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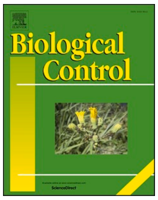

Laboratory and field trials reveal the potential of a gel formulation of entomopathogenic nematodes for the biological control of fall armyworm caterpillars (*Spodoptera frugiperda*)

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HIGHLIGHTS

- Entomopathogenic nematodes are highly lethal to fall armyworm caterpillars.
- Appropriate formulation of the nematodes is crucial for their above-ground application.
- A gel formulation of entomopathogenic nematodes proved as effective as chemical insecticides.
- Entomopathogenic nematodes can be used for the control of fall armyworm in maize.

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ABSTRACT

The fall armyworm (FAW), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) can cause tremendous yield losses in maize. Its invasion into Africa and Asia has dramatically increased the use of insecticides in maize agro-ecosystems. Safe, effective and readily available alternatives are urgently needed. Entomopathogenic nematodes (EPN) represent a promising and sustainable option to control fall armyworm caterpillars on maize. Commonly used against soil insect pests, EPN can also be applied to control above-ground pests if formulated appropriately. We explored the possibility to control FAW by incorporating the EPN species *Steinernema carpocapsae* into protective formulations that can be easily applied into the whorl of maize plants, where the caterpillars mostly feed. We tested this approach in laboratory cage experiments as well as in field trials. In the laboratory, treating maize plants with a low dose of *S. carpocapsae* (3000 infective juveniles per plant) formulated in a carboxymethyl cellulose (CMC) gel caused 100% mortality of FAW caterpillars and substantially reduced plant damage, whereas EPN applied in water or a surfactant-polymer-formulation (SPF) caused 72% and 94% mortality, respectively. Under field conditions, one-time treatments with *S. carpocapsae* applied in water, SPF or CMC decreased plant damage, but only the EPN-gel formulation significantly reduced FAW infestation. As compared to control, about 40% fewer caterpillars were found on plants treated with EPN formulated in the gel. Notably, the EPN-gel formulation was as effective as a standard dose of cypermethrin, a pyrethroid insecticide commonly used against FAW, in reducing FAW infestation. Repeated applications may be needed to reduce re-infestations by FAW across a whole cropping season depending on the local maize phenology and pest dynamics. These findings demonstrate that EPN, when properly formulated, are excellent candidates for the biological control of FAW, and can be a safe and sustainable alternative to synthetic insecticides.

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1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is native to the tropical and subtropical regions of the Americas (Luginbill, 1928). Since 2016, FAW has spread from the Americas to over 70 countries around the globe on three continents. It is predicted to expand its range even further (CABI, 2022; Cock et al., 2017; Day et al., 2017; Early et al., 2018; Goergen et al., 2016; Liu et al., 2020; Sharanabasappa et al., 2018). FAW is already a threat to global food security and has major socio-economic consequences for farmers and their families.

Although FAW is polyphagous and can feed on over 350 plant species (Montezano et al., 2018), it mainly causes severe damage to maize and can substantially reduce yields, causing tremendous economic losses (Baudron et al., 2019; Day et al., 2017; Hruska and Gould, 1997; Rwomushana et al., 2018; Wan et al., 2021). Due to its voraciousness as well as its exceptional migration capabilities, it currently threatens the food security of millions of people (Babendreier et al., 2020; Day et al., 2017; Rwomushana et al., 2018). To mitigate the impact of FAW, several control options are available including synthetic insecticides, biopesticides such as viruses (i.e. multiple nucleopolyhedrovirus) or the bacterium *Bacillus thuringiensis* (*Bt*), botanicals such as neem extracts, genetically modified crops that contain *Bt* toxins, but also mechanical control practices such as handpicking caterpillars, or cultural control such as push and pull cropping (Abrahams et al., 2017; Guo et al., 2020; Harrison et al., 2019; Wan et al., 2021). However, chemical insecticides have quickly become the backbone of FAW control in Africa and Asia, mainly due to unavailability of alternatives and due to governmental emergency programmes subsidising synthetic insecticides (Abrahams et al., 2017; Tambo et al., 2020). This situation has led to an enormous influx of insecticides in previously rarely treated maize-growing areas (Tambo et al., 2020; Yang et al., 2021). High frequency and broad-scale use of synthetic insecticides will have negative consequences for human health and the environment (Rani et al., 2021). Their application also substantially reduces populations of beneficial natural enemies and may lead to resistance in populations of FAW, obvious disadvantages compared to biological control agents. FAW resistance has already been reported to a variety of chemical insecticides (Wan et al., 2021), as well as to single *Bt* toxins (Blanco et al., 2016; Farias et al., 2014; Storer et al., 2010). Hence, there is an urgent need for readily available, safe, effective, and sustainable alternatives (Day et al., 2017).

Entomopathogenic nematodes (EPN) are tiny soil dwelling roundworms that can be found naturally in soils worldwide (Hominick, 2002). EPN can infest and kill a large variety of insects and are therefore commonly used as biological control in agriculture, mainly against soil pests (Campos-Herrera, 2015; Kaya and Gaugler, 1993; Koppenhöfer et al., 2020). Many EPN species or strains are also highly virulent to lepidopteran larvae, including FAW (Acharya et al., 2020; Andaló et al., 2010; Caccia et al., 2014; Fallet et al., 2022; Fuxa et al., 1988; Kaya and Gaugler, 1993; Koppenhöfer et al., 2020; Richter and Fuxa, 1990). Unlike many pesticides, EPN pose no risk to farmers or consumers, and hardly any risk to the environment (Ehlers and Hokkanen, 1996). They can be mass-produced (Ehlers, 2001) – also in Africa (Holmes et al., 2015) – and have the potential to be cost effective if formulated and applied correctly (Ehlers, 2001; Kagimu et al., 2017).

As soil-dwelling organisms, EPN are highly susceptible to desiccation, ultraviolet radiation and heat (Kagimu et al., 2017; Kaya and Gaugler, 1993; Lacey and Georgis, 2012), which limits their use against above-ground pests. To resolve these limitations, EPN have been, as many other biopesticides and chemicals, incorporated into formulations that protect them against these abiotic factors (Beck et al., 2013; Glazer et al., 1992; Glazer and Navon, 1990; Hiltpold et al., 2015; Navon et al., 1998; Schroer et al., 2005; Shapiro-Ilan et al., 2010; Shapiro-Ilan et al., 2012). Each formulation has its advantages and disadvantages, and each plant-pest system may need its own optimized solution.

We hypothesised that the maize-FAW pest system may be particularly well-suited for the application of EPN because FAW caterpillars mostly feed deep in the wrapped leaves of the whorl or on the cob under the husk leaves (Buntin, 1986; Labatte, 1993; Luginbill, 1928). Although well-suited for EPNs, such feeding behaviour makes the control of FAW caterpillars with conventional flat sprays of contact insecticides difficult (Pannuti et al., 2015). In contrast, EPN applied into the whorl of maize or directly onto the cobs will be able to actively forage for FAW caterpillars. Moreover, the leaves will protect the EPN from unfavourable abiotic factors, providing higher humidity, reduced temperature and less radiation exposure, as compared to an open surface. In order to ensure that EPN are well protected and to enhance their longevity and finally to assure good control efficacy, we aimed at incorporating EPN into formulations that are particular suitable for application onto maize plants. First, we formulated EPN in sand as well as in two types of alginate beads with unsatisfactory results. Then, we tested in a series of laboratory experiments a commercial surfactant-polymer-formulation (SPF) as well as a non-toxic carboxymethyl cellulose-based gel (Bampidis et al., 2020). Finally, the most promising formulations were evaluated in field trials. Our findings should significantly advance the development of a formulation that will offer practitioners a way to achieve safe, sustainable and effective control of FAW using EPN.

2. Material and methods

2.1. Origin and handling of nematodes

Steinernema carpocapsae (strain RW14-G-R3a-2) was isolated from soil samples in Rwanda in 2014 (Fallet et al., 2020; Machado et al., 2021; Yan et al., 2016) and was among the most effective strains in killing FAW caterpillars in screening tests involving 40 EPN strains, representing twelve species, originating from Rwanda, Mexico and commercial sources (Fallet et al., 2022). EPN were reared *in vivo* on larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) and stored in darkness at 12 °C (White, 1927). They were used within a week post emergence from *G. mellonella* cadavers.

2.2. Tested formulations

In preliminary laboratory assays (Fig. S1-S4), we compared the efficacy of eight EPN-formulations in killing FAW caterpillars on potted, three to four leaf stage, maize plants. These tested formulations were: (1) water, (2) sand, (3) alginate beads (as described in Kim et al., 2021), (4) a commercial bead (Nema-Caps®, Agrocaps SPRL, Gedienne, Belgium), (5) Navon's alginate gel (as described in Navon et al., 2002), (6) a gel we made from carboxymethyl cellulose (CMC) (Sigma-Aldrich, St. Louis, MO, USA), and two commercial liquid formulations, (7) an emulsifiable vegetable oil (Addit®, Koppert Biological Systems, Berkel en Rodenrijs, Netherlands) and (8) a surfactant-polymer-formulation (SPF) (Nemaperfect®, e-nema GmbH, Schwentinental, Germany). From these preliminary trials, we concluded that the most promising formulations were water (for its low cost and ease of use), the commercially available SPF (Nemaperfect®, for its ease of use and efficacy) and the CMC gel (for its high efficacy). These three formulations were further investigated in this study.

2.3. Efficacy of EPN formulations in reducing fall armyworm infestation and plant damage under laboratory conditions

2.3.1. Maize and fall armyworm

Maize plants (Hybrid CML203 × CML204, Rwanda Animal and Agricultural Resource Board, Huye, Rwanda) were grown in plastic pots (12 cm diameter × 8.5 cm height) using commercial potting soil (Classic, Einheitserdewerke Patzer, Sinntal-Altengronau, Germany) between March 6th and March 27th 2020. Plants were grown for four weeks in a greenhouse supplemented with artificial light (16:8h L:D, approx. 350 µmol * m^{-2} * sec^{-1}). They were watered twice a week with water supplemented with fertilizer as specified by the supplier (engrais liquid universel, Capito, Intercoop House & Garden Cooperative, Biel, Switzerland), and were used in experiments when they carried three to four fully developed true leaves (ca. 30–35 cm in height).

Spodoptera frugiperda caterpillars (FAW) were obtained from a colony at the University of Neuchâtel reared on artificial diet (Beet Armyworm Diet, Frontier Scientific, Newark, USA) under quarantine conditions (FOEN permit A140502).

2.3.2. Experimental procedure

The most effective Rwandan strain from our initial screening (Fallet et al., 2022), *S. carpocapsae* (strain RW14-G-R3a-2), was used to evaluate three different formulations against FAW in cage experiments under laboratory conditions. Two maize plants (Rwandan hybrid CML203 × CML204, Rwanda Animal and Agricultural Resource Board, Huye, Rwanda; 30–35 cm in height) were placed inside a net cage (60 cm in height × 40 cm in depth × 40 cm in width, Fig. 1) supplemented with artificial LED light (16:8h L:D, approx. 300 µmol * m^{-2} * sec^{-1}). Three third-instar FAW caterpillars (ca. 1 cm in length) were placed into the whorl of each plant. They were first allowed to feed on the plant for twenty-four hours, which was sufficient for the caterpillars to establish and cause foliar damage. We then applied 2 mL of a given formulation into the whorl of the two plants in a cage, using a 20 mL plastic syringe for the gel or a hand sprayer for SPF and water, respectively. EPN treatments consisted of ~ 3000 IJs applied in 2 mL (1500 IJs/mL) of either water, 0.2 % SPF or 3 % CMC gel. SPF and CMC were dissolved in 90 mL of water to final concentrations of 0.2 % (w/w) and 3 % (w/w) respectively by rapid stirring in a 200 mL beaker until completely dissolved. Then ~ 150′000 free living infective juveniles (less than one week old) in 10 mL of water were added. The same procedure was used to incorporate EPN in 90 mL of tap-water. Using a stereoscopic microscope, we confirmed that the formulations contained approximately 1500 IJs/mL. Formulations were kept in cool boxes until use, which occurred within 30 min. As controls, we treated plants with the same three formulations but without EPN. Every morning, 2 mL of water was vaporized above the whorl (ca. 15 cm distance) to mimic the effect of the dew. Six days post treatment, we evaluated plant damage using the Davis scale (Davis whorl & furl damage scale, Davis et al., 1992) as described in Toepfer et al. (2021), where a score of “0″ represents an intact plant while a score of “9” represents an almost completely destroyed plant. Plant damage was evaluated by one assessor only, who had no knowledge of the specific treatments being assessed. Subsequently we counted the number of surviving caterpillars. Five cages (ten plants) per treatment were used in each of three independent experiments (n = 15 cages; 30 plants per treatment in total).

2.3.3. Data analyses

Statistical analyses were performed using R version 4.1.2 (R Core Team, 2021). Given the complete separation of the data (100 % mortality in the gel + EPN treatment), we analysed caterpillar survival per cage using a General Independence Test (“coin” package; Hothorn et al., 2006). The survival of caterpillars in a cage (sum of the two plants) was used as the response variable, while treatment (each formulation with or without EPN) was used as an explanatory factor. Pairwise Two-Sample Permutation Tests (“rcompanion” package; Mangiafico, 2021) were used to compare treatments and were corrected for false discovery using the Benjamini & Hochberg method (1995). To determine differences between treatments, we calculated the efficacy of the EPN formulations as a percentage reduction of infestation as compared to their respective negative control (formulation without nematodes) (Eq. B.1).

The effect of treatments on plant damage was analysed using cumulative link mixed models (“ordinal” package; Christensen, 2019), followed by multiple comparisons (“emmeans” package; Lenth, 2021) corrected for false discovery using the Benjamini & Hochberg method (1995). The damage score given to a plant was used as the response variable, while treatment was used as a fixed factor and cage as a random factor (two plants per cage). To compare the proportion of plants with medium and high damage (Davis score higher than “3”) among treatments, we used a General Independence Test (“coin” package; Hothorn et al., 2006) and Pairwise Two-Sample Permutation Tests (“rcompanion” package; Mangiafico, 2021) corrected for false discovery using the Benjamini & Hochberg method (1995). The number of plants with medium and heavy damage per cage was used as the response variable, while treatment was used as an explanatory factor. To determine differences between treatments, we calculated the efficacy of the EPN formulations as a percentage reduction in the proportion of plants that suffered medium to heavy damage as compared to their respective control (formulation without nematodes) (Eq. B.2).

2.4. Efficacy of EPN formulations in reducing fall armyworm infestation and plant damage under field conditions

2.4.1. Field sites

We assessed the efficacy of formulations containing EPN in four maize fields in Southern Rwanda. Two fields (I and II) were located at the RAB Rubona Station in the district of Huye (GPS: S 02°28.827′, E 029°45.825′; altitude 1660 m.a.s.l.) and the two others (III and IV) in the district of Nyamagabe (GPS: S 02°28.539′, E 029°28.515′; altitude 2000 m.a.s.l.). During our experiment from mid-February to early-March 2020, the mean temperature recorded at the Rubona site was 21.3 ± 6.6 °C (mean ± sd; max = 38.5 °C; min = 13.3 °C) and was 20.3 ± 7.3 °C (mean ± sd; max = 38.7 °C; min = 7.3 °C) at the Nyamagabe site. At the Rubona site, the mean daily rainfall was 2.5 ± 6.2 mm

Fig. 1. Laboratory experimental setup. Two plants were placed in a net cage and subsequently infested by placing three third-instar caterpillars into the whorl. Twenty-four hours later, a given formulation was applied into the whorl. Five cages (ten plants) were used per treatment in each of three independent experiments.

(mean ± sd; max = 22.1 mm; min = 0 mm). Rainfall could not be measured at the Nyamagabe site.

2.4.2. *Maize and fall armyworm*

We planted maize (Rwandan hybrid CML203 × CML204, Rwanda Animal and Agricultural Resource Board, Huye, Rwanda) in the four fields measuring approximately 25 m by 25 between the 9th and 22nd of January 2020. Each field was fertilized with about 300 kg of manure. Maize plants were sown every 30 cm in rows separated by 70 cm, representing about 47′000 plants per hectare. Plants were grown for four to five weeks and were not treated with pesticides to ensure natural infestation by FAW. At the start of the experiment (before treatment), the maize plants in fields I, II, III and IV measured on average 28 ± 6.7, 23 ± 7.7, 13 ± 3.1 and 33 ± 9.9 cm in height (mean ± sd) and carried on average 7.8 ± 1.6, 7.7 ± 1, 5.9 ± 0.7 and 9.1 ± 1.1 leaves (mean ± sd), respectively. They were found to be infested by 1.3 ± 1.1, 1.5 ± 1.4, 0.15 ± 0.4, 1 ± 0.99 FAW caterpillars per plant (mean ± sd) in fields I, II, III and IV, respectively, not considering neonates.

2.4.3. *Experimental procedure*

We assessed the efficacy of formulations containing EPN in the four fields using a block design. To account for varying environmental conditions (i.e. surrounding habitats, exposition, etc…) as well as FAW infestation densities across the fields, each field was divided into four quadrants (Fig. 2). The quadrants were subdivided into six plots (3.3 m × 2.1 m) each with 48 plants. All plants within a plot were treated with one of six treatments (n = 4 plots per treatment per field; 16 plots per treatment in total). Plots were separated from each other by two untreated rows of plants that served as buffer (Fig. 2). Treatments consisted of applying 2 mL into the whorl of all plants. Treatments comprised: *S. carpocapsae* RW14-G-R3a-2 formulated in either (1) water, in (2) 0.2 % SPF, or in (3) 3 % CMC gel, (4) 5 % cypermethrin as positive control and (5) water without nematodes as negative control. In addition, we tested a second EPN species, *Heterorhabditis ruandica* Rw18_M−Hr1a, applied in water (6). *H. ruandica* was isolated from soil samples in Rwanda in 2018 (Fallet et al., 2020) and was slightly less effective than *S. carpocapsae* RW14-G-R3a-2 against FAW under laboratory conditions (Fallet et al., 2022). Despite this lower efficacy, we included *H. ruandica* because EPN may perform differently under field conditions compared to laboratory conditions. *H. ruandica* was reared as described above for *S. carpocapsae*. The EPN formulations were prepared as described above and 2 mL (1500 IJs/mL) was injected into the whorl of a plant with a 20 mL plastic syringe for the gel or a hand sprayer for SPF and water, respectively. The pyrethroid insecticide cypermethrin (Supra EC 50 g a.i. / litre, thus ca 5 % a.i. in product, ETG inputs ltd, India) was dissolved in water to a solution of 1.875 µL/mL (0.19 µg active ingredient per plant in a 2 mL spot spray).

We evaluated treatment efficacy in ten plants from one of the inner rows of each plot at five and ten days post treatment, as indicated in Fig. 2. Plant damage was assessed using the Davis scale (Davis whorl & furl damage scale, Davis et al., 1992) as described in Toepfer et al. (2021). Plant damage was evaluated by one assessor only, who had no knowledge of the specific treatments being assessed. Then we searched for surviving caterpillars. The occurrence (presence or absence) of small caterpillars (<0.5 cm) in each plant was recorded as a proxy for re-infestation (after treatments) by FAW, while the number of older caterpillars (caterpillars longer than 0.5 cm) was used to determine remaining FAW infestation levels as a proxy for treatment efficacy.

2.4.4. *Data analyses*

Statistical analyses were performed using R 4.1.2 (R Core Team, 2021). The number of FAW caterpillars per plot (sum of caterpillars in the ten assessed plants, excluding neonates) were analysed using generalized linear mixed-effects models ("lme4" package; Bates et al., 2015) with a negative binomial error distribution, followed by multiple comparisons ("emmeans" package; Lenth, 2021) corrected for false discovery using the Benjamini & Hochberg method (1995). The number of FAW caterpillars per plot was the response variable. Treatment (EPN formulations and cypermethrin) and field number were used as fixed factors, while quadrant was used as a random factor to account for the variation in the spatial distribution of FAW across the fields. To determine differences between treatments, we calculated their efficacy as a

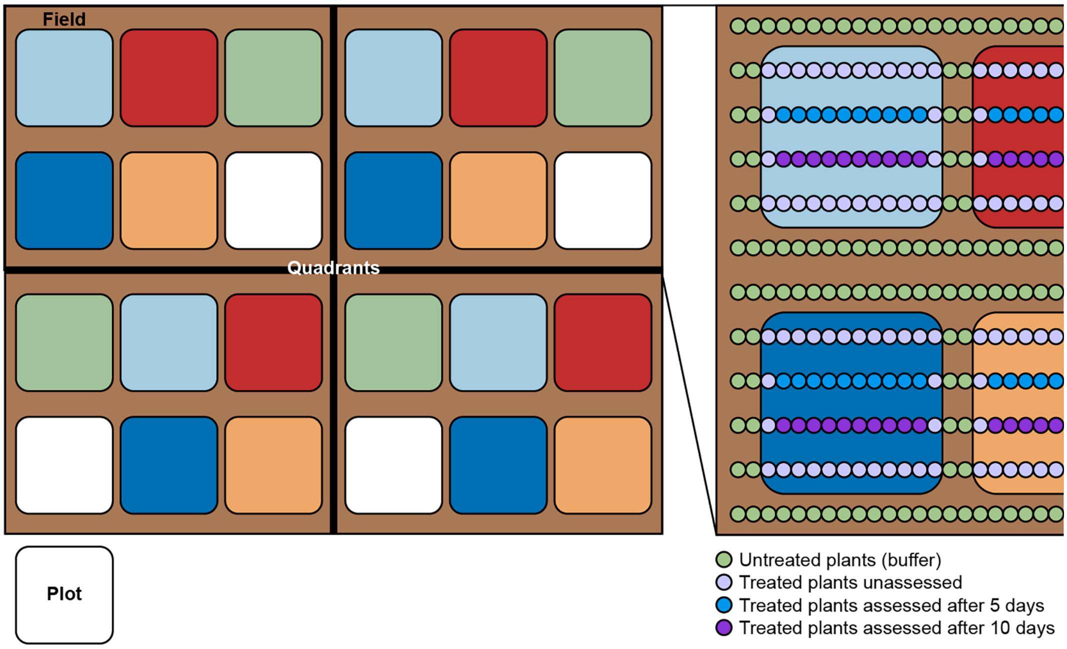

Fig. 2. Field experimental setup. Each field measured 25 m by 25 m. Maize was sown in 24 rows separated by 70 cm. Within rows, plants were separated by 30 cm. The fields were divided into four quadrants to account for the spatial variation of FAW across the fields. Quadrants were subdivided into six plots which were treated with a different treatment (coloured squares). Four plots were used per treatment in each field (squares of the same colour). The 48 plants in a plot were treated, but only ten plants inside one of the two inner rows of each plot were evaluated for damage and infestation by FAW at five (turquoise dots) and ten (purple dots) days post treatment. Two untreated rows of plants were kept between plots (green dots) as buffer.

percentage reduction in FAW infestation as compared to the control plots (water without nematodes) (Eq. B.3).

The damage score per plant was analysed using cumulative link mixed models ("ordinal" package; Christensen, 2019), followed by multiple comparisons ("emmeans" package; Lenth, 2021) corrected for false discovery using the Benjamini & Hochberg method (1995). The proportion of plants with a damage score higher than "3" as well as the re-infestation by neonates were analysed using generalized linear mixed-effects models ("lme4" package; Bates et al., 2015) with a binomial distribution, followed by multiple comparisons ("emmeans" package; Lenth, 2021) corrected for false discovery using the Benjamini & Hochberg method (1995). In these tests, the response variable was either the damage score attributed to each plant or the presence/absence of neonates on each plant. Treatment and field number were used as fixed factors, while quadrant and plot were used as random factors.

3. Results

3.1. Efficacy of EPN formulations in reducing fall armyworm infestation and plant damage under laboratory conditions

In a first step, we tested the effect of differently formulated EPN on fall armyworm (FAW) survival, by applying them into the whorl of maize plants under controlled laboratory conditions. Treating maize with formulated-EPN drastically reduced the number of surviving FAW caterpillars ($MaxT = 4.7, p < 0.001$; Fig. 3). The best result was obtained with 3000 IJs formulated in the CMC gel (0 % survival; Gel + EPN vs SPF + EPN: $p = 0.23$; Gel + EPN vs Water + EPN: $p = 0.002$; Gel + EPN vs Gel: $p < 0.001$), closely followed by the commercial SPF formulation of EPN (4 ± 13 % survival [mean ± sd]; SPF + EPN vs Water + EPN: $p = 0.02$; SPF + EPN vs SPF: $p < 0.001$). EPN applied in just water was the least effective among the EPN formulations, but still reduced the number of surviving FAW individuals when compared to its control (21 ± 20 % survival [mean ± sd]; Water + EPN vs Water: $p < 0.001$). The formulations without EPN did not affect fall armyworm survival (Water vs SPF: $p = 0.7$; Water vs Gel: $p = 0.17$; Fig. 3). As compared to their respective control (formulations without EPN), the efficacy of EPN formulated in gel was 100 %, in SPF 94 ± 18 % and in water 72 ± 27 % (mean ± sd).

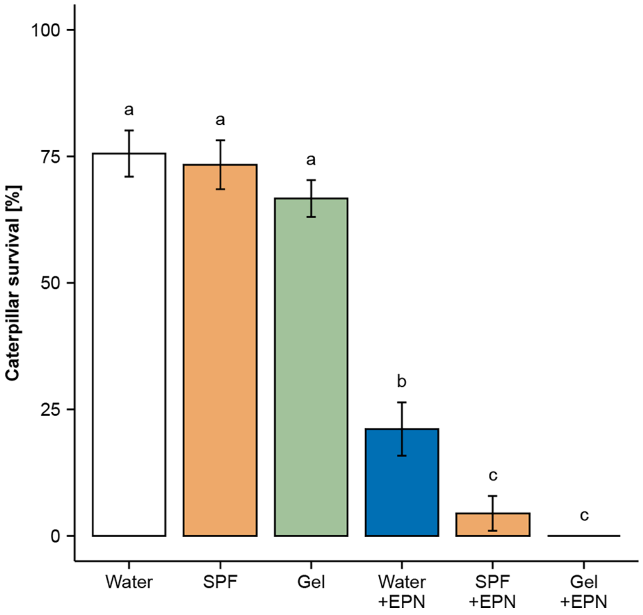

Fig. 3. Caterpillar survival (mean ± se) six days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 or formulations without nematodes into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 2 mL of formulation per plant. Five cages (each containing two plants) per treatment were used in each of three independent experiments (n = 15 cages; 30 plants per treatment). Letters above bars indicate significant differences ($p < 0.05$) between treatments according to a pairwise permutation test corrected for false discovery with the Benjamini & Hochberg method (1995).

We then evaluated to what extent the EPN formulations reduced plant damage caused by FAW by recording the overall damaged and determining the proportion of plants that suffered minor damage versus medium to heavy damage. EPN applied in water, SPF or gel all significantly reduced leaf damage caused by FAW ($\chi^2_{(5)} = 141, p < 0.001$; Fig. 4A). These treatments also reduced the proportion of plants with medium to heavy damage (a Davis score higher than three; MaxT = 5.4, $p < 0.001$; Fig. 4B). As compared to their respective control (formulations without EPN), EPN in SPF reduced medium and heavy damage (Davis scale > 3) by 100 %, in gel 93 % and in water 39 %.

3.2. Efficacy of EPN formulations in reducing fall armyworm infestation and plant damage under field conditions

We evaluated the effect of differently formulated EPN as well as cypermethrin on FAW caterpillar survival under field conditions five and ten days post application. Overall, treatments affected FAW infestations at both time points (five days: $\chi^2_{(5)} = 17, p = 0.005$; ten days: $\chi^2_{(5)} = 14, p = 0.01$; Fig. 5). Five days post treatment, FAW infestation was significantly reduced only by *S. carpocapsae* applied in gel (gel + *Sc* vs control: $p = 0.028$; Fig. 5A), and not by any other treatment. Ten days post treatment, FAW infestation was significantly reduced by *S. carpocapsae* applied in gel, as well as by cypermethrin (gel + *Sc* vs control: $p = 0.042$; cypermethrin: $p = 0.037$; Fig. 5B). As compared to the water control, *S. carpocapsae* applied in gel reduced FAW infestation by 41 ± 15 % [mean ± sd] after five days and by 34 ± 40 % after ten days, whilst cypermethrin achieved 35 ± 29 % and 41 ± 42 % reduction, respectively.

We then evaluated the effect of the differently formulated EPN as well as cypermethrin on plant damage (Fig. 6). Overall, plant damage was significantly affected by the treatments, at both five and ten days post application (five days post treatment: $\chi^2_{(5)} = 17, p = 0.005$; ten days post treatment: $\chi^2_{(5)} = 17, p = 0.005$; Fig. 6A and B). Medium and heavy crop damage were also found to be significantly reduced at both sampling dates (five days post treatment: $\chi^2_{(5)} = 12, p = 0.04$; ten days post treatment: $\chi^2_{(5)} = 17, p = 0.004$; Fig. 6C and D).

More specifically, five days post treatment, all the differently formulated *S. carpocapsae* as well as cypermethrin reduced FAW-leaf damages to maize plants (water + *Sc* vs control: $p = 0.014$; SPF + *Sc* vs control: $p = 0.04$; gel + *Sc* vs control: $p = 0.001$; cypermethrin vs control: $p = 0.014$; Fig. 6A). In contrast, *H. ruandica* (water + *Hb* vs control: $p = 0.19$; Fig. 6A) had no effect on leaf damage. *S. carpocapsae* in gel was the only treatment that significantly reduced medium and heavy crop damage (Davis score higher than three: gel + *Sc* vs control: $p = 0.029$; Fig. 6C).

Ten days post treatment, cypermethrin and each of the different formulations with *S. carpocapsae* reduced leaf damage (water + *Sc* vs control $p = 0.015$; SPF + *Sc* vs control: $p = 0.015$; gel + *Sc* vs control: $p = 0.037$; cypermethrin vs control: $p = 0.043$; Fig. 6B), whereas *H. ruandica* had no detectable effect (water + *Hb* vs control: $p = 0.67$; Fig. 6B). Only *S. carpocapsae* applied in water as well as the cypermethrin application significantly reduced medium and heavily damaged plants observed after 10 days (Davis score higher than three: water + *Sc* vs control: $p = 0.017$; cypermethrin vs control: $p = 0.039$; Fig. 6D).

Rapid re-infestation of maize plants by FAW was observed, which was evident from the occurrence of new neonate larvae on the plants. None of the treatments prevented these re-infestations (five days post treatment: $\chi^2_{(5)} = 5.4, p = 0.37$; ten days post treatment: $\chi^2_{(5)} = 4.5, p = 0.48$; Fig. 7).

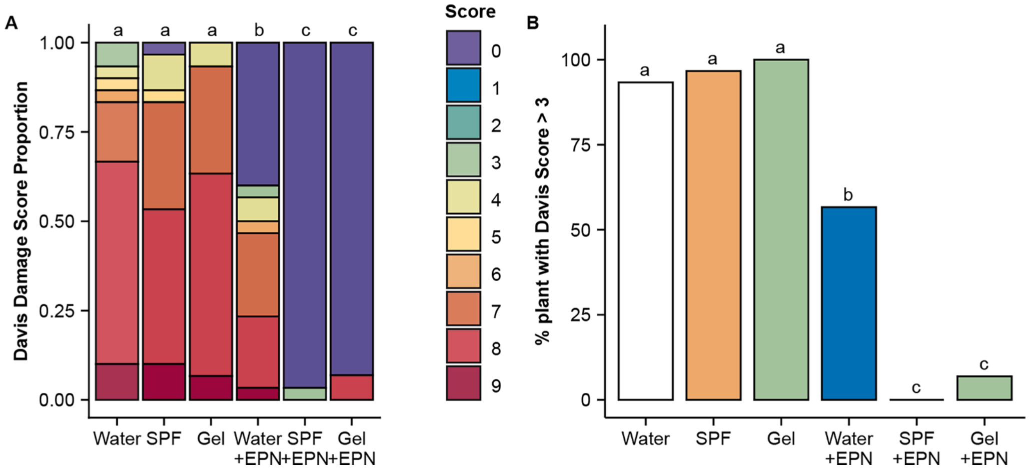

Fig. 4. Leaf damage on maize plants six days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 or formulations without nematodes into leaf whorls. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 2 mL of formulation per plant. Five cages (each containing two plants) per treatment were used in each of three independent experiments (n = 15 cages; 30 plants per treatment). Plant damage was assessed using the 0 to 9 Davis whorl & furl damage scale, where 0 represent absence of damage and 9 represents an almost completely destroyed plant. (A) Proportion of plants with a given Davis score within treatment. (B) Proportion of plants with medium to heavy damage. Letters above bars indicate significant differences ($p < 0.05$) between treatments according to (A) multiple comparisons or (B) a pairwise permutation test. Both tests were corrected for false discovery using the Benjamini & Hochberg method (1995).

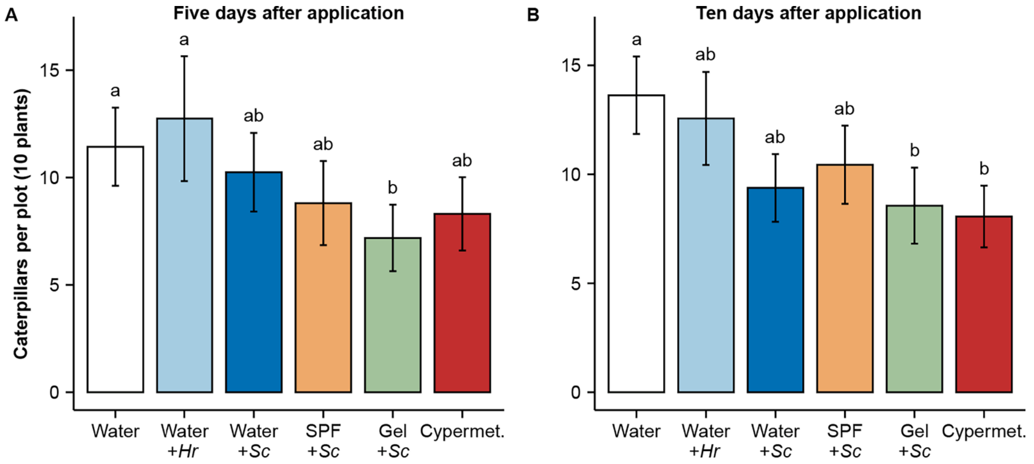

Fig. 5. Average number of fall armyworms per plot of ten plants five (A) and ten days (B) after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 (*Sc*) or *Heterorhabditis ruandica* Rw18_M−Hr1a (*Hr*) into leaf whorls of maize, as compared to application of water and a commonly used insecticide, cypermethrin (Cypermet.). Four experiments (=maize fields) with natural infestation of fall armyworms (4 plots per treatment per experiment; n = 16 plots per treatment) were carried out in the districts of Nyamagabe and Huye in southern Rwanda in 2020. About 3000 infective juvenile nematodes were applied in 2 mL of formulation per plant. Forty plants were assessed per treatment and per field at both five and ten days post treatment (160 plants per treatment and date). Letters above bars indicate significant differences ($p < 0.05$) according to multiple comparisons corrected for false discovery using the Benjamini & Hochberg method (1995).

4. Discussion

We show that the application of EPN has great potential for the effective biological control of FAW caterpillars in maize cultivation. This is highly encouraging as EPN offer a safe alternative to synthetic pesticides, as shown for belowground pests (Campos-Herrera et al., 2015; Ehlers, 2001; Ehlers and Hokkanen, 1996; Holmes et al., 2015; Kagimu et al., 2017).

As FAW larvae are above-ground pests, they rarely encounter soil-borne EPN and are therefore poorly adapted to resist them. This may explain why the caterpillars have been found so highly susceptible to many species and strains of EPN (Acharya et al., 2020; Fallet et al., 2022). EPN normally only reproduce inside an insect host, but they can also be mass produced in fermenters with cultures of their specific symbiotic bacteria or in semi-solid cultures. Hence, these tiny biocontrol agents EPN offer many advantages and it is shown here that the proper application of EPN also holds great promise as a strategy to fight FAW.

In standardised laboratory experimentation, we found that the whorl application of *S. carpocapsae* in a carboxymethyl cellulose gel, in a commercial surfactant-polymer-formulation, or in water killed respectively 100 %, 94 % or 72 % of FAW caterpillars on young maize plants (Fig. 3), thereby drastically reducing damage to the plants (Fig. 4). This required a dose of only 3000 IJs per plant which is far less than many other known usages of EPN in agriculture (Georgis, 1990; Toepfer et al., 2010; Toth et al., 2020). The application of the same formulations without EPN did not affect FAW mortality, proving that treatment effects were solely due to EPN.

The positive results from the laboratory also held true under field conditions. We found in our four fields in Rwanda, that EPN applications, if properly formulated, can effectively control FAW under typical maize field conditions, even under the high pest infestation levels (Figs. 5 and 6). Thirty years earlier, Richter and Fuxa (1990) had explored whether EPN can be used against FAW, but obtained inconsistent efficacy under field conditions. They found that the application of *Steinernema feltiae* in water into the whorl of maize seedlings only reduced FAW infestation in one of three experiments. Similarly, Garcia et al. (2008) did not observe a clear treatment effect when applying *Steinernema* sp. in water into the whorl of artificially infested maize. Using a similar approach, Negrisoli et al. (2010) achieved low efficacy with *Steinernema carpocapsae* and *Heterorhabditis indica*. We also did not

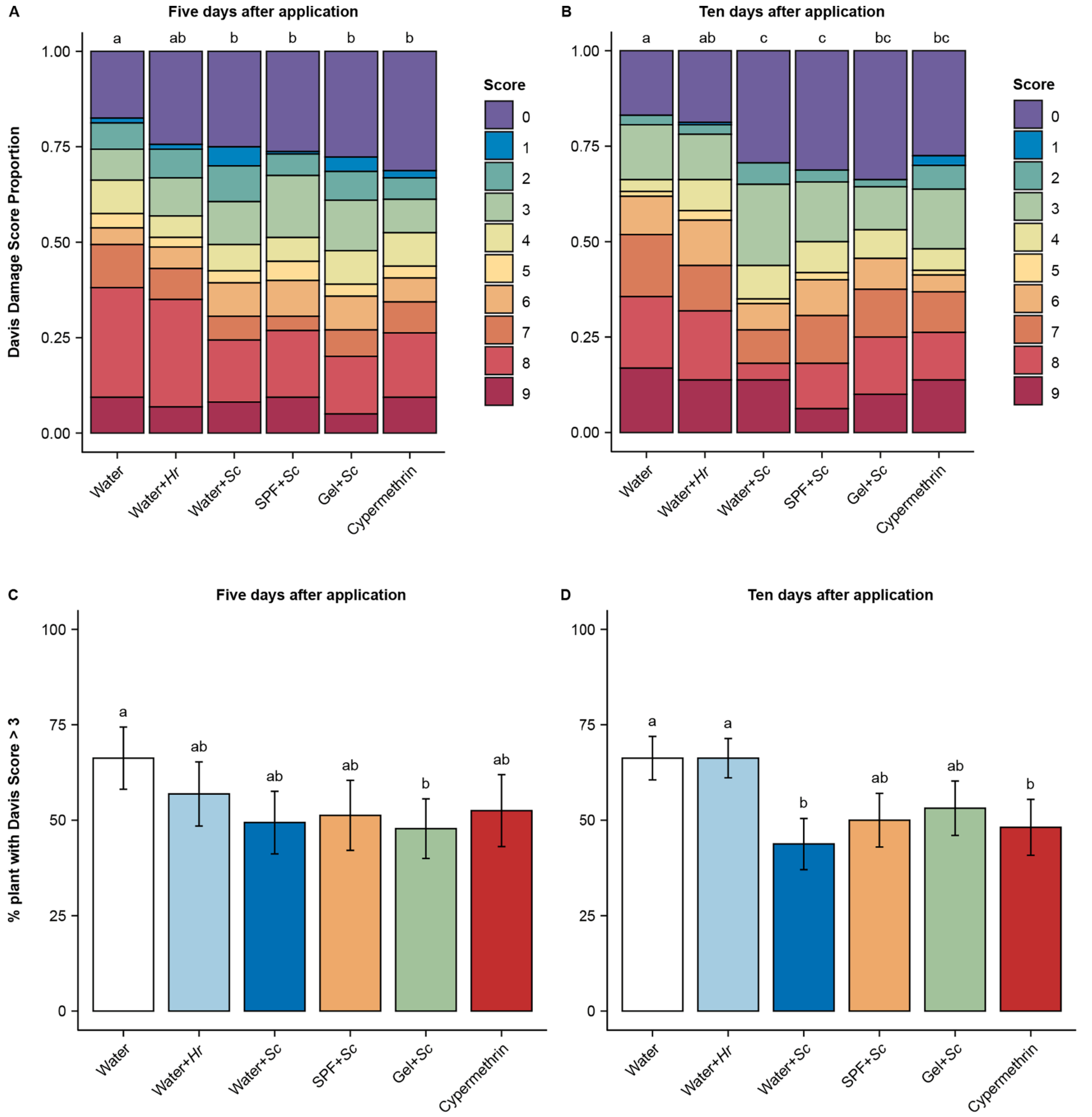

Fig. 6. Leaf damage at five (A and C) and ten (B and D) days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 (*Sc*) or *Heterorhabditis ruandica* Rw18_M−Hr1a (*Hr*) into leaf whorls, as compared to the application of water, as a control, and a commonly used insecticide cypermethrin. Four experiments (=maize fields) with natural infestation of fall armyworm caterpillars (4 plots per treatment per experiments, n = 16 plots per treatment) were carried out in the districts of Nyamagabe and Huye in southern Rwanda in 2020. About 3000 infective juvenile nematodes were applied in 2 mL of formulation per plant. Forty plants were assessed per treatment and per field at both five and ten days post treatment (n = 160 plants per treatment and date). (A and B) Proportion of plants within treatments with a given damage score according to the 0 to 9 Davis whorl & furl damage scale, where 0 represent absence of damage and 9 represents an almost completely destroyed plant. (C and D) Proportion of plants with medium to heavy damage. Letters above bars indicate significant differences ($p < 0.05$) between treatments according to multiple comparisons corrected for false discovery using the Benjamini & Hochberg method (1995).

achieve significant control of FAW in field conditions when treating plants with EPN formulated in water. However, in a recent study, Patil et al. (2022) showed that isolates of *H. indica* and *S. carpocapsae* from India can reduce FAW infestation and plant damage when sprayed with water onto maize plants. We show that a protective formulation of EPN may be needed to achieve consistent high control efficacies under field conditions.

Garcia et al. (2008), have shown that the addition of tensioactive agents to EPN hardly improves the efficacy of *Steinernema* sp. In our case, adding surfactant-polymer-formulation (SPF) to the EPN helped somewhat in the field (Figs. 5 and 6), but we achieved the best results with EPN in the cellulose gel (Figs. 5, 6 and S5). Despite an efficacy of 40 %, *S. carpocapsae* (strain RW14-G-R3a-2) applied in the gel was in fact just as effective in killing FAW and reducing leaf damage as the contact pesticide cypermethrin, which is commonly used against FAW throughout Africa and beyond (Uzayisenga et al., 2020). The specific properties of the gel formulation appear to contribute to the effectiveness of the EPN in controlling FAW. The EPN in water was the least effective treatment, which may be because it seeped out of the whorl during application, likely leading to the desiccation and death of the EPN. In contrast, the more viscous gel retained humidity, filled up the whorl and persisted on the plants for several days, which is largely sufficient for EPN to infest their host.

Not only the EPN-formulation but also the specific EPN species and strain were found to be important for the successful control of FAW under field conditions. The choice for *S. carpocapsae* (RW14-G-R3a-2) and *Heterorhabditis ruandica* (Rw18_M−Hr1a) for our study was based on extensive laboratory screening in which we compared the virulence of 40 EPN strains, representing twelve species, originating from Mexico, Rwanda, and a few from commercial sources (Fallet et al., 2022). Those

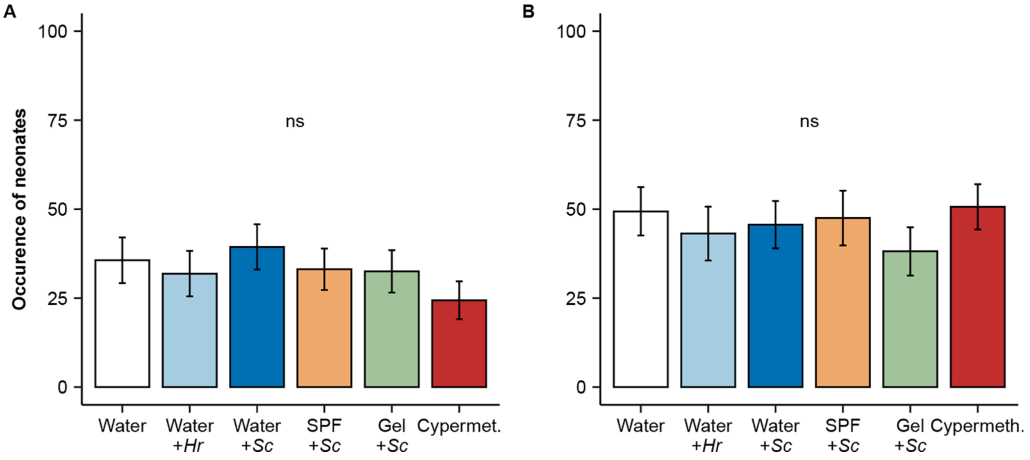

Fig. 7. Proportion of plant re-infested with fall armyworm neonates (A) five and (B) ten days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 (*Sc*) or *Heterorhabditis ruandica* Rw18_M−Hr1a (*Hr*) into leaf whorls, and in comparison to the application of just water and a commonly used insecticide cypermethrin. Four experiments (=maize fields) with natural infestation of fall armyworm caterpillars (4 plots per treatment per experiments, $n = 16$ plots per treatment) were carried out in the districts of Nyamagabe and Huye in southern Rwanda in 2020. About 3000 infective juvenile nematodes were applied in 2 mL of formulation per plant. Forty plants were assessed per treatment and per field at both five and ten days post treatment ($n = 160$ plants per treatment and date). No significant differences (ns; $p > 0.05$) were found between treatments using generalized linear mixed-effects models.

screenings showed that most EPN species and strains can kill FAW caterpillars, but certain strains, even within the same species, were found more infectious (Fallet et al., 2022). In addition, in the field, the harsh abiotic conditions will affect some species and strains more than others (Hiltpold, 2015; Shapiro-Ilan and Dolinski, 2015), and the differences in the killing efficiency of EPN strains will be more contrasting under field than under laboratory conditions. Although not significant, a trend suggests that *S. carpocapsae* (RW14-G-R3a-2) is more lethal to FAW in the field than *H. ruandica* (Rw18_M−Hr1a) when both species were applied with water (Figs. 5 and 6). This trend could be explained by the fact that *S. carpocapsae* is more tolerant than other EPN species to radiation (Gaugler et al., 1992; Shapiro-Ilan et al., 2015) and desiccation (Brown and Gaugler, 1997; Shapiro-Ilan et al., 2014). Further studies could evaluate the potential of different species and strains of EPN when applied in combination with protective formulations against FAW in field conditions.

EPN behaviour is yet another factor influencing their efficacy as biological control agents (Hiltpold et al., 2015; Shapiro-Ilan and Dolinski, 2015). Different species and strains of EPN are known to use varying foraging strategies along a continuum from ambushers to crusaders (Grewal et al., 1994). Some EPN, such as *S. carpocapsae* are ambushers and wait for the host to pass by (Lewis and Clarke, 2012), whereas *H. ruandica* appears to be a very motile EPN actively looking for its host. Considering the crusader strategy of *H. ruandica*, we could have expected that it actively orients towards FAW caterpillars in the maize whorl and be more effective than *S. carpocapsae*. This was not what we found. Possibly, *H. ruandica* is more exposed to lethal abiotic stresses when it actively searches for hosts and the use of an appropriate formulation such as the CMC gel could help to support a crusader type behaviour. Learning from our and other studies, it is evident that EPN candidates for biological control of the FAW, and other pests, need to be carefully selected based on traits that are crucial under field conditions.

The relative high number of FAW caterpillars that were still recovered in the EPN treated plots in our field experiments may have been the result of migration from the untreated buffer plants. Indeed, we observed large numbers of older larvae crawling among plots. This migrating behaviour is common for FAW, especially when plants are heavily infested (Pannuti et al., 2016), which was the case here. With this in mind, our results suggest that just one application of a low dose of *S. carpocapsae* applied in gel can already significantly reduce FAW infestation and prevent heavy damage. However, season-long crop protection may not be possible with just one application, regardless of it being EPN or a synthetic insecticide. Further studies will need to confirm that multiple treatments can indeed fully control FAW. For this, different levels of FAW infestations in different agricultural settings, ranging from small scale African farming to more extensive and commercial farming, should be considered.

In further steps, we also aim to improve the formulation. A first approach would be the incorporation of feeding stimulants to encourage FAW caterpillars to move towards and feed on the EPN-containing substrate. Other additives could protect EPN from harmful abiotic factors such as UV radiation and desiccation. Additional improvements might be achieved by artificial selection of EPN strains for enhanced longevity under field conditions with specific formulations. Selective breeding has been shown to greatly enhance specific traits in EPN, such as tolerance to desiccation and heat and responsiveness to foraging cues (Anbesse et al., 2013; Hiltpold et al., 2010; Mukuka et al., 2010; Perry et al., 2012). Another approach would be to increase the dose of EPN to a level that provides better FAW control. Normally, 2–4 billion EPN are applied per hectare to ensure sufficient pest control (Georgis, 1990; Toepfer et al., 2010), which is in sharp contrast to the ~ 3000 EPN per plant tested here (representing ca. 0.2–0.3 billion EPN/ha). Our experiments in the laboratory imply that this dose can rapidly achieve 100 % mortality of FAW on a plant (Fig. 3). Dose response tests have confirmed the high infectivity of the strains that we used here (Fallet et al., 2022). It may therefore be more beneficial to increase the number of applications, and stick to the relative low dose of EPN per application to ensure a low production cost.

In conclusion, our study represents a promising step towards the development of a safe, sustainable and effective alternative to chemical insecticides. Controlling FAW through the use of formulated EPN seems particularly realistic in an African and Asian context, where low tech manual labour is predominantly used in pest management efforts, allowing manual spot applications in maize fields. Moreover, given the availability of EPN in local soils and the relatively low number of EPN needed to control FAW, we envision that smallholder farmers, provided with specific training, could produce their own locally isolated EPN to fight the FAW in a practical, economically viable and environmentally friendly way. The EPN formulations should also be adaptable to large-scale high-tech application across commercial maize fields using high wheel precision farming machinery. Regardless of the application technology, we believe our findings clearly underpin the feasibility of using EPN based biocontrol products against FAW.

CRediT authorship contribution statement

Patrick Fallet: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Supervision, Project administration. **Didace Bazagwira:** Investigation, Writing – review & editing. **Julie Morgane Guenat:** Investigation, Data curation, Writing – review & editing. **Carlos Bustos-Segura:** Methodology, Formal analysis, Writing – review & editing. **Patrick Karangwa:** Resources, Writing – review & editing, Project administration. **Ishimwe Primitive Mukundwa:** Investigation, Writing – review & editing. **Joelle Kajuga:** Investigation, Resources, Writing – review & editing, Project administration. **Thomas Degen:** Visualization, Writing – review & editing. **Stefan Toepfer:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing, Project administration, Funding acquisition. **Ted C.J. Turlings:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.105086.

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