuberosum in Romania.

Key words*: Meloidogyne hapla, potato tubers, PCR, Sibiu, soil.*

INTRODUCTION

The potato crop (*Solanum tuberosum*) is particularly important worldwide, being considered a basic food crop whose production is constantly increasing. Potato tubers are exposed to a large number of diseases and pests that can significantly reduce production per hectare.

The National Phytosanitary Authority monitors Union quarantine and regulated non-quarantine pests, harmful to potatoes from domestic production, Union territory and imported from third countries. The nematode species monitored are: *Globodera rostochiensis* and *G. pallida*, *Meloidogyne chitwoodi* and *M. fallax*, as Union quarantine species (Annex II B of EU Reg. 2019/ 2072) as well as the non-quarantine species *Ditylenchus destructor* (Annex IV of EU Reg. 2019/ 2072. Among the quarantine nematode species carefully monitored phytosanitarily in Romania, only *Globodera rostochiensis* and *G. pallida* are present.

Numerous species of nematodes belonging to the genus *Meloidogyne* Göldi, 1892 (Tylenchida) (root-knot nematode - RKN) are considered major pests of the potato crop worlwide in terms of economic damage potato crops by reducing yield and affecting tuber quality with external deformations like gall and internal flesh necrosis. The genus *Meloidogyne* includes over 100 species, but only a few of them are considered agriculturally important pest species (Subbotin et al., 2021).

Meloidogyne chitwoodi, M. fallax and *M. hapla,* are species which are more adapted to temperate conditions.

In the past few years some papers had drawn attention about the presence of some species belonging to the genus *Meloidogyne* in Romania: *M. incognita* on *Impatiens* spp., *Hibiscus rosa sinensis* (Rădoi et al., 2019), *Mammillaria backebergiana*, *Apium graveolens*, *Beta vulgaris*, *Brassica oleracea*, *Brassica oleracea* var. *botritys*, *Capsicum*

annuum, *Cucumis sativus, Cucurbita pepo, Lactuca sativa, Lycopersicum esculentum* (Boroș et al., 2015; 2018); *M. hapla* on *Vaccinium myrtillus, Apium graveolens, Beta vulgaris, Cucurbita pepo, Daucus carota, Lactuca sativa, Lycopersicum esculentum, Pastinaca sativa, Petroselinum crispum* (Boroș et al., 2015; 2018); *M. arenaria* on *Vitis vinifera* (Boroș et al., 2018).

MATERIALS AND METHODS

The materials had consisted of soil and seed potato tubers originated from domestic crop and coming from an EU country. The sampling was done according to the Phytosanitary Monitoring Program of Potato Crop.

Detection

National Phytosanitary Laboratory - Nematology Unit received potato tubers samples (40 tubers/sample - Bernina, Levantina and Sanibel variety) sent by phytosanitary inspectors from Sibiu County, in 2021. The samples were from local potato seed production.

Also, in the framework of the annual official survey, in 2022, the seed potato tubers samples (200 tubers/sample) originating from The Netherlands, were sent to the laboratory for analysis by inspectors from Covasna Phytosanitary Office.

In each case, the potato tubers were peeled into 1 cm pieces and combined them to a bulk sample. The enzymatic solution (Pectinex and Celluclast) was used for recover immotile (females) stages of nematode species from plant tissues (EPPO Protocol PM 7/119 (1), 2013).

The females extracted from Sibiu potato tubers were incubated to obtain the juveniles (J_2).

The soil samples were taken immediately after harvesting of seed potato from 3 ha (Ostrojel parcel, Avrig locality, Sibiu county – GPS: coordinates 45.7305, 24.36198), in 2023. The samples were carried out according to national instructions of sampling a network model 10 x 20, each core containing 40 mL of soil from the top 25 cm of soil (Phytosanitary Monitoring Program of Potato Crop). The soil samples were mixed thoroughly and three final sub-samples of 200 mL were taken and were sent by the phytosanitary inspector to the National Phytosanitary Laboratory - Nematology Department.

The nematodes extraction from soil was performed using the Oostenbrink elutriator and the Baermann funnel (EPPO Protocol PM 7/119 (1), 2013) in order to collect the second stage juveniles (J2) *of Meloidogyne* genus.

Identification

The aqueous suspension resulting from the processing of soil samples and tubers was observed under stereomicroscopes (Zeiss Discovery V8, Leica MZ 125).

The identification was based on morphology of the 15 juveniles (J_2), 10 female perineal patterns and molecular analysis.

The suspicious specimens of *Meloidogyne* sp. juveniles (J2) were mounted in a drop of water and formaldehyde 4% (1:1) on the microscopic glass slides for morphological analysis. The identification was based on the following characters: total body length; tail length, hyaline tail length (Karssen, 2002; Hunt and Handoo, 2009).

The female perineal patterns were mounted on glycerine after removing the neck and eggs contents (Jepson, 1987) and identified after (Karssen, 2002; Hunt and Handoo, 2009).

Morphological and morphometrically observations were made by Leica DMLB microscope fitted with Leica FDC 295 camera.

Extraction of DNA from individual nematodes and molecular analysis

Genomic DNA was extracted from juveniles (J_2) isolated from soil and females isolate from potatoes tubers as described by Holterman et al. (2006), using worm lysis buffer (WLB) and incubate for 90 minutes at 65°C, followed by 5 minutes at 99°C.

IGS-based PCR test for *M. chitwoodi*, *M. fallax* and *M. hapla*

The molecular identification of *M. chitwoodi*, *M. fallax* and *M. hapla* with the conventional PCR was performed by amplifying a part of the IGS (Intergenic Spacer) region of the ribosomal DNA (rDNA) using following species-specific primers (Wishart et al., 2002):

MV1 5′-GGATGGCGTGCTTTCAAC-3′ 5S gene, JMV2 5′-

TTTCCCCTTATGATGTTTACCC-3′ IGS (*M. chitwoodi* and *M. fallax*) and JMV3 *M. hapla* 5′ - AAAATCCCCTCGAAAAATCCACC-3′ IGS. The PCR mix (total volume 50 µL) contained 1x buffer enzyme, 3 mM $MgCl_2$, 0.3 µM of each primer, 0.2 mM dNTPs (MP Biomedicals), 2 U Taq DNA polymerase (MP Biomedicals), and 2 µL DNA extract. Amplifications were performed using the following conditions: initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min 30 sec and a final step at 72°C for 10 min. The size of amplification products was determined by comparison with the molecular weight marker ladder 100 bp (DNA Ladder, Promega) following electrophoresis of 10 µl on a 1.5% agarose gel and data analysis was performed using GENi (Syngene).

PCR - Restriction Fragment Length Polymorphism (RFLP)

The ITS regions of rDNA were amplified using the forward primer 18S 5'-TTG ATT ACG TCC CTG CCC TTT-3' and reverse primer 26S 5'-TTT CAC TCG CCG TTA CTA AGG-3' (Vrain et al., 1992). The PCR mixture (total volume 25 µL) contained 1x buffer enzyme, 1.5 mM $MgCl_2$, 0.6 µM of each primer, 0.6 U Taq DNA polymerase (GoTaq DNA polymerase, Promega), 0.1 mM dNTPs (10 mM, Promega) and 5 µL DNA extract. A MasterCycler Pro S (Eppendorf) was used for amplification, and the reaction consisted of a denaturation step at 94°C for 1 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension step of 5 min 72°C. Following PCR, 10 µL of the amplified product was analysed by electrophoresis in a 1% agarose gel. Species-specific ITS–RFLP profiles for *Meloidogyne* were generated using DraI restriction enzyme (Zijlstra et al., 1995). Amplified DNA was digested with DraI restriction endonucleases (Fermentas) using an aliquot of 5 µL of the PCR product and 5U of enzyme, according to the manufacturer's instructions. Fragments were resolved by electrophoresis in 1.5-2% agarose gel. Data analysis was performed using GENi (Syngene) and 100 bp DNA Ladder (Promega) was used as a molecular size marker.

RESULTS AND DISCUSSIONS

The species *Meloidogyne hapla* was detected and identified in tuber samples (Bernica variety, class A) (Figure 1) from Sibiu County (2021).

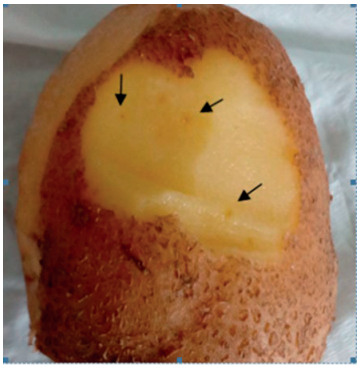

Figure 1. Infested potato tuber with *Meloidogyne hapla*

The identification was carried out morphologically on the adult female stage and the juvenile stage (J2).

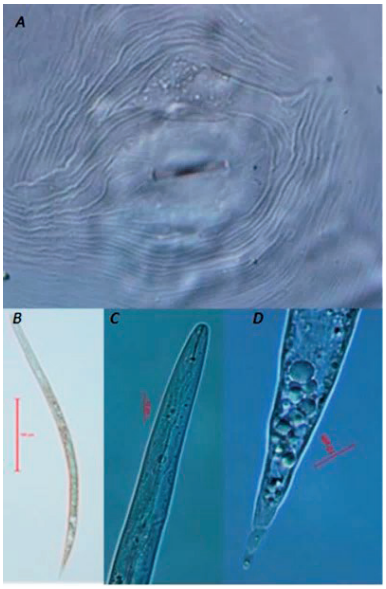

Figure 2. *Meloidogyne hapla*: (A) female perineal pattern; (B) whole specimen (bar = 100 µm); (C) anterior region (bar = 10 µm); (D) tail region (bar = 10 µm)

Perineal pattern of females was rounded with low dorsal arch, a characteristic punctuation near annus, lateral field present (Figure 2). Juvenile (J_2) measurements were revealed the following values: total body length 355 µm ± 14.9 (333 - 384), tail length 48 µm ± 2.1 (45 - 50), hyaline zone length 12 µm ± 1.1 (10-13), stylet length 11 µm ± 1.2 (10-13).

Following the processing of soil samples from Sibiu County (2023), J2 juveniles of *Meloidogyne* sp. were detected. To identify the species, the juveniles were placed in WLB and continued with molecular biology analyses. The identified species was *Meloidogyne hapla*.

In the samples of potato tubers from The Netherlands, sent by the Covasna Phytosanitary Office (2022), adult females of the genus *Meloidogyne* were detected (Figure 3).

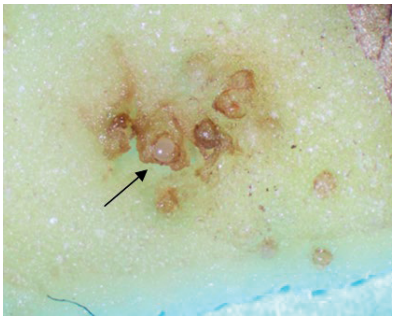

Figure 3. *Meloidogyne chitoodi* female on potato tuber

Females obtained after enzymatic extraction (Figure 4) were placed in WLB buffer and analysed by molecular biology.

Figure 4. *Meloidogyne chitwoodi* females

The quarantine species *Meloidogyne chitwoodi* was detected (Figure 5).

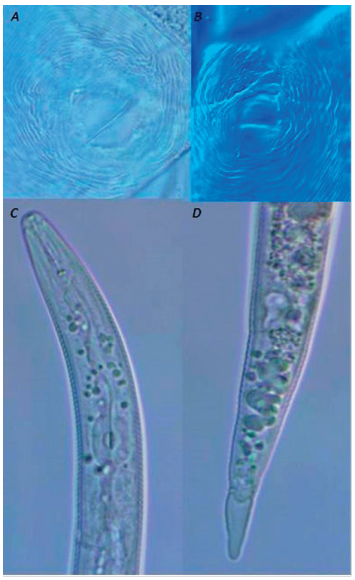

Figure 5. *Meloidogyne chitwoodi*: (A, B) female perineal pattern; (C) anterior region (D) tail region

IGS-based PCR test for *M. chitwoodi, M. fallax* and *M. hapla*

The JMV1, JMV2 and JMV3 primers allow a specific amplification fragment of approximately 540 bp for *Meloidogyne chitwoodi*, 670 bp for *Meloidogyne fallax* and 440 bp *Meloidogyne hapla* (Figure 6 and Figure 7).

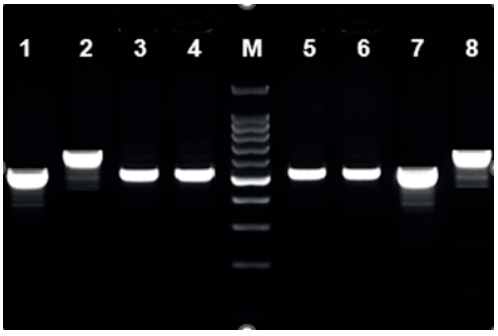

Figure 6. PCR amplification products generated using species-specific primer sets. M: 100 bp marker ladder. Lanes 1 and 7: *M.* (positive control); lanes 2 and 8: *M. fallax* (positive control); lanes 3-6: isolates from potatoes – *M. chitwoodi*

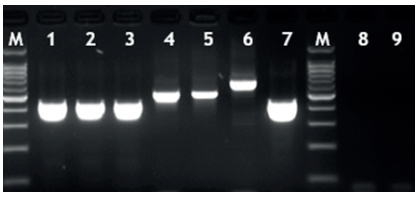

Figure 7. PCR amplification product generated using species-specific primer sets. M: 100 bp marker ladder. Lanes 1-3: isolates from soil – *M. hapla*; lanes 4: isolate from potatoes – *M. chitwoodi*; lanes 5: *M. chitwoodi* (positive control); lane 6: *M. fallax* (positive control); lane 7*: M. hapla* (positive control); lane 8: negative control extraction; lane 9: water control

PCR - Restriction Fragment Length Polymorphism (RFLP)

The 760 bp PCR product we obtained for the amplified ITS region with 18S and 26S primers (Zijlstra C. et al., 1995, 1997). After digestion PCR products with the DraI restriction enzyme, the isolates showed the following restriction patterns: 380 bp for *Meloidogyne hapla* and 660, 100 bp for *Meloidogyne chitwoodi*. The positive control of *Meloidogyne javanica* showed the 380, 220, 200, 180, 120 bp restriction patterns (Figure 8 and Figure 9).

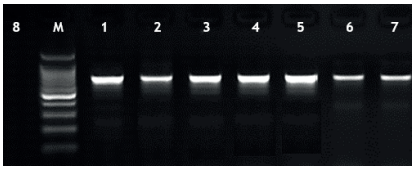

Figure 8. Typical amplification of 760 bp polymerase chain reaction (PCR) product from template of total DNA extracted from isolates of *M. hapla* and *M. chitwoodi.* M: 100 bp marker ladder. Lanes 1 *M. chitwoodi* (positive control); lanes 2-3: isolates from soil – *M. hapla;*lanes 4-5 isolates from potatoes – *M. chitwoodi*; lanes 6-7: *M. javanica* (positive control); lane 8: water control

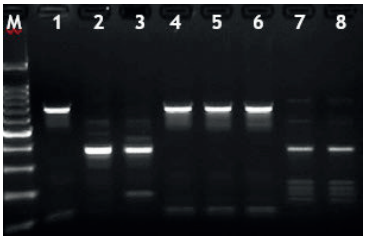

Figure 9. DraI restriction profiles products of *M. hapla* and *M. chitwoodi*. M: 100 bp marker ladder. Lane 1: *M. chitwoodi* (positive control); lanes 2-3: isolates from soil – *M. hapla*; lane 4-6: isolates from potatoes – *M. chitwoodi*; lanes 7-8: *M. javanica* (positive control)

CONCLUSIONS

Solanum tuberosum was recorded first time in Romania as new host for *Meloidogyne hapla*. *Meloidogyne chitwoodi* was detected in seed potato tubers originating from the Union territory (The Netherlands).

Rapid diagnosis of *Meloidogyne* species obtained at the (J_2), female or male stage is essential for management decision making or in support of plant health inspection services. PCR-RFLP was shown to be sensitive and specific but this technique requires an additional step compared to species-specific PCR (multiplex). Use the restriction enzymes leads to increase the cost of analyses.

The multiplex PCR protocol used represents an improvement in terms of reducing the time and material required for the diagnosis of the three species. Combined with an efficient, easy-to-use DNA extraction protocol, identification can be done in 1-2 days, having the potential to be used in routine molecular diagnostics, allowing rapid identification of the three species.

The survey has the aim of providing seed potatoes which are free from targeted nematodes and other pests that can be used safely for domestic use or for export.

The monitoring activities reveals nematodes that can be present in soil or goods which can be regulated or common species, but responsible for damaging of crops.

ACKNOWLEDGEMENTS

This work was carried out with the support of National Phytosanitary Authority.

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