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## **The incidence of the root-knot nematode *Meloidogyne incognita* and *Meloidogyne hapla* in Slovenia**

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### **ABSTRACT**

The root knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood, 1949 is one of the most harmful and most ubiquitous species in the genus *Meloidogyne*. It was detected very often in the greenhouses of northern Europe, but in the open field it is restricted to the southern parts of Europe. While this species has been recorded in almost all parts of the world and also in some parts of the former Yugoslavia, there is no data about its presence in Slovenia. *M. incognita* is able to parasitize about 700 plant hosts and varieties including the majority of economically important crops. In the late summer of 2002, nematodes of *M. incognita* were isolated from the roots of hot pepper plants, *Capsicum annum* L., from the greenhouse situated in Portorož at the Adriatic Coast, Slovenia. The nematode was morphologically identified as *M. incognita* and confirmed by isozyme gel electrophoresis (PhastSystem, Pharmacia). To our knowledge, this is the first report of *M. incognita* in Slovenia. Beside this finding, *M. hapla* was also found for the first time in the open field in Slovenia; until now it has been detected on different host plants grown only in greenhouses. In October 2002, *M. hapla* was isolated from galls of the sweet pepper grown in the open field in Ljubljana situated in the central part of Slovenia.

**Key words:** identification, isozyme gel electrophoresis, *Meloidogyne incognita*, *M. hapla*, morphology, nematodes

### **IZVLEČEK**

#### **PRVO POROČILO O NAJDBI OGORČICE KORENINSKIH ŠIŠK *MELOIDOGYNE INCOGNITA* V SLOVENIJI**

Vrsta *Meloidogyne incognita* (Kofoid and White) Chitwood, 1949, je ena najbolj škodljivih in najbolj razširjenih ogorčic koreninskih šišk, *Meloidogyne* spp. V območjih severne Evrope je bila večkrat ugotovljena v rastlinjakih, na prostem pa se v Evropi pojavlja predvsem v južnejših, toplejših predelih. Medtem ko so o omenjeni vrsti poročali že iz skoraj vseh območij sveta, tudi iz nekaterih predelov bivše Jugoslavije, pa o njeni navzočnosti v Sloveniji ni nobenih podatkov. Ogorčica *M. incognita* zajeda okoli 700 različnih rastlinskih vrst, vključno z večino gospodarsko pomembnih rastlin. Vrsto *M. incognita* smo pri nas prvič izolirali pozno

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poleti leta 2002 iz korenin paprike iz rastlinjaka v Portorožu. Ogorčico smo morfološko določili, identifikacijo pa potrdili z izoencimsko gelsko elektroforezo (PhastSystem, Pharmacia). Poleg že omenjene vrste smo v preteklem letu iz korenin feferonov v okolici Ljubljane prvič na prostem pri nas izolirali tudi vrsto *M. hapla*, o kateri navzočnosti v rastlinjakih se je v Sloveniji v preteklosti že poročalo.

**Ključne besede:** identifikacija, izoencimska gelska elektroforeza, *Meloidogyne incognita*, *M. hapla*, morfologija, ogorčice

## 1 INTRODUCTION

Root-knot nematodes, the genus *Meloidogyne*, represent a relatively small but economically important group of obligate plant parasites. They are distributed world wide and parasitize thousands of higher plant species including monocotyledons, dicotyledons, and herbaceous and woody plants (Eisenback and Hirschmann, 1991). They reproduce and feed within the roots and usually cause the formation of knots and galls on roots of susceptible host plants. The physiology of infected plants is disordered, crop yield is reduced and the quality of the plant products is affected. More than eighty nominal species have been described so far, about ten species are agricultural pests, four are major pests and distributed world wide in agricultural areas (Karsen, 2002).

Twenty root-knot nematode species have been detected in Europe so far, thirteen of them having been described from an European type locality (Karsen *et al.*, 1998). Most species were described from agricultural areas, except for *M. ardenensis*, *M. deconincki*, *M. litoralis*, *M. kralli*, *M. maritima* and *M. duytsi* (Karsen, 2002). From the five root-knot nematodes detected in former Yugoslavia only one species, *M. hapla*, was found in Slovenia until 2002.

Among all described root-knot nematodes, two species: *M. chitwoodi* and *M. fallax* were recently added to the European list of quarantine organisms, to prevent further distribution within Europe. They parasitize on monocotyledons and dicotyledons, including several crop plants as potatoes, carrots and tomatoes (Santo *et al.*, 1980; O'Bannon *et al.*, 1982; Brinkman *et al.*, 1996; Karsen, 2002).

Knowledge about the dissemination of *Meloidogyne* spp. in Slovenia is rather poor. Infrequently was reported about the presence of root-knot nematodes in Slovenia and only *M. hapla* was detected in some glasshouses in the regions of Čatež and Ljubljana. This was the reason for starting more intensive survey on *Meloidogyne* species in Slovenia. So far, two different species of the genus *Meloidogyne* were detected. The nematodes were identified with morphological and biochemical methods as *M. incognita* and *M. hapla*. Nematodes of *M. incognita* were isolated from the roots of paprika plants, *Capsicum annum* L., from the greenhouse situated in Portorož at the Adriatic Coast, Slovenia. *M. hapla* was found for the first time in the open field in Slovenia. It was isolated from galls of the sweet pepper grown in the open field in Ljubljana.

Many enzymatic studies have demonstrated that species within *Meloidogyne* genus can be differentiated using polyacriamide-gel electrophoresis (PAGE). PhastSistem

(Amersham Biosciences) enables biochemical approach for isozyme phenotyping using very thin (0.4 mm) polyacrylamide slab gel electrophoresis where relatively small amounts of enzyme (single female content) can be analysed. Combining malate dehydrogenase (MDH) and esterase (EST) phenotypes analyses is possible to distinguish between *M. incognita* and *M. hapla* (Dalmasso and Berge, 1987; Esbenshade and Triantaphyllou, 1985; Karssen *et al.*, 1995).

## 2 MATERIAL AND METHODS

### 2.1 Nematodes

Females, males and second stage juveniles of *M. incognita* and *M. hapla* were isolated from the infested Capsicum roots samples taken in greenhouse near Portorož and in open field in Ljubljana, respectively. Roots were placed in a Petry dish and poured over with 0.9% NaCl to prevent female bursting. Under dissecting microscope nematodes were isolated using scalpel and nematological needle.

### 2.2 Method 1: morphometrical identification

Isolated males and second juvenile stages were heat killed and fixed in trietanolamin-formalin (TAF) solution while analyzes of female parameters and perineal patterns were made on fresh isolated females. Nematodes were analyzed under microscope connected to personal computer (LUCIA image analyser system). From different life stages different morphological parameters were measured and compared with species characteristic parameters (Karssen, 2002). Identification of the species was made by combining morphometrics and isozyme phenotypes patterns.

### 2.3 Method 2: native PAGE and isozyme staining

#### 2.3.1 Preparation of the samples

Six young females of each species were isolated under dissecting microscope. After isolation females were rinsed with reagent-grade water and transferred to 12 sample-well stamp placed on ice bath. Each female was placed in sample well containing 0.5 $\mu$ l of extraction buffer (20% sucrose, 2% Triton X-100, 0.01% Bromphenol Blue) (Esbenshade and Triantaphyllou, 1985) and squashed with needle to release body content. Samples were loaded on two 12/0.3 sample applicator which were placed into applicator arms of the PhastSystem device. For reference species we used *Meloidogyne javanica* (lines 6 and 7).

#### 2.3.2 Electrophoresis and enzyme staining

Electrophoresis took place onto PhastGel gradient (8 - 25) gel with buffer system according to manufacture instructions. The following adapted program was used (Karssen *et al.*, 1995):

Sample appl. down at 3.2 Vh  
Sample appl. up at 3.3 Vh

Sep 3.1400 V 10 mA 2.5 W 10°C 10 Vh  
Sep 3.1400 V 1 mA 2.5 W 10°C 2 Vh  
Sep 3.1400 V 10 mA 2.5 W 10°C 125 Vh

After electrophoresis gel was stained for enzymatic activity in a Petry dish at 37°C with different staining solutions. MDH staining solution contained 0.05 g  $\beta$ -NAD, 0.03 g Nitro Blue Tetrazolium, 0.02 g Phenazine Methosulfate, 5.0 ml 0.5 M Tris pH 7.1, and 7.5 ml stock (10.6 g Na<sub>2</sub>CO<sub>3</sub> + 1.34 g L-malic acid in 100 ml water) dissolved in 70 ml of reagent-grade water. For EST activity we used staining solution contained 100 ml 0.1 M Phosphate buffer pH 7.3, 0.06 g Fast Blue RR salt, 0.03 g EDTA and 0.04 g  $\alpha$ -Naphthyl acetate dissolved in 2 ml

acetone (Karssen *et al.*, 1995). Incubation for MDH lasted 5 minutes, after that gel was twice washed with distilled water and further stained for EST activity for 30 minutes. After isozyme phenotypes patterns were clearly visible the enzymatic reaction was stopped by rinsing gels with distilled water and fixed for 5 minutes in a solution of 10% acetic acid, 10% glycerol and 80% distilled water.

### 3 RESULTS

Isozyme phenotype patterns (see Figure 1) were analyzed by calculating relative migration rates ( $R_m$ ). The  $R_m$ 's for MDH were 0.32 and 0.47 for *M. incognita* and *M. hapla*., respectively and 3.30 for *M. javanica* as reference. EST phenotype  $R_m$  was 0.56 for *M. incognita* and 0.57 for *M. hapla*. *M. javanica* gave tree banded EST pattern with  $R_m$ 's 0.55, 0.62 and 0.64. Species specific isozyme pattern supported morphological identification of species done on females perineal patterns (Figure 2 and 3) and measurements of nematode different life stages (see Table 1).

Table 1: Morphometrics (average, minimum and maximum) of *M. incognita* and *M. hapla* isolated from the roots of hot and sweet pepper in Slovenia.

Character	<i>M. incognita</i>			<i>M. hapla</i>		
	J2	Males	Females	J2	Males	Females
N	10	-	5	10	2	5
Body length [ $\mu\text{m}$ ]	404.5 (378.7- 416.8)		-	418.0 (389.0- 455.3)	1562.2 (1520.4- 1603.9)	-
Greatest body diameter [ $\mu\text{m}$ ]	13.9 (12.7- 15.0)		-	13.9 (12.8- 15.0)	42.3 (37.9- 46.7)	-
Body diam. at stylet knobs [ $\mu\text{m}$ ]	9.7 (9.3- 10.1)		-	9.5 (9.0- 9.9)	21.5 (20.8- 22.1)	-
Body diam. at anus [ $\mu\text{m}$ ]	10.5 (8.4- 12.0)	-	-	10.3 (9.5- 11.2)	-	-
Stylet length [ $\mu\text{m}$ ]	11.3 (10.3- 11.9)		16.5 (16.0- 16.7)	11.2 (10.2- 12.2)	17.2 (16.7- 17.6)	14.4 (12.5 - 15.9)
Dorsal gland opening [ $\mu\text{m}$ ]	2.6 (2.3- 2.9)		3.6 (3.1- 4.4)	3.1 (2.2- 5.1)	4.2 (3.6- 4.8)	3.7 (2.9 - 5.1)
Metacarpus length [ $\mu\text{m}$ ]	-	-	43.1 (35.6- 48)	-	-	50.2 (45.6 - 54.6)
Metacarpus diameter [ $\mu\text{m}$ ]	-	-	39.3 (32- 51)	-	-	50.6 (46.0 - 54.2)
Tail length [ $\mu\text{m}$ ]	51.5 (45.3- 56.8)	-	-	54.2 (49.2- 59.6)	-	-
Tail terminus length [ $\mu\text{m}$ ]	12.5 (10.8- 14.9)	-	-	17.0 (13.6- 19.9)	-	-
Spicule [ $\mu\text{m}$ ]	-		-	-	39.2 (35.2- 43.1)	-
a	29.0 (26.7- 32.1)		-	30.1 (28.2- 32.0)	37.2 (34.1- 40.1)	-
c	7.9 (7.2- 8.9)	-	-	7.7 (7.3- 8.6)	-	-
c'	5.0 (4.2- 6.8)	-	-	5.3 (4.8- 5.6)	-	-

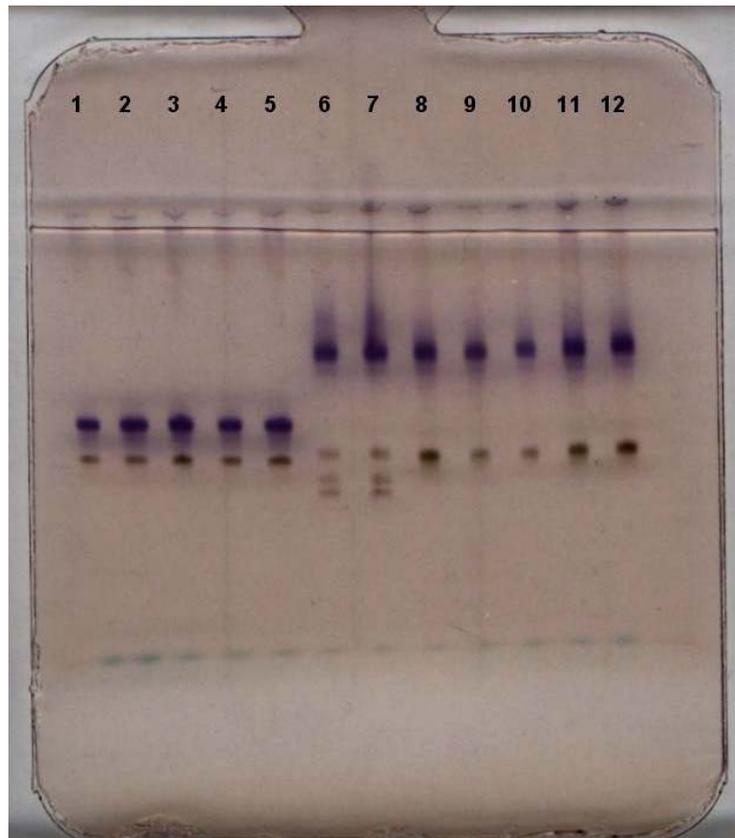


Fig. 1: Isozyme (EST and MDH) phenotype patterns of individual female of *M. hapla* (lanes 1,2,3,4,5), *M. incognita* (lanes 8,9,10,11,12) and *M. javanica* as reference (lanes 6,7).



Fig. 2: *Meloidigyne incognitia* female perineal pattern.

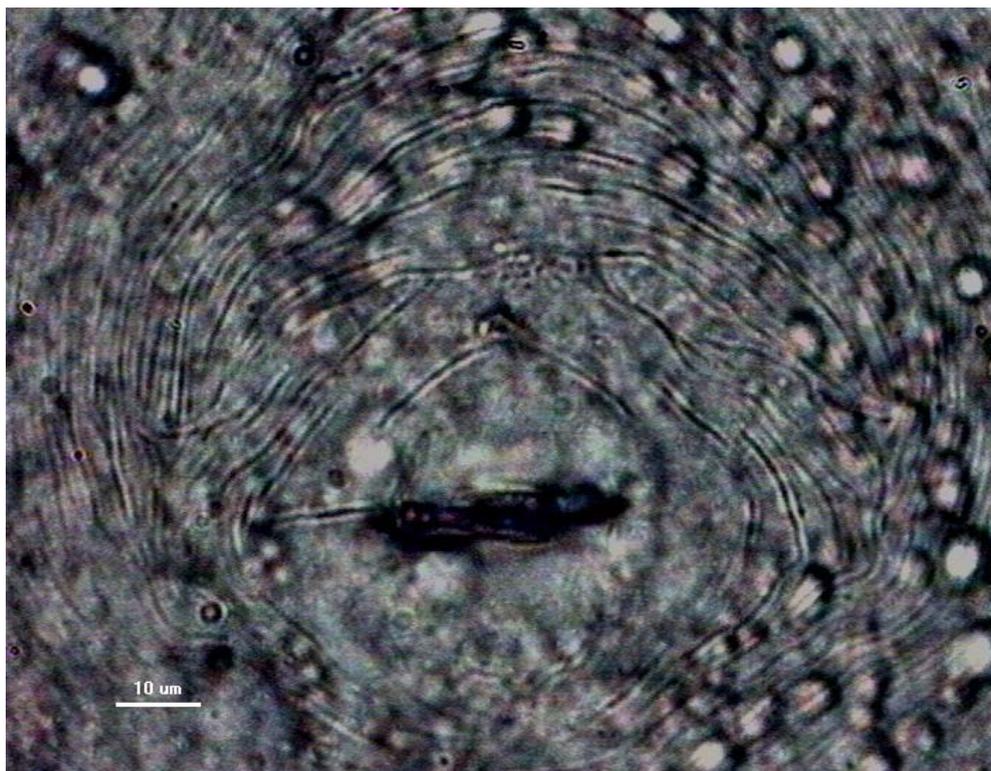


Fig. 3: *Meloidigyne hapla* female perineal pattern.

#### 4 DISCUSSION

M. J. Berkely (1855) was the first who correlated galls on glasshouse cucumber roots with nematodes. The first root-knot nematode was described in Europe as *Anguillula marioni* on *Onobrychis sativa* Lam. by Cornu in 1879 which was in 1884 synonymized with *Heterodera radiculicola* by Müller (cited in Karsen, 2002). At the end of the 19<sup>th</sup> century, root-knot nematodes were known from all parts of the world and their polyphagous behaviour were recognized.

Almost one century after the first report of root-knot nematodes, they were still marked as a single polyphagous species within the genus *Heterodera*, despite the clearly documented differences between root-knot and cyst nematodes by Marcinowski (1909) and Nagakura (1930) (cited in Klindić, 1968). In 1949 Chitwood separated the root-knot nematodes from *Heterodera*, re-erected the genus *Meloidogyne*, and described a generic diagnosis for the genus. This was the turning point in root-knot nematode taxonomy and the end of the period of confusion in taxonomical studies of this group of nematodes (cited in Klindić, 1968). Between 1949 and 1998 more than eighty nominal *Meloidogyne* species have been described, especially from North and South America, Africa, China and Europe (Karsen, 2002). *M. artiellia* was the first root-knot nematode described in Europe. Twenty root-knot nematode species have been detected in Europe so far, thirteen of them having been described from an European type locality (Karsen *et al.*, 1998).

Root-knot nematodes were also studied in several regions of former Yugoslavia. Protić mentioned the presence of the root-knot nematodes on tomato and egg plant roots in Hercegovina already in 1926 while Martinović in 1947 established the root-

knot nematodes on cucumber plants grown in one of glasshouses near Belgrade in Serbia (cited in Grujičić, 1971). Klindić (1955) reported about damages on red pepper caused by root-knot nematodes in Hercegovina. Her investigations were focused mainly in study of efficacy of DD nematicides. Grujičić (1959) reported about the presence of the *Meloidogyne* spp. on tomato (*Solanum lycopersicum* L.), red pepper (*Capsicum annuum* L.), cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), celery (*Apium graveolens* L.) and the weed species *Solanum nigrum* L. on the territory of Serbia where he examined the influence of temperature, moisture and soil on nematode population dynamics. In 1967 Grujičić established *M. naasi* on sugar beet, mangold, wheat and barley in Serbia. With all these crops the nematode appeared sporadically. Some reports about damages caused by root-knot nematodes, especially from the regions of Istria and Dalmatia, derived from Croatia; no species were identified (Maceljiski, 1967). In 1969 Klindić isolated *M. incognita*, *M. incognita* var. *incognita*, *M. arenaria*, *M. hapla* and *M. javanica* from different locations in Hercegovina. In 1970 Grujičić and Paunović for the first time detected *M. hapla* in the open field in Serbia. Grujičić also reported about the presence of *M. incognita*, *M. arenaria*, *M. acrita*, *M. hapla* and *M. javanica* on various vegetable plants, particularly in glasshouses in Serbia. Krnjaić (1977) reported about the spreading of *M. incognita* and *M. arenaria* in glasshouses in Macedonia.

At Agricultural Institute of Slovenia we have recently started more intensive survey on presence of *Meloidogyne* species in Slovenia. So far, we have found new locations of *M. hapla* and for the first time *M. incognita* in Slovenia (Širca *et al.*, 2003). Due to variety of soil and climate present in Slovenia we expect to find more *Meloidogyne* species in the future.

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