BioControl (2020) 65:613–621
https://doi.org/10.1007/s10526-020-10020-3

Field assay using a native entomopathogenic nematode for biological control of the weevil *Phyrdenus muriceus* in organic eggplant crops in Argentina

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Received: 20 November 2019 / Accepted: 4 May 2020 / Published online: 12 May 2020

Abstract The weevil *Phyrdenus muriceus* Germar (Coleoptera: Curculionidae) is an emergent pest of eggplant (*Solanum melongena* L.) crops in the humid Pampa of Argentina. Entomopathogenic nematodes (EPNs) constitute an effective and environmentally safe strategy within integrated pest management. The efficacy of a native EPN *Heterorhabditis bacteriophora* SUP isolate for the control of *P. muriceus* was evaluated. Larvae and adults were susceptible to the nematode in laboratory assays. Field evaluations indicated a slight reduction of *P. muriceus* populations, evidenced after a second application by a decrease of the foliar damage in the eggplant crops. Adults of *P. muriceus* parasitized by *H. bacteriophora* were observed in the field one month after the second application. The present study is the first assessment of EPNs against *P. muriceus* under field conditions, and the first report of their use in the humid Pampa, one of the major agricultural areas of Argentina.

Keywords Biocontrol · Curculionidae · Entomopathogenic nematodes · Heterorhabditidae

Handling Editor: Ralf Ehlers.

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Introduction

Phyrdenus muriceus Germar (Coleoptera: Curculionidae) is a pest that attacks almost all Solanaceae crops. This weevil has been recorded as a tomato (*Solanum lycopersicum* L.) pest in North America, Central America, Caribbean, and South America regions (Grousset et al. 2015). Also, it is an important potato (*Solanum tuberosum* L.) pest mainly in Brazil (Novo et al. 2002), Bolivia (Vreugdenhil et al. 2011), Colombia, Paraguay, Uruguay (Cisneros 1999) and in crops of cocona (*Solanum sessiliflorum* Dunal) from Perú (Novo et al. 2002).

In Argentina, *P. muriceus* has a special preference for eggplant, potato and tomato crops, in decreasing order, but also has the capacity to feed on wild Solanaceae, causing damage to both aerial and underground parts of the plant (Novo et al. 2002). During the last five years, it is considered to be an emerging pest that causes significant losses in the organic production of a main horticultural area of the central-eastern

region of Argentina (Eliceche 2019). In this region, the appearance of adults occurs at the beginning of spring, corresponding to pupae and adults who spend the winter in hibernating stages. The oviposition of the adults gives rise to the first generation of the year at the beginning of January. A late generation of adults is also observed at the end of March (Eliceche 2019). Females lay eggs in the soil-stem interface, and larvae create galleries in roots and young stems. Larvae and adults are responsible for the damage to the crops. Larvae feed on the roots affecting the absorption of water and nutrients causing progressive damage able to snap the stems of seedlings. Adults damage shoots and leaves leaving a characteristic "shot-hole" pattern on leaves by feeding (Kühne 2007).

The control of this pest is mainly performed through the use of chemical insecticides in excess, to control the larvae that are generally found inside the roots. However, in organic crops, where agrochemicals are not used, *P. muriceus* represents a problem.

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are considered good candidates for integrated pest management and sustainable agriculture. They are widely used in biological control programs due to a variety of attributes, such as: a wide range of hosts, the capacity of mass production, the viability of the application through the use of conventional equipment, the absence of harmful effects in non-target organisms and environmental compatibility (Griffin 2015). EPNs are obligate parasites and must locate suitable hosts to complete their life cycle. *Heterorhabditis* (Rhabditida: Heterorhabditidae) species are characterized as cruisers by active mobility, an ability to orientate to volatile long-range host cues and to find below-ground sedentary insects (Bal and Grewal 2015; Labaude and Griffin 2018). The life cycle of *P. muriceus* serves as an excellent window of opportunity for EPN penetration and infection.

Heterorhabditis bacteriophora Poinar SUP constitutes an indigenous nematode isolated from a horticultural zone of the central-eastern region of Argentina (Achinelly et al. 2017). Previous studies demonstrated mortality on other horticultural crop pests of the region (Eliceche 2019). In this context, the objective of the present work was to evaluate the efficacy of *H. bacteriophora* (SUP isolate) under laboratory and field trials for the control of *P. muriceus* on eggplant crops, within the framework of sustainable agricultural practices in Argentina.

Materials and methods

Nematode and insect colonies

The entomopathogenic nematode *Heterorhabditis bacteriophora* SUP was isolated from soil of tomato crops in the horticultural area of La Plata, Buenos Aires province, Argentina known as the La Plata green belt (34° 51′ 12″ S; 58° 04′ 45″ W). This agricultural zone, part of the humid pampean region, is characterized by a temperate climate with an annual average temperature of 15.9 °C and rainfall of *ca*. 1,100 mm. *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae were used to maintain EPNs (Shapiro-Ilan et al. 2012) and also as bait insects for persistence tests. Emerging infective juveniles (IJs) from infected insects were stored in cell-culture flasks at 16 °C during one week until they were used for bioassays (Stock and Goodrich-Blair 2012; Eliceche 2019). Bioassays were performed using IJs from the same offspring.

Adults of *P. muriceus* were manually collected from attacked eggplant (*Solanum melongena* L.) crops in organic fields of La Plata from different samplings. They were identified taxonomically by Dr. Del Río Guadalupe of the Division of Entomology in the Museum of La Plata, Argentina. Insects were maintained in the laboratory according to procedures described by Espul and Magistretti (1969). The adults were placed in a 50 ml plastic container, the base of which contained a disc of filter paper and a disc of absorbent cloth with a small strip that passed through a hole in the base to the outside for humidification. Silt loam soil obtained from the field previously sterilized in an oven for 20 min at 180 °C was placed inside the container over the absorbent cloth in addition to a tuber of potato as a source of food. The vessel was placed in a 100 mm diameter. Petri dish containing water that wetted the absorbent cloth for maintaining the moisture (10%, w/w) of the substrate within the container. The adults laid eggs on the tubers and hatched larvae fed and developed inside. Every ten days the adults were transferred to new containers.

Laboratory assays against larvae and adults

The efficacy of *H. bacteriophora* SUP against fourth-instar larvae and adults of *P. muriceus* was tested in the laboratory. Assays were performed in ten-multi-well plastic trays (5 × 5 × 2 cm) containing sterile silt loam soil up to a height of 2 cm moistened with sterile water (10%, w/w). An aqueous suspension of 0.5 ml containing the IJs was surface applied. Then, one insect was placed in each well and the container covered with a lid. For *P. muriceus* adults, three nematode concentrations: 5, 25 and 250 IJs cm^{-2} (corresponding to 100, 500 and 5000 IJs per well, respectively) and a control group with 0.5 ml of distilled water without nematodes were used. Due to limited material availability, two concentrations: 25 and 250 IJs cm^{-2} (500 and 5000 IJs per well, respectively) and a control group with 0.5 ml of distilled water without nematodes were used for *P. muriceus* larvae. Ten replicates were performed. The experiments were repeated three times using IJs from different batches.

The mortality by each stage and concentration was recorded daily during ten days post-exposure. Dead insects were put in a modified white trap (Kaya and Stock 1997) to corroborate the multiplication of the nematodes, according to Stock and Goodrich-Blair (2012). Cadavers were dissected when the emergence of IJs in white traps was not observed. For the adults the lethal concentration 50% (LC50) was calculated.

Field experiment

The trial was conducted in an organic eggplant crop (34° 48′ 17.6″ S, 58° 07′ 29.7″ W) north of La Plata, Buenos Aires, Argentina, at fifteen days of seedlings transplant to the soil. The crop was naturally infested by *P. muriceus*, which was evidenced by the insect damage produced during the feeding of adults on leaves and stems. Crops were arranged in three ridges with two rows of plants each, covered by plastic mulch and irrigated by channels with a total extension of 560 m^2. Plants were separated by 0.5 m of distance. Each ridge was divided into plots (1.5 m), with six 0.5-m-tall plants (64 treated plots and 64 control plots). A buffer zone of four plants was designated between experimental plots. The assignment of treatment and control plots (n = 64) was random. According to studies carried out with other entomopathogenic agents in the field (Campbell et al. 1984; Wraight and Ramos 2005), two consecutive applications were made, the first on November 10, 2016 (spring), and the second on December 29, 2016 (summer), coinciding with the emergence of the adults of *P. muriceus* at field. An aqueous suspension containing IJs of *H. bacteriophora* SUP was applied at a ratio of 100,000 m^{-2} by each treated plot, which was determined from preliminary assays (unpublished data).

The level of activity of the adults at each eggplant plant was estimated from the feeding damage to leaves according to Yan et al. (2013) and Gulcu et al. (2019). Adults leave a distinctive feeding pattern, causing circular shape "bites" on the leaf. The average number of holes per leaf due to the adults feeding in each plot was quantified. According to Mitidieri and Polack (2012), two leaves were removed from two plants selected randomly per each plot (treatment and control). A total of 512 leaves were examined per sampling, 256 for the total of treated plots and 256 for the total control plots. Because the evaluation was carried out in a field of family horticulture, roots were not monitored, since the removal of the attacked plants implied significant losses to the producer.

This procedure was performed once before the application from the 4 November 2016 and then every two–three weeks up to the 8 February of 2017. A total of six records were performed. If dead adults of *P. muriceus* were observed during the sampling they were transported to the laboratory and placed in modified white traps to determine parasitism by EPNs.

Persistence of IJs at field

Before and after application of IJs at field, the presence and activity of EPNs were examined by exposure of *T. molitor* larvae to the soil. This insect was used as host due to its availability and ease of breeding in the laboratory. One larva was individually caged in wire meshes (bait traps) and buried at random in eight treated plots and eight control plots for seven days (Yan et al. 2013). After this period cages were recovered and the dead larvae were transported to the laboratory to confirm successful EPN infection by IJs emergence in white traps or by dissection. The procedure was carried out monthly in a period from Oct 2016, before the first application, to Feb 2017 and then every two months up to Jun 2017. Simultaneously, silt loam soil samples (500 cm^3) moistened with

sterile tap water (10%, w/w) from the same plots were taken and exposed to *T. molitor* larvae in the laboratory to confirm inactivity of nematodes in negative plots. Ten larvae were individually exposed to soil samples in plastic containers with 100 cm^3 of soil at 25 °C. They were observed daily for a week and dead insects were dissected to evaluate EPN infectivity present in soil samples. The percentage of positive plots by EPNs in every time frame was calculated. If one larva from a sample soil, or bait trap, was confirmed to be infected successfully with nematodes, the plot was recorded as positive for the occurrence of EPNs (Susurluk and Ehlers 2008).

Statistical analysis

For laboratory experiments, one-way ANOVA was used to compare the percentage mortality of larvae and adults of *P. muriceus* followed by HSD Tukey's multiple means comparison procedure at P = 0.05. The mortality data were arcsine square root transformed before statistical analysis. A Probit analysis on the adults mortality data was carried out to assess the LC50. For this analysis, we used the Ecotoxicology Library V 1.0.1 (Gama 2015), implemented in R, and the Probit EPA function that simulates the EPA's proven EERD (Ecological Exposure Research Division).

In the field assay, significant differences between treatments for the foliar damage were determined using a t-test for independent samples. Before of analysis, a Shapiro–Wilk test (Shapiro and Wilk 1965) for normality (package stats version 3.4.0 in the R software) and a Levene's Test (Fox 2016) for homogeneity of variances (package car version 3.0-0 in the R software) were performed. Persistence data of EPNs in the field were subjected to proportion comparisons by Fisher's exact probability test. All data were analyzed by means of R software version 1.0.143 (R Core Team 2018).

Results

Nematode laboratory assays against *P. muriceus*

Heterorhabditis bacteriophora SUP killed the two stages (larvae and adults) of *P. muriceus* and was able to complete its life cycle which was evidenced for the emergence of IJs in the modified white traps. In larvae an infectivity of 0%, 74% and 84% was observed at a concentration of 0, 25 and 250 IJs cm^{-2}, respectively. The ANOVA test performed showed significant differences (F = 50.59; df = 2,6; P = 0.0001) between the concentrations evaluated. These differences were significant among the concentrations of 25 and 250 with respect to the controls (Tukey's test at $P < 0.05$) but non-significant between concentrations (Tukey's test at $P > 0.05$) (Fig. 1a). For adults, the infectivity was 0%, 17%, 20% and 50% at a concentration of 0, 5, 25 and 250 IJs cm^{-2}, respectively. The ANOVA test performed showed significant differences (F = 23.67; df = 3,8; P = 0.0002) among the concentrations evaluated. The concentration of 250 IJs cm^{-2} was significantly different from 0, 5 and 25 IJs cm^{-2} (Tukey's test at $P < 0.05$). However, the concentrations of 5 and 25 IJs cm^{-2} were not significantly different from the control (Tukey's test at $P > 0.05$) (Fig. 1b). Probit regression analysis for *P. muriceus* adults resulted in a regression coefficient (slope ± SE) of 0.588 ± 0.426, and a χ^2 value that was not significant ($\chi^2 = 0.575$, df = 1, P = 0.46), indicating a good fit of the regression line, with a LC50 value of 332 ± 0.31 IJs cm^{-2}.

Field release

Before the first release of IJs, the foliar damage compared by means of a t-test did not indicate significant differences among control and treated plots (t = 0.54, df = 62, P = 0.5931) (Fig. 2). After the first release, the foliar damage did not show significant differences between treatments and controls for the samplings of November 23, 2016 (t = -0.89, df = 62, P = 0.3782), December 14, 2016 (t = -0.48, df = 62, P = 0.6307) and December 29, 2016 (t = 1.05, df = 62, P = 0.2956). Nevertheless, subsequent to the second application, the foliar damage was significantly different between treated and control plots for the sampling of January 11, 2017 (t = 2.26, df = 62, P = 0.02705) and February 08, 2017 (t = 2.88, df = 62, P = 0.005438). An average of ten and 14 holes per leaf was observed in January for treated and not treated plots, respectively, with bigger damages during February, which showed an average of 24 and 40 holes per leaf for treated and not treated plots, respectively. *Phyrdenus muriceus* was not the only herbivore attacking eggplant crops during the assays,

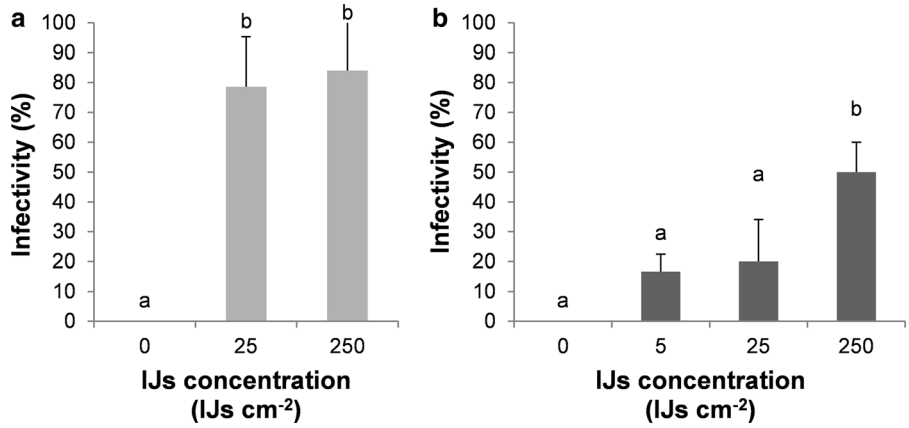

Fig. 1 Mortality percentage (means + SD) of larvae and adults of *Phyrdenus muriceus* by *Heterorhabditis bacteriophora* SUP under laboratory conditions. **a** Larvae mortality produced by three concentrations (0 IJs, 25 IJs and 250 IJs cm^{-2}); **b** Adults mortality produced by four concentrations (0 IJs, 5 IJs, 25 IJs and 250 IJs cm^{-2}). Bars with the same lowercase letter for each stage do not differ significantly according to Tukey's test at P = 0.05

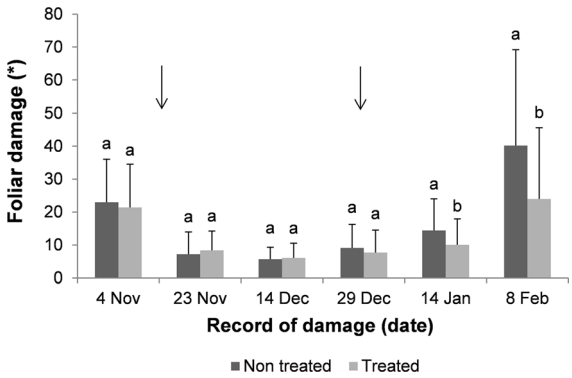

Fig. 2 Foliar damage (means + SD) produced by adults of *Phyrdenus muriceus* in eggplant plants treated and untreated (control) with *Heterorhabditis bacteriophora* SUP. Bars with the same lowercase letter for each date do not differ significantly according to t-test at P = 0.05. The arrows indicate the moments of application of IJs, 10 November 2016 and 29 December 2016. *Number of holes per leaf

but it was the most important in terms of damage to the crop. Additionally, dead adults of *P. muriceus* (n = 10) collected from treated plots in February were positive for infection by EPNs.

Persistence and infectivity of the *H. bacteriophora* SUP at field after releasing

Soil samples examined before releasing (Oct 2016) were negative for EPNs. This confirmed that the application site was free of EPNs that could potentially cause a misinterpretation of the final results.

Soil samples from treated plots were positive for nematodes from November to June (Fig. 3a), confirming the persistence and infectivity of the EPNs at the study site. The proportion of positive soil samples in treated plots showed no significant differences (Fisher's exact test) respect to control plots for records of December (P = 0.4783), January (P = 0.4667), April (P = 0.1818) and June (P = 0.1274). However, significant differences were detected in November (P = 0.01515) and February (P = 0.00905). In treated plots, the highest percentage of EPN-positive plots (83%) was observed in November, and then a reduction of 66% was recorded in December. After a second application, the percentage of positive soil samples increased in the successive samplings, reaching its maximum in February (67%), progressively reducing until June (35%) (Fig. 3a). The bait traps buried in the field in June were negative for parasitism. However, when soil samples were transported to the laboratory and exposed to *T. molitor* larvae, parasitism by *H. bacteriophora* was observed in 37.5% of soil samples, which was corroborated by the emergence of IJs from cadavers of *T. molitor* larvae from white traps. In the sampling of June a percentage of positive soil samples for EPNs (9%) was observed in control plots (Fig. 3b).

Discussion

Heterorhabditis bacteriophora SUP isolate possesses favorable attributes as an effective control agent of *P.*

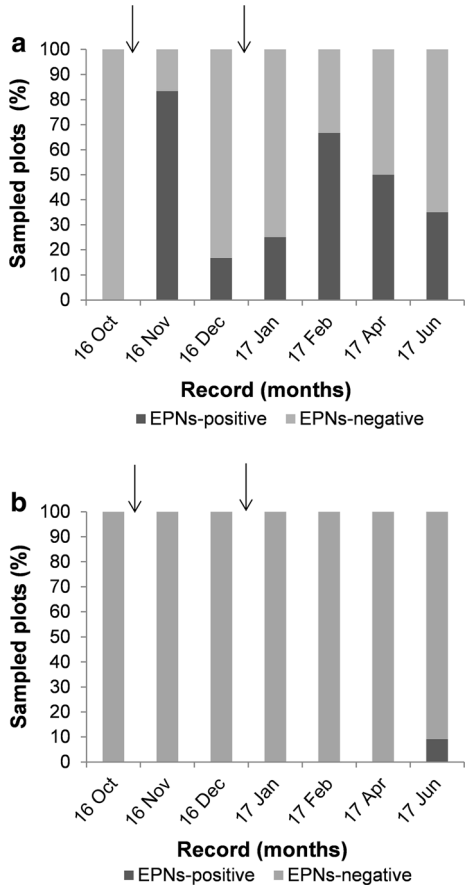

Fig. 3 Persistence of EPNs applied to the field. The percentage of positive and negative plots by EPN parasitism in *Tenebrio molitor* larvae in eggplant crops after two nematode applications (November and December 2016) is observed. **a** Treated plots with EPNs. **b** Untreated plots. The arrows indicate the moments of application of IJs (10 November 2016 and 29 December 2016)

muriceus. It was able to parasitize and kill larvae and adults of this weevil in the laboratory so as to complete its life cycle, which could contribute to its reproduction on the ground.

According to Campbell et al. (1984) and Wraight and Ramos (2005), consecutive applications are more effective in controlling insects characterized by overlapping generations. Moreover, they could counteract the effect of high mortality of IJs observed after the release of EPNs under natural conditions (Griffin 2015). In our study, the field evaluation indicated a slight reduction of the foliar damage caused by *P. muriceus* between treated and control plots after a second application. In addition, dead and infected adults of *P. muriceus* with *H. bacteriophora* from treated plots were collected. The increase in foliar perforations in February in both control and treated plants concurs with the emergence of adults of *P. muriceus* belonging to the first generation of the calendar year, as was reported by Espul and Magistretti (1969) and Eliceche (2019) in field observations.

Biotic and abiotic factors, such as predation, the presence of pathogens, ultraviolet light, temperature, and desiccation of the soil surface diminish the effectiveness of IJs in the field and its survival (Griffin 2015). In this way, soil moisture is essential for the movement and survival of the nematodes (Griffin, 2015). This factor may have been decisive in the efficiency of IJs in the field, due to the canal irrigation systems present in the study area. These channels are inefficient in the distribution of moisture in the soil (Demin 2014) which could explain the reduction in the percentage of positive plots for EPNs in December. Adults collected from the field parasitized by EPNs would allow us to infer that nematodes could be able to reproduce under field conditions in *P. muriceus* and/or that other hosts are present explaining the increment of positive soil samples after the second application.

IJs of *H. bacteriophora* SUP persisted seven months post-application. Parasitism in *T. molitor* larvae buried was only recorded up to five months, not showing activity posterior to this date in the field, but they were infective under laboratory conditions. The extreme changes of temperature can be lethal for infective juveniles in the soil, while less severe temperatures would simply inhibit mobility. Anyway, inactivation of IJs would result in both cases (Gaugler, 1988). The absence of parasitism observed in our study at the field in June would be attributable to the cooler temperatures (median temperature 10.6 °C for June-2017), corresponding to the winter months in this region. In this way, *H. bacteriophora* SUP isolate was not infective at a temperature of 11 °C under laboratory conditions, whereas 16 °C and 25 °C were the optimum temperatures for nematode infectivity. Moreover, the bacteria play an important role in the infectivity of EPNs being responsible for its pathogenesis. The absence of infectivity may also reflect the sensitivity of bacteria to these low temperatures (Griffin 1993). Similar results have been observed in other populations of nematodes inhabiting temperate climates (Grewal et al. 1994).

The presence of EPNs in control plots in June could be related to the dispersion of the nematodes from the treated plots to the control plots. This dispersion would be explained in part by the active movement of EPNs and/or through infected hosts, phoresis or irrigation systems (Koppenhöfer 2007). Even if data on the persistence of the nematodes were obtained, the present study did not provide information on the total number of IJs that survived in the field after the two applications, nor it allowed to determine if these IJs were able to reproduce in other hosts.

The concentration used for controlling *P. muriceus* was lower than recommended for applications in the field. Generally, to be effective, EPNs must be applied to soil at minimum rates of 250,000 IJs m^{-2} (Shapiro-Ilan et al. 2012). However, in determined conditions, a lower concentration may be effective (Shapiro-Ilan and Dolinski 2015). In this sense, a reduction of the foliar damage by these weevils was obtained with a concentration lower than recommended.

The susceptibility of adults to *H. bacteriophora* SUP was lower than in larvae. Similar results have also been reported for other strains of EPN in weevils of the Curculionidae family (Batalla-Carrera et al. 2016). *Heterorhabditis bacteriophora* SUP showed higher mortality in both larvae and adults, compared to *H. bacteriophora* (EG46) tested against *Curculio nucum* L. weevil that produced 8.3% mortality with a concentration of 50 IJs cm^{-2} (981 IJs per adult) and a mortality around 70% using a concentration of 50 IJs cm^{-2} (481 nematodes per larvae) (Batalla-Carrera et al. 2016). Meanwhile, its efficacy was lower than *H. bacteriophora* UWS1, which produced 100% mortality in larvae of *Otiorhynchus sulcatus* at concentration of 25 IJs cm^{-2} (315 IJs per larva) (Ansari and Butt 2011).

In conclusion, the results of laboratory and field bioassays indicate that *H. bacteriophora* SUP is a potential agent for the biological control of *P. muriceus*. A higher efficacy in the field was obtained after two applications, probably due to a major number of IJs present in the field as a result of a reproduction ability in *P. muriceus* and other insects. Because weevils are generalist herbivores that colonize crops during the first two weeks after eggplant seedling transplant, higher concentrations should be applied mainly at the first application to improve the reduction of the insect populations in the early crop stages, in which the damage generates significant economic losses. An integrated pest management (IPM) that combines the use of different biocontrol agents could improve the effectiveness, as reported by Shapiro-Ilan et al. (2004), who performed combined applications of the nematode *H. indica* and the fungus *Metarhizium anisopliae,* obtaining an additive effect in the control of *Curculio caryae*. Because *P. muriceus* was reported as natural host of *Beauveria bassiana* in Argentina (Nussenbaum and Lecuona 2012), the effect of its use combined with *H. bacteriophora* SUP should be considered as well as its interaction with other EPNs and fungus.

To our knowledge, the present work constitutes the first field evaluation of EPNs against the weevil *P. muriceus* and the first one in the humid Pampa region of Argentina representing a great contribution for the integrated pest management in this country. Further studies should be conducted to increase the effectiveness of nematodes in the field in order to reduce the level of economic damage. The present study is part of a project that seeks to encourage family farming to incorporate biocontrol strategies for pest management instead of synthetic insecticides.

Acknowledgements This study was partially supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Universidad Nacional de La Plata (UNLP). We are grateful to Dr. Graciela Minardi (CEPAVE), for statistical assistance. Brian Harkins native English speaker edited the final version of the manuscript.

Funding This study was funded by UE CONICET Research Projects 2017–2021.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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