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Survey of Entomopathogenic Nematodes on Agricultural Land in Croatia

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ABSTRACT

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are soil-dwelling nematodes that parasitize many insect species. They are a promising alternative to chemical insecticides in many crops. The aim of this work was to verify the occurrence of EPN on agricultural land in Croatia and to isolate native EPN that could be more effective in pest control than commercial strains. In the period from 2017 to 2019, a total of 135 soil samples were taken from 27 locations in 7 continental Croatian counties. Using the insect baiting technique, White trap and molecular biological analysis, *Steinernema carpocapsae* was detected for the first time in Croatia in two soil samples taken from a corn crop in the locality Karane and from an apple orchard in the locality Đeletovci. The analyzed DNA sequences were assigned the accession numbers MN982232 (isolate Karane) and MT114473 (isolate Đeletovci) in the GenBank database. The recovery frequency of EPNs was 1.48%. The abundance of nematodes in Croatian soils was 7.4%. Currently, *S. carpocapsae* has the status of a native species, which offers the possibility of field application in Croatia.

Keywords: EPNs, native species, Steinernematidae, *Steinernema carpocapsae*, biological pest control.

INTRODUCTION

Entomopathogenic nematodes (EPNs) are lethal obligate parasites of insects (Gaugler and Kaya, 1990; Bedding et al., 1993; Kaya and Gaugler, 1993; Liu et al., 2000) and are among the most important biological control agents of insect pests (Liu et al., 2000). EPNs include the families Heterorhabditidae and Steinernematidae (Chitwood and Chitwood, 1937). These nematodes are carriers of specific pathogenic bacteria, *Photorhabdus* spp. in the Heterorhabditidae and *Xenorhabdus* spp. in the Steinernematidae, which are released into the hemocell after the infective stage of the nematode enters the insect host (Forst et al., 1997; Liu et al., 2000).

The association between nematodes and their symbiotic bacteria is of fundamental importance for nematode infectivity, mass reproduction and authorization as biocontrol agents (Liu et al., 1999).

The importance of EPNs for biological control was first discovered in the 1930s in the United States of America (Laznik and Trdan, 2008). EPNs have been commercially available since the 1970s, and a high percentage of field trials with rhabditids resulted in significant reductions in pest population densities and successful crop protection (Mracek, 2002). In addition, no significant effects of EPNs on populations of collembolans and mites [Ishibashi et al. (1987), Georgis et al. (1991) cit. Somasekhar et al. (2002)] or non-target insects of the families Carabidae, Histridae, Staphylinidae and Gryllidae under field conditions [Georgis et al. (1991), Koch and Bathon (1993) cit. Somasekhar et al. (2002)] have been demonstrated. The reduction in abundance and diversity of the plant-parasitic nematodes and no negative effects on free-living nematodes, which are important for nutrient cycling, are also considered as positive non-target effects of EPN (Somasekhar et al., 2002).

EPNs have been found in a variety of soil types and habitats, including crops, pastures, forests and beaches (Liu and Berry, 1995). There are at least 100 species of EPNs

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worldwide (Liu et al., 2000), and Europe is the most intensively studied region (Hominick et al., 1996). As many chemical pesticides have been withdrawn from the market due to ecotoxicological problems, an increasing trend in the research and commercialization of EPNs can be observed worldwide. Strong demand from authorities, agricultural professionals and society across Europe has also driven collaborative research to accelerate the agroecological transition (INRAE, 2020).

The introduction of EPNs as beneficial organisms for pest control in a new area requires an initial investigation into the existence of native species as well as their identification (Emelianoff et al., 2008). The introduction of exotic EPN can lead to displacement of local populations and/or species, undermining natural diversity, and can also be insufficiently effective against local pests, due to non-adaptation to local environmental conditions (Miller and Barbercheck, 2001). Blackshaw and Newell (1987) demonstrated that the use of endemic EPNs in the field is likely to be most effective. The use of endemic EPNs may also have less risk to non-target organisms than introduced species (Blackshaw, 1988).

The aim of this study was to determine the natural distribution of EPN in Croatia and to detect endemic EPN. In this way, the effectiveness of endemic EPNs in pest control can be further tested and a path towards production without chemical pesticides is made possible.

MATERIAL AND METHODS

To investigate the presence and distribution of EPN, a total of 135 soil samples were collected over three years (2017-2019) from 27 locations in 7 continental Croatian counties from corn, soybean, potato and alfalfa fields, hazel, apple and cherry orchards and pastures (Table 1). An area of at least 2-4 m^2 was sampled at each sampling point. Soil samples were collected randomly (Orozco et al., 2014), five from each sampling point (crop). Each soil sample (approximately 1 kg) consisted of three subsamples taken at a depth of at least 15 cm. To obtain the EPN from the soil, the insect baiting technique (Bedding and Akhurst, 1975) was used. In the laboratory, 5 larvae of greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) in the last larval stage were inserted into perforated tubes in each soil sample. The bags with the soil samples were stored at room temperature (20 ± 3°C). After 1-10 days, all insects were collected, and parasitized cadavers with the typical grey-brown coloration were individually placed in a White trap (White, 1927) to allow emergence of infective juveniles (IJs). Developing nematodes were pooled for each sample and used to infect fresh *G. mellonella* larvae to induce reinfection and generate nematodes for identification and culture establishment. In reinfected larvae, a grey-brown coloration typical of *Steinernema* sp. was observed after one day. The harvested IJs from the parasitized cadavers were stored at 4°C in distilled water (Valadas et al., 2007).

Molecular characterization was performed to identify the isolated EPNs to species level. The genomic DNA was extracted from these samples using a commercially available isolation kit (Nematode DNA Extraction & Purification kit for Nematode Suspensions & Multiple Cysts, ClearDetections, Netherlands). The isolated genomic DNA was subjected to PCR to amplify the ITS-rDNA region using primers AB 28 and TW 81 and the PCR procedure described by Hominick et al. (1997). The PCR products were reisolated from the electrophoresis gel using the commercial E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, USA) and then purified using the QIAquick PCR Purification Kit (QIAGEN, USA). The samples were sequenced in the laboratory of Macrogen Europe (Netherlands). The nucleotide sequences obtained were entered into the GenBank genetic sequence database and compared with all nucleotide sequences publicly available in the National Center for Biotechnology Information (NCBI) database using the standard nucleotide BLAST search (www.ncbi.nlm.nih.gov).

Table 1. Distribution of soil sampling for EPNs isolation in Croatia (2017-2019)

County	Location	Position	Sampling area*	Soil sampling date
Koprivnica - Križevci County	Karane	46°01'08.8"N 16°31'31.7"E	corn	10.08.2017
	Lepavina	46°05'48.1“N 16°40'01.1“E	stubble (wheat or barley)	
	Glogovac	46°07'07.4“N 16°52'06.1“E	corn	
Bjelovar - Bilogora County	Garešnica	45°35'47.9“N 16°51'01.4“E	garden	03.08.2017
	Palešnik	45°38'35.3"N 16°59'20.8"E	stubble (wheat)	24.08.2017
	Velika Dapčevica	45°37'47.9"N 16°59'26.2"E	pasture	
	Milaševac	45°46'02.0"N 16°39'00.0"E	orchard (apple)	15.07.2019
Međimurje County	Lopatinec	46°25'59“N 16°22'59“E	garden	17.07.2019
	Nedelišće	46°25'59“N 16°22'59“E	orchard (apple)	17.07.2019
	Slakovec	46°24'22“N 16°22'37“E	pasture	17.07.2019
Virovitica - Podravina County	Rit	45°54'19.4"N 17°27'57.6"E	stubble	24.08.2017
	Gradina	45°51'09.6"N 17°32'00.4"E	soya	
	Veliki Rezovac	45°48'18.5"N 17°24'51.1"E	corn	
	Orahovica	45°21'59.1"N 17°51'01.2"E	pasture	30.05.2018
	Pčelić	45°48'47"N 17°30'22"E	orchard (apple)	19.07.2018
	Slatina	45°43'13.3"N 17°40'32.3"E	orchard (apple)	15.07.2019
	Bukovački Antunovac	45°38'41.9"N 17°47'08.2"E	orchard (apple)	15.07.2019
Vukovar - Srijem County	Tovarnik	45°10'35.9"N 19°10'19.9"E	corn	07.07.2017
	Tovarnik	45°10'00.3"N 19°08'25.5"E	orchard (apple)	02.09.2017
	Ilača	45°11'22.9"N 19°07'34.4"E	hazel	
	Đeletovci	45°10'31.3"N 18°59'39.9"E	orchard (apple)	15.07.2019
	Ilok	45°13'17.3"N 19°21'02.7"E	orchard (apple)	15.07.2019
	Tovarnik	45°10'51.1"N 19°09'31.6"E	orchard (cherry)	15.07.2019
	Lovas	45°12'39.2"N 19°09'00.7"E	alfalfa field	15.07.2019
City of Zagreb	Zagreb - Maksimir	45°49'30.4"N 16°01'54.4"E	potato	02.08.2017
Zagreb County	Bapča	45°44'41.5"N 16°06'29.9"E	orchard (apple)	15.07.2019
	Prečec	45°45'04.5"N 16°21'53.2"E	pasture	15.07.2019

* Soil samples were collected randomly, five from each sampling area/crop.

RESULTS AND DISCUSSION

EPN of the species *Steinernema carpocapsae* Weiser (Nematoda: Steinernematidae), identified for the first time in Croatia, was obtained from a soil sample taken in corn crop at the village of Karane (46°01'08.8"N, 16°31'31.7"E) in Koprivnica - Križevci County in 2017. In 2019, the same species, *S. carpocapsae*, was obtained from a soil sample taken in an apple orchard in the locality Đeletovci (45°10'31.3"N, 18°59'39.9"E) in Vukovar - Srijem County (Table 2). In this survey, the recovery frequency (number of samples with positive nematode findings per total number of samples) of EPN was 1.48% (2/135). The frequency of nematodes in Croatian soils (number of locations with positive nematode findings in relation to the total number of locations) was 7.4% (2/27 locations). Bait larvae of *G. mellonella* infected with isolates showed the grey-brown coloration typical of *Steinernema* sp.

Table 2. The results of survey of EPN on agricultural land in Croatia

County	Location	Position	Crop	Soil sampling date	Isolated EPN species
Koprivnica - Križevci County	Karane	46°01'08.8"N 16°31'31.7"E	corn	10.08.2017	*Steinernema carpocapsae* Weiser
Vukovar - Srijem County	Đeletovci	45°10'31.3"N 18°59'39.9"E	orchard (apple)	15.07.2019	*Steinernema carpocapsae* Weiser

Molecular characterization was performed to confirm the identification of the isolated nematodes obtained from the larvae of the wax moth. The DNA sequences obtained after sequencing the samples were compared with all nucleotide sequences available in the NCBI database using the standard nucleotide BLAST search. Sequences that showed a significant alignment and at least 96% identity (in the case of the sample from Karane) or at least 99% identity (sample from Đeletovci) belonged to the species *S. carpocapsae*. Therefore, the analyzed DNA sequences were assigned the accession numbers MN982232 (isolate Karane) and MT114473 (isolate Đeletovci) in the GenBank database for genetic sequences.

The recovery frequency of EPNs (*S. carpocapsae*) in our study in Croatian soils (1.48%) was higher than the recovery frequency of *S. carpocapsae* (0.83%) (Laznik et al., 2008), but lower than the recovery frequency of *Steinernema feltiae* (Filipjev) (2.5%) (Laznik et al., 2009) in Slovenia and of *S. feltiae* (2%), which was reported for the first time in Croatia (Majić et al., 2018). In the study by Razia and Sivaramakrishnan (2014), EPNs were similarly found in 1.6% of the soil samples collected. According to Orozco et al. (2014), the probability of finding insects naturally parasitized by EPNs in nature is less than 3%. This statement is consistent with all the data mentioned above. The frequency of EPN occurrence could be much higher, as in the soils of Oregon (11.8%) (Liu and Berry, 1995) or Ireland (10.5%) (Griffin et al., 1991), where similar results related to a similar sandy soil texture have been reported.

The spread of nematodes may be due to the presence of suitable hosts, vegetation, soil textures, and agricultural practices (Liu and Berry, 1995). The frequency of EPN occurrence may also be influenced by soil temperature and moisture, as these vary with the season (Akhurst and Bedding, 1986; Blackshaw, 1988; Griffin et al., 1991). In our study, *S. carpocapsae* was detected in corn fields and apple orchards, both croplands. Mracek and Webster (1993) found that nematodes occurred at sites where human impact was considerable and that no nematode-positive soil samples were found at sites where human impact was low. They suggested that this could be the result of insect pest outbreaks associated with crop monocultures. However, Liu and Berry (1995) found a significant correlation between habitat group and nematode recovery. The highest recovery rate was

found on marine beach soils, followed by forest and orchard soils. The recovery rate was lower in cropland groups such as the corn, wheat and vegetable group and the grass and pasture group. Human activities in croplands, such as the use of chemical pesticides and fertilizers, could have an unfavorable effect on the survival of EPN, so further studies are needed to confirm this.

Molecular characterization of EPN found in samples from the Croatian localities of Karane and Đeletovci identified the nematode species in both cases as *S. carpocapsae*. The nucleotide sequence of the Croatian isolate Karane (accession number MN982232) is 783 bp long and includes a partial sequence of the small subunit ribosomal RNA gene, a complete sequence of the internal transcribed spacer 1 and ribosomal RNA gene 5.8S, and a partial sequence of the internal transcribed spacer 2. The standard nucleotide BLAST search performed in GenBank revealed that the Croatian isolate Karane has a significant match and 96.96% identity with *S. carpocapsae* populations in France (accession number LR745198), the Czech Republic (KJ950291) and the Azores, Portugal (GQ421606). A high percentage match (96.76%) was also observed in sequences of *S. carpocapsae* found in the Veneto region of Italy (accession numbers LN624758 and LN624759) and the Azores, Portugal (GQ421607). The other Croatian isolate Đeletovci (accession number MT114473) is 1485 bp long and contains the partial sequence of the internal transcribed spacer 1, the complete sequence of the ribosomal RNA gene 5.8S and the internal transcribed spacer 2 as well as a partial sequence of the ribosomal RNA large subunit gene. The BLAST search via GenBank revealed a high percentage identity (99.49%) with *S. carpocapsae* found in the Azores, Portugal (accession number GQ421606). High similarity (99.36%) was also found for sequences of *S. carpocapsae* strains isolated in the Veneto region, Italy (LN624759), Switzerland (KJ818295) and Azores, Portugal (GQ421607), while a similarity of 99.10% was determined for *S. carpocapsae* populations found in the Veneto region, Italy (LN624758). A high percentage similarity (99.48 %) was also found for populations from Russia (KJ950291).

This study is the first report on *S. carpocapsae* in Croatia. EPN *S. carpocapsae* has a wide distribution in temperate regions and is one of the most common species in Europe and in many other parts of the world (Hominick, 2002). In Europe, *S. carpocapsae* is confirmed to occur in Austria, Bulgaria, the Czech Republic, France, Germany, Great Britain, Italy, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden and Switzerland (Hominick, 2002; Laznik et al., 2008). It is also the best studied, most available and most adaptive of all EPNs (Gaugler, 2002). In Croatia, apart from *S. carpocapsae*, only the species *Steinernema feltiae* Filipjev 1934 (Majić et al., 2018) has been recorded. In Slovenia, five species were found: *S. feltiae*, *S. carpocapsae*, *Steinernema kraussei* (Steiner), *Steinernema affine* (Bovien) and *Heterorhabditis bacteriophora* (Poinar) (Laznik et al., 2010), which were isolated from the soil using the same method (Bedding and Akhurst, 1975). Nguyen and Smart (1990a, 1990b) found that *G. mellonella* is not a good host for EPN *Steinernema scapterisci*, which cannot reproduce in *G. mellonella* larvae. For these reasons, our results should be viewed as a current assessment of the presence and abundance of EPN species in Croatia.

CONCLUSIONS

In this study, the EPN species *Steinernema carpocapsae* Weiser (Nematoda: Steinernematidae) was isolated for the first time in Croatia. The use of native nematodes may pose a lower risk to non-target organisms than introduced species and may also achieve even higher pest control efficiency than commercial strains. Furthermore, the detection of EPNs in Croatia will simplify the administrative formalities related to the import and use of commercial preparations. In order to identify as many species and strains of EPNs as possible, more samples need to be collected.

The presence of EPNs in Croatia indicates that the conditions in Croatia are favorable for EPNs and that their use for biological control of insect pests could be successful. It is also important to evaluate the effectiveness of the native EPN, as it is particularly valuable in the period of agro-ecological transition, when the use of chemical insecticides should be avoided.

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