insects

Article

Evaluation of field efficacy of *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and of synthetic insecticides for the control of western corn rootworm larvae

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Abstract: The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte [Coleoptera, Chrysomelidae], is an important insect pest of maize in North America and Central and Eastern Europe. In Central Europe the larvae emerge in May and its three instars feed intensively on maize roots in June, causing plant lodging that leads to loss of economic yield. A three year field experiment (2016-2018) was conducted to compare the effectiveness i) of soil applied granular insecticide based on the active ingredient tefluthrin, ii) of maize seeds dressed with thiacloprid and iii) of entomopathogenic nematodes (EPN) *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae, product Dianem), against WCR larvae. An additional treatment with soil conditioner (a.i. alcohol ethoxylate) mixed with EPN was performed in 2017 and 2018, to check for any increase of EPN effectiveness. Field tests were carried out in two fields infested naturally with a WCR pest population, one in Bučečovci (Eastern Slovenia) and the other in Šmartno pri Cerkljah (northern Slovenia). They exhibit dissimilar pedo-climatic conditions and soil pest pressures. The efficacy of the treatments was very similar at the two locations, despite the approximately five-fold lower WCR larvae pressure in northern than in eastern Slovenia, as well as being constant over time. The largest number of WCR beetles was observed in the negative control, followed by that of beetles subjected to thiacloprid treatment (insignificant decrease). Treatments with tefluthrin, *H. bacteriophora* and *H. bacteriophora* + soil conditioner decreased significantly the numbers of emerging adults. Treatments of thiacloprid, *H. bacteriophora* and *H. bacteriophora*, each with a soil conditioner, led additionally to significantly increased maize plant weights. Furthermore, EPNs were able to persist in maize fields for almost five months, in each? soil type. It was concluded that control of WCR larvae in maize, using entomopathogenic nematode *H. bacteriophora*, is as effective as ~~that subjected to~~a tefluthrin treatment, and could thus offer a sustainable WCR biological control management option in Europe.

Keywords: *Diabrotca v. virgifera*; inundative biological control; entomopathogenic nematodes; *Zea mays*; tefluthrin; thiacloprid

1. Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte [Coleoptera, Chrysomelidae], was assigned the status of an insect pest of corn (*Zea mays* L.) in the early twentieth century [1] and is still an important and challenging pest worldwide. Organochlorine insecticides were first used against WCR by farmers in the USA in 1949, but the pest developed resistance within five years [2]. After that, first carbamate and organophosphate, then pyrethroid insecticides were used for rootworm control, but evolution of the resistance of adult WCR to these insecticides has been reported [3–5]. L~~Much l~~ater, genetically engineered maize (Bt maize), that produces Cry toxins derived from the bacterium *Bacillus thuringiensis* (*Bt*) [6,7], has been ~~in use since~~used in 2003 in the USA. However, in 2009, the first WCR population showing resistance to Cry3Bb1 corn and mCry3A corn was discovered [8]. Additionally a WCR population with resistance to Cry34/35Ab1 corn was confirmed in 2016 which is a consequence of different mechanisms including field–evolved resistance and through cross-resistance [9].

In Europe, crop rotation, as an agricultural practice, remains effective as a tool for managing WCR [10–12]. However, in the western United States Corn Belt, crop rotation is considered to have limited value for WCR management because the species has lost its fidelity for corn and lays eggs in fields planted with other crops [13,14]. In addition to crop rotation, soil or foliar insecticide applications are frequently used in the EU as management options against WCR, especially in fields with high population pressure. Use of foliar insecticides against WCR adults in Slovenia, as well as in some other European countries, is problematic due to i) lack of machinery for foliar application of pesticides in maize, ii) dispersed arable land (small fields), iii) prohibition of aerial application of pesticides, iv) difficulties by official registration of proper efficacy pesticides. Seed coating and application of soil insecticides appears to be less invasive and is thus favored in maize protection programs [15]. Pyrethroid insecticides are one of the few chemical options for controlling corn rootworm larvae. Especially common is the use of the soil insecticide tefluthrin [16]. Tefluthrin is a synthetic pyrethroid insecticide targeting soil-dwelling pests and is applied in granular form at the time of planting as an in-furrow treatment. The addition of tefluthrin mainly affects WCR larvae, resulting in a significantly lower adult emergence rate [17], reduction of maize root damage and therefore reduced ~~a~~ ~~percentage of~~ lodged plants [18]. Neonicotinoid insecticide seed treatments are commonly used in pest management systems because seed-applied insecticides can control various underground pests simultaneously. After the introduction in 2013 of restrictions on the use of some neonicotinoid active substances (Regulation (EU) No 485/2013), thiacloprid, has been recommended for maize seed treatment, because of its lower toxicity to honey bees [19].

Development of behavioral and physiological insecticide resistance and withdrawal of many insecticides due to the non-target effects against soil-dwelling arthropods, together with the adverse effects on the environment and on human health, have fueled research for environmentally friendly alternatives for WCR control. In Europe, various methods have been investigated for reducing the WCR population. For example, pheromone mating disruption using 8-methyl-2decanolpropanoat [20], hybrids possessing native resistance and tolerance to WCR [21], attract and kill strategy [22] have been developed, but their implementation is challenging and have not been adopted by many growers.

Kuhlmann and vaen der Burgt (1998) recommended that biological control options should be considered for WCR management in Europe. Laboratory screening tests of eight entomopathogenic nematode (EPN) species available in Europe showed their ability to infest the larval stages of WCR [24], among which the species *H. bacteriophora* was the most promising candidate as a biological control agent [25]. It reduced the population of the WCR larvae by up to 65 % and the lodging of plants by up to 60 % in field trials, which is comparable to the activity of soil insecticides [26–28]. ~~The first record of~~ *H. bacteriophora* *was firstly determined* in Slovenia ~~was~~ in August 2008 [29]. After its discovery, Slovenia became one of the countries where the use of nematodes as ~~a means of~~ biological protection agent is sanctioned by law also for outdoors application. Establishing the presence of entomopathogenic nematodes in Slovenia was first studied by Laznik and Trdan (2012). Besides inundative EPN use, the efficacy of entomopathogenic fungi (EPF) for WCR larvae management has been studied [31]. Field efficacy assessment of EPF, EPN and insecticides revealed that EPF application shows lower efficacy than EPN or insecticides [28]. Evaluation of potential non-target effects of these biological and chemical control agents was investigated by Babendreier et al. (2015). However, a more recent field study showed that a combination of entomopathogens with chemical insecticides performed better than either product alone [33].

Despite *H. bacteriophora's* great potential as a biological control agent for controlling WCR larvae in the soil [12,34–36], its efficacy was often highly variable due its persistence, virulence and survival in different field soils being affected by dissimilar edaphic and climatic conditions [37,38]. Besides their susceptibility to UV-radiation [39], high temperatures and desiccation [40], moisture condition is one of the crucial factors that affect nematode activity and survival in the soil [37]. Various commercial products have been developed to reduce surface tension and improve soil hydration, making more water available for plants. However better distribution of water in the soil profile may also enhance dispersal of EPNs and help prolong their survival [41]. The objectives of this study were thus threefold: i) comparison of the field efficacy of *H. bacteriophora* and chemical control options commonly used in maize for controlling WCR and wireworm larvae; ii) investigation of the potential efficacy of *H. bacteriophora* increase when blended with a soil conditioner (alcohol ethoxylate); and iii) assessment of the persistence of *H. bacteriophora* in the soil.

Commented [LA1]: I would delete that because you never mention the result of an application against wireworms further..

2. Materials and Methods

2.1 Field sites and experimental set-up

The three consecutive years (2016-2018) of field studies were carried out in a maize growing area in Eastern Slovenia at Bučečovci (site 1) and Northern Slovenia, Šmartno pri Cerkljah (site 2) (Table 1). Both field sites had a natural pest infestation of Western corn rootworm (WCR) (site 1 since 2004, site 2 since 2008) and were four years in monoculture production of maize. Weeds were controlled at both locations after the emergence of corn (BBCH 12-14), using herbicide Lumax (a.i. S – Metolachlor, Terbutilazin and Mezotrion) at a rate of 3.5 l / ha.

Table 1. Characteristics of the field experiment.

Field	Site 1: Eastern Slovenia,	Site 2: Northern Slovenia, Šmartno

	Bučečovci	**pri Cerkljah**
Experiment location	46 °35'07"N 16 °06'37"E	46 °15'08.8"N 14 °29'54.7"E
Date of sowing/nematode application	22th April 2016	11th May 2016
	26th April 2017	17th May 2017
	9th May 2018	
Maize seed	Chapalu (Agrosaat)	LG 34.90 (Agrosaat)
Field size	0.14 ha	0.14 ha
Soil types	silty loam texture	sandy loam texture

Both fields were ploughed and harrowed before sowing. At site 1 corn seeds were sown by Monosem NC classic pneumatic planter, at an inter-row spacing of 70 cm and an intra-row spacing of 16-17 cm, resulting in a theoretical stand of 85.000 plants per ha (Table 2). The maize seeds were sown at site 2 by Gaspardo pneumatic planter using the same procedure. Each treatment comprised 4 rows, each of 100 m length. The middle two were used for assessments that were performed in 5 replicates separated by approx. 20 m. Each replicate had a surface of 0.028 ha; the total size of each experimental field was 0.14 ha. In both regions, flight dynamics of WCR adults were recorded using pheromone PAL traps (Csalomon, Hungary) within an official pest monitoring program. Traps were placed in the maize fields in mid-June and inspected weekly until October. Pheromone bait and sticky traps were replaced monthly. Participation and soil temperatures at depths of 5 and 10 cm were recorded daily from April to September of each study year.

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Commented [LA3]: You do not show the result of those recordings?

Table 2. WCR management on two field sites in three consecutive years (2016-2018). After the active ingredient (a.i.) the commercial product is mentioned (in parenthesis). *Site 1* – Bučečovci E Slovenia; *site 2* – Šmartno pri Cerkljah, N Slovenia.

Field site	**Date of application**	**Treatment**	**Dose**
1	22th April 2016	1. Untreated maize seed (Control)	85.000 plants/ha
		2. *H. bacteriophora* (Dianem)	2x10⁹ nematodes /ha with 200 l water
		3. a.i. Tefluthrin (Force 1,5 G)	12 kg/ha (1.5 % a.i.)
		4. a.i. Thiacloprid (Sonido FS 400)	0.125 l / 50.000 seeds (40 % a.i.)
	26th April 2017	1. Untreated maize seed (Control)	85.000 plants/ha
		2. *H. bacteriophora* (Dianem)	2x10⁹ nematodes /ha with 200 l water
		3. a.i. Tefluthrin (Force 1,5 G)	12 kg/ha (1.5 % a.i.)
		4. a.i. Thiacloprid (Sonido FS 400)	0.125 l / 50.000 seeds (40 % a.i.)
		5. *H. bacteriophora* + soil conditioner (Dianem + Transformer)	5 l/ha, 20 % w/w alcohol ethoxylate
	~~9th May 2018~~	1. Untreated maize seed (Control)	85.000 plants/ha

		2. *H. bacteriophora* (Dianem)	2x10^9 nematodes /ha with 200 l water
		3. a.i. Tefluthrin (Force 1,5 G)	12 kg /ha (1.5 % a.i.)
		4. a.i. Thiacloprid (Sonido FS 400)	0.125 l / 50.000 seeds (40 % a.i.)
		5. *H. bacteriophora* + soil conditioner (Dianem + Transformer)	5 l /ha, 20 % w/w,20 % alcohol ethoxylate
2	11th May 2016	1. Untreated maize seed (Control)	85.000 plants/ha
		2. *H. bacteriophora* (Dianem)	2x10^9 nematodes /ha with 200 l water
		3. a.i. Tefluthrin (Force 1,5 G)	12 kg/ha (1.5 % a.i.)
		4. a.i. Thiacloprid (Sonido FS 400)	0.125 l / 50.000 seeds (40 % a.i.)
	17th May 2017	1. Untreated maize seed (Control)	85.000 plants/ha
		2. *H. bacteriophora* (Dianem)	2x10^9 nematodes /ha with 200 l water
		3. a.i. Tefluthrin (Force 1,5 G)	12 kg/ha (1.5 % a.i.)
		4. a.i. Thiacloprid (Sonido FS 400)	0.125 l / 50.000 seeds (40 % a.i.)
		5. *H. bacteriophora* + soil conditioner (Dianem + Transformer)	5 l/ha, 20 % w/w alcohol ethoxylate

2.2 Treatments

Treatment with entomopathogenic nematodes (EPN). The product Dianem® is based on an entomopathogenic nematode hybrid of European and US strains of *Heterorhabditis bacteriophora* Poinar, which is produced by e-nema GmbH (Schwentinental, Germany). The free-living stage of *H. bacteriophora*, known as the infective juvenile (IJ), was shipped by the producer to the Agricultural Institute of Slovenia (AIS). Upon arrival, they were stored in the fridge at 7-9 ℃ in darkness until use. A week before application, subsamples of nematodes were taken to evaluate the quality of the shipment batches by assessing the mortality of larvae of *Galleria mellonella* (Lepidoptera, Pyralidae). Three plastic cups (diameter 40 mm, height 60 mm) per nematode shipment batch were filled with

200 g of moist, sterilized sand to which five larvae and 100 IJ were added. A mortality of 80-100 % *G. mellonella* larvae was observed after one week in darkness and at 22 ℃ for all nematode batches. This was considered sufficiently high for testing. The IJ were applied as liquid formulations with application rates of 2 × 10^9 IJ per ha in 200 liters of water. Suspension of the IJs was applied at sowing, directly into the seed furrows at a depth of 5-6 cm using a special EPN application system developed at the Agricultural Institute of Slovenia. The EPN application system consists of a 30 l tank, a 12 V pump and distribution pipes. The tube for the application of EPN is aligned to each row unit on the planter, which allows the direct application into the vicinity of the sown maize kernel of the EPN suspension during sowing. The tractor speed at the time of nematodes application was 3 km / h.

Treatment of the EPN in combinations with soil conditioner. Transformer® (Oro Agri International Ltd., Nederland) is a soil conditioner formulated to improve distribution, penetration, infiltration and retention of water in the soil, resulting in reduced water logging, run-off and increased root formation. It was applied at a rate of 150 ml per 30 l water together with EPN.

Commented [LA4]: So in sum 1000ml per hectar (when 200 l H20 per hectar was used)?

Treatment with thiacloprid. Maize seeds were treated with the neonicotinoid thiacloprid (Sonido FS 400; Bayer AG, Germany) at a dose of 0.125 l / 50.000 seeds.

Treatment with tefluthrin. The tefluthrin based granular soil insecticide Force 1,5 G (Syngenta Crop Protection AG, Switzerland) was applied at a rate 12 kg per hectare into the seed furrows during sowing using a micro granular applicator.

Control treatment. The seeds of maize hybrids LG 34.90 (Limagrain, France) and Chapalu (RWA AG, Austria) were treated with bird repellent Korit 420 FS (a.i. 42 % ziram) and fungicides Flowsan FS (a.i. thiram) and Maxim XL 035 FS (a.i. fludioxonil and metalaxyl-M) at the dose 12.5 ml / 50.000 seeds.

2.3 Evaluation of treatment efficacy

Field efficacy of treatments was assessed by counting the number of emerged WCR beetles in adult emergence cages. In each treatment, five emergence cages, approx. 20 m apart were installed over the inner two rows before adult emergence was expected in mid-June. Twelve whole, uncut maize plants were covered by a one square meter gauze cage (1000 mm x 1000 mm x 2300 mm in size) in 2016 and 2017. The emerged beetles were caught and counted using an unbaited Pherocon AM yellow sticky trap (Tr éc é, USA), placed 1.5 m above the soil in the middle of the emergence cage. The yellow sticky traps were replaced monthly from June to September. In 2018 a pyramidal designed emergence trap developed by Rauch et al. (2016) was used. The main difference is that the installation of this trap relies on cutting maize plants at a height of 20 cm, shortly prior to the beetles' expected emergence.

Commented [LA5]: By exhauster and additionally the sticky traps? If only the sticky traps were used please discuss that in detail. Adults could have been died on the soil surface or leaves, so not fixed on the plates…the control interval is during the peak quiet long- so already decomposing of insect, soil particles on it or when raining etc…?

2.4 Evaluation of maize root damage, plant lodging, fresh plant weight and yield

The root systems (250 x 250 x 200 mm) of ten maize plants were dug out from each treatment to measure root damage at the end of July in 2016, 2017, and 2018. Soil was initially removed from the root systems by gently shaking the roots and, secondly, using a high-pressure water spray. Damage was rated using the 0.00–3.00 node injury scale [43]. The suggested economic threshold level in conventional grain maize is 0.25 according to this scale. The percentage of lodged plants, or plants exhibiting typical WCR larvae damage (i.e. 'goose necks'), was assessed in August by counting the symptomatic plants in the two middle rows of each treatment along the whole length of the experimental plots. Prior to harvest, in mid-September, fresh plant weight and corn ears weight were measured. Five randomly selected whole plants with corn ears per replicate, thus twenty-five per treatment (n=5), were cut at the stem base and weighed using a portable scale (DZD, model DJ30KL, G&G GmbH, Germany). The total number of corn ears per plant was counted and weighed separately for determining yield.

2.5 Assessment of nematode persistence in soil

In order to study the persistence of applied nematodes *H. bacteriophora* in the soil, soil samples were taken monthly from the time of application until September, at two depths 5-10 cm and 10-15 cm. Three 0.2 l soil samples were taken randomly with a garden spatula in the EPNs, EPNs with soil conditioner and control treatment. Naturally occurring indigenous EPN populations at the experimental field sites were assessed in a control treatment. Each sample was put into a 250 ml covered plastic box and transported to the laboratory in a portable cool box. The presence or absence of nematodes was determined by using an insect baiting technique [44]. Three larvae of *Tenebrio molitor* (Coleoptera, Tenebrionidae) and three larvae of *Galleria mellonella* (Lepidoptera, Pyralidae) were placed in each cup containing a soil sample and used as bait for the EPNs. Mortality of larvae was assessed during 2 weeks of incubation in a growth chamber in darkness at 21 ℃ and 77 ± 3 % relative humidity. Dead insect larvae were placed into a nematode emergence trap [45] for another two weeks for potential propagation of nematodes.

2.6 Data analysis

The number of WCR beetles caught on yellow sticky traps in emergence cages was analyzed separately for each year and location by ANOVA and Dunnett's Multiple Comparison Test. The entire three year dataset was analyzed by the general linear model (GLM), where the effect of treatment (thiacloprid, tefluthrin, *H. bacteriophora*, *H. bacteriophora* + soil conditioner, control), location (site 1 - Bučečovci, site 2 – Šmartno pri Cerkljah), year (2016, 2017, 2018) and the interaction treatment × location was analyzed on the parameters: number of emerged beetles, fresh plant weight and yield. Further, Fisher's least significance difference (LSD) procedure at the 95 % confidence level was used to discriminate between the treatments within the three year dataset. The analyses were performed with the statistical software Statgraphics Centurion XVI (StatPoint Technologies, Inc., The Plains, VA, USA) and GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). The number of replicates (n) is indicated in the figure or table captions.

3. Results

3.1 Insect pest pressure at two experimental sites

The average emergence of western corn rootworm (WCR) beetles from pest infested soil in the field cages in the control plots was approximately fivefold lower at site 2 (Šmartno pri Cerkljah, Northern Slovenia; 18.8 ± 7.4 in 2016 and 21.6 ± 4.4 in 2017) than at site 1 catches (Bučečovci, Eastern Slovenia; 90.0 ± 10.7 in 2016 and 110.2 ± 20.8 in 2017). The average catches in 2018 at site 1, where different emergence traps were used, were approximately twofold lower than in 2016 and 2017 at site 1: 43.7 ± 1.2.

In contrast, the official pest monitoring survey, where PAL pheromone traps (Csalomon, Hungary) were used to catch flying males, showed similar catches at the two sites (Figure 1). Furthermore, according to the seasonal dynamics in the prior monitoring of the WCR outbreak, it was established that the first specimens appear each year at the end of June, while the peak of outbreak occurs at the beginning of August. WCR were observed on individual fields until the end of October [46].

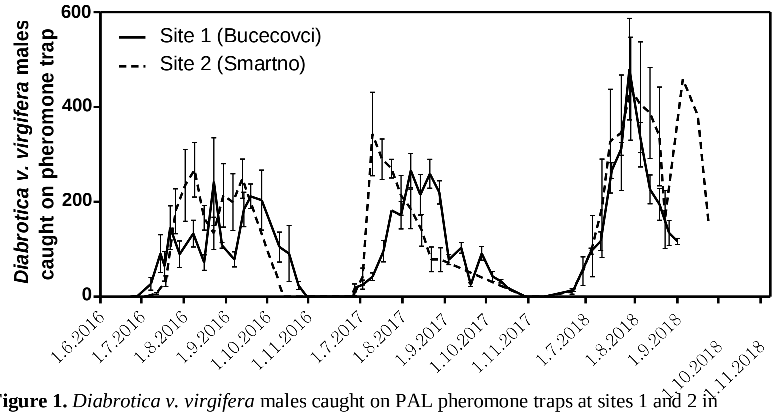

Figure 1. *Diabrotica v. virgifera* males caught on PAL pheromone traps at sites 1 and 2 in 2016-2018 (n=3 for site 1 and n=9 for site 2).

3.2 Treatment efficacy

The results of the general linear model and the LSD procedure showed a significant effect of treatment ($F_{4, 114}$ = 4.73; P = 0.0015), year ($F_{2, 114}$ = 40.62; P = 0.0000) and location ($F_{1, 114}$ = 154.89; P = 0.0000), but not of the interaction of treatment × location ($F_{4, 114}$ = 1.79, P = 0.1368) on the number of emerged beetles. The highest number of WCR beetles was caught in the negative control, followed by the treatment with thiacloprid (insignificant decrease). Treatments with tefluthrin, *H. bacteriophora* and *H. bacteriophora* + soil conditioner significantly decreased the number of emerged beetles and were statistically indistinguishable according to the GLM and LSD analyses. The ANOVA and Dunnett's Multiple Comparison Test showed a significant reduction of the number of emerged beetles in all treatments compared to control at site 1 in 2018, treatments tefluthrin, *H. bacteriophora* and *H. bacteriophora* + soil conditioner in 2017 at site 1 and *H. bacteriophora* + soil conditioner at site 2 in 2017 (Figure 2, Table 3).

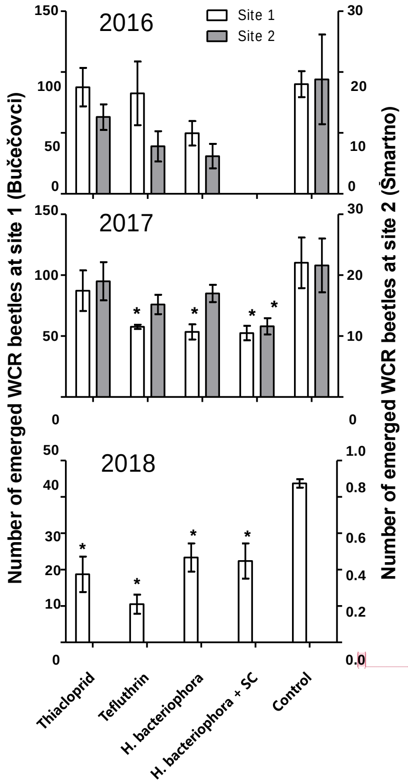

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Figure 2. The number of WCR beetles that emerged and were caught on the yellow sticky trap in the 1 m^2 emergence cages on both field sites in the three consecutive years (2016-2018) of field experimentation. Graphs show average and standard errors (n=25 for all treatments, except 15 for *H. bacteriophora* + SC). Asterisks indicate significant differences from the respective controls (P < 0.05). *SC* – soil conditioner based on alcohol ethoxylate.

The (numerically) highest treatment efficacy, i.e. average reduction of emerged beetles in different treatments compared to control emergence, was calculated for the treatment *H. bacteriophora* + soil conditioner (49.2 %), followed by treatment *H. bacteriophora* (46.2 %), and tefluthrin (44.1 %), which all significantly decreased the number of emerged beetles. In the treatment with thiacloprid an insignificant efficacy of 25.2 % was observed (Table 3).

Table 3. Treatment efficacy in three consecutive years at two experimental sites. Efficacy is expressed as average reduction of beetles that emerged in different treatments compared to control emergence [%]. N=25 for all treatments, except 15 for *H. bacteriophora* + soil conditioner. Columns not sharing the same letters are significantly different. *SE* – standard error; *nd* – no data.

Year	**Site /**	**Thiacloprid**	**Tefluthrin**	***H.***	***H. bacteriophora***	**Control**

Treatment				***bacteriophora***	**+ soil conditioner**	
2016	1	2.7%	8.4%	44.7%	nd	0.0%
	2	33.0%	58.5%	67.0%	nd	0.0%
2017	1	20.9%	47.7%	51.5%	52.5%	0.0%
	2	12.0%	29.6%	21.3%	46.3%	0.0%
2018	1	57.2%	76.0%	46.6%	48.8%	0.0%
	2	nd	nd	nd	nd	nd
Average efficacy ± SE		25.2 ± 9.4%	44.1 ± 11.7%	46.2 ± 7.4%	49.2 ± 1.8%	0.0 ± 0.0%
LSD multiple range test		BC	AB	A	AB	C

3.3 Evaluation of maize root damage, plant lodging, fresh plant weight and yield

The WCR natural population density in the soil was not high enough to cause root damage above the economic threshold. Results of the assessment of larvae damage on maize roots by node injury scale [43] showed no statistical difference between treatments. Only two lodged plants were observed in the thiacloprid treatment at the site 1 (Bučečovci) experiment on 11th August 2016; elsewhere no lodging was observed. In 2017 several plants exhibited typical WCR damage symptoms (i.e. 'goose necks'), but the damage was transient as the plants recuperated. In 2018, corn lodging wasn't evident in the experimental plots.

The results of the general linear model and the LSD procedure have shown a significant effect of treatment ($F_{4, 94}$ = 4.27; P = 0.0034), and location ($F_{1, 94}$ = 237; P = 0.0000), but not year ($F_{2, 94}$ = 1.82; P = 0.169) or the interaction of treatment × location ($F_{4, 94}$ = 0.11, P = 0.9784) on fresh plants' weight. The lowest plant weight was measured in the negative control, followed by treatments with tefluthrin, *H. bacteriophora*, *H. bacteriophora* + soil conditioner and thiacloprid (all treatments except tefluthrin significantly increased plant weight). The yield, assessed as the mass of ears from the plants that were previously weighed, was affected significantly by factor location ($F_{1, 94}$ = 161; P = 0.0000) and by year ($F_{2, 94}$ = 14.99; P = 0.0000) but not treatment ($F_{4, 94}$ = 2.27; P = 0.0689), or by the interaction of treatment × location ($F_{4, 94}$ = 0.25, P = 0.907) (Table 4).

Table 4. Effect of different treatments on fresh plant weight and yield. Yield was assessed by weighing ears from five plants. Data presented are averages ± standard error (n=20 for all treatments, except n=15 for *H. bacteriophora* + soil conditioner). Treatments not sharing the same letters are significantly different.

	Mass of 5 plants [kg]			**Yield [kg]**		
	Site 1	Site 2	LSD test	Site 1	Site 2	LSD test
Thiacloprid		***H. bac***	***terio p***	***hora***	2.79 ± 0.18	2.61 ± 0.18
Tefluthrin					2.45 ± 0.18	

5.16 ± 0.18	.23 ± 0.07	A	1 1.97 ± 0.07	A BC ABC
4.78 ± 0.18	.11 ± 0.07	BC	1 1.82 ± 0.07	
5.10 ± 0.18	.20 ± 0.07	AB	1 1.91 ± 0.07	

***H. bacteriophora* + soil conditioner**	2.80 ± 0.18	5.06 ± 0.25	AB	1.29 ± 0.07	1.92 ± 0.09	AB
Control	2.20 ± 0.18	4.53 ± 0.18	C	1.08 ± 0.07	1.84 ± 0.07	C

3.4 Assessment of persistence of nematodes (EPN) in the soil

The presence of naturally occurring EPN was not confirmed. Persistence of applied EPNs was defined as the percentage of infected *G. mellonella* and *T. molitor* larvae in tested soil samples. Results varied significantly varied over the period of monitoring of EPN persistence monitoring. Throughout the duration of the experiment (2016-'18), the presence of EPNs has been confirmed at first sampling dates after application of EPN at both sites in all treated plots. ~~More infected~~More infected soil samples were found when taken from site 1, where, one month post application, up to 89 % of larvae used as EPN baits were infected with nematodes. With the exception of year 2017, decreasing larval mortalities in later periods indicate a decrease of EPN population or their pathogenicity. However, some soil samples taken up to 141 days after application of *H. bacteriophora* still contained virulent EPN. The highest infectivity rates were recorded in 2017 at site 1 where, 83 days after application, the addition of soil conditioner resulted in 90 % mortality at 5-10 cm and 83 % in soil samples taken from
10-15 cm soil depth. On the other hand, a lower level of mortality was observed for soil samples collected at site 2 where, in 2016, the presence of EPN was confirmed only at the first sampling date, when 33 % of the larvae were infected at a depth of 10-15 cm. Mixing *H. bacteriophora* solution with soil conditioner generally increased insect baits' mortality rate in soil samples taken in 2017 and 2018 (
Table **5**).

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Table 5. EPN persistence, expressed as percentage of infected insect cadavers used as insect baits. Larvae of *T. molitor* and *G. mellonella* were used as EPN bait and placed on soil samples collected at two depths at both experimental sites; nd – no data.

Site / Year	**Days post Treatment**	**Treatment / depth of sampling**			
		H. bacteriophora		***H. bacteriophora* + soil conditioner**	
		5-10	**10-15**	**5-10**	**10-15**
Site 1					
2016	26	78	89	nd	nd
	61	11	22	nd	nd
	83	11	33	nd	nd
2017	30	73	37	50	43
	57	0	0	10	37
	83	50	63	90	83
	113	8	8	25	13
	141	4	8	4	0
2018	20	13	27	40	27
	41	13	7	7	13
	57	0	0	0	22

	69	0	7	0	0
	83	13	13	3	3
	100	13	0	0	17
	128	0	0	13	0
Site 2					
2016	28	0	33	nd	nd
	49	0	0	nd	nd
	84	0	0	nd	nd
2017	22	4	0	7	11
	57	33	10	60	50
	114	8	4	13	8

4. Discussion

The results of the three year field study, performed on naturally WCR-infested maize fields at two pedo-climatically different locations with dissimilar (soil) pest pressures, shows that the use of biocontrol, product based on entomopathogenic nematode *H. bacteriophora* can significantly reduce WCR adult emergence (46.2 ± 7.4% reduction), which is at par with the chemical control represented by the use of the granular soil insecticide tefluthrin (44.1 ± 11.7% reduction) (Figure 2, Table 3). Similar findings were reported when biological and chemical based managements were compared [28,47]. However, in contrast to our experiment, these studies were performed using artificially infested maize plants with WCR eggs, resulting in severe damage to untreated maize plants, but not to the nematode-treated maize plants in which the root damage was kept below the economic thresholds [28,47]. Our study, where the natural WCR larval population in the soil did not cause significant root damage [43], did not allow us to evaluate potential lodging prevention, although it showed that *H. bacteriophora* controlled WCR larvae as well as tefluthrin, one of the commonly used chemicals for WCR Management in Europe and the USA [48,49]. The data recorded in the present study for the agent tefluthrin are contrary to the observations of [50,51], where application of tefluthrin soil insecticide application made no significant impact on the emergence of WCR. Therefore, this inconsistency can be the result of precipitation on the effectiveness of soil insecticide and WCR emergence interactions, as assumed by previous research [52], and of other environmental factors which can influence the efficacy of soil insecticide [16,53].

The efficacy of biological as well as of chemical treatments has varied significantly over the years (Figure 2), as it is influenced by several environmental factors that may affect nematode or insecticide efficacy in the soil. For example, soil moisture conditions [54] and soil type [55] affect EPN infectivity and its persistence in the soil. Furthermore, the characteristics of different soil types [37,55,56] as well as of soil temperatures [57,58] and the absence of larvae or alternative hosts [57,59] can impact on the efficacy of EPNs. However, such variable results are often observed in field WCR biocontrol studies [28,56,60].

The numbers of beetles emerging in the field cages were significantly affected by treatment as well as by year and location. The effect of 'year' can be attributed to the change of the field cages used at site 1 in 2018 According to [42] they affect the maize plants much more (i.e. the plants are cut at ca. 20 cm at trap set-up) than the traps used in years 2016 and 2017, where the plants were left intact. This could mean that only the peak beetle emergence was assessed at site 1 in 2018 whereas, in the trap design used in 2016 and 2017, the late emerging beetles were also caught. In addition, factor location significantly affected the number of emerging beetles. This is due mainly to different below ground pest pressure and is probably connected to the field history and different climatic and edaphic conditions at the two sites. Besides the difference in soil types, the WCR was already observed at site 1 in 2004, whereas at site 2 it had been present since 2008 [46]. However, the interaction of treatment and location was insignificant, proving that the treatments gave similar results at the two locations in the three year experiment, showing that the nematodes (and tefluthrin) performed equally well under dissimilar pest pressures and pseudo-climatic conditions, and that the two trap designs gave comparable results.

Treatments evaluated in the present study (*H. bacteriophora*, *H. bacteriophora* + soil conditioner and thiacloprid), except that of tefluthrin, led significantly to increased fresh plant weight. In the similar study conducted in Hungary [47] investigated the application of entomopathogenic nematodes and a soil insecticide based on active ingredients: tefluthrin, cypermethrin and chlorpyrifos. In most circumstances, all these agents were found to be similarly effective on larval control and increased yield slightly. The application of *H. bacteriophora* in the soil successfully reduced the root damage caused by WCR larvae and by plant lodging, by up to 60 % [26]. Further, the application at planting of soil insecticides to the furrows at planting can prevent larval damage leading to yield benefits [61]. A significant positive impact of tefluthrin on the grain yield of transgenic Bt corn hybrids was reported from studies in North Dakota at a site with high WCR infestation levels [50]. In some other studies, tefluthrin did not provide a yield benefit consistently [62], but improved root protection from larval feeding injury at high larval feeding pressure [63] and significantly increased delays in WCR emergence as compared with Bt maize alone [17]. However, due to a variety of findings, treatments can be affected by biotic and abiotic factors, as discussed earlier [16,50].

Applied *H. bacteriophora* nematodes were detected almost four months after application in sandy loam texture at site 2 and almost five months after applications in silty loam texture at site 1, which is *in sync* with previous research [32,34,58]. These studies showed that applied nematodes were able to survive in the soil for approximately one month without their host, which hatches 2-4 weeks after maize sowing and EPN application. They also showed that *H. bacteriophora* survived for up to 2-5 months in the soil of maize fields [58] while its persistence after application on golf courses [64] and in alfa alfa fields [65] was up to 2 years. Moreover, our experiment showed that nematodes, regardless of the soil types, reduce emergence of WCR beetles. These results support the findings that *H. bacteriophora* has the potential to reduce WCR larvae in different soil types [56]. It was shown that the efficacy of entomopathogenic nematodes can be higher in soils with high clay and silt content than in sandy soils [56]. This is in sync with our observations in 2017 where *H. bacteriophora* performed better at site 1 (silty loam texture) than at site 2 (sandy loam texture).

Among other abiotic factors, soil moisture content is one of the most important factors limiting persistence and infectivity of EPNs [37], which tend to be most infective under moderate soil moisture conditions [54]. The ability of EPN to persist under longer desiccating conditions varies between species, depending on their ability to adapt metabolic activity and recover after exposure to unfavorable moisture conditions [54]. A recent study showed that combining *H. bacteriophora* with adjuvants such as soil conditioners may enhance the performance of nematodes against soil-dwelling insects [41]. In our study the addition of surfactant did not increase infection rates in general, although some results obtained from site 2 in 2017 indicate that addition of surfactant may improve the persistence and effectiveness of infective juveniles in soil compared with regular water application treatment.

One of our aims was to evaluate the efficacy of commonly used maize seeds coated with thiacloprid on WCR. Although the results showed that thiacloprid has affected the number of emerged adults in the emergence cages (Figure 1, Table 3), it did not affect WCR larvae significantly. Furthermore it was much less effective than tefluthrin, which is commonly used against WCR larvae in Europe; therefore we consider it an inappropriate option for WCR larvae control.

The average number of WCR beetles caught on an unbaited Pherocon AM yellow sticky trap (Trécé, USA) in emergence cages, from pest infested soil in the control plots, was approximately fivefold lower at site 2 (Šmartno pri Cerkljah, Northern Slovenia) than at site 1 (Bučečovci, Eastern Slovenia. The official WCR monitoring program, however, in which PAL pheromone traps are used to capture WCR males, has shown similar pest densities at both sites. Our study thus shows that pest pressure in the soil did not correlate with official (aerial) pest monitoring. However, abiotic factors like climate and soil conditions can affect the natural mortality factors in each development stage of WCR, which can influence the generational mortality of WCR populations as was already concluded
by Toepfer and Kuhlmann (2006).

Commented [LA8]: I think here the method has to be discussed in detail. The cages are influencing the sticky traps? The development stage of the plant influences the catches (if there is flowering or cobs the adults could be more attracted on them, also the time of the day influences their movements, ..) . Normally there should be even more caught in the untreated control as you get male and female on unbaited sticky traps whereas on the official monitoring program, where PAL pheromone tras are use just males are there, no?

5. Conclusions

We conclude that WCR larvae management in maize using EPN *H. bacteriophora* could offer a sustainable WCR biological control management option in Europe. The three year study showed that the effectiveness of chemical products against biological products has varied over the years as it was affected by several abiotic factors. EPNs managed the WCR larvae population as effectively as a commonly used chemical agent tefluthrin, while thiacloprid was found to be less effective and therefore assessed as inappropriate for WCR larvae control. EPNs were proved to be able to persist in maize fields for almost five months regardless dissimilar pedo-climatic conditions. The results suggested sandy loam soil to be more favorable compared to silty loam soil type for EPNs treatment with soil conditioner.

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