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Virulence of new strain of *Heterorhabditis bacteriophora* from Croatia against *Lasioptera rubi*

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Abstract: Soil samples from 100 cultivated and natural sites were assessed for the presence of entomopathogenic nematodes. Heterorhabditid nematodes were recovered from three soil samples during spring months, with the overall positive sample rate of 3%. The isolates of entomopathogenic nematodes were identified as three different strains conspecific with *Heterorhabditis bacteriophora* (Heterorhabditidae). They were found from natural sites and vineyard, while no recovery occurred from intensively cultivated agricultural fields. The morphometrical characteristics of infective juveniles and males showed differences between all Croatian strains and from the original description. *Heterorhabditis bacteriophora* ISO9 was bioassayed on *Lasioptera rubi* (Cecidomyiidae) (the raspberry gall midge) larvae at different nematode concentrations under laboratory conditions. The significantly highest mortality was observed in treatments with 50 and 200 infective juveniles per insect larvae within 8 days after inoculation. This is the first report of entomopathogenic nematodes of the family Heterorhabditidae from Croatia, and susceptibility of *L. rubi* larvae to entomopathogenic nematodes. The Croatian strain *H. bacteriophora* ISO9 was proved to possess strong insecticidal properties against *L. rubi* larvae.

Keywords: entomopathogenic nematode; raspberry gall midge; bioassay; biocontrol

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are obligate and lethal insect parasites used as a tool in the biological control of important soil-dwelling pests and pests that occur in other cryptic habitats (Lacey & Georgis 2012). *Lasioptera rubi* Schrank (Diptera: Cecidomyiidae), the raspberry gall midge larvae feed within plant tissue of various species of *Rubus* (Rosaceae), creating abnormal plant growths called galls. The gall midge larvae are found in colonies inside the galls, feeding in clusters. The interior of the galls is lined with fungal mycelium introduced by infected larvae during hatching. Gall midges feed on associated fungal mycelium (Rohfritsch 2008.). According to OEEP/EPPO certification schemes for *Rubus* and its hybrids, *Lasioptera rubi* is considered as a severe pest. *L. rubi* requires preventive monitoring measures and compulsory chemical control measures in conditions of high pest populations. The most important management strategy is removal of the infested canes subsequently upon harvesting, prior to the emergence of adult stage (Tanasković & Milenković 2011). The plants mechanically wounded due to the cane removal are consequently more susceptible to other pathogens. Gall midges of the family Cecidomyiidae are difficult to control since

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many species create malformations in plants where they stay hidden. Entomopathogenic nematodes of the family Heterorhabditidae have been tested against several economically important gall midges. Corlay *et al.* (2007) reported *Heterorhabditis bacteriophora* to be able to cause 90–100% mortality of the swede midge *Contarinia nasturtii,* an important pest of cruciferous crops. High virulence of three species of EPNs against the swede midge was reported by Evans *et al.* (2015) as well. The same authors found that *H. bacteriophora* alone and in combination with the entomopathogenic fungus *Metarhizium brunneum* caused the highest mortality of all insect stages under different temperature regimes. Powell & Webster (2004) reported a significant reduction in the emergence of adults of the aphid predator *Aphidoletes aphidimyza* when pupae in soil were exposed to *H. bacteriophora*. In mushroom crops, the gall midge *Heteropeza pygmaea* was eradicated when *H. heliothidis* (=*bacteriophora*) was applied to compost (Richardson 1987). The low efficiency of *H. bacteriophora* was observed in the control of the brassica pod midge *Dasyneura brassicae* (Nielsen & Philipsen 2005).

The genus *Heterorhabditis* currently comprises 16 species, with *H. bacteriophora* as a type species which has the widest geographical distribution among heterorhabditids (Hunt & Nguyen 2016). This species is associated with moderate temperature profile, however a broad range of temperature and desiccation tolerance has been observed due to strain specificity (Mukuka *et al.* 2010; Shapiro-Ilan *et al.* 2012). For these reasons, bioecological traits and virulence of indigenous EPNs species against insect pests of local and global importance should be bioassayed in order to reveal the potential of the nematode as a biocontrol agent (Burnell & Stock 2000). The first report of EPNs from Croatia was in 2018 (Majic *et al.* 2018). In the region of Southeastern Europe, EPNs are reported from seven of 11 countries (Majic *et al.* 2018). In this paper, we report EPNs of the family Heterorhabtididae for the first time from Croatia. Furthermore, we conducted laboratory bioassays to report the susceptibility of *L. rubi* larvae to EPNs for the first time.

MATERIAL AND METHODS

Survey and identification of nematodes. Soil samples were collected from the valley region in Slavonia and Baranya, Croatian northeastern part of the Panonian basin in 2016 and 2017, from April till the end of June, and from September till the end of November. In total, 100 soil samples were collected from cultivated fields, meadows, woodland habitats and swamp sites. At each sampling site, soil was collected within an area of 10 m^2 using a hand shovel from the upper 3–30 cm layer, near the plant root zone or at a distance of 1 m from the tree trunk. A sample within each site consisted of 1 kg of soil randomly taken from five subsamples. Samples were placed in polyethylene bags (to prevent dehydration) and transported to a laboratory in polystyrene box (to avoid temperature stress). Within two days from collection, the samples were processed. The soil was thoroughly mixed and ca. 100 g of soil was taken from each sample, for analyses of soil type, organic matter and pH (Table 1).

The insect baiting technique used in this study was similar to the previously described methods (Kaya & Stock 1997) with modification of the insect host. Larvae of *Achroia grisella* Fab., the lesser wax worm were used as bait insect.

Nematode progeny was used for identification and establishment of culture (Kaya & Stock 1997). Stock cultures are maintained in the Laboratory of Entomology and Nematology, Faculty of Agrobiotechnical Sciences Osijek (Croatia). Twenty individuals from adults (males) and infective juveniles

Table 1. Locations, soil characteristics, natural habitats, and sampling time of three positive sites for *Heterorhabditis bacteriophora* in Croatia

Isolate	Location	Coordinates	Soil type	Organic matter (%)	pH (H_2O)	Habitat	Vegetation	Sampling date
ISO9	Vučedol	45°20'13.4"N 19°03'29.8"E	Loam	4.03	7.84	natural	mixed deciduous	June 2016
ISO12	Sotin	45°19'02.2"N 19°04'23.3"E	Silty loam	3.28	7.25	natural	mixed deciduous	May 2016
ISO15	Principovac	45°11'51.4"N 19°21'10.5"E	Silty clay loam	1.72	7.16	agricultural land	vineyard	June 2016

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(IJs) were heat-killed with hot water (60°C), fixed, transferred to anhydrous glycerin and examined under an Olympus BX50 microscope equipped with differential interference contrast optics and digital image software (Olympus LCmicro 2.1). The following morphometric data of body measurements of first-generation males and IJs are presented in Table 2: total body length, tail length, spicule length, gubernaculum length, distance from anterior end to excretory pore, value of the ratio of total body length to tail length, value of the ratio of distance from anterior end to excretory pore and distance from anterior end to the base of oesophagus, and value of the ratio of distance from anterior end to excretory pore and tail length. These morphological characteristics were considered as the most reliable for *H. bacteriophora* (Hunt & Nguyen 2016). The morphometric data are presented in Table 2.

Genomic DNA was extracted from single individuals and a polymerase chain reaction (PCR) was performed to multiply ITS (internal transcribed spacer) region using primers TW81 and AB28 according to Hominick *et al*. (1997). The PCR products were re-isolated from 1% TAE-buffered agarose gel using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). Re-isolated sample was sequenced in the Laboratory of Agricultural Biotechnology Centre in Gödöllő, Hungary. Sample DNA sequence was compared with sequences of the species *Heterorhabditis* from GenBank using BLAST search in the National Centre for Biotechnology Information (NCBI). The sequences were deposited in GenBank under accession numbers MG944244 (ISO9), MG952285 (ISO12) and MG952286 (ISO15).

Sample DNA sequences were used for phylogenetic analysis. ITS1, ITS2, and 5.8S rRNA gene sequences were aligned using CLUSTAL_X 2.0. The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length 0.76662943 is presented. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 346 positions in the final dataset. Evolutionary analysis was carried out in the MEGA5 software package.

Bioassays. The raspberry gall midge larvae were collected in June and July 2017 from an infested raspberry plantation near Osijek, Croatia (45°34'01.0"N and 18°42'11.2"E). More than 200 infested canes were brought to a laboratory where galls were dissected longitudinally and insect last instar larvae were collected.

H. bacteriophora ISO9 were reared in last instar larvae of the lesser wax moth as described by Majic *et al.* (2018). After harvesting from White traps, nematodes were used in an experiment within 2 to 4 weeks prior to experimentation. Nematode concentrations were prepared in aqueous suspensions using distilled water (Laznik *et al*. 2010). The sterile Petri dishes with a double layer of wetted filter papers were randomly inoculated with 50, 200, 500, and 1 000 IJs per *L. rubi* larvae in 1 ml of sterile distilled

Table 2. Comparative morphometric data of *Heterorhabditis bacteriophora* (Croatian isolates and known species)

Isolate	Male				Infective juvenile					
	n	SL	GL	D%	*n*	L	T	EP	c	E%
ISO9	20	41 (36–44)	20 (19–24)	128 (113–140)	20	572 (537–650)	90 (82–102)	97 (86–106)	6.6 (5.5–7.0)	120 (105–128)
ISO12	20	41 (39–45)	20 (18–23)	118 (110–132)	20	590 (550–661)	98 (89–110)	90 (79–100)	6.1 (5.2–7.1)	101 (95–117)
ISO15	20	44 (42–48)	22 (21–25)	120 (115–136)	20	601 (543–670)	97 (87–112)	91 (81–105)	6.2 (5.7–7.2)	108 (101–120)
*Heterorhabditis bacteriophora**		40 (36–44)	20 (18–25)	117		588 (512–671)	98 (83–112)	103 (87–110)	6.2 (5.5–7.0)	112 (103–130)

SL – spicule length; GL – gubernaculum length; D% – EP/distance from anterior end to the base of oesophagus × 100; L – body length; T – tail length; EP – distance from anterior end to excretory pore; c – L/T; E% – EP/T × 100; *after Poinar (1990); all measurements represent mean and range in μm

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water. To test the virulence of a Croatian strain of *H. bacteriophora*, ten last instars of the raspberry gall midge larvae were placed in the dishes one hour later. In control Petri dishes, insect larvae received 2 ml of sterile distilled water without nematodes. All treatments were replicated five times, and the whole experiment was repeated once. The dishes were incubated at 22°C (80% RH) in the dark in a rearing chamber. Insect mortality was recorded in a two-days interval during a 14-day post treatment period. Insect cadavers were placed individually on modified White traps and monitored in a 30-day period for the offspring emergence.

Percentage data of insect mortality was Abbott-corrected and tested for normality (Kolmogorov-Smirnov test) and homogeneity (Levene's test). As a result, the data were square-root transformed, and back-transformed for presentation purpose. Comparisons between the means of the treatments were performed using a one-way ANOVA. When significant differences were found, multiple comparisons were performed using Tukey's studentised range test (HSD) ($P < 0.05$). Analyses were performed using the SAS v9.3 software (SAS Institute, Cary, USA).

RESULTS

Insect parasitic nematodes were isolated from 20 out of 100 soil samples (20%). Out of the 20 positive samples, heterorhabditid nematodes were recovered from three soil samples, with an overall positive sampling rate of 3% (Isolates 9, 12, and 15) (Table 1). The heterorhabditid nematodes were recovered only in May and June from disturbed and undisturbed habitats; however, two out of the three isolates were recovered from soils under mixed deciduous trees and one from a vineyard. Soil samples with negative nematode recovery were taken from different natural and cultivated habitats. Natural habitats (42% of samples) with negative sampling results were meadows, forests (oak, black locust, willow, and poplar), shrubs along the roadside and swamp grass, while negative cultivated sites (58% of samples) were annual (sunflower, maize, barley, wheat, lucerne, oilseed rape, sugar beet, carrot, cabbage, and potato) and perennial crops (black raspberry, vineyard, sweet cherry, hazelnut and walnut). *Heterorhabditis bacteriophora* was not found in intensively cultivated agricultural soils. Soil characteristics of the positive sites are classified as loamy soils with alkaline reaction thatvaried in organic matter content (from 1.72% to 4.03 %).

Molecular and morphological identification showed that nematodes belong to the family Heterorhabditidae, and all three isolates are conspecific with *H. bacteriophora* (Poinar 1990) (Table 2 and Figure 1).

The phylogenetic relationship of the studied isolates of *H. bacteriophora* and homologous sequences of the same genus from the GenBank is presented in Figure 1. The phylogenetic tree revealed three major

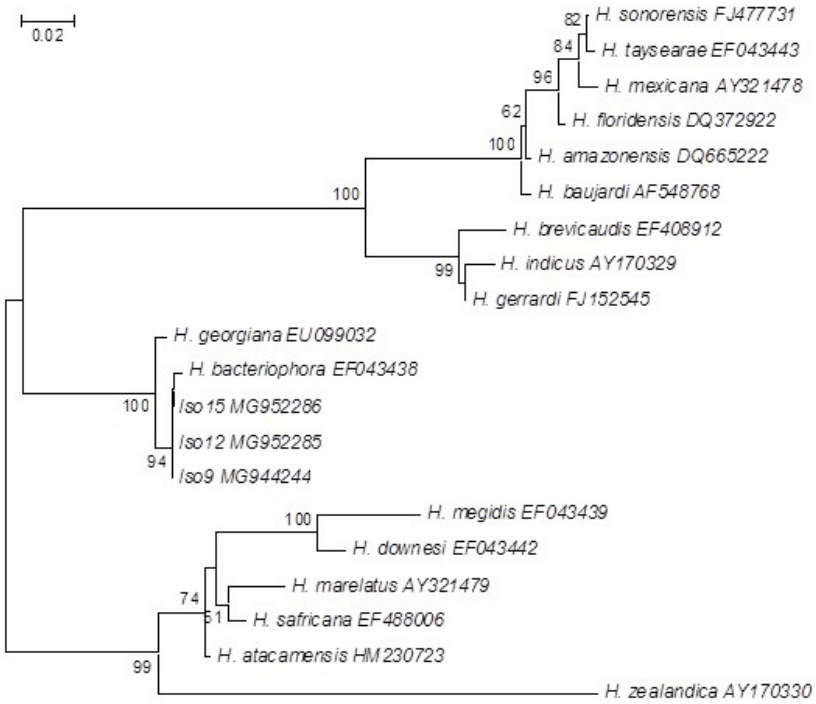

Figure 1. Evolutionary relationships of new Croatian entomopathogenic nematode isolates (Iso9, Iso12, and Iso15)

The evolutionary history was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site

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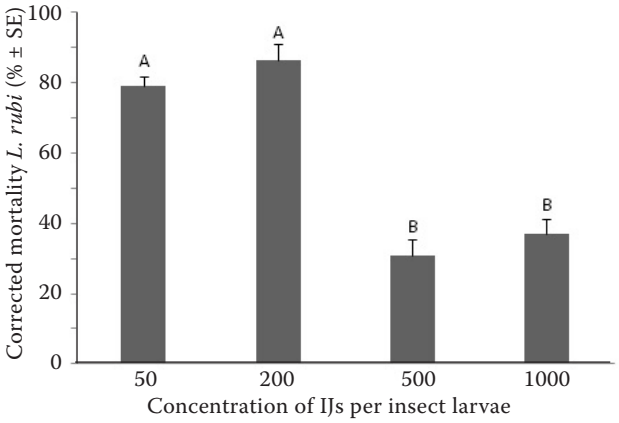

Figure 2. Corrected mortality (means ± SE) of *Lasioptera rubi* last instar larvae (n = 10) at different concentrations of *Heterorhabditis bacteriophora* (Croatian strain ISO 9)

The experiment was replicated five times and repeated once; different letters above bars indicate statistical significance (Tukey, $P < 0.05$)

clades: (I) megidis-downesi-marelatus-safricana-atacamensis-zealandica; (II) bacteriophora-georgiana; (III) sonorensis-mexicana-floridensis-amazonensis-baujardi-brevicaudis-indicus. All Croatian isolates were grouped with two species *H. bacteriophora* and *H. georgiana*. Overall, the three major clades are supported by a high bootstrap value ranging from 99% to 100%.

In the virulence test of *H. bacteriophora* (the Croatian strain ISO9), significantly different insect mortality was observed between the treatments. Application of 50 and 200 IJs per insect larvae resulted in 79.01 and 86.42% insect mortality, respectively (Figure 2). Treatments with the application of 500 and 1 000 IJs per insect larvae caused significantly lower insect mortality of 30.86 and 37.04%, respectively. The highest mortality (86%) was observed within 8 days after inoculation in the treatment with 200 IJs per insect larvae. All cadavers contained abundant populations of offspring 18 days after inoculation.

DISCUSSION

Our results are the first report of natural occurrence of EPNs of the family Heterorhabditidae, namely *H. bacteriophora* in Croatia. We recovered *H. bacteriophora* from habitats varying in ecological parameters indicating a possible wider distribution in Croatia. *Heterorhabditis bacteriophora* is ubiquitous in diverse habitats, and has been documented from all agricultural parts of the world (Hunt & Nguyen 2016). From ex-Yugoslavian republics, Croatia's neighbouring countries, *H. bacteriophora* was the only heterorhabditid species reported and found only in Slovenia (Laznik *et al.* 2009) and Bosnia and Herzegovina (Iqbal *et al.* 2016). In Hungary, the bordering country to Croatia's continental region, *H. bacteriophora*, *H. megidis*, and *H. downesi* are reported (Tóth 2006). We recovered heterorhabditids only during late spring, in May and June, while in autumn months we did not find any positive sample. This species is associated with moderate temperature conditions and its population densities in agroecosystem may change over time, as a response to various biotic and abiotic factors (Glazer 2002). The absence of seasonal patterns of EPNs is reported from other studies as well (Glazer *et al.* 1996). Majic *et al.* (2018) recovered *Steinernema feltiae* in Croatia in October, from potato field and fallow site. This may indicate that the observed seasonal patterns and habitat preference in this study could be species specific. We assume that many negative samples occurred due to the sampling error and patchy distribution of EPNs as well. Mráček *et al.* (2005) suggested that soil samples should be baited and processed in a laboratory under different temperatures in order to the achieve the more successful extraction rate of EPNs. Koppenhöfer and Fuzzy (2006) reported that *H. bacteriophora* was negatively affected by acid conditions and was not influenced by different loams. Our results are in agreement with the latter since we found this species in alkaline (pH > 7.1) and loamy soils. Croatian strains of *H. bacteriophora* were recovered from soils varying in organic matter content (1.72–4.03), indicating that it did not influence the recovery of heterorhabditid nematodes. Our results are similar to the report by Hazir *et al.* (2003) who recovered the same species from soils with organic matter content ranging from 1.5 to 4.2. Tarasco *et al.* (2015) reported that *H. bacteriophora* was present in all soil types in surveyed areas in Italy.

The variability of morphometric characters of Croatian strains of *H. bacteriophora* species was observed within the strains and with the descriptions of Poinar (1990). Body length of IJs of *H. bacteriophora* ISO9 was comparatively shorter, while strains ISO12 and ISO15 were longer than the originally described ones. The IJs tail lengths of strain ISO9 were also smaller. We found the greatest distance from anterior end to excretory pore in ISO9 (97 µm) compared to ISO12 (90 µm) and ISO15 (91 µm), but all Croatian strains

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had this characteristic shorter than the original description (107 µm). Nguyen and Smart (1995) reported that the body size of EPNs varied significantly with the time of harvest, and the longest nematodes could be observed at the first harvest, and became shorter with subsequent harvests. Same authors suggested that different insect hosts used in rearing could influence the nematode morphometrics. In our study, an alternative host (*A. grisella*) to standard *Galleria mellonella* was used, and this could be the reason for observed morphological variations as well. The temperature regime could also influence the body length of EPNs. Hazir *et al.* (2001) reported variations in the longest body length of IJs at 8°C, with a decreasing tendency at higher temperatures. Males in our study also showed variations. All Croatian strains of *H. bacteriophora* were measured to have a longer spicule thea the original description, however the longest among the strains and known species was found in strain ISO15. The value of D% (distance from anterior end to excretory pore and distance from anterior end to the base of oesophagous ratio) was comparatively larger for all strains than in the original description. The phylogenetic analyses showed the clear monophyly of a group of all Croatian isolates with the known species *H. bacteriophora* and *H. georgiana*. Nguyen *et al*. (2008) reported that *H. bacteriophora* and *H. georgiana* are in close relationship. Iqbal *et al*. (2016) constructed a phyllogram with heterorhabditid species and found similar results to our study, the close relationship of *H. bacteriophora* strain with *H. georgiana*. It is very difficult to distinguish these two species. In this study, using ITS region, the pairwise similarity between *H. georgiana* and ISO9, ISO12, ISO15 is 97.4%, while between *H. bacteriophora* and ISO9, ISO12, ISO15 it is 99.9%. Comparative morphometrics of males reveals that the gubernaculum length of Croatian *H. bacteriophora* strains (on average from 20 to 22 µm) is shorter compared to the same characteristic in *H. georgiana*, which is usually 25 µm long. Furthermore, males of Croatian *H. bacteriophora* strains have a higher value of D% than *H. georgiana* males. It is also notable that *H. georgiana* is an American species, there is not any data on its occurrence from Europe.

This is the first report of *L. rubi* susceptibility to EPNs. This pest proved to be susceptible to *H. bacteriophora* ISO9. The highest mortality (86%) was observed in a treatment with 200 IJs per insect larvae. The low mortality in treatments with the highest nematode population densities may be due to intraspecific competition. Too many IJs may reduce nematode fitness and overall potential to kill the insect host (Denno *et al.* 2008). Optimal number of IJs for penetration and development is insect host species dependent (Půža & Mráček 2005). For *G. mellonella* it was found that more than approximately 200 IJs per insect larvae inhibit the development of EPN offspring, since all nematodes die inside the host (Koppenhöfer & Kaya 1995). Contrary to our results, Corlay *et al.* (2007) tested higher EPN concentrations against another cecidomid species, and found that the inoculation of swede midge larvae with 1 000 IJs of *H. bacteriophora* caused more than 90% mortality under laboratory conditions. Powell and Webster (2004) reported *A. aphidimyza* to be the most susceptible at the highest IJ concentrations, since more than 80% larval mortality was observed when *H. bacteriophora* was applied in concentrations of 100 and 1 000 IJs per insect larvae under laboratory conditions. Compared to the other studies of dose-response bioassays of susceptibility of cecidomid insect species, in our study *L. rubi* larvae were observed to have a lower carrying capacity of EPNs. Larvae of *L. rubi* parasitised by *H. bacteriophora* ISO9 developed typical dark red colour and tissue were mummified. We found a large progeny from all cadavers, and this confirms that conditions inside the insect hosts and larval stage of *L. rubi* suits *H. bacteriophora* ISO9 for development.

Entomopathogenic nematodes are most commonly applied against soil-dwelling insect pests with low persistence in intenive agricultural fields (Cruz-Martínez *et al.* 2017). *Lasioptera rubi* pest does not have the soil-dwelling stage. By subsurface injection of *H. bacteriophora* ISO9 inside the insect-induced galls, further development of the pest could be prevented and savoided. The galls formed by *L. rubi* larvae on the host plant are an easily recognisable, cryptic, high moisture environment that offers nematodes protection from UV light, suggesting that EPNs may be efficiently and effectively targeted (Shapiro-Ilan *et al*. 2012). Evans *et al*. (2015) reported that the majority of EPNs applied to plant apical meristem against the swede midge were found alive inside plant pseudogalls 72 h after initial application, proving the EPN ability to survive inside the galls induced by insects. However, the same authors reported that EPNs ensure significant insect mortality only under laboratory conditions, while in foliar application no significant efficacy of treatments was observed.

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Nielsen and Philipsen (2005) found that brassica pod midge was unaffected by EPNs under laboratory conditions, since there were no significant reductions in the emergence of adults after the insect immature stages were exposed to EPNs.

Lasioptera rubi is widely distributed all over Europe to the far east of Russia and Japan (Yegorenkova & Yefremova 2016). Scarce information is available about the presence of natural enemies of this pest in the field. In Europe, *Platygaster pelias, Aprostocetus rubi*, and *Torymus eadyi* are associated hymenopterous parasitoids of *L. rubi* (Yegorenkova & Yefremova 2016). Farmers in Europe mostly rely on mechanical control measures which reduce plant fitness and yields. More research is required to demonstrate *H. bacteriophora* ability to infect and kill different insect' life stages over a range of environmental conditions. The virulence of EPNs observed under laboratory conditions may indicate the success of field application, however, the results are not always comparable (Laznik *et al*. 2010). Furthermore, multitrophic interactions between EPNs, *L. rubi* and its symbiotic fungi should be determined. The results of our study for the control of *L. rubi* larvae under laboratory conditions are encouraging. Due to the adopted application equipment and enhanced nematode formulations, insect pests well hidden inside the plants may be successfully supressed by aboveground application of EPNs (Shapiro-Ilan *et al*. 2012). It is necessary to evaluate the efficacy of different EPNs species against *L. rubi* after subsurface injection of EPNs inside the galls or direct spraying of plants. Entomopathogenic nematodes could become an important tool in nurseries, biological and greenhouse farming systems against *L. rubi*. It should be taken in consideration to prevent pest adult emergence and possibly prevent subsequent infestations.

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