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ORIGINAL PAPER

A novel approach for the biological control of invasive Bagrada bugs with entomopathogenic nematodes

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Abstract

The Bagrada bug, *Bagrada hilaris* Burmeister (Hemiptera: Pentatomidae), an invasive pest of Palearctic origin, has become a problem in the Western Hemisphere, attacking brassica and other crops. The Bagrada bug was first reported in Chile in 2016, and despite the availability of some efficacious synthetic insecticides, *B. hilaris* is a growing problem. Currently, few international studies have been performed regarding the biological control of *B. hilaris*. However, entomopathogenic nematodes (EPNs) are potential candidates, as they cause mortality to other pentatomids. Of the six native Chilean EPN isolates, *Steinernema feltiae* CH4 caused the highest mortality in *B. hilaris* adults (97.5%). In a second assay, the mortality of *B. hilaris* adults increased with higher doses and extension of the post-application time of this isolate, with 96.9 and 100% at 96 and 120 h after application, respectively. A probit analysis indicated that the lethal dose 50% (LD50) dropped from 60.7 to 4.4 IJ/insect at 48 and 168 h, respectively. In a semifield study, a single application of this EPN with an adjuvant achieved approximately 60% mortality of the bugs after 480 h on rocket plants, and the damage to leaves was significantly lower (29%) than the damage to leaves of control plants (54.3%). These novel results represent progress in the use of EPNs for the control of foliage pests and are also the first approach of successful control of *B. hilaris* with EPNs in any crop. Further field studies will allow the development of integrated pest management programmes using EPNs as biological control agents for *B. hilaris* populations.

Keywords *Bagrada hilaris* · *Steinernema* · *Heterorhabditis* · Biological control · Brassica · Integrated pest management

Key message

- In the laboratory, the entomopathogenic nematode (EPN) *Steinernema feltiae*, native isolate CH4, caused 97.5% mortality in *B. hilaris*.
- LD50 ranged between 69.7 and 4.4 IJ/insect in a period of 48–168 h.
- Under semifield conditions, a single application of EPN with an adjuvant achieved 60% mortality of *B. hilaris*.
- Additionally, EPNs have enormous potential for the control of foliage pests and may become a powerful alternative for *B. hilaris* control.

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Introduction

The Bagrada bug, *Bagrada hilaris* Burmeister (Hemiptera: Pentatomidae), is a pest that attacks a wide range of host plants (Palumbo et al. 2016), mostly belonging to the Brassicaceae family (Reed et al. 2013; Huang et al. 2014), but species from other families are also affected, including Solanaceae, Fabaceae, Poaceae, Malvaceae, Amaranthaceae, Cucurbitaceae and Apiaceae (Anwar et al. 1973; Dharpure 2002; Sabyasachi et al. 2013). This bug is native to Asia and Africa, and it invaded Europe, Australia, the

Middle East, Southeast Asia, the USA and Latin America in the last decade (Guarino et al. 2008; Reed et al. 2013; Sánchez-Peña 2014; Carvajal et al. 2019). The Bagrada bug has been considered a serious pest of Brassicaceae in India and Pakistan since the early 1990s (Vekarta and Patel 1999), in California and Arizona since 2010 (Palumbo et al. 2015) and in Mexico since 2014 (Barrera-López et al. 2019). In 2016, *B. hilaris* was first reported in the Metropolitan Region (MR) of Chile, attacking *Brassica rapa* L. (Faúndez et al. 2016). Since then, in only three seasons, the Bagrada bug has spread more than 1000 km, invading other regions, including Valparaiso in 2017 and Coquimbo, O'Higgins and Atacama and Maule in 2018 (Faúndez et al, 2018). In addition to brassica crops, in severely infested areas, the Bagrada bug has also been detected attacking other vegetable, legume and cereal crops, with maize being the most affected (Chilean Agricultural and Livestock Service (SAG) 2019, personal communication).

Some reasons for the success of this pest are its great invasive capacity and polyphagous nature. One female can lay up to 400 eggs on the soil, plant leaves or stems, and its life cycle can last only 21 days if the temperature is favourable. Nymphs and adults feed on leaves, stems, flower buds and fruits through their piercing–sucking mouthparts and damage plant tissues by the effect of their salivary enzymes (Palumbo and Natwick 2010). In severe infestations, plants die after wilting or grow deformed because of the loss of their apical meristems (Huang et al. 2014). The strong aggregation behaviour of this bug can aggravate damage (Reed et al. 2013), especially at early stages of the crop where loss can be total.

Due to the huge impact of this pest in Chile, in 2017, SAG established mandatory control and declared a phytosanitary emergency, authorizing the use of 34 synthetic insecticides. (In April 2019, six new molecules were included.) However, in an evaluation conducted by the National Institute of Agricultural Research (INIA), only 12 insecticides from the original list achieved more than 65% control in the field (INIA 2018, personal communication). Despite the availability of these products, the problem has not been solved, and *B. hilaris* continues expanding its distribution every season.

Furthermore, in the MR of Chile, this bug hibernates in large numbers in urban schoolyards or food storehouses (Faúndez et al, 2018). The Chilean Institute for Public Health has recommended its control, but the presence of humans or food complicates the situation, as no insecticides are registered for this pest in those habitats (SAG 2019, personal communication).

Currently, in all countries where *B. hilaris* has invaded, its control is based mainly on synthetic insecticides (Joseph, 2018; Grettenberger and Joseph, 2019). Despite the efficacious knockdown effect and residual control of some products such as pyrethroids, the pest continues to be a growing problem, and the literature does not inform any other methods that could replace the use of synthetic insecticides, partially or totally (Grettenberger and Joseph, 2019). In addition, in some *B. hilaris* populations elsewhere, resistance to insecticides has been detected, such as in India on mustard crops (Dhingra 1998) and Italy, where caper crops are sprayed annually 4–5 times and the efficacy of some synthetic insecticides has decreased in consecutive seasons (Guarino et al. 2007). In the case of organic systems, pesticide options have much lower efficacy and shorter residuals, making management more challenging, and it is necessary to apply combined products in multiple applications in time intervals of 4–7 days (Lloyd and Grettenbergerb 2018). Regarding other control methods, no single tool is enough for adequate control. For example, colour or pheromone traps or trap crops attract *B. hilaris* but do not significantly reduce populations, and no other lure has been identified that is more attractive than brassica crops (Shimat 2014). Similarly, essential oils do not deter *B. hilaris* except for geraniol, but no field assays have been performed yet (Joseph 2017). Therefore, a multidimensional integrated pest management IPM approach may reduce populations and crop damage, including several cultural strategies and biological control.

Concerning biological control agents (BCAs), it is known that *B. hilaris* can be parasitized or preyed upon by other insects. Recent studies have described parasitoid wasps from the genera *Trissolcus* and *Gryon* (Platygastroidea: Scelionidae) (Mahmood et al. 2015) and *Ooencyrtus* (Chalcidoidea: Encyrtidae) (Mahmood et al. 2015; Triapitsyn et al. 2020) as possible BCAs targeting pest eggs. A further study showed that *Gryon gonikopalense* (Hymenoptera: Scelionidae) parasitized 50–60% of eggs (Martel et al. 2019). Additionally, several isolates of entomopathogenic fungi such as *Beauveria bassiana* (Barrera-López et al. 2019), *B. pseudobassiana, Metarhizium anisopliae* and *Isaria fumosorosea* (Barrera-López et al. 2020) and *Zoophthora radicans* (Torres et al. 2016) have been evaluated successfully, causing high levels of mortality in *B. hilaris*. However, all these studies were conducted under laboratory conditions and are still in early stages, and no products based on microorganisms are commercially available for the control of this pest.

Among the BCAs commonly used commercially, entomopathogenic nematodes (EPNs) have enormous potential against numerous insect pests from different orders and other pentatomids. For example, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* have been shown to be capable of killing 65–83% of nymphs and adults of *Halyomorpha halys* (Gorgadze et al. 2017). Similarly, different isolates of *Heterorhabditis* achieved up to 76% mortality of *Dichelops melacanthus* adults (Aparecida et al. 2015), and *Steinernema* sp. applied at doses of 100 IJ/insect achieved 100% mortality of *Nezara viridula* nymphs within 72 h in laboratory trials (Pervez and Ali 2011). No studies thus far

have evaluated EPN against *B. hilaris*, but some evidence of their effect on these pentatomids suggests a potential for its control.

Based on the capability of EPN to kill pentatomid species, the aims of the present work were: (1) to select potential native Chilean isolates of EPN for the control of *B. hilaris;* (2) to establish some infection parameters of the EPN selected on *B. hilaris*; and (3) to evaluate the effects of post-application time and adjuvant on this isolate on *B. hilaris* in potted rocket, *Eruca sativa*, under semifield conditions.

Materials and methods

Insect source

Adults and nymphs of *B. hilaris* were collected directly from different weeds around the university campus located at 33° 34"12'S; 70° 37"59'W between January and May 2019. The insects were placed in plastic containers and used on the same day for the experiments.

Nematode culture

In the same period of time, non-commercial isolates of *Steinernema feltiae* (CH4 and LR), *S. unicornum* (CH3 and Vilchez), *Heterorhabditis atacamensis* (31,873) and *H. bacteriophora* (31,868) were cultured on last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) following Dutky et al. (1964). The infected larvae were incubated at 20 °C. The infective juveniles (IJs) of EPNs were collected using white traps (White 1927) and stored at 10 °C for a maximum of one week before setting the experiments.

Susceptibility of Bagrada hilaris to different species/isolates of EPN

A series of Petri dishes was prepared with two different concentrations of these EPN species/isolates. One millilitre containing 1000 and 2000 IJ was poured on a filter paper disc placed inside the Petri dish, and 20 adults or nymphs of *B. hilaris* were placed after the nematodes. The Petri dishes were then sealed and placed in an incubator at 20 °C in darkness. After 120 and 480 h, dead insects were counted, moved to containers and later dissected to confirm infection by EPNs. The experiment was repeated four times. The percentages of mortality were analysed by a general linear model (GLM; $\alpha = 0.05$) using EPN species/isolate, IJ dose (concentration) and *B. hilaris* development stage as variables, and their relationships were also analysed for significance. In this case, no correction was performed because no insects died in the control treatment.

Infection parameters of Steinernema feltiae (CH4 isolate) against Bagrada hilaris adults

A 24-multiwell plate was filled with moistened sterile sand (15% v/v; grain size between 850 and 75 µm). Randomly, 0, 2, 5, 9, 16, 30 or 50 IJ of *S. feltiae* (CH4 isolate) was poured in a well over the sand in 50 µL of water. A single *B. hilaris* adult was placed after the IJs and the plates closed and incubated at 20 °C. The mortality of the insects was assessed every 24 h for a week, and then cadavers were dissected under a stereoscope to confirm nematode infection. Each treatment was replicated 24 times, and the experiment was repeated four times ($n = 96$ insects per dose). The mortality data were analysed using the Chi-squared test for proportions (X^2) due to the binomial nature of the results (dead/alive). Lethal dose *vs.* time of exposure analysis was performed by logit regressions in 50% (LD 50). For significant results, a Tukey family post hoc test was performed ($\alpha = 0.05$).

Semifield assay

The experimental arena consisted of one pot (15 × 15 cm) with sterilized soil and five rocket plants protected by a 20-cm-tall acrylic and muslin cylinder. When the plants had grown five leaves, 20 *B. hilaris* were placed inside each experimental arena, and 15 mL of a treatment material was applied on the foliage of the plants using a manual sprayer. The treatments consisted of: (1) water (control treatment); (2) water + IJ; (3) water + IJ + Tween 80 at 1% v/v; (4) water + IJ + Tween 80 at 2% v/v; and (5) bifenthrin 10 EC at a concentration of 1.25 mL/L. In treatments (2), (3) and (4), 2,000 IJ of *S. feltiae* (isolate CH4) was used. The arenas were maintained under a glasshouse, and dead *B. hilaris* were removed every 24 h until completing 168 h. After 480 h, experimental arenas were dissembled, all dead insects were collected, and damage to plants was evaluated using the image processing package "Fiji" (Schindelin et al. 2012). After incubating all dead insects for five days at 20 °C, they were dissected to verify the presence of EPNs. Each treatment was replicated six times, and the experiment was repeated twice. Temperature and relative humidity were recorded daily during the experimental period, observing averages of 10.9 °C (min. 3.1–max. 18.6) and 60.8% (min. 33.3–max. 88.3), respectively.

The percentage of mortality was corrected using Sun–Shepard's formula for non-uniform populations because some insects were lost in the experiment (probably escaped) (Püntener 1981). The corrected percentage

of mortality was angularly transformed prior to analysis by one-way ANOVA ($\alpha = 0.05$). The results are presented untransformed (mean ± SE).

Results

Susceptibility of Bagrada hilaris to different species/isolates of EPN

The interaction between the EPN species/isolate and *B. hilaris* development stage was significant ($F_{(1, 24)} = 3.23$; $p = 0.023$), as were the independent factors EPN species/isolate ($F_{(5, 24)} = 23.1$; $p < 0.001$) and *B. hilaris* development stage ($F_{(1, 24)} = 146.0$; $p < 0.001$) (Fig. 1). In general, all isolates evaluated were more virulent to *B. hilaris* adults than nymphs, but *S. feltiae* (isolate CH4) reached the highest values of mortality, killing up to 97.5 ± 1.4% of the adults and 55 ± 10% of the nymphs after 120 h of exposure to EPN. On the other end, *B. hilaris* was less susceptible to *H. bacteriophora* (isolate 31,868), which killed only 45 ± 5% of the adults and 15 ± 1% of the nymphs. The IJ doses used in the experiment (1000 and 2000 IJ/mL) did not show any difference in the mortality rates of either adults or nymphs ($F_{(1, 24)} = 0.68$; $p = 0.416$).

Infection parameters of Steinernema feltiae (CH4 isolate) against Bagrada hilaris adults

The mortality of *B. hilaris* adults increased with higher doses of EPNs applied and the time of post-nematode exposure (Table 1). This difference was evident from 48 h after application, where the highest doses (30 and 50 IJ/insect) achieved 49.9 ± 2.1 and 51.0 ± 2.1% mortality, respectively. At 120 h, the only treatment that caused 100% mortality was 50 IJ/insect, whereas the rest of the doses caused a maximum of 83.3 ± 3.4%. The probit analysis indicated that the LD50 was dependent on the post-application time (Fig. 2), decreasing as the exposure time increased. At 48 h, 40.2 IJ/insect was necessary to kill 50% of the bugs ($\chi^2_{(4)} = 13.01$; 95% CI [32.3–48.2]; $p = 0.01$), whereas at the end of the experiment (168 h), the number of EPNs dropped to 4.4 IJ/insect ($\chi^2_{(4)} = 13.40$; 95%CI [3.1–8.5]; $p = 0.01$).

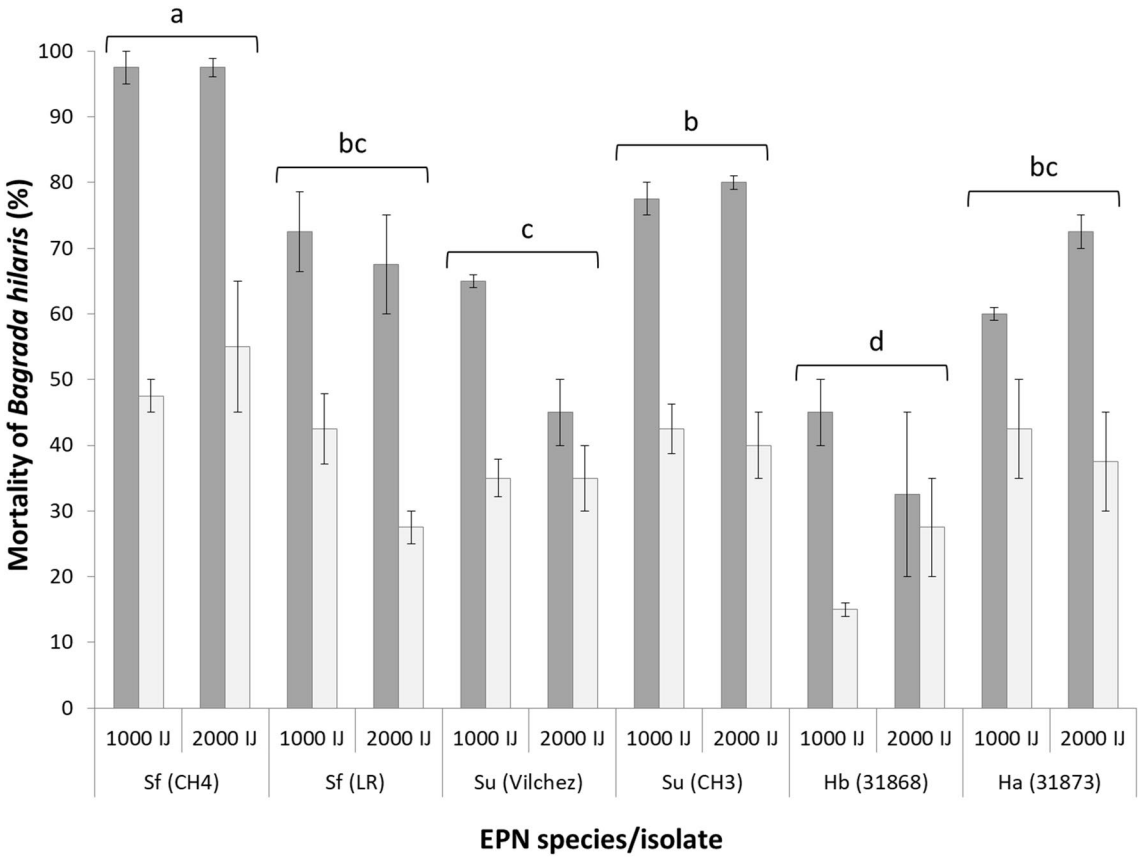

Fig. 1 Percentage of mortality (mean ± SE) of adults (dark grey bars) and nymphs (light grey bars) of *Bagrada hilaris* exposed to different entomopathogenic nematode species. Sf = *Steinernema feltiae*; Su = *Steinernema unicornum*; Ha = *Heterorhabditis atacamensis*; Hb = *Heterorhabditis bacteriophora*. Different letters indicate significant differences between EPN species/isolates ($\alpha = 0.05$)

Table 1 Percentage of mortality (mean ± SE) of *Bagrada hilaris* adults exposed to different doses of *Steinernema feltiae* (strain CH4) over time (time of exposure)

Treatment	% of mortality (mean ± SE) over time of exposure					
IJ per insect	48 h	72 h	96 h	120 h	144 h	168 h
2	14.6 ± 2.4 d	28.1 ± 2.1 d	30.2 ± 2.1 e	33.3 ± 3.4 d	36.5 ± 4.0 e	36.5 ± 4.0 e
5	21.8 ± 3.9 c	33.3 ± 3.4 c	40.6 ± 5.24 d	47.9 ± 2.4 c	47.9 ± 2.4 d	53.1 ± 2.1 d
9	21.9 ± 6.3 c	31.3 ± 5.4 cd	43.8 ± 5.4 cd	45.8 ± 3.4 c	53.1 ± 5.2 c	61.5 ± 8.6 c
16	42.7 ± 2.1 b	42.7 ± 2.1 b	47.9 ± 2.4 c	47.9 ± 2.4 c	47.9 ± 2.4 d	66.7 ± 5.9 c
30	49.9 ± 2.1 a	75 ± 0 a	81.3 ± 2.4 b	82.3 ± 2.1 b	83.3 ± 3.4 b	83.3 ± 3.4 b
50	51.0 ± 2.1 a	75.0 ± 3.4 ab	96.9 ± 2.1 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
$X^2_{(df=5)}$ value	17.9	43.5	75.98	78.3	75.35	73.57
P value	0.003	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001

Different letters indicate significant differences among doses (columns) $\alpha = 0.05$

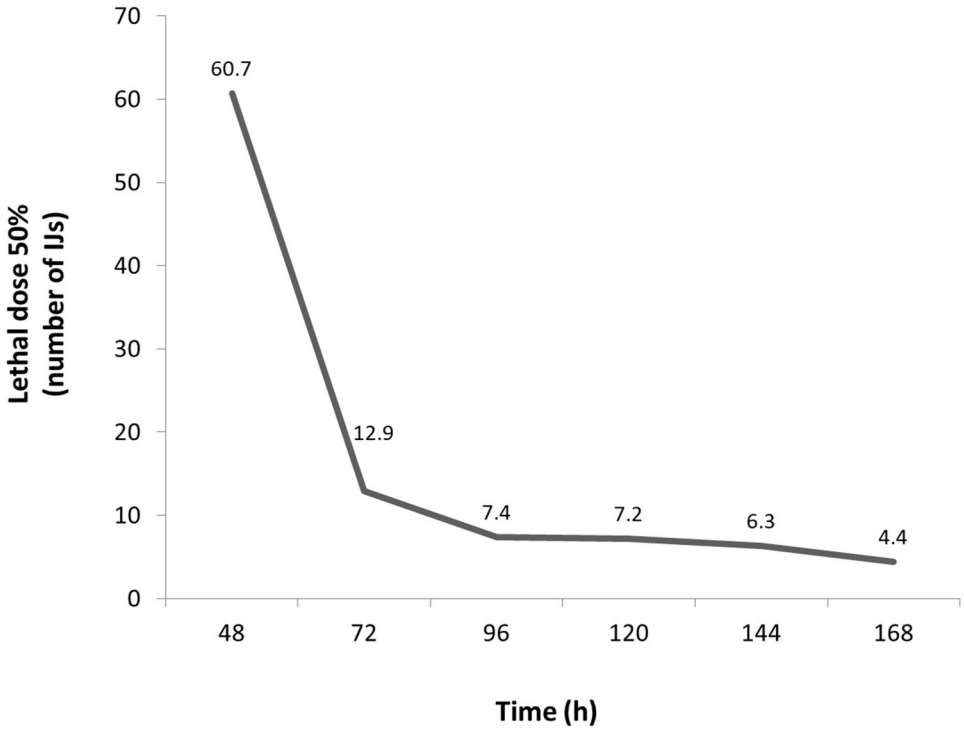

Fig. 2 Lethal dose 50% (LD50) of *Steinernema feltiae* (isolate CH4), i.e. the number of IJs capable of causing death in 50% of the treated *Bagrada hilaris* adults over 168 h

Semifield assay

At 120 h post-application, the mortality of *B. hilaris* varied among treatments ($F_{(3, 24)} = 81.41$; $p < 0.001$; Fig. 3). While the synthetic insecticide killed 100% of the insects, the maximum mortality rate produced by EPNs was 25.4 ± 5.7% (EPN + Tween 1%). However, after 480 h, the mortality of *B. hilaris* produced by EPNs increased significantly ($F_{(3, 24)} = 16.5$; $p < 0.001$). In this case, the EPNs applied with Tween 1% and 2% achieved higher mortality rates (60 ± 9.9% and 48.9 ± 7.8%, respectively) than the EPNs applied with no adjuvants (32.4 ± 6.9%; Fig. 3). In relation to leaf damage, significant differences were observed between treatments ($F_{(4, 30)} = 6.41$; $p < 0.001$), where the highest leaf area damage was observed in the control (water) treatment (54.27 ± 8.77%). The best biological control treatment was EPNs + Tween 1%, with 29.42 ± 9.84% of leaf area damaged, which was significantly lower than the leaf damage of the plants treated with water. The lowest percentage of leaf area damaged by *B. hilaris* was achieved in the treatment with the synthetic insecticide (4.5 ± 1.51%; Fig. 4).

Discussion

In Chile, registered biocontrol products have not been tested against *B. hilaris*. Additionally, products based on EPNs are imported from Europe and sold for the control of dipteran and coleopteran pests. The present results suggest that EPNs have the potential to become an alternative

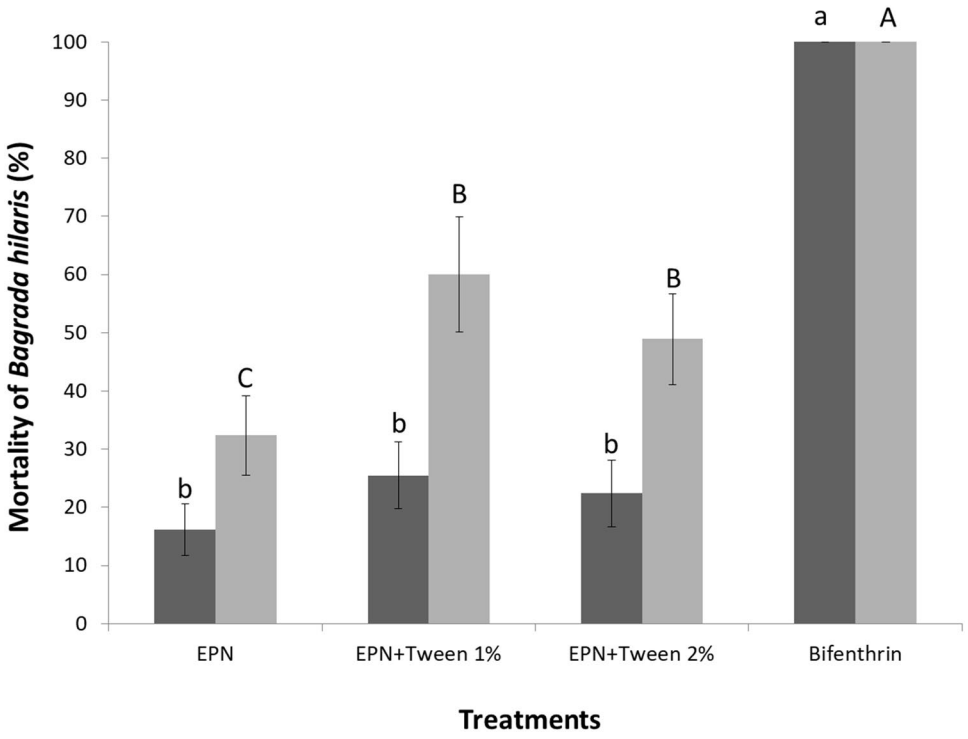

Fig. 3 Percentage of mortality of *Bagrada hilaris* (mean ± SE) after 120 h (dark grey bars) and 480 h (light grey bars) post-treatment in a semifield pot assay (five rocket plants/pot) caused by EPN *Steinernema feltiae* (isolate CH4). Different letters (lower case = 120 h, capital = 480 h) indicate significant differences between treatments ($\alpha = 0.05$)

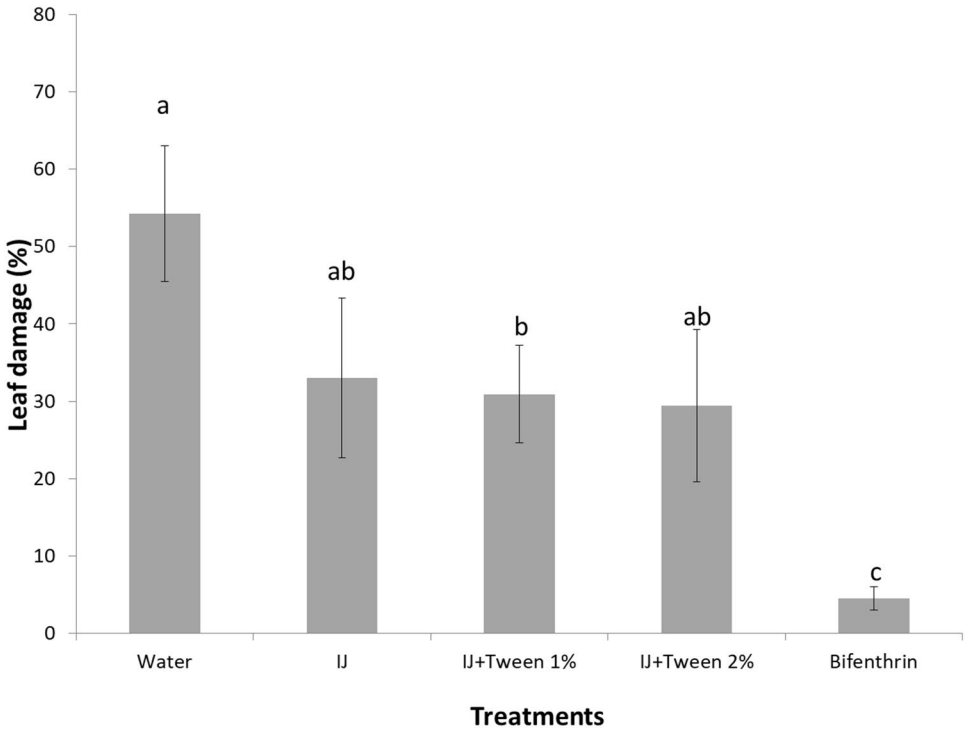

Fig. 4 Percentage of leaf damage produced by *Bagrada hilaris* (mean ± SE) after 480 h in a semifield pot assay (five rocket plants/pot) treated with the EPN *Steinernema feltiae* (isolate CH4) with and without an adjuvant, bifenthrin and water. Different letters indicate significant differences between treatments ($\alpha = 0.05$)

to control *B. hilaris* for vegetable growers in Chile, as all isolates evaluated, from both *Heterorhabditis* and *Steinernema*, were capable of killing *B. hilaris* adults and nymphs, with *S. feltiae* (isolate CH4) being the most effective isolate controlling them. We also observed that all EPN isolates caused higher mortality rates to *B. hilaris* adults than nymphs. This difference could be related to the smaller size of the latter (less than 5 mm), as the size of the host is known to affect the penetration and development of EPNs (Bastidas et al. 2014). However, in the case

of the pentatomid *Halyomorpha halys*, nymphs were more susceptible to EPNs than adults (Gorgadze et al. 2017). One of the factors affecting mortality by EPNs is their affinity with the insect host (Campos-Herrera and Gutiérrez 2014); therefore, other species/isolates not evaluated in this opportunity may be even more effective.

As far as we are concerned, this is the first attempt to control *B. hilaris* with EPNs, and the results suggest that EPNs could be included in integrated management programmes. In North America, many farmers apply multiple sprays of synthetic insecticides at young stages of plant development to achieve non-damaged products and even shift to non-brassica crops to avoid losses (Reed et al., 2013; Palumbo et al., 2016; Grettenberger and Joseph, 2019). This situation is also occurring in Chile, where farmers have also increased the number of insecticide applications, raising their costs, the negative impact on the environment and consumers, and risking fines. Therefore, it is quite likely that more effective solutions will be adopted by Chilean farmers. In addition, microbial biological control agents offer several other advantages in terms of innocuousness and sustainability (Lacey et al. 2001), opening stricter markets such as organics to their products. In terms of adaptation to the local conditions, cost of storage, transport and legal restrictions, native EPNs also present advantages compared to imported biocontrol agents.

In our study, a single application of *S. feltiae* (isolate CH4) demonstrated, in both laboratory and semifield conditions, high levels of control against *B. hilaris*. On potted rocket plants, EPNs with the adjuvant Tween 80 (1% v/v) were capable of controlling 60% of the bugs, reducing leaf damage to 30%. This level of control is close to the 65% that INIA defined as efficient for the authorized synthetic insecticides for this pest.

The use of adjuvants with EPNs has been tested in several studies. Their results have been contrasting and depend on nematode and insect species, type and concentration of adjuvant and environmental conditions, among other factors (Broadbent and Olthof 1995; IJn et al. 2004; Schroer et al. 2005; Shapiro-Ilan et al. 2012; Bellini and Dolinski 2012; Rezaei et al. 2015; Noosidum et al. 2016). For example, Lacey and Chauvin (1999) reported no effect of Tween 80 on improving the activity of *S. carpocapsae* for the control of codling moth larvae, *Cydia pomonella* (Lepidoptera: Tortricidae). However, in our work, the addition of this adjuvant doubled EPN activity against *B. hilaris* adults. As this is an aboveground insect, testing and including different adjuvants such as antidesiccants, UV protectants, surfactants and/or humectants are mandatory tasks to improve the efficiency and persistence of EPNs in future experiments.

In our study, the best control was achieved with bifenthrin (100% mortality). Similarly, in a study by Palumbo et al. (2015), although none of seven different synthetic insecticides achieved 100% mortality at any time during 120 h post-application, bifenthrin was the most effective, reaching 90% mortality.

The high rate of mortality observed in our study could be attributed to the *B. hilaris* population used in our assays being collected from a campus of the University of Chile where no insecticides have been applied for their control. Therefore, it is unlikely that they have developed resistance to synthetic insecticides yet, as they have in other places (Guarino et al. 2008), but more research is required in the country to establish any level of resistance of *B. hilaris* populations to the most frequently used insecticides.

The Bagrada bug has become a very complicated insect to control in brassica and other crops worldwide. Currently, the only available tool is the use of synthetic insecticides, and alternative management tactics are not commercially available yet (Huang et al. 2014). As the problem regarding these bugs is relatively new in the occidental hemisphere, only some fungi (Torres Acosta et al. 2016; Barrera-López et al. 2019) and parasitoids (Martel et al. 2019) have been evaluated as possible BCAs, but these experiments have only been conducted in laboratory trials. One single application of the EPN isolate *S. feltiae* (CH4) on potted plants achieved a high control level. Likely, a management plan considering more than one application at the right moment will improve control further. We believe that the combined timely action of EPNs and other BCAs must be addressed in the near future to optimize the control of this serious pest.

Authors' contribution

GL, EA and ESB conceived and designed the research. AS and MH conducted experiments. ESB and GL analysed data. ESB, GL and EA wrote the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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