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Efficacy of the EPNs (*Heterorhabditis bacteriophora* Poinar, 1976) on sugar beet larvae (*Bothynoderes punctiventris* Germar 1824; Coleoptera: Curculionidae) in field conditions

Učinkovitost entomopatoge nematode (EPN) (*Heterorhabditis bacteriophora*) u suzbijanju ličinki repine pipe (*Bothynoderes puntiventris* Germar 1824; Coleoptera: Curculionidae) u poljskim uvjetima

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ABSTRACT

The effectiveness of entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* Poinar, 1976 (Nematoda: Heterorhabditidae) infective juveniles (IJs) on sugar beet weevil (*Bothynoderes punctiventris* Germar, 1824 (Coleoptera: Curculionidae)) larvae was investigated during two-year (2014-2015). The field study was conducted to assess EPNs potential for use in practice. In field experiments, three doses of nematodes were used and an untreated control. In the conditions of moderate larval attack (average infestation of 0.28 larvae per plant), EPNs showed clear dose response being highly effective (92.86% control) when applied in the highest dose. Although the results indicated that *H. bacteriophora* could have a satisfactory mortality effect on sugar beet weevil larvae, further investigations are needed in order to better determine the optimal dose and application timing. If effective, EPNs could be used as one of the tools in a strategy which aims to reduce sugar beet weevil population levels in a wider area.

Keywords: biological control, sugar beet weevil, entomopathogenic nematodes, Heterorhabditidae

SAŽETAK

Provedeno je dvogodišnje istraživanje (tijekom 2014. i 2015.) s ciljem utvrđivanja učinkovitosti infektivnog ličinačkog stadija entomopatogene nematode (EPN) *Heterorhabditis bacteriophora* Poinar, 1976 (Nematoda: Heterorhabditidae) na ličinke repine pipe te mogućnost primjene u praksi. U poljskim pokusima primjenjene su tri doze EPN i netretirana kontrola. U uvjetima umjerene brojnosti repine pipe (prosječna brojnost 0,28 ličinki pipa po biljci šećerne repe), EPN su bile visoke učinkovitosti (92,86% u odnosu na kontrolu), primjenjene u najvećoj dozi. Iako rezultati ukazuju na zadovoljavajući učinak mortaliteta ličinaka repine pipe, potrebna su daljnja istraživanja kako bi se detaljnije moglo odrediti optimalno vrijeme primjene kao i doza EPN. Primjena EPN bi u budućnosti mogla biti dio strategije suzbijanja repine pipe na širem području.

Ključne riječi: biološko suzbijanje, repina pipa, entomopatogene nematode, Heterorhabditidae

INTRODUCTION

The sugar beet weevil, *Bothynoderes punctiventris* Germar, 1824 (Coleoptera: Curculionidae) is an important pest of sugar beet throughout the central, eastern and south-eastern parts of Europe (Hoffmann, 1966 cited in Tóth et al., 2006, p. 125). Weevils, as adults, damage young sugar beet plants in their most sensitive period, from germination to the development stage of 3-4 leaf pairs (Maceljski, 2002) and may completely destroy sugar beet fields. Thus, very often repeated sowing is needed. Larvae cause the most damage between June and September (when sugar beet has 6-9 and more leaf pairs), at a soil depth of 0-15 cm, at soil temperatures between 16°C and 25°C and soil water content of up to 15% (Susurluk, 2008). In Croatian conditions damage caused by larval feeding usually doesn't have an economic importance. Due to a warmer climate in the last ten years (DHMZ, 2013) sugar beet weevil became a growing problem in eastern Croatia. Also, to the population growth of pests contributed the absence of secondary effects of insecticides and the intensive cultivation of sugar beet (Bažok et al., 2012).

Entomopathogenic nematodes (EPNs) or insecticidal nematodes are harmless to humans, animals and plants (Potter, 1998). In the USA and other countries, the increased use of EPNs, due to their high efficiency and many advantages (Smart, 1995) compared to chemical preparations, is stimulated in order to restrict the use of pesticides (Grewal et al., 2005; Ehlers, 2007). Numerous biological control studies using EPNs have been conducted in order to establish their efficacy on various pests (Blackshaw, 1988; Nguyen and Smart, 1990; Hominick and Briscoe, 1990; Miduturi et al., 1996; Elawad et al., 1999; Susurluk et al., 2001; Hazir et al., 2003). EPNs of the genera Steinernema and Heterorhabditis are pathogenic to a large number of insect pests attacking many economically important crops (Poinar, 1990). Infective juveniles (IJs) of these genera are successfully used for the control of soil-borne insect pests (Ehlers, 1996). The possibilities of application of nematodes against the sugar beet weevil and commercialization of these products were explored (Susurluk, 2008).

The conventional approach to sugar beet weevil control aims to control adults as the most damaging insect stage. In the past, the conventional chemical control of sugar beet weevil was conducted by using chlorinated hydrocarbons (Čamprag, 1984), organophosphorus (OP) insecticides (Radin, 1983) and in combination with pyrethroid OP insecticides (Bažok et al., 2012). Strict registration and authorization procedures for insecticides in the EU led to a narrowing of the spectrum of insecticides registered for suppressing pests, including sugar beet weevil. Today, the number of permitted active insecticide substances for sugar beet weevil control has been significantly reduced. Active substances that had permission for use against sugar beet weevil in 2016 in Croatia were acetamiprid, cypermethrin, chlorpyrifos and lambda cyhalothrin (Bažok, 2016). In 2017, this number declined to only two substances: thiamethoxam and lambda cyhalothrin, with latter already having its permit abolished, and it can be used until the inventory sales in the current year (Bažok, 2017). Due to the specific morphology of sugar beet weevil adults, its feeding habits, the time and mode of pesticide application, even permitted insecticides often do not give the desired effect. Therefore, it is often necessary to repeat the treatments (Bažok et al., 2013), which is not in accordance with Integrated Pest Management (IPM) principles, nor with rational use of plant protection products to which the modern agriculture aims.

Accordingly, it is necessary to change the approach of the weevil control from an individual field approach toward controlling the larger affected area (Drmić et al., 2017). It is necessary to develop a strategy aimed to suppress the weevil population on the whole endangered area and to combine all available tools and methods avoiding the use of classical chemical control measures. One of the methods that comply with IPM principles is the use of EPNs as means of biological control. The use of EPNs aims to control the larval stage of the sugar beet weevil and is not aiming to prevent the damage in the year of application. However, this may contribute to population reduction in the next year, which will lead to the overall reduction of pest abundance.

This study aims to assess the potential of EPNs for use in practice.

MATERIALS AND METHODS

Field data

The commercially available product Nematop (e-nema GmbH, Germany) was tested in field trials in 2014 and 2015. The experiments were carried out in commercial sugar beet fields located in the eastern part of Croatia, classified in texture mark as silty clay soils. Chemical properties of researched fields are shown in table 1. The total field size in 2014 was 105 ha (field coordinates 45°11'46.02"N, 19°09'7.24"E) and in 2015 field size was 4.06 ha (field coordinates 45°10'44.93"N, 19°08'50.29"E). The fields were chosen based on the presence of smaller ground elevations or surface unevenness's, because the sugar beet weevil prefers these spots for laying eggs. In 2014 the field trial was carried out in a sugar beet field seeded with KWS hybrid Serenade, while in 2015 the sugar beet field was sown with Strube hybrid Tesla. In 2014. sugar beet was sown on the 7th April and in 2015. on the April 11th. For the purpose of the experiments, the isolated parts of the fields were divided into smaller plots, each covering 18 m in width (36 rows of sugar beet) and 15 m in length (270 m^2 in size). The average number of sugar beet plants per plot was 3375 plants in 2014 and 3000 plants in 2015.

Table 1. Chemical soil properties of researched fields

pH		%		Al-mg/100 g		$CaCO_3$
H_2O	nKCl	Humus	N	P_2O_5	K_2O	%
8.42	7.24	2.70	0.14	29.70	26.50	10.20

Treatments

Since the sugar beet weevil has one generation per year and larvae develop in the soil from May until September, the treatments were applied once in each year, at the time of the first larval appearance, i.e. 10 to 15 days after the copulation was observed. Weevils initiate multiple copulation (Čamprag, 1984) which was visual inspected on sugar beet plants and surrounding soil in researched fields. For trials three different doses of Nematop were used as well as one untreated control, as shown in Table 2.

The date of the treatments was determined by observing the sugar beet weevil emergence and copulation in the field every week, starting from mid-March in both years. The EPN application was carried out on 10th May 2014 and 1st June 2015.

The treatments were applied with trailed sprayer Amazone UG 3000 Special, which has working width of 18 m (36 rows of sugar beet) and water container capacity of 3.000 l. The amount of water required for one treatment was determined by a test done with clean water in a separate part of the field, so the appropriate dose of nematodes was calculated to be applied, representing a consumption of 370 l/ha. In order to achieve optimal soil moisture prior to application of nematodes, of the same amount of clean water was applied with the same sprayer covered on the whole field plot. Following the water application, different doses of nematodes were applied on experimental plots, while the control plot was sprayed again with clean water. After the nematode application, the whole field plot was once again sprayed with clean water, i.e. each treatment plot was sprayed with 1110 l/ha (per spaying 370 l/ha), first and third time with clean water and in-between one application containing EPNs. Each treatment was replicated five times. Two days after the treatments in 2014, inter row cultivation was carried out on the sugar beet field, while in 2015 it was carried out twice before the treatment, on 5th and 18th May.

Table 2. Trial treatments included in the field experiments in Tovarnik (2014-2015)

Treatment no.	Product	Dose	Dose
1	Nematop	3[a]	81[b]
2	Nematop	5[a]	135[b]
3	Nematop	7[a]	189[b]
4	Control	0	0

[a] Million nematode IJs/10 m^2
[b] Million nematode IJs applied in 100 l of water per 270 m^{-2}

Efficacy assessments

Before the experiments, visual inspections were performed on selected fields to determine the number of sugar beet weevil adults present in the field. The inspections were done on 22nd April 2014 and 20th April 2015. At that time, the sugar beet plants were in a phonological growth stage 15-19 according to BBCH scale (Meier et al., 1993), meaning that in that developmental stage 5-9 or more leaves were unfolded. The wooden quadrate (1 m^2) was randomly thrown on the surface and all sugar beet weevils found within the square were counted. The same method was applied in autumn to assess the sugar beet weevil abundance after the adults have emerged.

Four weeks after nematode application the visual inspections of plants started. Visual inspections were carried out every 12 to 16 days what corresponded with Julian days 158, 171, 188, 202, 214 and 230. A total of six visual inspections were done. At every field examination, from each replication five sugar beet roots were randomly selected, dug from the soil and inspected. Thus, from each treatment 25 plants were surveyed, i.e. a total of 100 plants per inspection. In the beginning, plants were dug and visually inspected for larvae presence. When the roots began to gain weight, in addition to visual inspection, a root dissection was performed in order to determine the number of larvae on the surface and inside the sugar beet roots. The number of larvae per root and the stage of larval development were observed.

Meteorological data

Meteorological data on average monthly air temperature, average monthly soil temperature at 10 cm depth and total monthly precipitation in the area of Tovarnik were acquired for five months (from May until September) for both years, 2014 and 2015. Meteorological data were provided by the Croatian Meteorological and Hydrological Service and included data from the nearest meteorological station (located in Gradište, 45 km away from the study area: 45°52'N, 18°58'E).

Data analysis

The number of larvae per sugar beet plant determined for every treatment in each field inspection, as well as the total number of larvae counted during the whole experiment period was subjected to variance analysis (ANOVA). The results (number of larvae) were then submitted to Tukey's HSD tests to compare mean values using ARM 9 software (ARM 9 GDM software). Based on the number of larvae found in the treatment and in the untreated control, the efficacy of the treatment was determined according to Abbott's formula (Abbott, 1925). Efficacy was determined only for the treatments in which significant reduction of larval population was established based on the results of ANOVA.

RESULTS

The average monthly air and soil temperatures in 2014 were highest in July (air: 22.3 °C; soil: 24.9 °C), while the total monthly precipitation was measured highest in September (205.4 mm) (Figure 1).

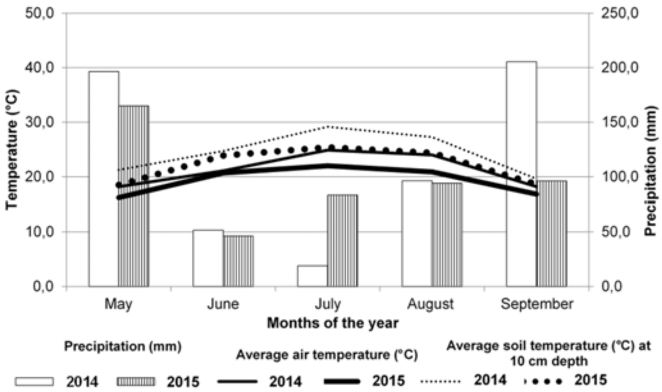


Figure 1. Prevailing climatic conditions in Tovarnik (from May till September) 2014 and 2015

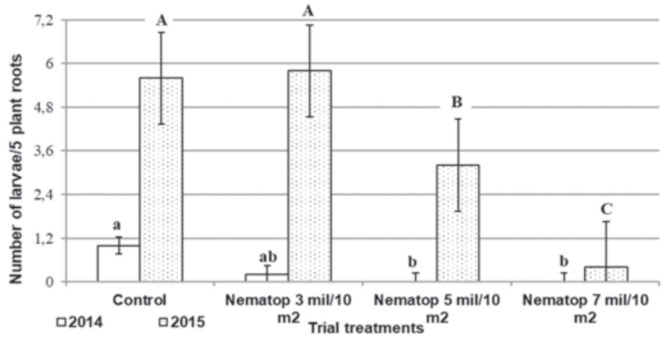
In 2015 the average monthly air temperature was highest (22.3 °C) in August, while the soil temperature was measured highest (28.3 °C) in July. The highest (165 mm) total monthly precipitation was observed in May 2015.

The number of sugar beet weevil adults determined prior the application of Nematop in 2014 was 0.75±0.1 weevil/m^2 (7,500±1,000 adults/ha). The total number of sugar beet weevil larvae found in the field experiment in 2014 was very low (18 larvae). The number of larvae established on sugar beet plants in 2014 was significantly lower than in 2015 at all inspection dates until the mid-August when larvae completed their development (Table 3).

The average infestation of the study field before the application of Nematop in 2015 was 0.5±0.06 weevil/m² (5,000±600 adults/ha). The infestation determined during the last visual inspection in autumn was 1.75±0.5 weevil/m². The total number of sugar beet weevil larvae counted in the whole field experiment during 2015 was 73 larvae.

Significant reduction of sugar beet weevil larvae in 2014 has been observed after the application of EPN in the doses of 5 and 7 million IJs/10 m² while the dose of 3 million IJs/10 m² did not result with significant reduction in the number of larvae compared to the control (Figure 2). No significant reduction in the number of larvae was established after the application of EPN in dose of 3 million IJs/10 m² in 2015 either. The doses of 5 and 7 million IJs/10 m² proved to be effective and ensured significant reduction in number of larvae compared to the control.

In 2014, the efficacy of the lowest applied dose of EPNs was 57% and doses of 5 and 7 million IJs/10 m² resulted with the efficacy of 85.7% and 100%, respectively. However, the larval density was very low. In the condition of much higher, but still moderate larval density, in 2015, the lowest dose of EPNs did not result with significant reduction of larvae. The efficacy of EPNs applied at a dose of 5 million IJs/10 m² was 44.82%, and the efficacy of the highest dose was 96.6%.



Means followed by the same letter are not significantly different (P>0.05; Tukey's HSD); small letters refer to differences among treatments in 2014; capital letters refer to differences among treatments in 2015. HSD, P>0.05=0.857 (for 2014), HSD P>0.05= 1.894 (in 2015)

Figure 2. Number of sugar beet weevil (*B. punctiventris*) larvae after the application of *H. bacteriophora* in a two-year field experiment in Tovarnik

DISCUSSION

Increased rainfall in April and May negatively affects egg lying, probably due to disruption of weevils' activities, and may also lead to egg deterioration or development difficulties of egg and larval stages (Maceljski, 2002). The amount of rainfall in April and May 2014 was 214 mm. In the same period in 2015, the amount of rainfall was about twice as low (119 mm) than in 2014. Therefore, less rainfall in 2015 could have enabled oviposition and led to higher infection of larvae than in 2014. The ratio of autumn compared to spring population of adults was 3.5 in 2015 and 1.5 in 2014. In 2015, weather conditions were more favorable for sugar beet weevil. However, the population growth in both years was much below the growth which could be expected if weather conditions were optimal. Based on the available data on pest biology (Drmić and Bažok, 2015) and average number of adults determined on study fields (7,500±1,000 adult's/

Table 3. The number of larvae (±SE) found on sugar beet plants (5 plants per sample) in Tovarnik at different inspection dates in 2014 and 2015 and the results of statistical analysis

Year	Trials inspection dates (Julian days) in 2014 and 2015					
	June 7th (158)	June 20th (171)	July 7th (188)	July 21th (202)	August 2nd (214)	August 18th (230)
2014	0.50±1.00 b	0.50±1.00 b	0.25±0.50 b	1.00±0.82 b	0.50±1.00 b	0
2015	3.25±0.96 a	3.75±0.96 a	4.50±1.29 a	3.75±0.96 a	3.50±0.58 a	0
LSD p=5%	1.523	0.796	1.523	1.523	2.250	ns

*Means followed by the same letter are not significantly different according to Tukey's HSD test (P=0.05)

** ns = nonsignificant

ha in 2014 and 5,000±600 adults/ha in 2015) the infestation between 2.5 and 3.75 larvae per plant could be expected. In spite of the fact that the initial population of adult weevils in 2014 was higher (0.75±0.5 adults/m^2) compared to 2015 (0.5±0.06 adults/m^2), the infestation of plants in the trial was much higher in 2015 than in 2014. However, in 2015 the maximal number of larvae found in untreated plot was 7 larvae on 25 plants, which makes an average infestation of 0.28 larvae per plant. The maximum infestation on the untreated plot was on 6th July 2015. These data indicate that the amount of rainfall (as observed in May), although much lower than in 2014, still might have exceeded the optimal conditions for the sugar beet weevil larval development.

Observed results indicate high efficacy of all three doses of EPNs in the condition of very low attack intensity. Also, EPNs show a dose response in the conditions of moderate attack intensity. In such conditions the lowest dose was not effective while the highest dose resulted in 96.6% of the efficacy. The dose of 5 million IJs/10 m^2 is recommended by the producer for the control of other weevils and it is clear that it did not result in satisfactory efficacy in the conditions of moderate (or even low) attack. Therefore, the question of the efficacy of the moderate dose could be raised.

Soil depth and temperature have an important influence on sugar beet weevil infection and mortality caused by the nematode *H. bacteriophora* (Susurluk, 2008). The efficacy of this nematode species increases as the soil temperature rises. *Heterorhabditis bacteriophora* shows the highest performance at soil temperatures between 20 °C and 25 °C, and between 5 cm and 10 cm of depth, where the larval stages are mobile (Čamprag, 1984). Temperatures <8 °C and >40 °C are lethal to most EPNs (Griffin, 1993; Grewal et al. 1994). The soil temperatures detected during this research were in the optimum range for the nematode *H. bacteriophora*. Other species of nematodes, such as *Steinernema feltiae* Filipjev and *Steinernema weiseri*, according to Mrácek et al. (2003) perform better at lower temperatures (15 °C), but are not effective on sugar beet weevil as *H. bacteriophora* (Susurluk, 2008).

Since EPN affects only the larval stages of weevils, the critical point to achieve success is the application timing. According to the literature, the oviposition chiefly occurs at the end of May and beginning of June, and the larvae hatch on the third day after eggs being laid (Rozsypal, 1930). EPNs were applied ten to fifteen days after the copulation was observed, to make sure the eggs had hatched. In both years the oviposition started earlier than it is reported in the literature. It is possible that EPNs were applied when the oviposition started and that most of the oviposition occurred later. Thus, it might have been that at the time of EPN application most of the eggs hadn't been laid yet and the nematodes did not have enough available larvae to attack. Later on when eggs were laid, the number of nematodes might have declined as a result of many mortality factors (edaphic factors, predation, infection by antagonists, depletion of energy and desiccation) during the post application period (Duncan and McCoy, 1996; Smits, 1996). When applying EPNs targeted was the first larval stage because it was assumed that this stage is more susceptible to EPN attack as it is the case with some other species (Mannion and Jansson, 1992; Trdan et al., 2009). Sugar beet weevil larva passes through five developmental stages (Čamprag and Mihajlović, 1973), which might raise the question which of these stages is the most sensitive for the penetration and effectiveness of nematodes? New laboratory researches are needed in order to determine the most sensitive larval stage and the optimal period of treatment after oviposition.

The number of adults which entered the overwintering stage, detected in autumn on investigated fields, was 1±0.2 weevil/m^2 in 2014 and 1.75±0.5 weevil/m^2 in 2015. Both numbers are considered as medium infestation in long-term forecasts (Stojanović et al., 1971).

Low larval population in 2014 prevents from making reliable conclusions about the efficacy of the EPNs against sugar beet larvae. However, the results achieved in 2015 show clear dose response and indicate that EPNs could have a satisfactory effect on the larvae. For making more reliable conclusions additional research are needed.

The use of EPNs for sugar beet weevil control focuses on the prevention of larvae, which actually rarely damage the root (Maceljski, 2002). Therefore, it is not a strategy for preventing damage on one field in a particular season. This is a measure that is aimed for the suppression of the adult population and should be implemented within a well-developed strategy, in which a number of different joint measures should be developed to suppress the population of pests in a wider area. This kind of suppression is very expensive but has a long-term impact. However, it is unlikely that the farmers themselves would fund this measure. Therefore, this measure will have to be organized and (semi)financed by the organizers of production (sugar factories).

CONCLUSIONS

The results showed that EPN *Heterorhabditis bacteriophora* has a potential in suppressing the sugar beet weevil. Practice of suppressing EPN in not yet ready for use in practice and additional research, both in the laboratory and in the field, are needed.

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