Turkish Journal of Zoology

Volume 45 | Number 8 Article 2

1-1-2021

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TÜBİTAK

Turkish Journal of Zoology

http://journals.tubitak.gov.tr/zoology/

Research Article

Turk J Zool
(2021) 45: 347-355
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doi:10.3906/zoo-2106-38

The lure of hidden death: development of an attract-and-kill strategy against *Agriotes obscurus* (Coleoptera: Elateridae) combining semiochemicals and entomopathogenic nematodes

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Received: 29.06.2021 • **Accepted/Published Online:** 04.08.2021 • **Final Version:** 31.08.2021

Abstract: Wireworms are polyphagous soil-dwelling pests that are hard to control. Attract-and-kill strategies, combining attractive semiochemicals with biocontrol agents, have great promise to control insect pests. We hypothesized that the combination of plant semiochemicals and entomopathogenic nematodes (EPNs) in an attract-and-kill system could greatly enhance the nematodes' efficiency against wireworms. We evaluated the potential of alginate beads loaded with plant extracts and EPNs to control *Agriotes obscurus*. We tested the efficiency to kill wireworms or to reduce their feeding activity when combining potato tuber extracts as attractants with any of seven different EPN populations. While a direct application of EPNs on wireworms did not reduce the feeding activity nor increased their mortality, the combination of attractants and EPNs encapsulated in alginate beads resulted in attraction and consumption of the EPNs and caused up to 50% wireworm mortality with the EPN species *Steinernema carpocapsae*. Beads with EPNs caused a significant reduction of the wireworms' feeding activity. This study shows that wireworms feeding on EPN-containing beads have their feeding activity and survival negatively affected. Considering their long developmental time and the survival capability of EPNs in the soil, implementing this attract-and-kill system in the field might be a suitable strategy for the long-term management of wireworms.

Key words: Attract-and-kill, Coleoptera, entomopathogenic nematodes, encapsulation, feeding attractants

1. Introduction

Wireworms (Coleoptera: Elateridae) are common polyphagous soil-dwelling pests that are responsible for important economic losses (Jansson and Seal, 1994). They feed on roots and tubers of cereals, potatoes, sugarcane, and some ornamental plants (Parker and Howard, 2001). Even a low density of larvae can cause severe commercial damage (Furlan, 1989; Parker and Howard, 2001). Neonicotinoids coated on seeds were effective in controlling wireworms; however, they are now mostly banned from the European market. The few effective alternatives are costly or need to be used in multiple applications, with the corresponding cost for the farmer (Jactel et al., 2019) and environmental risks as well as the development of resistance. Several alternatives to pesticides have been proposed to control wireworms' damage, including agricultural practices, microorganisms and semiochemicals (Traugott et al., 2015; Barsics et al., 2017; la Forgia and Verheggen, 2019).

Entomopathogenic nematodes (EPNs) belonging to the genera *Steinernema* (Rhabditida: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) are widely distributed in the soil worldwide (Campos-Herrera, 2015). They are able to kill their hosts rapidly, making them valid biocontrol agents in integrated pest management programs and organic production (Shapiro-Ilan et al., 2012). EPNs are soil organisms able to parasite many insects (Lacey et al., 2015). Their infective juveniles (IJs) stage is the one responsible for the infection of the host (Koppenhöfer, 2007). The infective juveniles (IJs) penetrate hosts through natural openings (mouth, spiracles and anus) or through the cuticle (Campbell and Gaugler, 1991). Inside the host, IJs release their symbiotic bacteria (*Xenorhabdus* spp. for Steinernematidae and *Photorhabdus* spp. for Heterorhabditidae) that induce septicemia. The bacteria also support the nematodes in the suppression of microbial competitors (Hazir et al., 2003) and in scavenging competition (Blanco-Pérez et al., 2017; 2019). Normally, the insect host dies within 24–48 h (Burnell and Stock, 2000), making them excellent biological control agents that can be used against various

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arthropods in annual and perennial agroecosystems (Campos-Herrera, 2015).

Despite great success in various systems against insect pests, EPNs show low efficiency in controlling wireworms (la Forgia and Verheggen, 2019). Indeed, previous studies have investigated the biocontrol potential of EPN against wireworms, but, overall, the success was less than 75% (Lacey et al. 2015), and in many cases, registering moderate to low efficiency (Ansari et al., 2009; Campos-Herrera and Gutiérrez, 2009; la Forgia et al., 2020). This limited ability to control wireworms might be due to the fact that wireworms have evolved defense mechanisms including an efficient immune system to cope with a pathogen infection (Rahatkhan et al., 2005). Their very thick cuticle prevents tunneling through the exoskeleton. This creates a strong physical barrier against EPNs that lower the chances of invasion in the haemolymph. In addition, other physical opening like biforate spiracles, strong anal musculature and narrow, densely-haired pre-oral cavities, prevent the entrance of EPNs and the subsequent infection (Eidt and Thurson, 1995; Lewis et al., 2015). All combined, these factors explain the current limited effectiveness of EPNs against wireworms (Esther and Huiting, 2007).

To overcome these limitations, Schalk et al. (1993) suggested that EPNs could be associated with other control agents to compensate for their low efficiency in penetration in the host or overcoming the immune system under certain ecological scenarios. In fact, EPNs have difficulties to reach wireworms if they are not applied at the right depth. To overcome this limitation and improve EPNs' efficiency, their association with attractants like semiochemicals has been suggested (Ansari et al., 2009; la Forgia and Verheggen, 2019). Indeed, it has been suggested to use plant roots extracts and volatiles that affect the orientation of wireworms (Barsics et al., 2017; Gfeller et al., 2013; Johnson and Gregory, 2006) in attract-and-kill stragegies.

In a previous study, we compared the efficiency of two EPN species against wireworm (la Forgia et al., 2020). The recorded mortality attained 8.3% with *Steinernema carpocapsae* B14 and 16.7% *Heterorhabditis bacteriophora* 09.43 after 10 days exposure. Then, the ingestion of EPNs encapsulated with potato extract in alginate beads by *Agriotes sordidus* (Coleoptera: Elateridae) showed that the beads led to an increase in mortality of 16.7% and 41.7%, for the *S. carpocapsae* B14 and *H. bacteriophora* 09.43, respectively. In absence of the potato extract, mortality decreased only by 0% and 8.3%, respectively, demonstrating the importance of incorporating a feeding stimulant in such formulations.

The second limitation is that EPN formulations are mainly used on "classical spraying" of nematodes in the irrigation systems or similar inundative approaches (Shapiro-Ilan et al., 2015). New application approaches based on encapsulations of different nature might enhance the entrance and release of the EPN in the soil. Kaya and Nelsen (1985) were the first to encapsulate *Heterorhabditis bacteriophora* and *Steinernema feltiae* in calcium alginate beads. Progressively, several studies reported on the fight against the western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) with EPNs formulations (Hiltpold et al., 2012; Kim et al., 2014; Jaffuel et al., 2020; Kim et al., 2021). The treatments protected maize plants from *D. v. virgifera* damage, when applied at right time of the season.

To overcome the cited difficulties within a perspective to limit wireworm infestation, the aim of this case study was to investigate the possible attract-and-kill system by combining plant semiochemicals (as attraction cues) and EPNs (as the kill factor). We hypothesized that EPNs oral ingestion could increase wireworms' mortality, and this ingestion will be increased when specific attractants are added to the bead. In order to test this, we compared treatments with EPNs infection through direct application on wireworms and infection through larval feeding on alginates beads with encapsulated nematodes.

2. Materials and methods

2.1. Wireworms

Larvae of *Agriotes obscurus* (Coleoptera: Elateridae) were collected in maize and potato fields in France in 2019. Each larva was isolated in a single rearing box of 80 mL in order to avoid cannibalism. The rearing substrate consisted in a mixture of vermiculite and potting soil (1/1, moisturized at 16.5% w/v). Germinating meadow seeds (*Hordeum vulgare* and *Triticum aestivum*) were sown in each box (0.13–0.16 g, Prelac Bio, SCAR, Belgium) as a food source. The larvae selected for the behavioral assays were at least 10 mm in length (second instar), which corresponds to the most threatening instar to crops (Furlan, 2004). Four days before the bioassays, they were transferred to 80 mL vials filled with vermiculite and raw potting soil. Larvae exhibiting reduced activity, including larvae in the pre-molting phase (with lateral white stripes and scuffed mandibles) and post-molting phase (with a light-colored cuticle and soft and light-colored mouth-appendages) were discarded (Furlan, 2004).

2.2. Potato extract preparation

Based on previous assays, we selected potato tuber extracts as a source of attractant inside alginate (la Forgia and Verheggen, 2017). A full tuber was cut and covered with aluminum foil, then squeezed by hand. The potato juice was collected and used right away in order to limit oxidative and bacterial degradations.

2.3. Nematode populations

Seven commercial and non-commercial EPNs populations were tested: three *H. bacteriophora: H. bacteriophora* (e-nema, Schwentinental, Germany), *H. bacteriophora* AM203 (Algarve, Portugal) and *H. bacteriophora* RM102 (la Rioja, Spain); two *S. carpocapsae: S. carpocapsae* (e-nema, Schwentinental, Germany) and *S. carpocapsae* B14 (Barcelona, Spain); two *S. feltiae*: *S. feltiae* AM25 (Algarve, Portugal), *S. feltiae* RM107 (la Rioja, Spain). The EPNs populations were first reproduced in waxworm larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae). Briefly, three *G. mellonella* larvae were placed on a filter paper inside a 55 diam. Petri dish. A EPNs solution of 300 µL was applied on the larva. After four days, dead *G. mellonella* were transferred to individual white traps in order to collect the IJs progeny. The IJs were mixed from different cadavers. Ten days later, water was added in the white traps, and the first IJs were emerging within the next 24 h. The IJs were collected from the cadaver during a maximum time of four days in order to avoid the collection of the EPNs with a lower infectivity (Griffin, 2015). Emerging nematodes were pooled into one cell-culture flask and stored at 14 °C for further use. Each population was reproduced in *G. mellonella* before the new infection. Nematodes of < 14 days after emergence were employed in any of the experiments.

2.4. Feeding experiment

The EPNs populations were spread on each larva on a Petri dish of 55 mm diam. (VWR, Belgium) with a water suspension of 100 µL adjusted to 250 IJs (equivalent to 10.5 IJs/cm^2). Each larva was placed in a Petri dish with 15 g of sand and a slice of potato (var. Annabelle, 2 g weight, 4 cm length and 2 cm wide) and infected right after. Mortality was checked every week. All wireworm's cadavers were individually placed in white traps (White, 1927) to check for the presence of EPNs inside the insects. After twenty days, cadavers with no emerging IJs were dissected in order to verify their occurrence.

Twenty-four replicates per EPN population were carried out as well as 24 controls (larvae sprayed with water only). The larvae of *A. obscurus* were checked every week during eight weeks in order to register the number of holes or tunnel made on a potato, the molting and the potato slice consumption after EPNs infection.

2.5. Attract-and-kill experiment

The protocol of the alginate beads was adapted from Hiltpold et al. (2012). We previously determined that a maximum of 10% of the potato extract could be added without significantly changing bead shape and structure. Then, a choice test was carried out under darkness to simulate soil conditions. Petri dishes 55 mm diam. were prepared with autoclaved washed sand with 10% humidity (Ø 1–4 mm, Hubo, Belgium) and with a single starved wireworm inside (starvation of one week). The stock solution was made with 1 g of alginate per 100 mL. This alginate solution was dropped in a $CaCl_2$ solution (20 g of Calcium in 1 L of water). A syringe of 1 mL with a 4.5 mm diameter (Henke Sass Wolf, Tuberkulin, Germany) was used to take the alginate and drop beads of 100 µL. The EPNs, when needed, were inserted in the alginate, and the EPNs solution was dropped in the $CaCl_2$. The beads were left polymerizing for 20 min. We determined that a maximum of 10% of the potato extract could be added without significantly changing the bead formation. One bead per petri dish was placed individually on a marked spot in the Petri dish and covered with sand to maintain humid conditions as long as possible.

One single bead containing 1000 IJs was placed on a marked spot in the Petri dish and was covered with humid sand (16%) to maintain humidity. Based on the previous experiment (feeding activity), two EPNs populations were tested in this attract-and-kill experiment: the e-nema strain of *S. carpocapsae* and *S. feltiae* AM25. For each of the EPN populations, the experiment evaluated four treatments: (i) beads with EPNs and potato extract (n = 24), (ii) beads with EPNs only (n = 24), (iii) beads with extract only (n = 12), and (iv) control beads with water (n = 12). Larvae's feeding activity (larva that eat) and vitality (larva able to move and displace) were checked once a week per eight weeks. The feeding activity was estimated by the counting of the number of holes on the food source.

2.6. Statistical analyses

Statistical analyses were carried out using R (v. 0.98.1102). Wireworm's mortality was assessed by recording the dead (value = 1) or alive (value = 0) status of each wireworm in each Petri dish. Data were analyzed using a chi-squared test for binomial distribution. Feeding activity (percentage of eating wireworms and number of holes in the potato) as well as mortality and molting were analyzed using generalized linear mixed-effects models (R package lme4) with the week of measurement as repetition unit and with the population of nematodes as factor. In the case of the screening with beads, the addition of potato extract was also added as a factor. To the exception of the number of holes that followed a normal distribution, the models were based on a binomial distribution. When relevant, a Tukey post-hoc test was performed to identify differences between treatments. We confirmed that the data of the two independent trials per experiment and species could be pooled by two ways ANOVA (data not shown). Statistical differences were assessed for $p < 0.05$. We used least square means ± S. E. as descriptive statistics.

3. Results

After direct application of EPN on wireworms, the EPNs populations reduced the proportion of actively eating

wireworms (χ^2 = 24.89, p < 0.001) and increased their mortality (χ^2 = 57.76, p < 0.001 – Figure 1). The populations of *S. feltiae* AM25 and *S. carpocapsae* e-nema were the ones inducing the highest mortality (after 8 weeks) in wireworms, with 50% and 28%, respectively. A Tukey post-hoc test revealed that *S. feltiae* AM25 was responsible for a mortality that is significantly higher than with any other populations. Mortality with *S. carpocapsae* e-nema population was the second highest observed, though the difference was not significant with the other *S. carpocapsae* population. The EPNs population did not have any significant effect on the number of holes made in the food source and on the proportion of molting wireworms (χ^2 = 12.06, p = 0.10 and χ^2 = 0.02, p = 0.99, respectively).

3.1. Attract-and-kill experiment

The use of alginate beads with encapsulated nematodes affected the feeding behaviour of wireworms (χ^2 = 56.77, p < 0.001). Both EPNs populations *S. feltiae* AM25 and *S. carpocapsae* e-nema, significantly reduced the wireworms feeding activity (Tukey: z = -6.12; p < 0.001 and z = –5.74; p < 0.001, respectively) (Figure 2), with *S. carpocapsae* e-nema showing a stronger effect than *S. feltiae* AM25 (Tukey: z = –2.42; p = 0.04). The addition of potato extract increased the effects observed with *S. feltiae* AM25 population (Tukey: z = 3.91; p < 0.001), but did not impact those observed with *S. carpocapsae* e-nema.

The association of nematodes and potato extracts reduced the number of holes made by wireworms in the potato that was given as food source (S. carpocapsae e-nema: χ^2 = 73.62, p < 0.001; S. feltiae AM25: χ^2 = 5.03, p = 0.02, respectively – Figure 3). The two strains of nematodes significantly reduced the food consumption by wireworms compared to the control (Tukey: z = –8.32; p < 0.001 and z = –7.12; p < 0.001, respectively) but did not differ from each other (Tukey: z = –1.46; p = 0.31).

Exposing wireworms to alginate beads with nematodes but without attractants did not impact wireworms' mortality (χ2 = 0.96; p = 0.62 – Figure 4). The addition of potato extracts to the EPN-containing beads increased wireworms' mortality (χ2 = 6.50; p = 0.01 – Figure 4).

The molting of wireworms was neither affected by nematodes (χ^2 = 0.17, p = 0.92 – Fig. (5) nor by the addition of potato extract (χ^2 = 0.10, p = 0.75 – Figure 5).

4. Discussion

Wireworms have strong natural barriers that allow them to resist EPNs' infection. Here, we have demonstrated combining EPN-containing alginate beads with a plant extract can enhance the killing power of EPN against wireworms, most likely by facilitating the ingestion of EPN. Identifying specific plant-derived compounds that serve as feeding stimulants may further enhance the infection and killing potential of this formulation (la Forgia et al., 2020). The EPNs exit from the infected wireworms is also very hard. Usually, infected cadavers presented an important swelling in their ventral side, suggesting that the cuticle prevented them from exiting. Because of the particularly tough cuticle, we hypothesized that EPNs will have to find specific weak spots to enter wireworms. In this context, the size of EPNs may be an important factor to be taken into

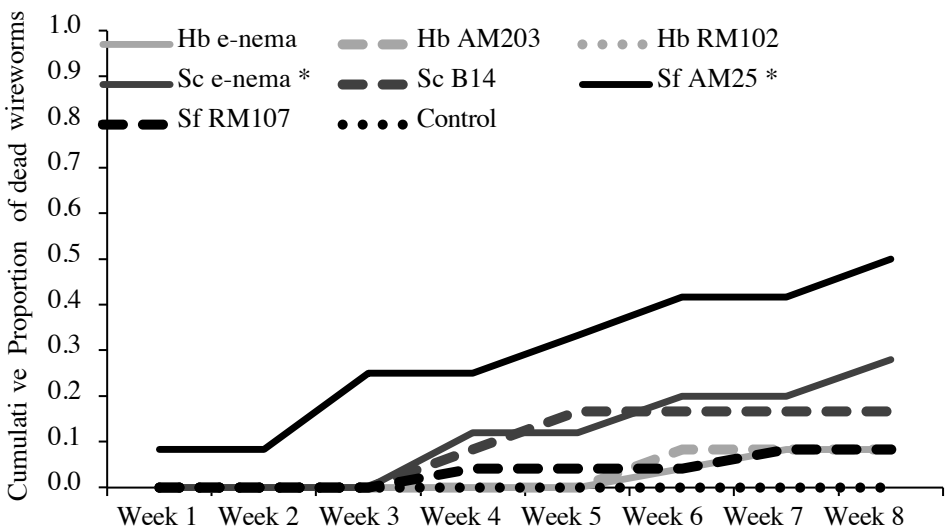

Figure 1. Proportion of dead wireworms over time after the application of different nematode strains (χ^2 = 57.76, p < 0.001). Hb stands for *Heterorhabditis bacteriophora,* Sc stands for *Steinernema carpocapsae,* and Sf stands for *Steinernema feltiae*. Control corresponds to absence of EPNs. The stars indicate that the concerned strains are responsible for a significantly higher mortality (Tukey HSD; p < 0.05).

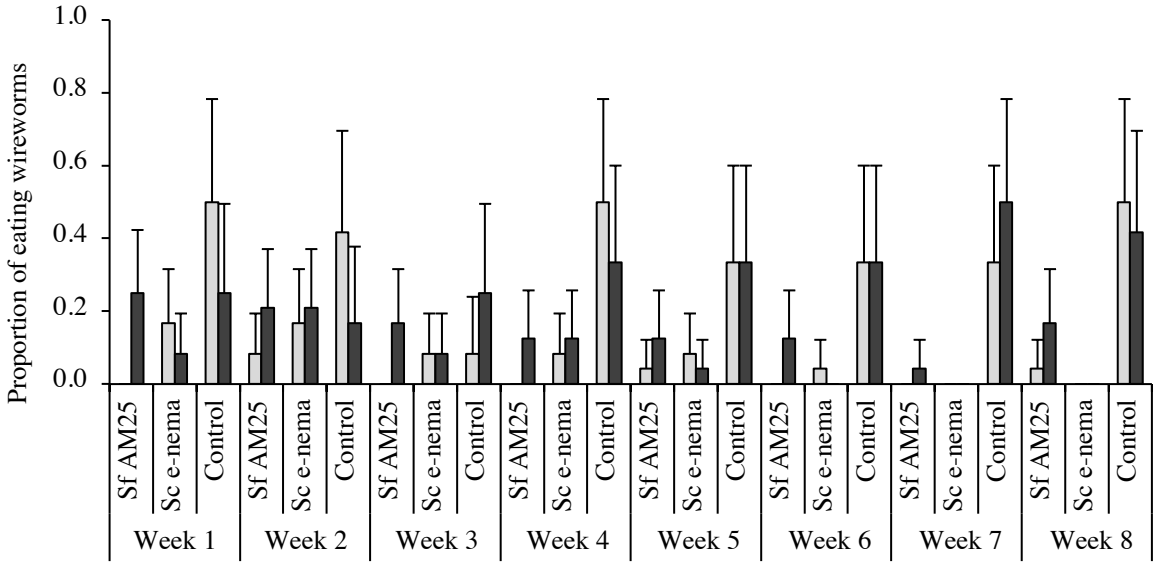

Figure 2. Proportion of eating wireworms over time after the application of two different nematode strains ($\chi^2 = 56.77$, $p < 0.001$). Light bars correspond to the treatments without potato extract, and the dark bars correspond to the treatments with addition of potato extract. Error bars represent 95% confidence intervals.

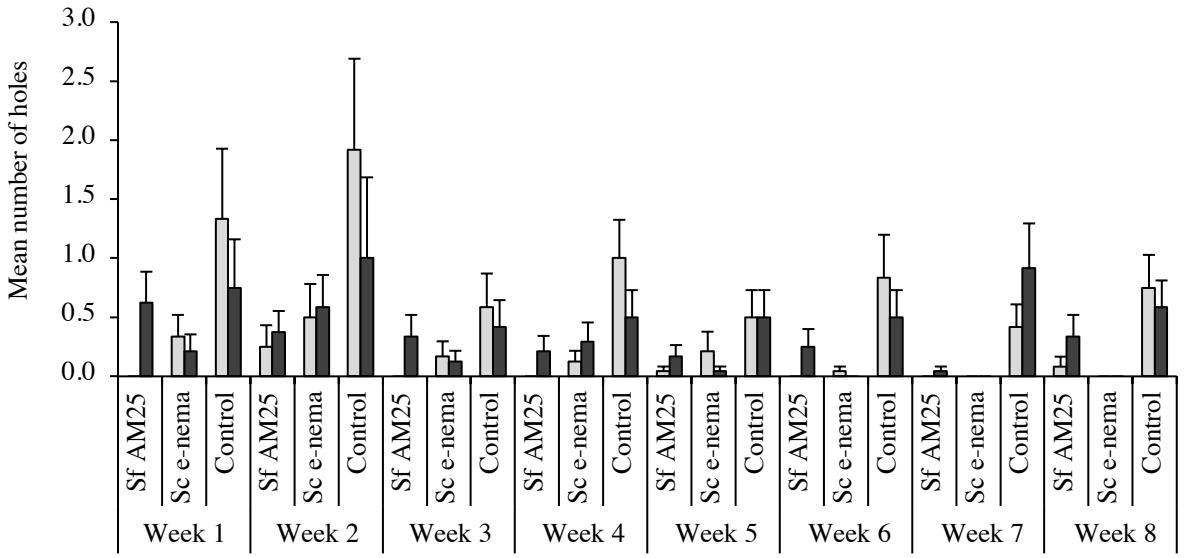

Figure 3. Mean number of holes in the potato food source after infection according to nematode populations across time ((*S. carpocapsae* e-nema: $\chi^2 = 73.62$, $p < 0.001$; *S. feltiae* AM25: $\chi^2 = 5.03$, $p = 0.02$, respectively). Light bars correspond to the treatments without potato extract, and the dark bars correspond to the treatments with addition of potato extract. Error bars represent the standard error of the mean.

consideration as well. Spiracular openings are sensitive entry points for IJs in many insect species (Forschler and Gardner, 1991), and they should be considered as a possible entry points into wireworms as well. The oral and anal openings are the most vulnerable (Mráček and Růžička, 1990), but the size of the mouth, the crushing action of mandibles, the sclerotized foregut and the peritrophic membrane may still prevent successful infections (Eidt and Thurston, 1995).

The populations *S. feltiae* AM25 and *S. carpocapsae* e-nema led to the highest mortality rate. A very recent study by Sandhi et al. (2020) compared ten different EPNs against the wireworm *Limonius californicus*, and showed that *S. carpocapsae* (All and Cxrd populations) and *S. riobrave* (355 and 7–12 populations) killed 60%–70% of *L. californicus* larvae in four weeks with a charge of 700 IJs (25 IJs/cm^2), 1400 IJs (50 IJs/cm^2), 2800 IJs (100 IJs/cm^2), and 5600 IJs (200 IJs/cm^2) in laboratory conditions. On the

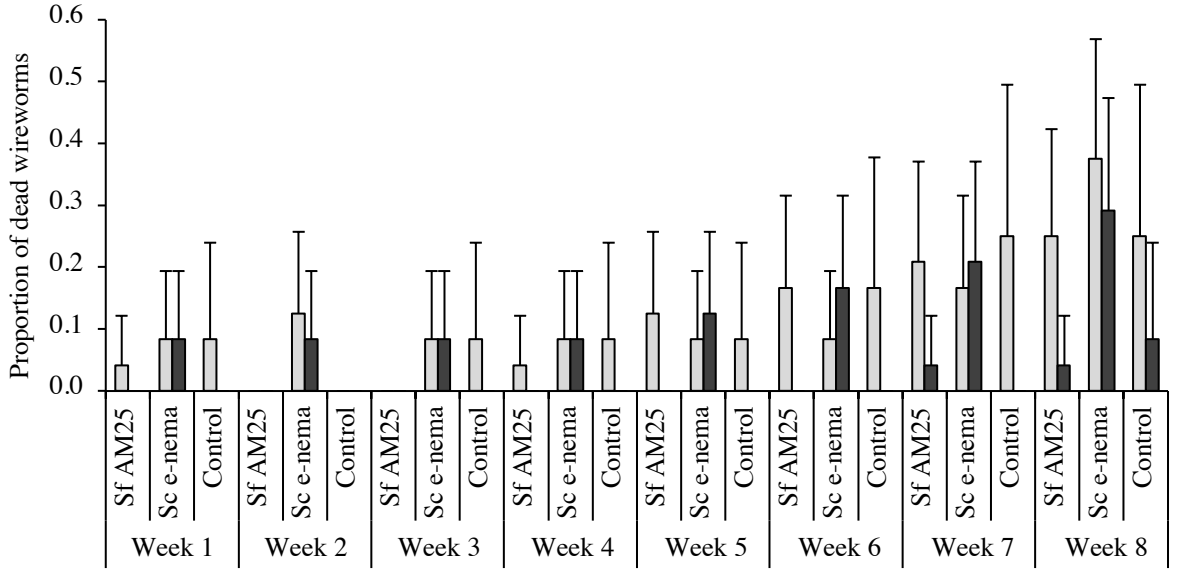

Figure 4. Proportion of dead wireworms according to nematode populations across time. Light bars correspond to the treatments without potato extract ($\chi 2 = 0.96$; $p = 0.62$), and the dark bars correspond to the treatments with addition of potato extract ($\chi 2 = 6.50$; $p = 0.01$). Error bars represent 95% confidence intervals.

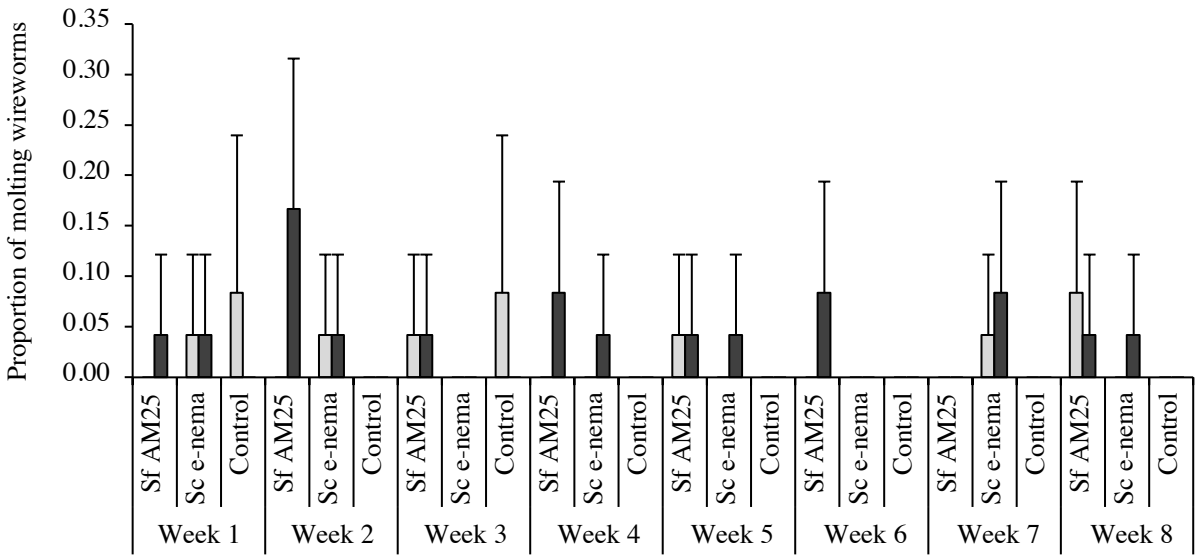

Figure 5. Proportion of molting wireworms according to nematode population across time ($\chi^2 = 0.17$, $p = 0.92$). Light bars correspond to the treatments without potato extract, and the dark bars correspond to the treatments with addition of potato extract. Error bars represent 95% confidence intervals.

contrary, Campos-Herrera and Gutiérrez (2009) found that a Spanish EPN isolate (*S. feltiae*) killed <10% *A. sordidus* in 12 days, confirming results of previous studies (Eidt and Thurston, 1995; Zhao et al., 1996). Relatively strong virulence has been found for *H. bacteriophora* (strain UWS1), causing a 67% mortality of *A. lineatus* larvae three weeks after inoculation (Ansari et al., 2009). Morton and Garcia-Del-Pino (2016) tested the susceptibility of *A. obscurus* to *S. carpocapsae* (B14) and found that initial EPN concentration is critical, observing 13.3% mortality at the rate of 50 IJs/cm^2, and increasing to 75.6% when the concentration of IJs were 100 IJs/cm^2. It is noteworthy that we had <50% mortality, but the concentration of 10 IJs/cm2 were 5 and 10 times lower than those tested by Morton and García del Pino (2016) with the same EPN population.

Encapsulating EPNs in alginate beads may increase their shelf life as well as their survival in the field by offering them shelter and protection against environmental stress such as drought, UV or pathogens (Kim et al., 2014; 2021). Longer living nematodes will result in a longer lasting effect on wireworm mortality and feeding activity.

The formulations with only EPNs did not impact the number of holes on the food source during the feeding damage experiment, suggesting a constant feeding even after EPNs infection. The difficulty of penetrating the wireworms through the physical barriers of the cuticula, combined with a strong immune system of the host, may have resulted in very low rates of successful infections by the directly applied nematodes and, consequently, allowed the wireworms to maintain a high feeding activity. It would be important to confirm this observation with future studies. In fact, a low infection rate may not affect the feeding capacity of the wireworm population. This may be different if the wireworms ingest the EPNs, and it may result in a decrease of the feeding performance, possibly limiting the damage to the crop.

None of the nematode populations by themselves significantly increased mortality, but the addition of potato extracts did. In addition to attracting the wireworms, it is possible that the extract also put the nematodes in a state of quiescence, an additional factor to take into consideration. Quiescence slows down EPN metabolism and increases their longevity with a minimum loss of infectivity (Grewal, 2000; Kim et al., 2021). Indeed, plant exudates can induce reversible quiescence in EPNs while favoring long-term storage of EPNs (Hiltpold et al., 2015). Hence, a potential quiescence effect of the potato extract could explain long term wireworm mortality, as it would make the nematodes less active. It would also explain the higher mortality of wireworms during the last week of the assays, confirming a longer EPNs survival when quiescent.

The cost-efficiency of EPNs within an attract-and-kill system was evaluated by Shapiro-Ilan et al. (2002). The factors that could affect the commercial success, for aerial application, will depend on the plant model and other biological and ecological factors but also abiotic factors like temperature, soil moisture and UV exposure. All these factors will affect the EPNs' ability to locate and infect hosts (Griffin, 2015). Most important is to select the most effective EPN strain against the target pest (high virulence) and the compatibility with the environmental conditions and application technology. The costs and benefits will also have to be compared to other control measures and needs to be seen in light of the farmers' needs to control the insect pest within the constraints of environmental policies. A positive aspect is the generally low cost of EPNs registration due to fewer safety requirements (Birch and Glare, 2020).

Further studies are needed to fully understand the role of potato extract in the observed long term in wireworm mortality. The longer lasting EPNs activity may also be explained by the properties of the bead system itself. For instance, tests on the use of capsules containing *H. bacteriophora* in the field against rootworms have shown that the IJs had a high survival rate in the capsules and that this formulation was more effective than water sprays (Hiltpold et al., 2012; Kim et al., 2021).

5. Conclusions and future directions

That EPNs can cause significant wireworm mortality was already known (Morton and Garcia-del-Pino, 2016), but ours appears to be the first study to show that EPNs can negatively affect wireworm feeding activity. Supplementing the formulation with an attractive plant extract enhanced the control potential of the EPNs considerably. Our results suggest that encapsulated EPNs combined with potato extract can mitigate the wireworms' negative impact on crops for at least two months. The attractiveness of the potato extract had already been demonstrated with olfactometer assays in which the choice for and feeding activity on the alginate beads were observed (la Forgia et al., 2020). In the future, the attraction to beads should also be compared using other attractants like maize roots extract, in order to optimize and validate the attract-and-kill system with the alginate beads. The improvement of the attractiveness could indeed increase the feeding and, consequently, the mortality. Further improvements may be achieved by combining EPNs with entomopathogenic fungi in the beads, which may further the main increase mortality, especially in field conditions.

Acknowledgments

The authors thank Ricardo Machado from the University of Bern (Switzerland), Selcuk Hazir from the Aydın Adnan Menderes University (Turkey) and Fernando García del Pino from the Universidad Autónoma de Barcelona (Spain) for providing the nematodes populations that were used in the experiments. The authors also thank to Mickaël Gaillard for the statistical help. The results presented in this study are part of the PhD thesis by the first author DLF, supported by a Ph.D. grant from Coordinated Integrated Pest Management in Europe (C-IPM), project ElatPro. RCH is awarded by Ramon y Cajal contract award (RYC-2016-19939) from the Government of Spain.

Author contributions

DLF concepted the idea and conducted the experiments. DLF, FV and TT wrote the manuscript. DLF and FV analysed the data. TT, PB and RCH helped in the nematodes rearing, infections and results interpretation. All authors read and approved the manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

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