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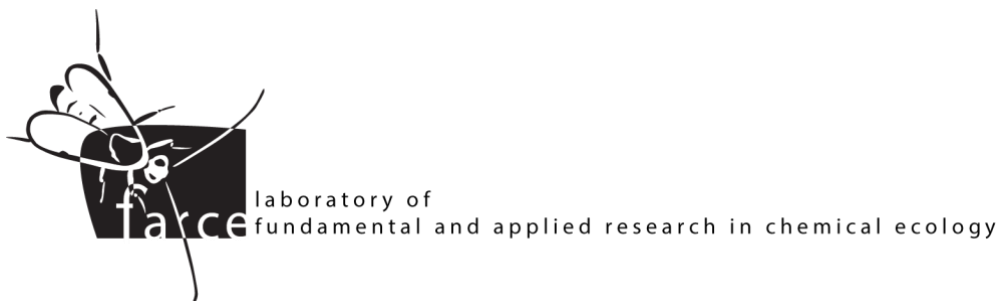
A novel strategy to control the fall armyworm with entomopathogenic nematodes

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Summary

The fall armyworm (*Spodoptera frugiperda*, Smith; Lepidoptera: Noctuidae) is an insect species native to the Americas known to be a major agricultural pest. It can feed on a large variety of plants, but is particularly devastating to maize. The recent invasion of the fall armyworm in Africa and Asia has wreaked havoc in maize cultivation, leading to a tremendous increase in the use of harmful synthetic insecticides, which are often only marginally effective. This limited efficacy is mainly due to the specific feeding behaviour of the fall armyworm caterpillars, which are mostly found deep within the wrapped leaves of the maize whorl, making them difficult targets for conventional contact insecticides. In addition, the fall armyworm has rapidly developed resistances to a wide range of synthetic insecticides as well as to some *Bacillus thuringiensis* (*Bt*) toxins commonly employed against insect pests in some regions of the world. Effective, safe and sustainable control alternatives are desperately needed to ensure food security as well as to preserve beneficial organisms, such as pollinators, and the environment.

Several biological control agents, such as entomopathogenic nematodes, can be quite effective in killing armyworms, but their application on plant leaves is challenging. Entomopathogenic nematodes are parasites that specifically target insects. They live in soils around the world and are commonly used as biological control agents for decades, notably because they have the ability to actively locate, infect and swiftly kill insects. As soil organisms, nematodes are particularly sensitive to the abiotic conditions found aboveground and on foliage, such as desiccation and UV radiation. The aim of this thesis is to address these challenges by exploring novel solutions to apply and protect entomopathogenic nematodes on maize leaves to ensure effective as well as sustainable control of the fall armyworm.

We showed in **Chapters 1** and **2** that locally isolated nematodes represent excellent candidates against the fall armyworm. They also offer the advantage of preventing the risk of introducing foreign species in a target country, associated with the use of often non-native commercially available nematodes. In **Chapter 3**, we explored different methods to formulate nematodes for aboveground application. We found a here-developed carboxymethyl cellulose gel formulation to be quite effective, killing 100% caterpillars in laboratory conditions and significantly reducing FAW infestations in preliminary field trials in Rwanda. In **Chapter 4**, we showed that throughout a full maize growing season in Rwanda, nematodes formulated in the gel consistently limited plant damage. In addition, the nematode-gel was significantly more effective than the commonly used insecticide cypermethrin in reducing armyworms infestation. This led to an increased grain yield as compared to untreated control plants. Lastly, we demonstrated in **Chapter 5** that the addition of affordable, readily available, natural substances to the gel formulation prolonged the survival and effectiveness of nematodes exposed to UV radiation. This could translate into an increased efficacy of

the nematode-gel formulation in realistic farming conditions as well as contribute to expanding the use of nematodes against aboveground pests.

This thesis demonstrates the potential of locally isolated entomopathogenic nematodes for fall armyworm control and represents a step towards the sustainable management of this devastating pest.

Key words: Biological control, Integrated Pest Management; sustainable crop protection, invasive species, maize, food security

Résumé

La chenille légionnaire d'automne (*Spodoptera frugiperda*, Smith ; Lepidoptera : Noctuidae) est une espèce d'insecte originaire des Amériques connue pour être un ravageur agricole majeur. Elle peut se nourrir d'une grande variété de plantes, mais elle est particulièrement dévastatrice pour le maïs. L'invasion récente de la chenille légionnaire d'automne en Afrique et en Asie provoque des ravages considérables dans la culture du maïs, entraînant une augmentation importante de l'utilisation d'insecticides synthétiques nocifs, dont l'efficacité est souvent marginale. Cette efficacité limitée est principalement due au comportement alimentaire spécifique de ces chenilles. Elles se trouvent principalement cachées en profondeur dans les feuilles enroulées du maïs, ce qui en fait des cibles difficiles pour les insecticides de contact conventionnels. En outre, la chenille légionnaire d'automne a rapidement développée des résistances à une large gamme d'insecticides synthétiques ainsi qu'à certaines toxines de *Bacillus thuringiensis* (*Bt*) couramment utilisées contre les insectes nuisibles dans certaines régions du monde. Des solutions de lutte efficaces, sûres et durables sont primordiales pour garantir la sécurité alimentaire et préserver les organismes bénéfiques, tels que les pollinisateurs, ainsi que l'environnement.

Plusieurs agents de contrôle biologique, tels que les nématodes entomopathogènes, peuvent être très efficaces contre des chenilles, mais leur application sur les feuilles des plantes est difficile. Les nématodes entomopathogènes sont des parasites qui ciblent spécifiquement les insectes. Ils peuplent les sols du monde entier et sont couramment utilisés comme agents de lutte biologique depuis des décennies, notamment parce qu'ils ont la capacité de localiser activement, d'infecter et de rapidement tuer des insectes. En tant qu'organismes du sol, les nématodes sont particulièrement sensibles aux conditions abiotiques aériennes, et notamment présentes sur le feuillage, telles que la dessiccation et le rayonnement UV. L'objectif de cette thèse est de relever ces défis en explorant de nouvelles solutions pour appliquer et protéger les nématodes entomopathogènes sur les feuilles de maïs, afin d'assurer un contrôle efficace et durable de la chenille légionnaire d'automne.

Nous démontrons dans les **chapitres 1 et 2** que des nématodes isolés localement représentent d'excellents candidats contre la chenille légionnaire d'automne. Ils offrent également l'avantage d'éviter le risque d'introduction d'espèces exotiques dans une région cible, associé à l'utilisation de nématodes commerciaux souvent non indigènes. Dans le **chapitre 3**, nous explorons différentes méthodes de formulation des nématodes pour une application foliaire. Une formulation développée lors ce travail à base de gel de carboxyméthylcellulose s'est avérée très efficace, tuant 100 % des chenilles en laboratoire et réduisant de manière significative l'infestation des plantes lors d'essais préliminaires sur le terrain au Rwanda. Dans le **chapitre 4**, nous évaluons, au long d'une saison complète de croissance du maïs au Rwanda, l'efficacité de nématodes formulés dans le gel. Les résultats démontrent que, formulés dans le gel, les nématodes ont

systematiquement limité les dommages aux plantes. En outre, ce traitement a été nettement plus efficace pour réduire l'infestation des plantes que la cyperméthrine, un insecticide couramment utilisé, et a conduit à une augmentation du rendement par rapport aux plantes contrôles non traitées. Enfin, nous avons démontré au **chapitre 5** que l'ajout de substances naturelles et facilement disponibles dans le gel prolongeait la survie et l'efficacité des nématodes exposés aux rayons UV, et ceci à un coût abordable. Cette avancée pourrait se traduire par une efficacité accrue des nématodes dans des conditions agricoles réelles et contribuer à étendre l'utilisation des nématodes contre les ravageurs foliaires.

Cette thèse démontre le potentiel des nématodes entomopathogènes natifs, isolés localement, pour lutter contre la chenille légionnaire d'automne et représente une étape vers la gestion durable de ce ravageur dévastateur.

Mots clés : Lutte biologique, lutte intégrée contre les ravageurs, protection durable des cultures, espèces envahissantes, maïs, sécurité alimentaire

Introduction and Outline

Maize: an important staple crop in Africa and Asia

Maize is one of the most widely grown crops in the world and a major staple food (FAO, 2023a). It accounts for almost half of the proteins and calories consumed in eastern and southern Africa and is a major feed for animals (Abrahams et al., 2017). In sub-Saharan Africa, 37 million hectares of maize are grown annually, while Asia accounts for 32% of the maize produced globally (Hruska, 2019). In both continents, maize is mostly grown by smallholder farmers (<2 ha), based on family labour (Hruska, 2019). Hence, maize plays a primary role in food-security and farmers' livelihoods (Shiferaw et al., 2011).

The fall armyworm: biology & ecology

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith; Lepidoptera: Noctuidae) is a lepidopteran native to the tropical and subtropical regions of the Americas (Luginbill, 1928), where it is considered one of the major pests in agriculture (Fig.1; Buntin, 1986; Kenis et al., 2022).



Figure 1: A fall armyworm moth (left) and a sixth-instar caterpillar (right). Sources: Left) Wikipedia.org; Right) Neil Villard.

Adults are nocturnal (Sparks, 1979). At night, females release pheromones to attract males and mate (Sparks, 1979). Mated females usually oviposit on the underside of leaves, but eggs can also be observed on non-organic supports, such as window panes or even airplanes (Porter and Hughes, 1950; Sparks, 1979). One female can lay up to 1000 eggs, usually in masses of approximately 150 eggs (Johnson, 1987; Vickery, 1929). Once hatched, caterpillars will feed gregariously, but will disperse gradually as they grow to become solitary as early as the second instar (Vickery, 1929). Pannuti et al., (2016a) observed that three days after hatching, less than 10% of neonate caterpillars remained on a plant. They usually disperse via crawling or ballooning, which consist of using silks to hang from a leaf to reach another (Sokame et al., 2020). Dispersing caterpillars are mostly found within one meter, but no more than two meters away from the hatching site (Pannuti et al., 2016b). Nonetheless, it is likely that individual caterpillars can disperse much further as they can be swept away by the wind while ballooning. Caterpillars feed both during the day and

at night, mostly in protected areas, such as deep into the wrapped leaves of the whorl, or onto the cobs under the husk leaves (Buntin, 1986; Labatte, 1993; Luginbill, 1928). This feeding behaviour makes FAW caterpillars difficult to control with conventional contact insecticides (Pannuti et al., 2016a).

FAW caterpillars are cannibalistic (Chapman et al., 2000; Luginbill, 1928; Vickery, 1929). Chapman et al., (2000) observed that cannibalism accounted for 40% of the mortality when two or more fourth-instar caterpillars were placed on an individual maize plant. The authors proposed that FAW cannibalistic behaviour might represent a strategy to increase individual survival. Indeed, by reducing densities of caterpillars through cannibalism, cannibalistic caterpillars benefit from reduced direct competition, lower predation and/or parasitism (due to reduced natural enemy attraction) and benefit from a more effective refuge (less damaged plant). However, it has also been demonstrated that cannibalistic caterpillars suffered lower survival, lower body weight, slower development rate and increased virus transmission than non-cannibalistic caterpillars (Chapman et al., 1999a, 1999b). Due to this cannibalistic behaviour, there is usually no more than one or two late-instar caterpillars per plant in the native range of FAW, but several overlapping generations and multiple late-instar caterpillars can be observed in the invasive range (Capinera, 2002; Kenis et al., 2022).

Usually caterpillars complete six instars, where the fifth and sixth-instar account for 16% and 77% of the food intake respectively (Luginbill, 1928). Pest control should then occur before the fifth-instar to effectively protect plants. Sixth-instar caterpillars migrate into the soil to pupate (Sparks, 1979).

At dusk, insects emerging from their pupa crawl to the soil surface and deploy their wings (Sparks, 1979). Moths can disperse over hundreds of kilometres, with a reported back-tracked flight of 1600 km in just 30 hours (Rose et al., 1975). By being carried away on weather fronts, FAW migrate seasonally from the southern USA or Mexico to Canada at a rate of 500 km per generation (Sparks, 1979; Johnson, 1987; Mitchell et al., 1991).

In warmer regions, the life cycle of FAW takes approximately 30 days, but can be as short as 19 days under laboratory conditions at 35°C (Barfield et al., 1978; Sparks, 1979). FAW has no diapause mechanism and cannot survive for long when temperatures are below 15°C (Barfield et al., 1978; Sparks, 1979). Therefore, FAW can only overwinter in warm regions.

FAW is polyphagous and can feed on more than 350 plant species belonging to 76 families (Montezano et al., 2018). In cultivated crops, it is mainly known to cause tremendous damage and economic losses in maize cultivation, but it is also an important threat to other crops such as rice, sorghum, soybean and cotton (Fig. 2; Kenis et al., 2022).



Figure 2: Damage caused by the fall armyworm in Rwanda. Source: Patrick Fallet.

With its good ability to migrate, a short generation time and a high reproductive rate, FAW has a high colonizing potential (Johnson, 1987) and is therefore listed as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO, 2023a).

The fall armyworm: a devastating pest of maize

The first report of FAW outside the Americas occurred in 2016, where the pest was detected in West and Central Africa (Fig. 3A; Goergen et al., 2016). It is likely that these first populations were carried by aircraft from the USA (Kenis et al., 2022). Within a short time, FAW presence was confirmed in most sub-Saharan countries of the continent (Cock et al., 2017; Day et al., 2017), and in Asia two years later (Sharanabasappa et al., 2018). It was discovered for the first time in Europe (Cyprus) in 2023 (EPPO, 2023b). To date, FAW has spread from the Americas to over 80 countries around the globe and will likely continue to expand its range (FAO, 2023b). Indeed, predictive distribution models stress the potential for further migration of FAW. For instance, the majority of European countries present environmental conditions favourable for its seasonal migration (Fig. 3B; du Plessis et al., 2018; Early et al., 2018; Liu et al., 2020; Paudel Timilsena et al., 2022; Wang et al., 2023).

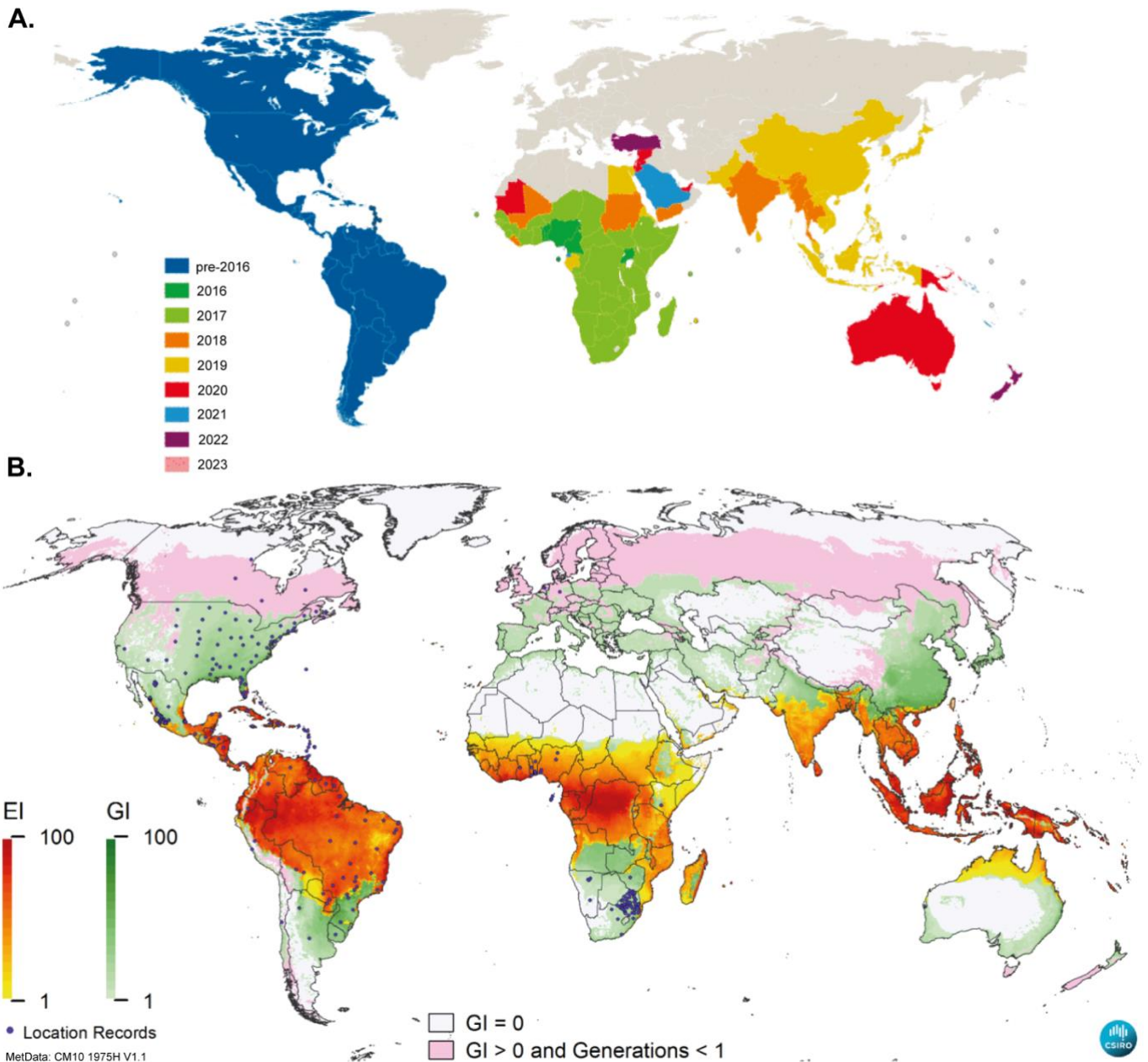


Figure 3: Current (A) and potential (B) distribution of the fall armyworm. Ecoclimatic Index (EI) describes the potential suitability for persistence; Growth Index (GI) describes suitability for population growth. Sources: A) FAO, 2023b: <https://www.fao.org/fall-armyworm/faw-monitoring/faw-map/en>; B) Du Plessis et al., 2018.

The arrival of FAW in Africa and Asia has caused severe damage in maize and threatens the livelihood of millions of farmers (Fig. 2; Day et al., 2017; Rwomushana et al., 2018; Baudron et al., 2019; Babendreier et al., 2020; Kenis et al., 2022). In ten African countries alone, annual yield losses are estimated at 16 thousand tons, representing USD 6 billion (Day et al., 2017), while households affected by FAW are more prone to suffer from hunger (Tambo et al., 2021). The invasion of FAW has led to a tremendous increase in synthetic insecticide use (Matova et al., 2020; Yang et al., 2021; Kenis et al., 2022). For instance, nearly USD 2 million worth of insecticide was distributed in Zambia via a subsidised governmental program to

face FAW in 2017 (Hruska, 2019), whereas maize cultures used to be only rarely treated. In China, FAW has led to a three-fold increase in insecticide use (Yang et al., 2021). Importantly, FAW rapidly developed resistance to many synthetic insecticides (Yu, 1991; Carvalho et al., 2013; Van den Berg and du Plessis, 2022), as well as to single *Bacillus thuringiensis* (*Bt*) proteins (Storer et al., 2010; Farias et al., 2014; Blanco et al., 2016), indicating that these control strategies may not be effective indefinitely. The increasing occurrence of resistance, together with the potential negative impacts of synthetic insecticides on the environment, populations of beneficial natural enemies and public health has prompted the call for urgently developing more sustainable and benign control practices (Day et al., 2017; Tambo et al., 2020). This is particularly important considering that most smallholder farmers often cannot afford and do not wear protective equipment (Tambo et al., 2020; Kenis et al., 2022).

Extensive research has been conducted to develop safe and sustainable alternatives to synthetic insecticide against FAW and was widely reviewed (e.g., Abrahams et al., 2017; Harrison et al., 2019; Wan et al., 2021; Kenis et al., 2022). Among these, entomopathogenic nematodes (EPN) are highly promising.

Entomopathogenic nematodes: promising alternatives

Entomopathogenic nematodes are pathogens of insects commonly used as biological control agents (Kaya and Gaugler, 1993). They are microscopic soil dwelling roundworms that belong to the families Steinernematidae and Heterorhabditidae (Fig. 4). They have a mutualistic association with specific bacteria, primarily from the genera *Xenorhabdus* and *Photorhabdus*, which are carried within their intestines. The lifecycle of EPN goes through distinct stages, starting with the infective juveniles (IJs), which are the infective stage and are free-living in the soil (Kaya and Gaugler, 1993). These IJs actively seek out suitable insect hosts, relying on chemical and physical cues (Hiltpold et al., 2010; Turlings et al., 2012; Zhang et al., 2021). For long-range orientation, the EPN may exploit volatile chemicals released by roots from plants that are damaged by insect herbivores (Rasman et al., 2005). At closer range, they rely more on cues emitted by the hosts themselves. Once in contact with an insect, IJs penetrate the body through natural openings or intersegmental membranes of the cuticle. Within the insect host, the EPN release the symbiotic bacteria into the host's hemocoel, which is the start of the parasitic phase (Fig. 4). The bacteria contribute to the nematodes' pathogenicity by producing toxins and degrading host tissues, facilitating the nematodes' survival and reproduction. During the parasitic phase, the nematodes reproduce, and their population rapidly increases within the host. After completing several reproductive cycles, the nematodes enter the infective stage once again, emerging from the host cadaver to seek new hosts and continue their lifecycle (Stock, 2015).

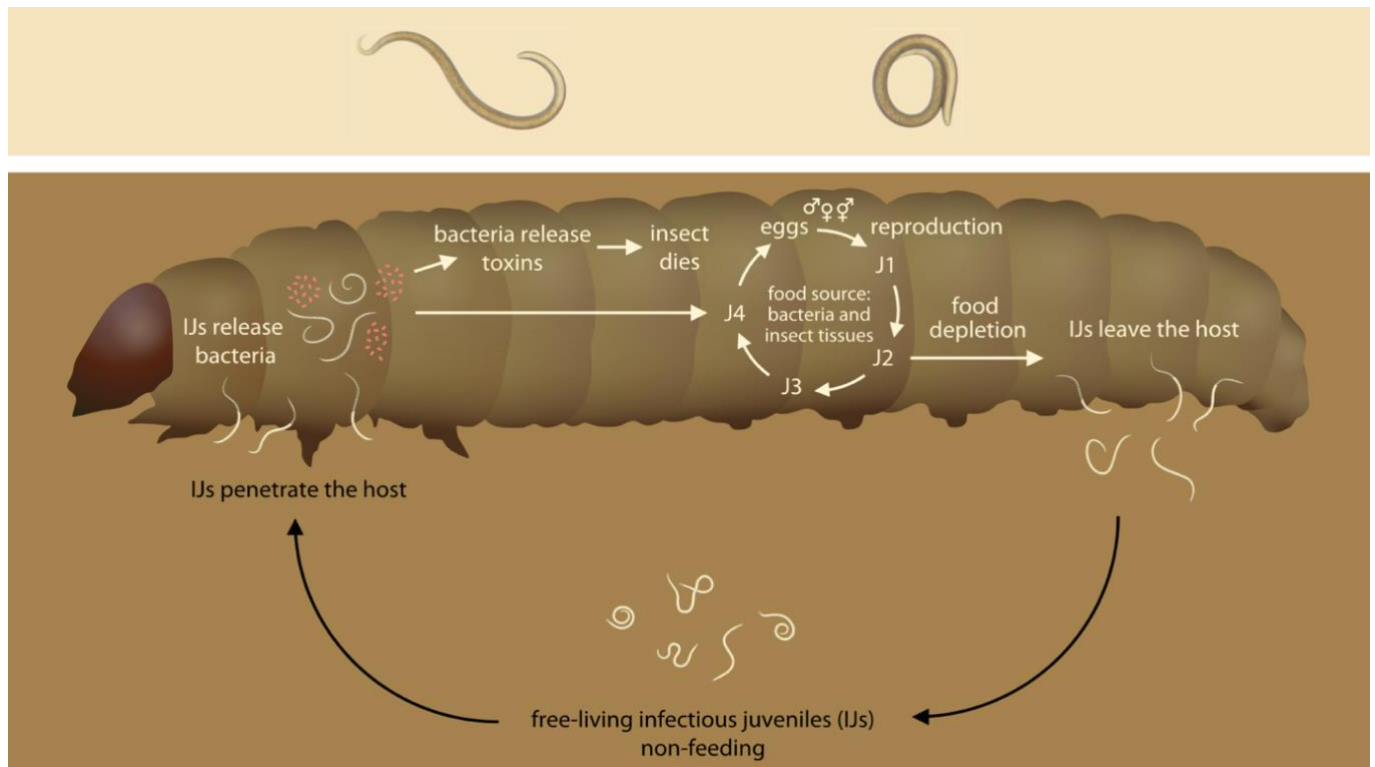


Figure 3: Entomopathogenic nematodes. Top) Two infective juvenile nematodes; Bottom) Life cycle of entomopathogenic nematodes. Sources: Top) Neil Villard; Bottom) Thomas Degen and Geoffrey Jaffuel, adapted from Stock (2015).

The last few decades, the use of entomopathogenic nematodes (EPN) has emerged as an effective biological control strategy (Grewal, Ehlers and Shapiro-Ilan, 2005; Lacey and Georgis, 2012). Their effectiveness is attributed to their ability to persist in the soil and to their pathogenicity towards a variety of insects, but they also offer several distinct benefits over other control methods:

- **Active search of hosts:** EPN have a keen ability to actively seek out their hosts. This makes them suitable candidates against pests located in concealed habitats that are usually difficult to control, such as borers beneath tree barks or FAW deep within maize whorls.
- **Local strains and production:** EPN can be isolated and mass-produced locally (Holmes et al., 2015; Fallet et al., 2020), facilitating their registration and commercialization as plant protection products and limiting the risk of introducing foreign species in a target country. Furthermore, they can be cost-effective when formulated appropriately (Ehlers, 2001).
- **Safety:** EPN pose no harm to farmers, livestock or plants and have minimal adverse effects on the environment and non-target organisms (Ehlers and Hokkanen, 1996).
- **Combined pest management:** The compatibility of EPN with other biological control agents and many synthetic insecticides presents opportunities for synergistic pest management strategies. Indeed, EPN have been successfully applied in combination with beneficial fungi or bacteria (e.g.,

Imperiali et al., 2017; Sáenz-Aponte et al., 2020), with reported synergistic interactions (e.g., Li et al., 2021).

- **Resistance management:** The likelihood of pests developing resistance to EPN is minimal due to the multitude of toxins released by the EPN and their symbiotic bacteria upon infection.

EPN are commonly used against belowground pests. They are typically applied in liquid solutions onto the soil or into protected areas such as tree bark, stem bases or dense vegetation. EPN have been successfully employed in various agricultural systems, including horticulture, fruticulture, and to some extent field crops, orchards and forestry. The efficacy of EPN in controlling pests such as white grubs, root weevils and the soil stage of various lepidopteran and dipteran pests has been frequently demonstrated (Lacey and Georgis, 2012; Campos-Herrera, 2015; Koppenhöfer et al., 2020).

While EPN are primarily known for their effectiveness against soil-dwelling pests, the application of EPN against aboveground pests has also been considered (Lacey & Georgis, 2012; Shapiro-Ilan and Dolinski, 2015; Hiltbold, 2015). The aboveground conditions pose several challenges to the successful application of EPN. As soil organisms, EPN are particularly sensitive to desiccation, heat and UV radiation (Kaya and Gaugler, 1993; Lacey and Georgis, 2012). Nonetheless, a number of recent studies have highlighted their potential as aboveground pest control agents (e.g., Tomar et al., 2022; Sáenz-Aponte et al., 2020; Wakil et al., 2023; Shapiro-Ilan et al., 2016; El Aimani et al., 2021). EPN have been found to be effective against pests such as caterpillars, leaf miners, thrips and beetle caterpillars. For example, EPN applications were as effective as synthetic insecticides against the tomato leaf miner (*Tuta absoluta*; Meyrick) or against the lesser peachtree borer (*Synanthedon pictipes*; Grote & Robinson). In these cases, the EPN were applied through sprays to foliage or directly onto bark wounds (Shapiro-Ilan et al., 2016; El Aimani et al., 2021).

Several studies have shown that certain strains of EPN are highly effective in infecting and killing FAW caterpillars (Fig. 5; Fuxa et al., 1988; Andaló et al., 2010; Acharya et al., 2020; Fallet et al., 2022a). First attempts to use EPN against FAW explored the possibility of spraying EPN with water, or in combination with adjuvants, onto the leaves of maize plants. These trials resulted in inconsistent or low efficacy, mainly due to the limited retention of EPN on plants post-application, rapid desiccation of the nematodes, or the absence of substrate allowing nematodes to move towards the caterpillars (Richter and Fuxa, 1990; Garcia et al., 2008; Negrisoli et al., 2010; Patil et al., 2022; Fallet et al., 2022b).



Figure 4: Left) Fall armyworm caterpillars killed by nematodes. Right) Nematodes emerging from a dead caterpillar. Sources: Neil Villard and Patrick Fallet.

Thesis outline

The main objective of this thesis is to overcome the existing limitations in the use of EPN against FAW. We explored novel strategies to formulate and apply EPN to ensure that they remain on plants post-application, facilitate their search and infection of caterpillars and ultimately increase their effectiveness (Fig. 6).

In **Chapter 1**, we isolated EPN from both agricultural and semi-natural habitats in Rwanda. This allowed us to use native EPN strains, eliminating the risk of introducing foreign species in the Rwandan ecosystem and avoiding the legislation hurdles associated with such introduction. In **Chapter 2**, we compared the virulence of EPN from Rwanda, Mexico or obtained from commercial sources. The objective was to determine whether EPN isolated in the invasive range of FAW can be as effective as commercially available strains or strains that have co-evolved with FAW (from its native range). In **Chapter 3**, we explored different approaches to formulate and apply EPN onto maize plants. The efficacy of each EPN-formulation was evaluated in laboratory assays. The most effective formulations were subsequently tested in preliminary field trials. **Chapter 4** represents the evaluation of the most promising formulations throughout a full growing season of maize in Rwanda. In **Chapter 5**, we investigated the potential of natural substances to protect EPN from the harmful effects of UV radiation to further improve the efficacy of the EPN formulations.



Figure 5: A gel formulation of nematodes (left) and its application using a caulking-gun (right). Sources: Stefan Toepfer.

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Chapter 1

A Rwandan survey of entomopathogenic nematodes that can potentially be used to control the fall armyworm

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Abstract

Native to the Americas, fall armyworm (FAW; *Spodoptera frugiperda*, Lepidoptera: Noctuidae) recently invaded most of Africa and large parts of Asia. Its caterpillars cause tremendous damage to maize. Effective control measures, which do not harm humans or the environment, are needed. We intend to use native, locally well-adapted, entomopathogenic nematodes to control FAW caterpillars. Therefore, we conducted a survey of nematodes in small-holder farming and semi-natural habitats in Rwanda. The survey yielded two nematode isolates, which were molecularly assigned to the *Heterorhabditis bacteriophora* group, but might represent a new species.

Key words: *Spodoptera frugiperda*, invasive species, biological control, maize, food security

Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is native to the Americas, where it is a major agricultural pest, particularly in maize (Early *et al.*, 2018). In early 2016, FAW was reported for the first time in Africa (Goergen *et al.*, 2016). Rapidly, FAW spread to all sub-Saharan countries, where it became an important threat to food security (Day *et al.*, 2017). In the 12 major maize-producing countries of Africa, damage by FAW may result in 20 to 50% yield loss, valued at 2.5 to 6 billion USD per year (Day *et al.*, 2017). Hence, effective, safe and sustainable control measures are needed to minimize FAW damage.

Entomopathogenic nematodes (EPN) are microscopic soil-dwelling worms that can infest and kill a large variety of insects (Kaya & Gaugler, 1993). They have been widely used in the biological control of belowground pest insects. EPN are also highly effective at killing aboveground lepidopteran caterpillars, such as FAW (Fuxa *et al.*, 1988; Kaya & Gaugler, 1993). EPN usage is considered largely safe for the environment (Kaya & Gaugler, 1993) and they can be locally mass-produced in Africa (Keith *et al.*, 2015). Therefore, EPN represent good candidates to control FAW.

Our aim is to incorporate EPN in a formulation that can be applied into the whorl of maize where FAW caterpillars spend most of their time feeding (Labatte, 1993). We intend to use native strains of EPN to assure that: i) the applied EPN are well adapted to local conditions, ii) to avoid the introduction of non-native material, and iii) to comply with national plant protection product legislation. To obtain such strains, we conducted a survey for EPN in Rwanda using a standard insect baiting technique and used molecular methods to identify the isolated EPN.

Material and Methods

Survey area and sample collection

We conducted a survey in the South, West, and Kigali provinces of Rwanda in April 2018. We collected 208 soil samples in 35 locations at small-holder farms (118 samples) and semi-natural habitats (90). Small-holder farm habitats included maize fields (26), other cereal crops (18), vegetables (20), tuber crops (26), orchards (14), tea (4) and coffee (10) shrub lands. Semi-natural habitats included eucalyptus forests (8 samples), pine forests (20), tropical forests (2), grasslands (36), lakeshores (8) and river benches (16).

Sample collection was performed as described in Yan *et al.*, (2016). Briefly, two or three habitats were sampled at each location. For each habitat, two mixed soil samples were collected. First, the upper 5cm layer of soil was removed. Then, twice three 500ml soil samples were taken and each set mixed. Subsequently 400ml fractions were taken and transported in perforated plastic containers.

Entomopathogenic nematode isolation

EPN were isolated from soil samples using a baiting technique (Bedding and Akhurst, 1975). Briefly, five sixth-instar *Galleria mellonella* (Lepidoptera: Pyralidae) larvae were placed inside each soil container and incubated at room temperature (20-26°C). As the larvae tend to crawl to the soil surface, containers were turned upside-down every second day. Samples were checked for dead larvae after four and eight days. Dead larvae were transferred to nematode traps (White, 1927) and checked daily during three weeks. Emerging EPN infective juveniles were collected in culture flasks. In agreement with Rwanda Agriculture and Animal Resources Development Board (RAB; Kigali, Rwanda), isolates were transported to the University of Neuchâtel (Switzerland) where they are reared on *G. mellonella* under quarantine conditions. RAB also maintains the isolates at the biocontrol facility in Rubona, Rwanda.

Entomopathogenic nematode identification

EPN identification was carried out as described in Machado *et al.*, (2018), using the primers 18S (5'-TTGATTACGTCCCTGCCCTT-3') and 26S (5'-TTTCACTCGCCGTTACTAAGG-3'), and sequenced by Sanger sequencing (Microsynth AG, Balgach, Switzerland). The ITS-based evolutionary history of *Heterorhabditis* nematodes was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

Results and Discussion

Out of the 208 soil samples collected, two yielded EPN (0.96%), referred to as Rw18_M-Hr1a and Rw18_M-Hr1b. The two positive samples were collected from the base of a small valley near Mubuga

village (District of Karongi; West province of Rwanda, S02°07.890', E029°19.528', 1570m a.s.l.). The samples were taken from a sandy, moist habitat along a river bench 75% covered with sweet potato.

Phylogenetic reconstruction suggests that the two Rwandan isolates belong to the *Heterorhabditis bacteriophora* clade, but form a distinct group with 97% bootstrap support (Figure 1). They share 99.3% sequence similarity with the *H. bacteriophora* reference strain, and are identical to the strain Rw14_N-C4a, previously isolated in Rwanda by Yan *et al.*, (2016). This high similarity suggests that they should be classified as *H. bacteriophora*. However, the slight, but clear phylogenetic separation of the Rwandan nematode strains from the most closely related nematodes *H. bacteriophora*, *H. georgiana*, and *H. beicherriana* suggests that they might represent a new species. To determine if the Rwandan EPN truly represent a new species, further studies based on morphology and cross-breeding analysis are needed (Dix *et al.*, 1992).

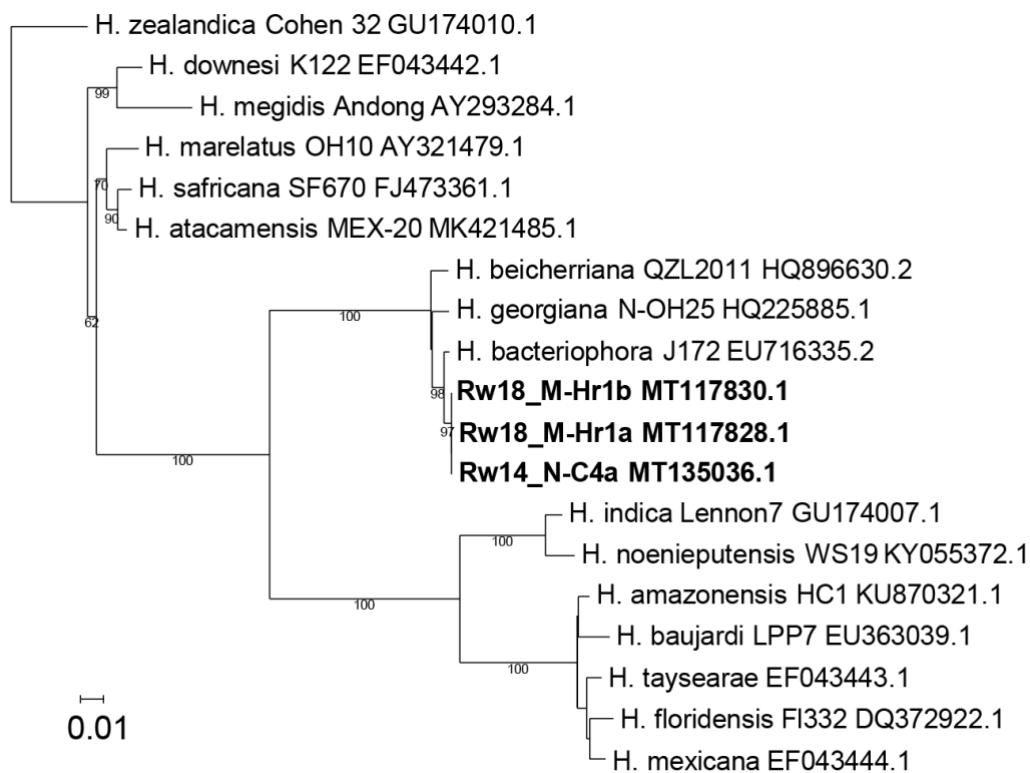


Figure 1. Taxonomic placement of Rwandan entomopathogenic nematodes Rw18_M-Hr1a, Rw18_M-Hr1b and Rw14_N-C4a. The optimal tree with the sum of branch length = 0.57108882 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 19 nucleotide sequences. There were 710 positions in the final dataset. Rwandan entomopathogenic nematodes are indicated in bold.

The Rwandan EPN are currently tested for pathogenicity against different FAW instars. The most pathogenic strain will be incorporated in a formulation and subsequently applied onto maize. Primary results show that this approach has good potential to control FAW.

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Chapter 2






Comparative screening of Mexican, Rwandan and commercial entomopathogenic nematodes to be used against invasive fall armyworm, *Spodoptera frugiperda*

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Article

Comparative Screening of Mexican, Rwandan and Commercial Entomopathogenic Nematodes to Be Used against Invasive Fall Armyworm, *Spodoptera frugiperda*

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Simple Summary: The fall armyworm is a devastating insect pest of maize that has recently spread from the Americas to Africa and Asia. Synthetic insecticides are currently being used excessively to fight this pest. Safe, effective and more sustainable alternatives are urgently needed. We explore the use of beneficial entomopathogenic nematodes to control the fall armyworm. These tiny soil-born roundworms are lethal parasites of insects, including caterpillars such as the fall armyworm. We tested forty nematode strains from either the native range of the fall armyworm (Mexico), or the area of invasion (Rwanda), and commercial strains. We found that certain strains of local nematodes from the area of invasion can be as effective in infecting and killing fall armyworm as commercial strains or those from the armyworm's native range. These findings will aid the development of locally acceptable and effective biological control products.

Abstract: The fall armyworm (FAW), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) is an important pest of maize originating from the Americas. It recently invaded Africa and Asia, where it causes severe yield losses to maize. To fight this pest, tremendous quantities of synthetic insecticides are being used. As a safe and sustainable alternative, we explore the possibility to control FAW with entomopathogenic nematodes (EPN). We tested in the laboratory whether local EPNs, isolated in the invasive range of FAW, are as effective as EPNs from FAW native range or as commercially available EPNs. This work compared the virulence, killing speed and propagation capability of low doses of forty EPN strains, representing twelve species, after placing them with second-, third- and sixth-instar caterpillars as well as pupae. EPN isolated in the invasive range of FAW (Rwanda) were found to be as effective as commercial and EPNs from the native range of FAW (Mexico) at killing FAW caterpillars. In particular, the Rwandan *Steinernema carpocapsae* strain RW14-G-R3a-2 caused rapid 100% mortality of second- and third-instar and close to 75% of sixth-instar FAW caterpillars. EPN strains and concentrations used in this study were not effective in killing FAW pupae. Virulence varied greatly among EPN strains, underlining the importance of thorough EPN screenings. These findings will facilitate the development of local EPN-based biological control products for sustainable and environmentally friendly control of FAW in East Africa and beyond.

Keywords: biological control; integrated pest management; East Africa; maize; invasive species; food security

1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) is a major pest of maize originating from the tropical and subtropical regions of the Americas [1]. FAW is polyphagous, but prefers grasses, particularly maize, sorghum and rice [1]. In early 2016, FAW was reported in West Africa and rapidly spread across the continent and then further into Asia [2–5]. On both continents, it causes tremendous crop damages and yield losses [5–9]. To mitigate the impact of FAW, governments launched emergency programs and subsidized synthetic insecticides, which quickly became the backbone of FAW control in the invasive range [10–12]. Because of the environmental harm and health risks caused by these measures, safe, sustainable and effective alternative FAW control strategies are urgently needed [5].

Biological control of pest insects using entomopathogenic nematodes (EPN) has been implemented with success for several decades, particularly against belowground pests [13]. EPN are tiny soil-dwelling roundworms that are naturally present in soils worldwide [14]. They can be isolated from soil samples, mass produced and then applied to control pest insects. Importantly, they are safe for farmers or consumers, and cause virtually no harm to the environment [15,16]. EPNs can be particularly virulent to lepidopteran caterpillars, including FAW, but the virulence against a target insect greatly varies between EPN species and strains [17–23]. Hence, EPN screenings are necessary to identify promising EPNs for specific biological control programs [20].

We have launched a project to use EPNs against FAW caterpillars, by applying EPN formulations into the whorl of maize plants. We intend to use locally isolated EPNs to avoid introducing foreign organisms. This is important, as it is increasingly evident that EPN species are not always correctly identified [24,25]. Misdiagnosing closely related EPN species increases the risk of accidentally introducing non-native EPNs in a targeted region, as has been reported previously [26].

The aim of the current study was to compare the virulence and killing speed of different species and strains of EPN to identify promising EPN candidates for biological control of FAW in East Africa. We used EPNs from different areas of origin that were either locally isolated in Rwanda and in Mexico, or that were obtained from commercial sources. We hypothesized that EPN strains from Mexico, where FAW is native, may have evolved higher virulence than EPNs from Rwanda, in the invasive range [27], while commercial strains are expected to be the most virulent, as they have been selected for this trait.

We compared forty strains of EPN, representing twelve species, in three steps. In the first step, we compared the virulence and the killing speed of twenty-nine Mexican EPN strains, representing five species, against third-instar FAW caterpillars. The five most virulent Mexican strains (four species) were selected for the second step, in which they were compared with five Rwandan (three species) and six commercial strains (six species). In the third step, the five most promising EPN from the second step (one Mexican, two Rwandan and two commercial EPN) were tested at three concentrations (5, 25, 125 EPN per FAW) against second-, third- and sixth-instar caterpillars as well as against pupae of FAW. In addition, we evaluated reproductive output of each of these EPN strains in FAW caterpillars. The overall aim was to determine the level of virulence of specific EPN strains against FAW and to lay the basis for their development as biological control agents. An additional objective was to clarify whether such efforts should focus on EPN from the area-of-origin of the pest, or whether commercial EPN or EPN native to the target region are just as effective.

2. Materials and Methods

2.1. Origin and Handling of the Fall Armyworm and Nematodes

Spodoptera frugiperda (FAW) caterpillars were reared on artificial diet (Beet Armyworm Diet, Frontier Scientific, Newark, NJ, USA) under quarantine conditions (OFEV permit A140502) at the University of Neuchâtel, Switzerland.

Forty entomopathogenic nematodes strains, representing twelve species, were used in this study (Table 1). They were either collected from soil samples in Mexico [28], Rwanda [29,30] or obtained from commercial sources (e-nema GmbH, Schwentental, Germany; Guangdong Academy of Sciences, Guangdong, China). EPN were reared in vivo on last-instar *Galleria mellonella* L. (Lepidoptera: Pyralidae). They were stored at 12 °C in the dark and used in experiments within ten days post emergence.

Table 1. Origin, strain, species and source of entomopathogenic nematodes.

Origin	Strain	Species (Authority)	Source
Commercial	NA (Dianem [®])	<i>Heterorhabditis bacteriophora</i> (Poinar)	Provided by e-nema GmbH, Schwentental, Germany
	NA	<i>Steinernema abbasi</i> (Elawad, Ahmad and Reid)	
	All (Nemastar [®])	<i>Steinernema carpocapsae</i> (Weiser)	
	NA (Nemaplus [®])	<i>Steinernema feltiae</i> (Filipjev)	
	H06 (HR-HB [®])	<i>Heterorhabditis beicherriana</i> (Xing-Yue, Qi-Zhi, Nermut, Puza and Mracek)	Provided by Guangdong Academy of Sciences, China
	LN2 (HR-HI [®])	<i>Heterorhabditis indica</i> (Poinar, Karunakar and David)	
Mexico	MEX-20	<i>Heterorhabditis atacamensis</i> (Edgington, Buddie, Moore, France, Merino and Hunt)	Collected by P. Bruno [28]
	MEX-14	<i>Heterorhabditis bacteriophora</i>	
	MEX-16		
	MEX-17		
	MEX-21		
	MEX-22		
	MEX-23		
	MEX-29		
	MEX-30		
	MEX-31		
	MEX-32		
	MEX-33		
	MEX-34		
	MEX-35		
	MEX-36		
	MEX-37		
	MEX-38		
	MEX-42		
	MEX-43		
	MEX-44		
MEX-45			
MEX-46			
	MEX-25	<i>Heterorhabditis mexicana</i> (Nguyen, Sharpiro-Ilan, Stuart, McCoy, James and Adams)	
	MEX-26		
MEX-47			
	MEX-39	<i>Heterorhabditis zacatecana</i> (Machado, Bhat, Abolafia, Muller, Bruno, Fallet, Arce, Turlings, Bernal, Kajuga, Waweru and Toepfer)	
	MEX-40		
	MEX-41		
	MEX-15	<i>Steinernema riobrave</i> (Cabanillas, Poinar and Raulston)	
Rwanda	RW14-N-C4a	<i>Heterorhabditis ruandica</i> (Machado, Bhat, Abolafia, Muller, Bruno, Fallet, Arce, Turlings, Bernal, Kajuga, Waweru and Toepfer)	Provided by RAB, Rwanda [29]
	RW14-G-R3a-2	<i>Steinernema carpocapsae</i>	
	RW14-M-C2b-1	<i>Steinernema</i> sp. (closely related to <i>S. feltiae</i>)	
	Rw18_M-Hr1a	<i>Heterorhabditis ruandica</i>	Provided by RAB, Rwanda [30]
	Rw18_M-Hr1b		

2.2. Step 1: Screening of Mexican Entomopathogenic Nematodes against the Fall Armyworm

The virulence and killing speed of the 29 Mexican EPN strains (five species) were evaluated on third-instar FAW caterpillars. One strain of *Heterorhabditis atacamensis*, twenty-one of *Heterorhabditis bacteriophora*, three of *Heterorhabditis zacatecana* (newly described by [25]), three of *Heterorhabditis mexicana* and one of *Steinernema riobrave* were used (Table 1). The virulence of each Mexican EPN strain was compared to the virulence of the commercial EPN *Steinernema carpocapsae* strain All, which was used as the positive control. In total, eighteen to twenty-four caterpillars were exposed to each EPN strain in three independent experiments.

Briefly, caterpillars were weighed and then individually placed in an arena (4 cm long × 4 cm wide × 2.5 cm height) containing a filter paper folded on the sides (5.5 cm in diameter) and artificial diet (ca. 1 cm³). The average weight (± SD) of the third-instar caterpillars was 10.6 ± 1.7 mg (n = 760). Ten infective juveniles (IJs) were applied in 400 µL of water in each arena (10 IJs per caterpillar). As control, only water was applied. Arenas were stored in the dark at 24 ± 1 °C, and caterpillar mortality was recorded daily for seven days. Mortality was assessed and corrected using Abbott's formula [31] as follows:

$$\frac{\text{survival in the control} - \text{survival in the treatment}}{\text{survival in the control}} \times 100$$

The killing speed of the most virulent EPNs, which were selected to be used in the second experiment, was compared amongst one another. These were *H. bacteriophora* (two strains: MEX-17, MEX-35), *H. mexicana* (two strains: MEX-47, MEX-25), *H. zacatecana* (MEX-41) as well as *S. riobrave* (MEX-15).

To evaluate the reproduction success of the EPNs (= propagation), cadavers were collected and weighed daily. They were then individually placed in nematode traps [32] for three weeks. The IJs that had emerged were then counted under a stereoscopic microscope in five replicates per cadaver. Because the size of the cadavers differed greatly (as some nematodes killed caterpillars faster than others), and with it the number of IJs that could emerge from each cadaver, the reproduction was expressed as the number of IJs per mg of the cadaver (IJs * mg⁻¹). The reproduction of different EPN was not compared statistically, as different EPN species differ considerably in the number of offspring per se. It was only used to determine whether each EPN strain could reproduce in FAW and to determine how many IJs can emerge from a cadaver. The reproduction of strains that killed fewer than five caterpillars was not evaluated.

2.3. Step 2: Comparing the Virulence of Mexican, Rwandan and Commercial Entomopathogenic Nematodes against the Fall Armyworm

The virulence and killing speed of five Mexican (highly efficient EPNs as per previous screening), five Rwandan, and six commercial EPN were evaluated using third-instar FAW caterpillars. The experiment was conducted as described above with each individual caterpillar exposed to ten IJs of each EPN strain. The average weight (± SD) of the caterpillars was 10.3 ± 1.3 mg (n = 408). The Mexican EPN that were selected based on the previous screening results, included two strains of *H. mexicana* (MEX-25, MEX-47), two of *H. bacteriophora* (MEX-17, MEX-35), one of *H. zacatecana* (MEX-41) and one of *S. riobrave* (MEX-15). The Rwandan EPN were three strains of *Heterorhabditis ruandica* (RW14-N-C4a, Rw18_M-Hr1a, Rw18_M-Hr1b), one of *S. carpocapsae* (RW14-G-R3a-2) and one undescribed species *Steinernema* sp. (closely related to *Steinernema feltiae*; RW14-M-C2b-1). The commercial EPN were *S. carpocapsae* All (Nemastar[®]), *H. bacteriophora* (Dianem[®]), *Steinernema abbasi*, *Steinernema feltiae* (Nemaplus[®]), *Heterorhabditis beicherriana* H06 (HR-HB[®]) and *Heterorhabditis indica* LN2 (HR-HI[®]). In total, sixteen to twenty-four caterpillars were exposed to each EPN strain in three independent experiments.

The virulence of each EPN strain was compared to the virulence of the commercial EPN *S. carpocapsae* All, which was used as positive control. The killing speed of a few highly effective strains, which were selected to be used in further experimentation, were

compared amongst one another. These EPNs were among the most virulent and fast killing strains originating from Mexico, Rwanda or commercial sources: *S. carpocapsae* strain All, *S. abbasi*, *S. carpocapsae* RW14-G-R3a-2, *H. ruandica* Rw18_M-Hr1a and *H. zcatecana* MEX-41. *H. zcatecana* MEX-41 was not the most effective Mexican strain, but it was selected for experiment three because it is closely related to *H. ruandica* as well as to *Heterorhabditis bacteriophora*. The latter is commonly used in commercial products. The reproduction success of each EPN was evaluated as described above.

2.4. Step 3: Assessing the Dose-Dependent Effectiveness of the Most Promising Entomopathogenic Nematodes against Different Stages of the Fall Armyworm

Five of the most promising EPN strains were selected, from the preceding two steps, with at least one strain from each origin (Mexican, Rwandan or commercial). The Rwandan EPN were *S. carpocapsae* RW14-G-R3a-2 and *H. ruandica* Rw18_M-Hr1a (formerly considered to be *H. bacteriophora* [25,30]). The Mexican EPN was *H. zcatecana* MEX-41. The commercial EPN were *S. carpocapsae* All (Nemastar®) and *S. abbasi*. The virulence and the killing speed of these strains were tested against second-, third- and sixth-instar FAW caterpillars as well as against FAW pupae. The experiment was conducted as described above, but using three concentrations of IJs: 5, 25 and 125 IJs per arena (individual caterpillar or pupa). To ensure that pupal mortality was not caused by EPN killing emerging adults (when the adult cracks open its pupa), five days post inoculation, pupae were plunged for a few seconds in bleach (0.5%), rinsed twice with water and transferred to clean arenas without EPN. The average weight (\pm SD) of the caterpillars was 1.2 ± 0.1 mg ($n = 413$), 6.2 ± 2.6 mg ($n = 416$), 447 ± 151 mg ($n = 410$) for second-, third- and sixth-instar, respectively. It was 252 ± 38 mg ($n = 400$) for the pupae. The virulence and the killing speed were compared amongst the EPN strains. Due to the low mortality of pupae, the killing speed of EPN was not evaluated for this developmental stage. In total, nineteen to thirty-one FAW individuals were exposed to each treatment (strains and concentrations) in three independent experiments.

The reproductive success of each EPN on FAW caterpillars was evaluated as described above. Since too few pupae died, reproductivity was not evaluated for the pupal stage.

2.5. Data Analyses

Statistical analyses were performed using R version 4.1.0 (R Core Team 2021).

Due to the complete separation of the data (100% or 0% mortality in some treatments), EPN virulence was analyzed using Bayesian generalized linear models (“arm” package [33]) with a binomial error distribution. In steps one and two, mortality of caterpillars was used as the response variable, while treatment and replication (each experiment was replicated three times) were used as fixed factors. The virulence of each strain was then compared to the virulence of the commercial EPN *S. carpocapsae* All, using many-to-one comparisons (“emmeans” package [34]) corrected for false discovery with the Benjamini and Hochberg method [35]. In step three, mortality of caterpillars was used as the response variable, while treatment, concentration (number of IJs used), FAW stage, and replication (each experiment was replicated three times) were used as fixed factors. Interaction effects between fixed factors that were not significant were removed from the model. Virulence was compared amongst EPN strains within each pair of concentration and insect stage using Bayesian generalized linear models with a binomial error distribution, followed by multiple comparisons (“emmeans” package [34]) corrected for false discovery using the Benjamini and Hochberg method [35].

EPN killing speed was analyzed using Cox proportional hazards regression models (“survival” package [36]) followed by multiple comparisons of survival curves (“survminer” package [37]) corrected for false discovery with the Benjamini and Hochberg method [35]. In steps one and two, the survival of FAW caterpillars was used as the response variable, while treatment and replication (each experiment was replicated three times) were used as fixed factors. Only the most effective strains (see Methods above) were compared in the multiple comparisons. In step three, the survival of FAW caterpillars or pupae was

used as the response variable, while treatment, concentration (number of IJs used), FAW stage and replication (each experiment was replicated three times) were used as fixed factors. Interaction effects between fixed factors that were not significant were removed from the model. Multiple comparisons of survival curves were performed within each pair of concentration and insect stage.

In steps one and two, statistical analyses were not performed on EPN reproduction, as EPN species produce different numbers of offspring per se. EPN reproduction was only used to determine whether each EPN strain could reproduce on FAW and to evaluate the number of IJs that could emerge from a cadaver. In step three, the effects of both inoculation dose as well as development stage of the caterpillars at death on EPN propagation were evaluated using zero-inflated regression models for count data with a Poisson error distribution (“pscl” package [38]).

3. Results

3.1. Step 1: Screening of Mexican Entomopathogenic Nematodes against the Fall Armyworm

All 29 Mexican EPN strains were able to infect and kill FAW caterpillars. The application of ten infective juveniles significantly reduced third-instar caterpillar survival ($\chi^2_{(29)} = 76, p < 0.001$; Figure 1). The positive control, the commercial *Steinernema carpocapsae* All, was among the most virulent strains and caused $88 \pm 12.5\%$ (mean \pm SE) mortality. Three of the Mexican strains were as virulent as *S. carpocapsae* All ($p > 0.05$): *Heterorhabditis zacatecana* MEX-41 ($71 \pm 4.2\%$), *Steinernema riobrave* MEX-15 ($58 \pm 8.3\%$) and *Heterorhabditis mexicana* MEX-47 ($55 \pm 12.6\%$; $p > 0.05$). All the other Mexican strains were less virulent than *S. carpocapsae* All ($p < 0.05$). Notably, EPN virulence varied greatly within the same species. For instance, *H. bacteriophora* MEX-17 was four times more virulent than *H. bacteriophora* MEX-33 (Figure 1).

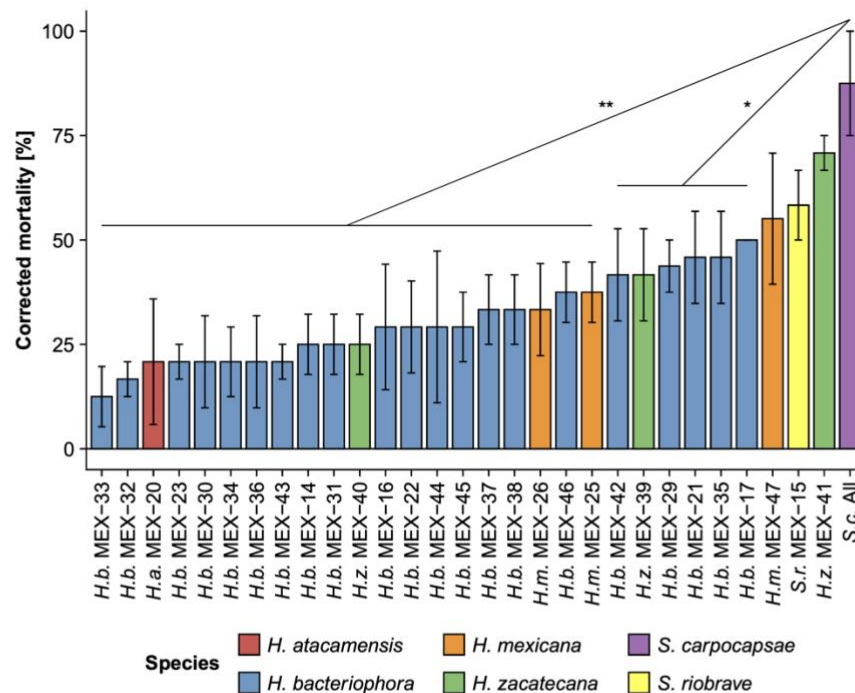
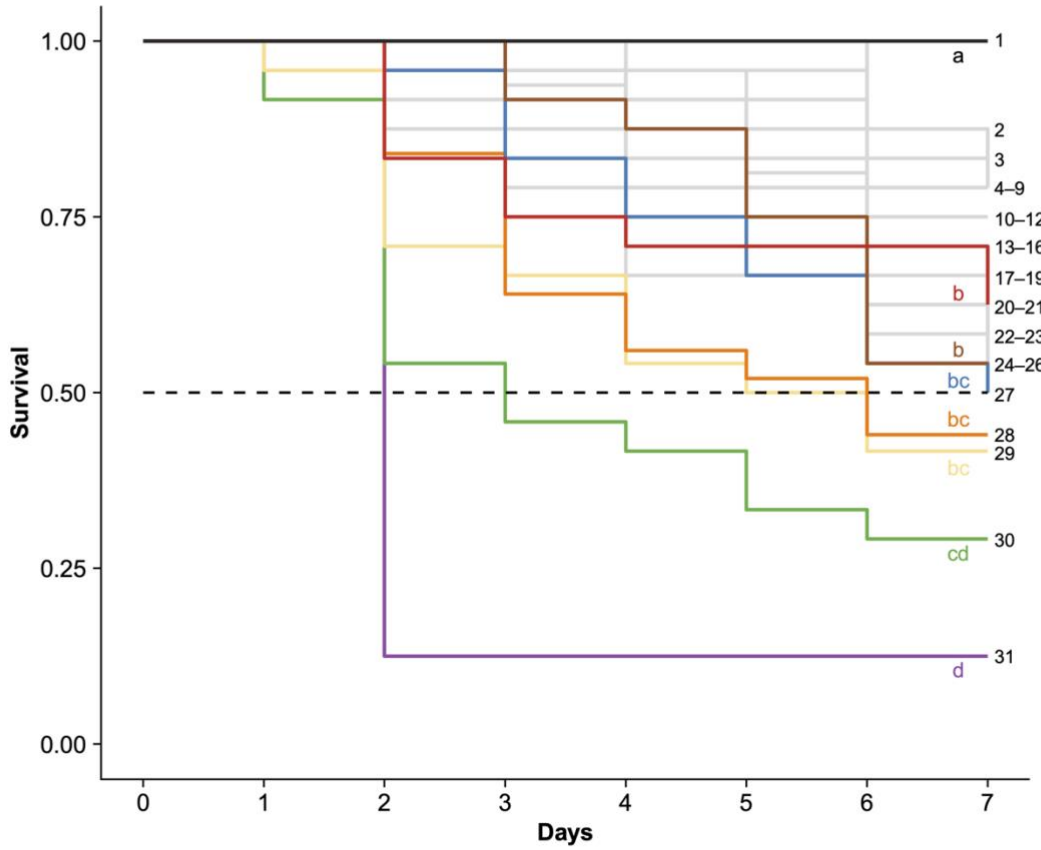


Figure 1. Virulence (mean \pm SE) of Mexican entomopathogenic nematode strains (MEX) on third-instar *Spodoptera frugiperda* caterpillars in small arena laboratory bioassays. Mortality was evaluated seven days post inoculation with ten infective juvenile nematodes per caterpillar. The virulence of each Mexican strain was compared to the commercial *Steinernema carpocapsae* strain All (positive control). Stars (*) indicate significant differences obtained from many-to-one comparisons corrected for false discovery using the Benjamini and Hochberg method (no star: non-significant, *: $p < 0.05$, **: $p < 0.01$).

EPN species and strains differed in their killing speed of third-instar FAW caterpillars ($\chi^2_{(29)} = 51, p = 0.008$; Figure 2). The commercial *S. carpocapsae* All as well as the Mexican *H. zacatecana* MEX-41 were the fastest in killing caterpillars with an LT_{50} of two and three days, respectively. The other strains needed more time than *S. carpocapsae* All to kill FAW caterpillars ($p < 0.05$).



Strains

1	Control	8	<i>H.b.</i> MEX-36	15	<i>H.b.</i> MEX-44	22	<i>H.b.</i> MEX-42	29	<i>S.r.</i> MEX-15
2	<i>H.b.</i> MEX-33	9	<i>H.b.</i> MEX-43	16	<i>H.b.</i> MEX-45	23	<i>H.z.</i> MEX-39	30	<i>H.z.</i> MEX-41
3	<i>H.b.</i> MEX-32	10	<i>H.b.</i> MEX-14	17	<i>H.b.</i> MEX-37	24	<i>H.b.</i> MEX-29	31	<i>S.c.</i> All
4	<i>H.a.</i> MEX-20	11	<i>H.b.</i> MEX-31	18	<i>H.b.</i> MEX-38	25	<i>H.b.</i> MEX-21		
5	<i>H.b.</i> MEX-23	12	<i>H.z.</i> MEX-40	19	<i>H.m.</i> MEX-26	26	<i>H.b.</i> MEX-35		
6	<i>H.b.</i> MEX-30	13	<i>H.b.</i> MEX-16	20	<i>H.b.</i> MEX-46	27	<i>H.b.</i> MEX-17		
7	<i>H.b.</i> MEX-34	14	<i>H.b.</i> MEX-22	21	<i>H.m.</i> MEX-25	28	<i>H.m.</i> MEX-47		

Figure 2. Killing speed of Mexican nematodes strains (MEX) of *Heterorhabditis bacteriophora* (*H.b.*), *H. mexicana* (*H.m.*), *H. zacatecana* (*H.z.*) and *Steinernema riobravae* (*S.r.*) when placed with third-instar *Spodoptera frugiperda* caterpillars in small arenas. Mortality was evaluated over seven days post inoculation with ten infective juvenile nematodes per caterpillar. The commercial nematode *Steinernema carpocapsae* (*S.c.*) All was used as positive control. To facilitate readability, only a few highly virulent strains, which were selected for further experimentation, are highlighted in colors. Less virulent strains are represented in grey. The dotted horizontal line represents 50% survival. Letters indicate significant differences ($p < 0.05$) obtained from multiple comparisons corrected for false discovery using the Benjamini and Hochberg method.

All Mexican strains successfully reproduced in FAW caterpillars (Table S1).

3.2. Step 2: Comparing the Virulence of Mexican, Rwandan and Commercial Entomopathogenic Nematodes against the Fall Armyworm

EPNs significantly reduced third-instar FAW caterpillar survival ($\chi^2_{(16)} = 122, p < 0.001$; Figure 3). The commercial *S. carpocapsae* strain All and *Steinernema abbasi* appeared to be the most lethal (*S. carpocapsae* All vs. *Steinernema abbasi*: $p > 0.05$) and killed $96 \pm 4.2\%$ and $87 \pm 0.9\%$ (mean \pm SE) of caterpillars, respectively. The Rwandan *S. carpocapsae* (RW14-G-R3a-2; $54 \pm 17\%$), the Mexican *S. riobrave* (MEX-15; $54 \pm 17\%$), as well as the commercial *H. indica* (LN2; $61 \pm 7.4\%$) were nearly as virulent as *S. carpocapsae* All ($0.01 < p < 0.05$). The other strains were found to be less virulent ($p < 0.01$).

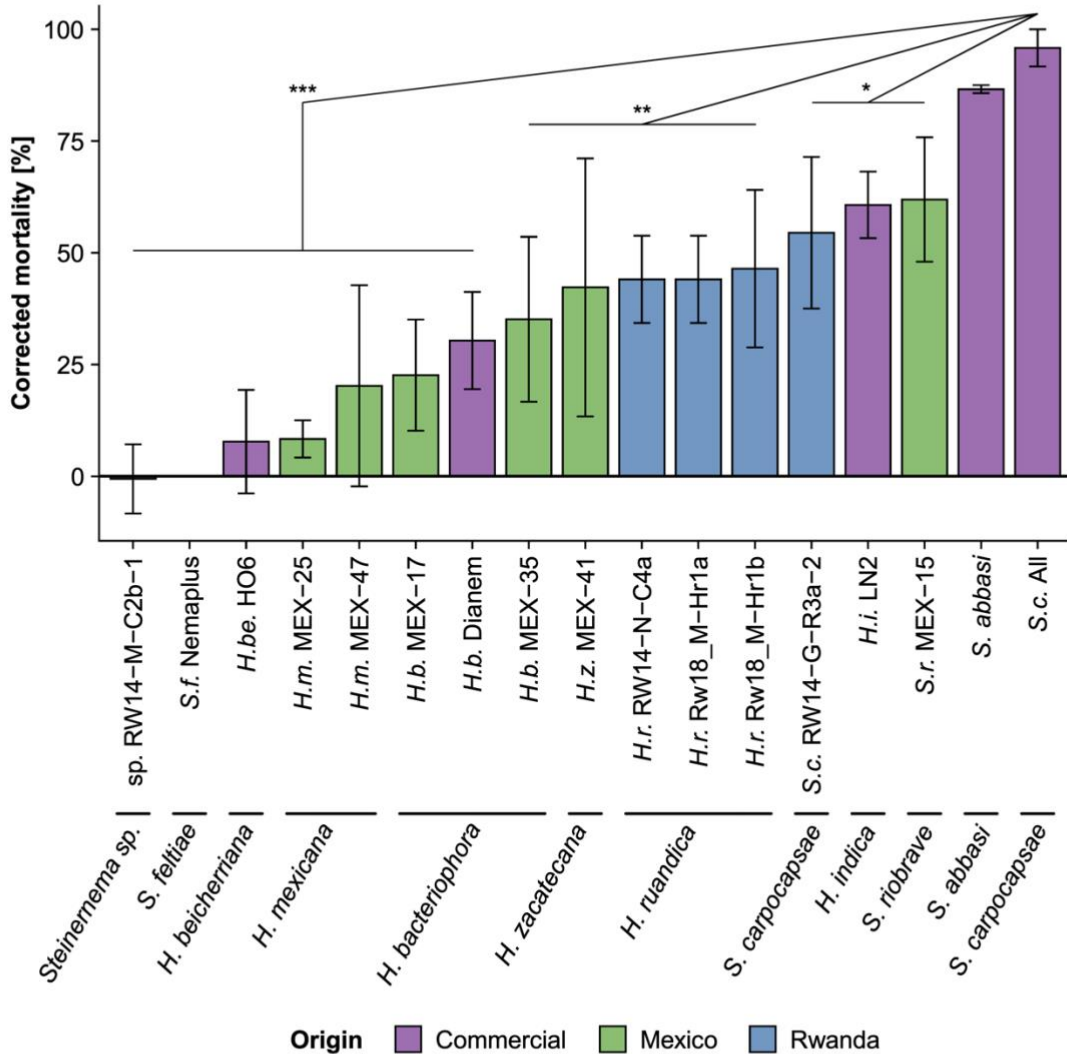


Figure 3. Virulence (mean \pm SE) of Mexican, Rwandan and commercial entomopathogenic nematode strains when placed with third-instar *Spodoptera frugiperda* caterpillars in small arenas. The mortality of individual caterpillars was evaluated seven days post inoculation with ten infective juvenile nematodes per caterpillar. The virulence of each strain was compared to the virulence of the commercial *Steinernema carpocapsae* strain All, which served as the positive control. Stars (*) indicate significant differences obtained from many-to-one comparisons corrected for false discovery using the Benjamini and Hochberg method (no star: non-significant, *: $p < 0.05$, **: $p < 0.01$, *** and $p < 0.001$).

Mexican, Rwandan and commercial EPN species and strains differed in their speed at which they killed third-instar FAW caterpillars ($\chi^2_{(17)} = 89, p < 0.001$; Figure 4). The commercial *S. carpocapsae* strain All and *S. abbasi* were the fastest in killing caterpillars (*S. carpocapsae* All vs. *S. abbasi*: $p > 0.05$). Both strains killed 50% of the caterpillars within

two days. The Rwandan *S. carpocapsae* RW14-G-R3a-2 was as fast as *S. abbasi* and reached LT_{50} after three days (*S. carpocapsae* RW14-G-R3a-2 vs. *S. abbasi*: $p > 0.05$). The fastest Mexican EPNs were *S. riobrave* MEX-15 (LT_{50} after four days) and *H. zacatecana* MEX-41 (50% mortality not reached after seven days). The Rwandan *H. ruandica* Rw18_M-Hr1a appeared slightly faster than the other strains of this species.

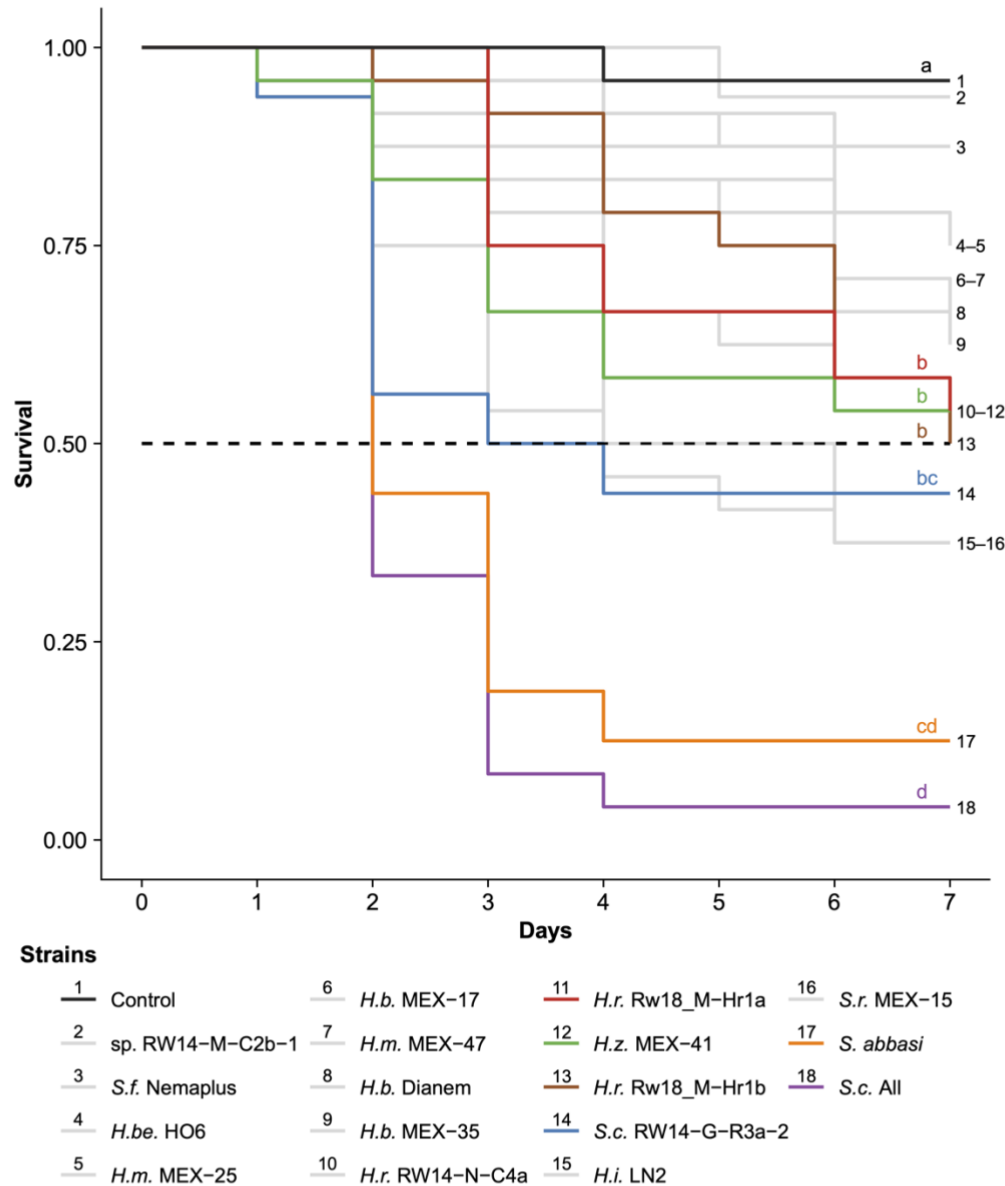


Figure 4. Killing speed of Mexican, Rwandan and commercial nematodes strains on third-instar *Spodoptera frugiperda* caterpillars in small arenas. *Heterorhabditis bacteriophora* (*H.b.*), *H. mexicana* (*H.m.*), *H. beicherriana* (*H.be.*), *H. zacatecana* (*H.z.*), *H. indica* (*H.i.*) and *H. ruandica* (*H.r.*), and *Steinernema feltiae* (*S.f.*), *Steinernema riobrave* (*S.r.*), *S. abbasi* and *S. carpocapsae* (*S.c.*). The mortality of individual caterpillars was evaluated over seven days post inoculation with ten infective juvenile nematodes per caterpillar. The commercial nematode *Steinernema carpocapsae* strain all was used as positive control. To facilitate readability, only a few highly virulent strains, which were selected for further tests, are highlighted in colors. The other strains are represented in grey. The dotted horizontal line represents 50% survival. Letters indicate significant differences ($p < 0.05$) obtained from multiple comparisons corrected for false discovery using the Benjamini and Hochberg method.

All tested EPN successfully reproduced in FAW caterpillars (Table S2), except for *Steinernema feltiae* and *Steinernema* sp. RW14-M-C2b-1, which killed too few caterpillars to evaluate their reproductivity.

3.3. Step 3: Assessing the Dose-Dependent Effectiveness of the Most Promising Entomopathogenic Nematodes against Different Stages of the Fall Armyworm

All EPN reduced FAW survival in dose-response experiments ($\chi^2_{(5)} = 293, p < 0.001$; Figure 5). FAW survival was different for different concentrations of EPN applied ($\chi^2_{(2)} = 99, p < 0.001$), as well as for the different FAW developmental stage ($\chi^2_{(3)} = 63, p < 0.001$). We found significant interactions between insect developmental stage and either EPN concentration or EPN treatment (Stage_Treatment $\chi^2_{(15)} = 232, p < 0.001$; Stage_Concentration: $\chi^2_{(6)} = 60, p < 0.001$).

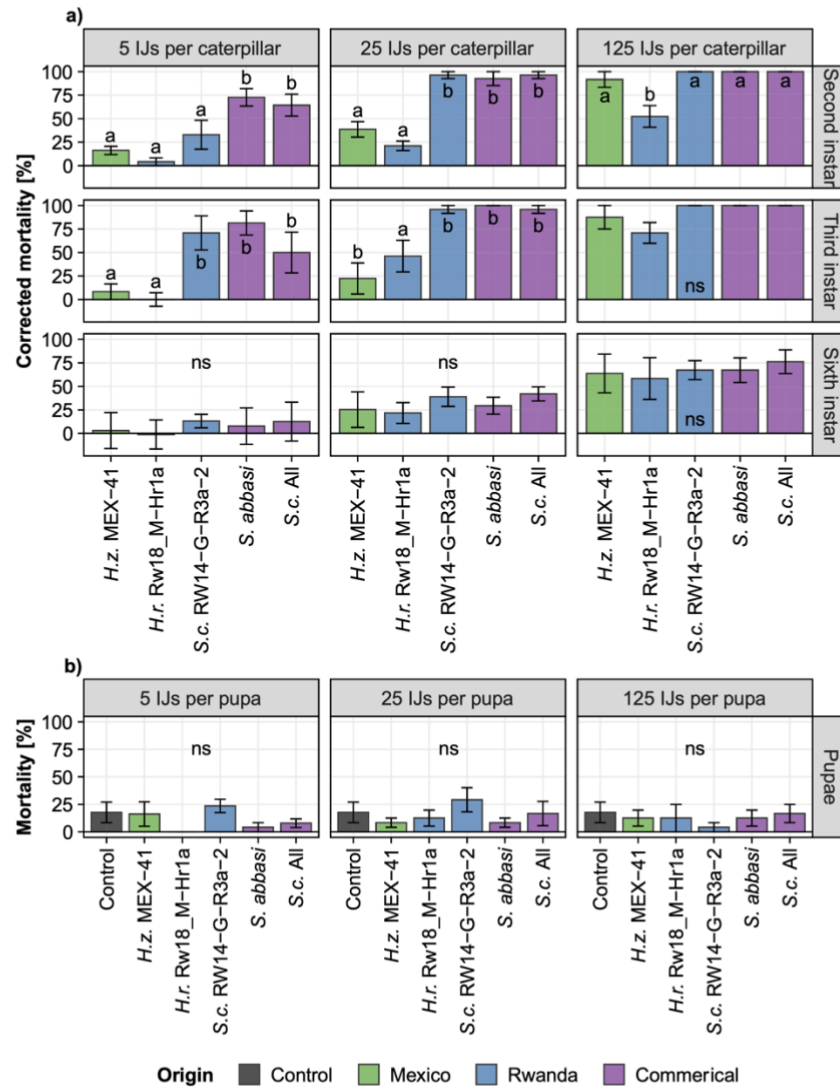


Figure 5. Virulence (mean \pm SE) of the most promising Mexican, Rwandan and commercial entomopathogenic nematode strains on (a) second-, third- and sixth-instar caterpillars as well as on (b) pupae of *Spodoptera frugiperda* in small arenas. *H. zacatecana* (H.z.), *H. ruandica* (H.r.), *Steinernema abbasi* and *S. carpocapsae* (S.c.). Mortality was evaluated seven days post inoculation with either 5, 25 or 125 infective juvenile nematodes per caterpillar (a) or pupa (b). The virulence of each strain was compared to one another using multiple-comparisons corrected for false discovery using the Benjamini and Hochberg method. Letters indicate significant differences ($p < 0.05$) between treatments (ns = non-significant differences).

Of all concentrations and FAW stages tested, the most virulent and equally effective EPN strains were the commercial *S. carpocapsae* All and *S. abbasi*, as well as the Rwandan *S. carpocapsae* RW14-G-R3a-2 ($p > 0.05$; Figure 5). The latter Rwandan strain was as effective as the two commercial strains ($p > 0.05$) on every development stage of FAW and at every tested concentration, except when five IJs were applied against second-instar caterpillars ($p < 0.05$; Figure 5). These strains were followed by *H. zcatecana* MEX-41 ($p < 0.001$). The Rwandan *H. ruandica* Rw18_M-Hr1a appeared to be the least virulent strain (Rw18_M-Hr1a vs. MEX-41: $p < 0.05$; Rw18_M-Hr1a vs. Steinernatid strains: $p < 0.001$).

None of the tested EPN strains affected the survival of FAW pupae at the three tested concentrations of EPNs as compared to the control treatment with water only ($p > 0.05$; Figure 5b).

The killing speed of the most promising Rwandan, Mexican and commercial EPN differed significantly ($\chi^2_{(5)} = 287, p < 0.001$; Figure 6) and was dependent on EPN concentration ($\chi^2_{(2)} = 156, p < 0.001$), as well as the caterpillar developmental stage ($\chi^2_{(2)} = 30, p < 0.001$). Across all concentrations and stages tested, the fastest strains in killing FAW were the commercial strains *S. carpocapsae* All and *S. abbasi* as well as the Rwandan *S. carpocapsae* RW14-G-R3a-2 ($p > 0.05$; Figure 6). The Mexican *H. zcatecana* MEX-41 was slower ($p < 0.001$), and the Rwandan *H. ruandica* Rw18_M-Hr1a was the slowest EPN in killing FAW (Rw18_M-Hr1a vs. MEX-41: $p < 0.05$; Rw18_M-Hr1a vs. Steinernatid strains: $p < 0.001$).

At the highest concentration of EPN used (125 IJs per caterpillar), the three fastest EPNs (*S. carpocapsae* All, *S. abbasi* and *S. carpocapsae* RW14-G-R3a-2) killed 50% of either second- or third-instar caterpillars within one or two days (Figure 6). They needed three days to reach LT_{50} on sixth-instar caterpillars. The Mexican *H. zcatecana* MEX-41 killed 50% of second-, third- and sixth-instar caterpillars within three, two and four days, respectively, while the Rwandan *H. ruandica* Rw18_M-Hr1a needed seven, three and six days.

At the lowest concentration (5 IJs per caterpillar), the two commercial strains *S. carpocapsae* All and *S. abbasi* killed 50% of second-instar caterpillars within three or four days, respectively. None of the other nematodes caused more than 50% mortality of second-instar caterpillars within the seven-day exposure time. On third-instar caterpillars, the commercial strains *S. carpocapsae* All and *S. abbasi* as well as the Rwandan *S. carpocapsae* RW14-G-R3a-2 needed five, three and four days, respectively, to kill 50% of the caterpillars, while *H. ruandica* Rw18_M-Hr1a and *H. zcatecana* MEX-41 never reach 50% mortality. None of the EPN killed more than 50% of the sixth-instar caterpillars within seven days, when only five IJs were used.

All strains could reproduce in all FAW caterpillar stages tested, but not in pupae (Table S3). The dose of EPN used to inoculate the caterpillars as well as the insect stage at death, both positively affected EPN propagation (EPN dose: $\chi^2_{(2)} = 845, p < 0.001$; insect stage: $\chi^2_{(3)} = 3959, p < 0.001$). Notably, older larvae yielded more IJs than smaller ones per unit of weight. At a dose of five IJs per caterpillar, *S. carpocapsae* All and RW14-G-R3a-2 did not reproduce in sixth-instar caterpillars. At the same dose, the reproduction of *H. ruandica* strain Rw18_M-Hr1a in second- and third-instar caterpillars could not be evaluated as this nematode killed too few caterpillars. On average, a sixth-instar caterpillar (306 ± 89 mg at death [mean \pm SD]) inoculated with 125 IJs of *H. ruandica*, *H. zcatecana*, *S. abbasi* or *S. carpocapsae* produced $312'400 \pm 131'249$ (N = 15), $188'023 \pm 117'192$ (N = 15), $105'667 \pm 644'424$ (N = 18), $81'653 \pm 62'120$ (N = 36) IJs per larva (mean \pm SD), respectively.

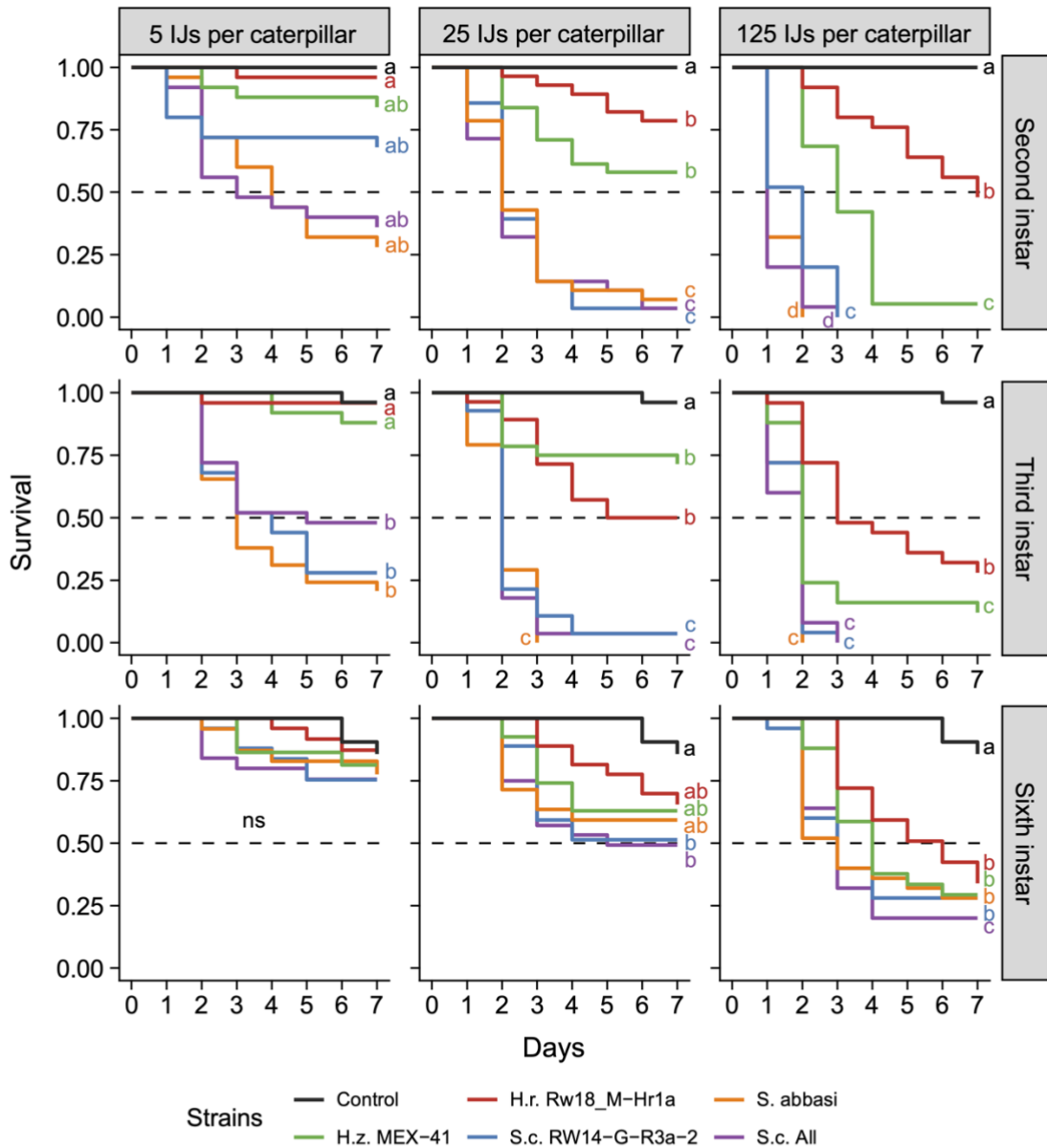


Figure 6. Killing speed of the most promising Mexican, Rwandan and commercial entomopathogenic nematode strains on second-, third- and sixth-instar caterpillars in small bioassay arenas. *H. zatecana* (H.z.), *H. ruandica* (H.r.), *Steinernema abbasi* and *S. carpocapsae* (S.c.). The mortality of individual caterpillars was evaluated over seven days post inoculation with either 5, 25 or 125 infective juvenile nematodes per caterpillar. The dotted horizontal line represents a probability of 50% survival. Letters indicate significant differences ($p < 0.05$) obtained from multiple comparisons corrected for false discovery using the Benjamini and Hochberg method (ns = non-significant differences).

4. Discussion

We found that entomopathogenic nematodes (EPN) isolated from the invasive range of the fall armyworm (FAW) can be as effective in killing it as either the Mexican EPN that have likely co-evolved with FAW or as commercial EPN. For example, with a concentration as low as 25 nematodes per caterpillar, the Rwandan *Steinernema carpocapsae* RW14-G-R3a-2 was as virulent as the best performing Mexican and commercial EPN strains tested (Figure 5). This demonstrates that locally isolated EPN can be excellent candidates for the development of local biological control solutions against FAW. Although most EPN species are considered cosmopolitan, and therefore often do not require specific registration, the

use of local strains may reduce potential risk of using genetically distinct alien strains or species as biocontrol agents in a target region.

In line with previous studies, we showed that EPN can be highly virulent against FAW caterpillars under laboratory conditions [17–19,39–41]. We further confirm here that the virulence of EPN on FAW varies importantly not only among species [17,22,40,41], but also among strains within a species [19]. For instance, we observed that *Heterorhabditis bacteriophora* strain MEX-17 was four times more virulent than *H. bacteriophora* MEX-33 (Figure 1). Similarly, Andalo et al. [17] reported that *Steinernema riobrave* was poorly effective against FAW, even at high concentrations (500 infective juveniles (IJs) per caterpillar), whereas we show that another *S. riobrave* was among the most effective strains in the present study (Figures 1–3; strain MEX-15). The high variability in virulence between as well as within species indicates that thorough EPN screenings are essential before implementing biological control programs using EPN against FAW, and other insect pests.

The results show that the mortality of FAW caterpillars increases, as expected, with the concentration of EPN applied, but decreases with caterpillar age (instar), and that EPN cannot successfully infect pupae (Figure 5). It is known that pupae are often more resistant than other immature stages [42–45], but our results are inconsistent with Acharya et al. [22] who reported a 67% reduction in the emergence rate of FAW pupae after being exposed for five days to 600 IJs of *S. carpocapsae*. Similarly, Fuxa et al. [19] observed 20% mortality within three days of FAW pupae exposed to 40 IJs of *Steinernema feltiae*. We used either similar EPN concentrations [19] or exposure times and EPN species [22], implying that these discrepancies in pupal mortality are explained by the use of different EPN strains. However, for these previous studies it is not excluded that lingering EPN actually infected the emerging adults rather than the pupae. By dipping the pupae in a weak bleach solution before adult emergence, we eliminated this possibility. The infectiousness of EPN against adult lepidopteran has seldom been studied, but the few reports indicate that the insects are indeed susceptible to EPN at this stage [42,46,47].

With a concentration as low as 125 nematodes per caterpillar, the EPNs were found to be highly virulent (Figure 5). Such low, but effective concentrations are encouraging, as normally billions of EPN are applied per hectare in biological control programs [48,49]. It is realistic to envision that the application of just a few thousand EPN per maize plant suffices to significantly control FAW. Indeed, we have since found that the application of only 3000 EPNs per plant can be as effective as the use of a chemical insecticide (cypermethrin 5%) in controlling FAW under field conditions [50].

Effective FAW control not only demands highly virulent strains, but it will also require strains that are resistant to aboveground abiotic environmental factors. The EPN will have to be applied onto plant foliage in order to reach the caterpillars. In this aboveground environment EPN are exposed to, for them, unusually harsh and unfavorable conditions in terms of temperatures, UV light and risk of desiccation. Identifying highly infectious EPN strains that can tolerate these abiotic factors would further increase their potential as effective biocontrol agents against FAW under field conditions [51–54].

5. Conclusions

We show in this study that EPN isolated in the area of invasion of FAW can be as effective as area-of-origin or commercial EPNs to control FAW. The most virulent Rwandan EPN was *S. carpocapsae* RW14-G-R3a-2, which killed FAW caterpillars within just a few days (Figure 6). This killing power is a clear advantage over other biological control agents, such as entomopathogenic fungi, which usually need five to nine days to kill caterpillars and cannot actively search for their target [19,55–57]. Overall, we demonstrate that locally isolated EPN can be promising candidates for the biological control of FAW in invasive regions such as East Africa, and that our screening approach offers possibilities for developing countries to produce specific biocontrol products without relying on external sources.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/insects13020205/s1>, Table S1: Reproduction of Mexican nematodes on third-instar fall armyworm caterpillars, Table S2: Reproduction of Rwandan, Mexican and commercial nematodes on third-instar fall armyworm caterpillars, Table S3: Reproduction of the most promising Rwandan, Mexican and commercial entomopathogenic nematode strains on second-, third- and sixth-instar caterpillars.

Author Contributions: Conceptualization, P.F., S.T. and T.C.J.T.; methodology, P.F., S.T. and T.C.J.T.; formal analysis, P.F.; investigation, P.F. and L.D.G.; resources, R.A.R.M., P.B., J.S.B., P.K., J.K., B.W., D.B., S.T. and T.C.J.T.; data curation, P.F. and L.D.G.; writing—original draft preparation, P.F.; writing—review and editing, L.D.G., R.A.R.M., P.B., J.S.B., P.K., J.K., B.W., D.B., T.D., S.T. and T.C.J.T.; visualization, P.F. and T.D.; supervision, S.T. and T.C.J.T.; project administration, P.F., J.K., P.K., S.T. and T.C.J.T.; funding acquisition, S.T. and T.C.J.T. All authors have read and agreed to the published version of the manuscript.

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Supplementary Material

Supplementary Table S1. Propagation of Mexican nematodes in fall armyworm caterpillars (mean \pm SD). Individual third-instar caterpillars were exposed to ten infective juvenile nematodes in small arenas in laboratory. Reproduction was evaluated in nematode traps three weeks post mortality of the caterpillars in three independent experiments. The reproduction of strains that killed less than five caterpillars was not evaluated. Note: Nematodes killing speed varies importantly among strains (Figure 2). Hence, at the time of death, the caterpillars are not all at the same stage.

Species	Strain	IJs/mg \pm SD	Number of cadavers
<i>S. carpocapsae</i>	All (Nemastar [®])	372 \pm 298.5	13
<i>H. mexicana</i>	MEX-26	361 \pm 111.6	8
<i>H. bacteriophora</i>	MEX-30	333 \pm 59.4	5
<i>H. bacteriophora</i>	MEX-36	327 \pm 58.2	5
<i>H. zacatecana</i>	MEX-40	325 \pm 80.6	6
<i>H. bacteriophora</i>	MEX-21	316 \pm 95.7	11
<i>H. bacteriophora</i>	MEX-22	309 \pm 99.4	7
<i>H. bacteriophora</i>	MEX-16	299 \pm 48.8	7
<i>H. zacatecana</i>	MEX-41	297 \pm 164.1	17
<i>H. bacteriophora</i>	MEX-43	294 \pm 43.5	5
<i>H. bacteriophora</i>	MEX-37	291 \pm 155.4	8
<i>H. zacatecana</i>	MEX-39	290 \pm 184.2	10
<i>H. bacteriophora</i>	MEX-44	286 \pm 52.9	7
<i>H. bacteriophora</i>	MEX-46	283 \pm 134.7	9
<i>H. mexicana</i>	MEX-47	282 \pm 159.1	12
<i>H. bacteriophora</i>	MEX-38	282 \pm 64.8	8
<i>H. bacteriophora</i>	MEX-35	273 \pm 73.3	11
<i>H. atacamensis</i>	MEX-20	272 \pm 41.1	5
<i>H. bacteriophora</i>	MEX-34	259 \pm 157.1	5
<i>H. bacteriophora</i>	MEX-31	249 \pm 62.2	6
<i>H. bacteriophora</i>	MEX-17	249 \pm 120.7	12
<i>H. bacteriophora</i>	MEX-42	232 \pm 137.4	10
<i>H. mexicana</i>	MEX-25	227 \pm 150.3	9
<i>H. bacteriophora</i>	MEX-32	212 \pm 145.7	4
<i>H. bacteriophora</i>	MEX-14	211 \pm 104.5	6
<i>H. bacteriophora</i>	MEX-45	202 \pm 163.0	6
<i>H. bacteriophora</i>	MEX-29	200 \pm 137.5	7
<i>H. bacteriophora</i>	MEX-23	186 \pm 105.6	5
<i>S. riobrave</i>	MEX-15	119 \pm 162.0	12

Supplementary Table S2. Reproduction of Mexican, Rwandan and commercial nematodes in fall armyworm caterpillars (mean \pm SD). Individual third-instar caterpillars were exposed to ten infective juvenile nematodes in small arenas. Reproduction was evaluated in nematode traps three weeks post mortality of the caterpillars in three independent experiments. The reproduction of strains that killed less than five caterpillars was not evaluated. Note: Nematodes killing speed varies importantly among strains (Figure 4). Hence, at the time of death, the caterpillars are not all at the same stage.

Species	Strain	IJs/mg \pm SD	Number of cadavers
<i>H. indica</i>	LN2 (HR-HI [®])	426 \pm 176.0	15
<i>H. ruandica</i>	Rw18_M-Hr1b	422 \pm 85.4	12
<i>H. ruandica</i>	Rw18_M-Hr1a	349 \pm 178.5	11
<i>S. abbasi</i>	NA	345 \pm 212.3	12
<i>H. zacatecana</i>	MEX-41	337 \pm 149.4	10
<i>H. bacteriophora</i>	MEX-17	310 \pm 88.1	6
<i>H. bacteriophora</i>	NA (Dianem [®])	303 \pm 51.2	8
<i>H. ruandica</i>	RW14-N-C4a	290 \pm 188.6	11
<i>S. carpocapsae</i>	All (Nemastar [®])	280 \pm 249.4	23
<i>H. mexicana</i>	MEX-47	246 \pm 199.7	6
<i>H. bacteriophora</i>	MEX-35	237 \pm 174.5	8
<i>S. riobrave</i>	MEX-15	176 \pm 177.3	14
<i>S. carpocapsae</i>	RW14-G-R3a-2	171 \pm 285.0	8

Supplementary Table S3. Propagation of the most promising Mexican, Rwandan and commercial entomopathogenic nematode strains (mean \pm SD). Individual second-, third- or sixth-instar caterpillars were exposed to either 5, 25 or 125 infective juvenile nematodes in small arenas. Reproduction was evaluated in nematode traps three weeks post mortality of the caterpillars in three independent experiments. The reproduction of strains that killed less than five caterpillars was not evaluated (NA). Note: Nematodes killing speed varies importantly among strains (Figure 6). Hence, at the time of death, the caterpillars are not all the same stage.

Inoculation of 2 nd instar caterpillars		Propagation EPN dose (IJs/arena)					
Species	Strain	5		25		125	
		IJs/mg \pm SD	N	IJs/mg \pm SD	N	IJs/mg \pm SD	N
<i>S. abbasi</i>	NA	27 \pm 70.5	17	247 \pm 254.6	20	339 \pm 293.4	19
<i>S. carpocapsae</i>	All (Nemastar [®])	78 \pm 153.5	16	288 \pm 342.8	21	181 \pm 244.0	19
<i>H. zacatecana</i>	MEX-41	240 \pm 199.3	4	397 \pm 234.5	7	268 \pm 222.8	15
<i>S. carpocapsae</i>	RW14-G-R3a-2	205 \pm 301.0	5	134 \pm 197.2	19	250 \pm 318.9	16
<i>H. ruandica</i>	Rw18_M-Hr1a	NA		187 \pm 180.5	5	340 \pm 177.3	11

Inoculation of 3 rd instar caterpillars		Propagation EPN dose (IJs/arena)					
Species	Strain	5		25		125	
		IJs/mg \pm SD	N	IJs/mg \pm SD	N	IJs/mg \pm SD	N
<i>S. abbasi</i>	NA	122 \pm 172.4	21	253 \pm 238.0	22	264 \pm 205.0	21
<i>S. carpocapsae</i>	All (Nemastar [®])	114 \pm 178.1	11	165 \pm 204.8	26	329 \pm 227.2	25
<i>H. zacatecana</i>	MEX-41	NA		235 \pm 201.5	7	217 \pm 184.5	19
<i>S. carpocapsae</i>	RW14-G-R3a-2	48 \pm 124.5	18	124 \pm 175.9	24	308 \pm 224.6	24
<i>H. ruandica</i>	Rw18_M-Hr1a	NA		259 \pm 174.6	12	366 \pm 179.8	17

Inoculation of 6 th instar caterpillars		Propagation EPN dose (IJs/arena)					
Species	Strain	5		25		125	
		IJs/mg \pm SD	N	IJs/mg \pm SD	N	IJs/mg \pm SD	N
<i>S. abbasi</i>	NA	72 \pm 150.9	5	305 \pm 283.2	11	376 \pm 219.3	18
<i>S. carpocapsae</i>	All (Nemastar [®])	0 \pm 1.2	6	168 \pm 206.0	14	309 \pm 154.4	20
<i>H. zacatecana</i>	MEX-41	614 \pm 477.2	4	752 \pm 278.9	10	582 \pm 374.0	17
<i>S. carpocapsae</i>	RW14-G-R3a-2	0 \pm 0.3	6	178 \pm 231.1	13	252 \pm 192.4	18
<i>H. ruandica</i>	Rw18_M-Hr1a	NA		732 \pm 552.7	9	876 \pm 435.8	16

Chapter 3

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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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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Above-ground pest control

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 agroecosystems. 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; Rwo-mushana 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; Rwo-mushana et al., 2018). To mitigate the impact of FAW, several control options are available including synthetic insecticides, bio-pesticides 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; Hiltbold 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 particularly 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, Gedinne, 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, Schwentental, 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. $350 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$). 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. $300 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$). 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 $21.3 \pm 6.6 \text{ }^\circ\text{C}$ (mean ± sd; max = 38.5 °C; min = 13.3 °C) and was $20.3 \pm 7.3 \text{ }^\circ\text{C}$ (mean ± sd; max = 38.7 °C; min = 7.3 °C) at the Nyamagabe site. At the Rubona site, the mean daily rainfall was $2.5 \pm 6.2 \text{ mm}$



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 \pm 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 \times CML204, Rwanda Animal and Agricultural Resource Board, Huye, Rwanda) in the four fields measuring approximately 25 m by 25 m 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 \pm sd) and carried on average 7.8 ± 1.6 , 7.7 ± 1 , 5.9 ± 0.7 and 9.1 ± 1.1 leaves (mean \pm 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 \pm 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 \times 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 μ L/mL (0.19 μ g 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

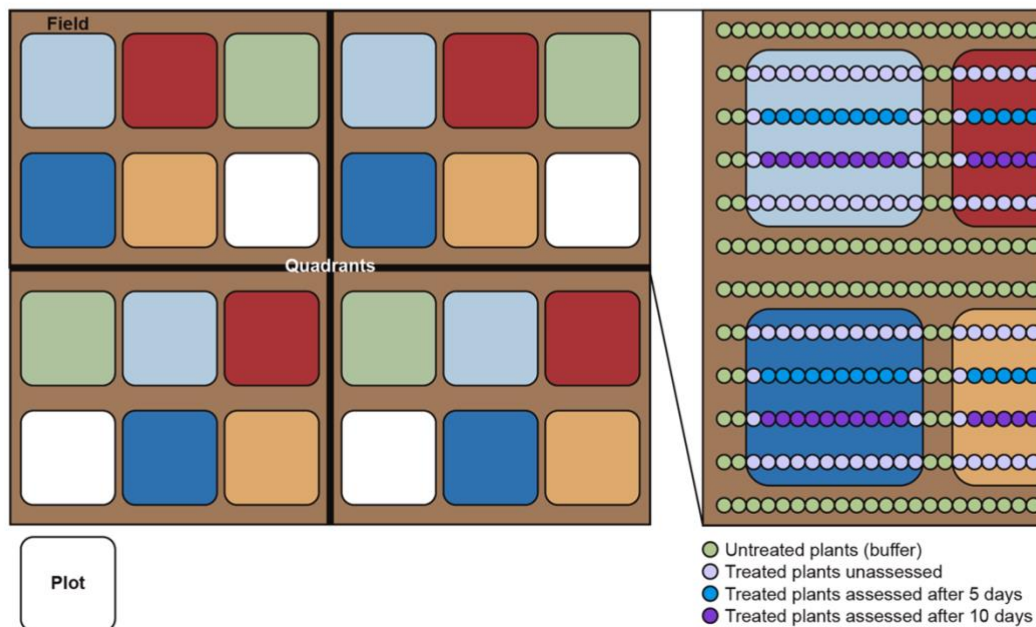


Fig. 2. Field experimental setup. Each field measured 25 m by 25 m. Maize was sown in 24 rows separated by 70 cm. Within rows, plants were separated by 30 cm. The fields were divided into four quadrants to account for the spatial variation of FAW across the fields. Quadrants were subdivided into six 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 ($4 \pm 13\%$ survival [mean \pm 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

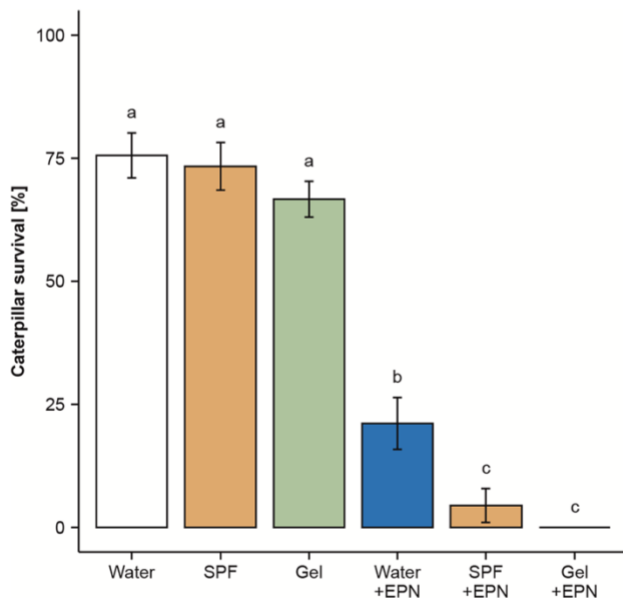


Fig. 3. Caterpillar survival (mean \pm se) six days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 or formulations without nematodes into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes 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).

($21 \pm 20\%$ survival [mean \pm 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 $94 \pm 18\%$ and in water $72 \pm 27\%$ (mean \pm sd).

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 $41 \pm 15\%$ [mean \pm sd] after five days and by $34 \pm 40\%$ after ten days, whilst cypermethrin achieved $35 \pm 29\%$ and $41 \pm 42\%$ 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).

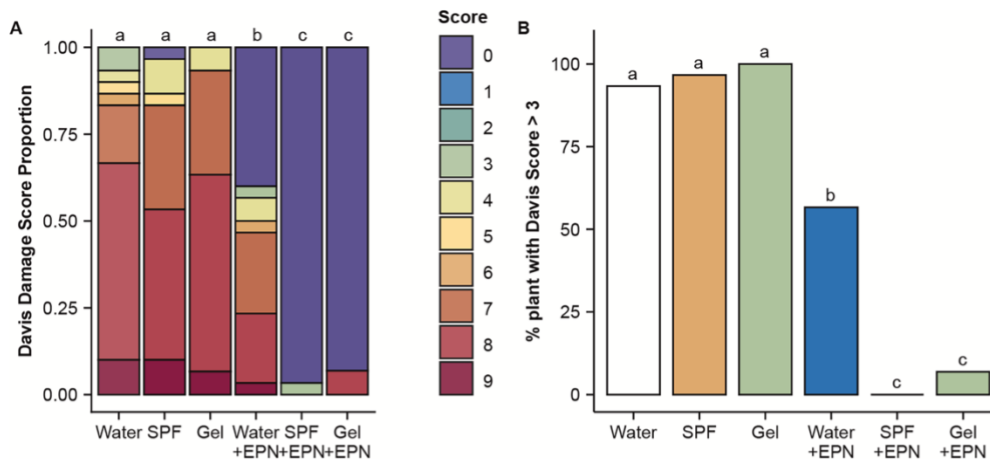


Fig. 4. Leaf damage on maize plants six days after applying differently formulated *Steinerma carpocapsae* RW14-G-R3a-2 or formulations without nematodes into leaf whorls. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 2 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 treatment differences ($p < 0.05$) using the [Benjamini & Hochberg method \(1995\)](#).

ments according to (A) multiple comparisons or (B) a pairwise permutation test. Both tests were corrected for false discovery using the [Benjamini & Hochberg method \(1995\)](#).

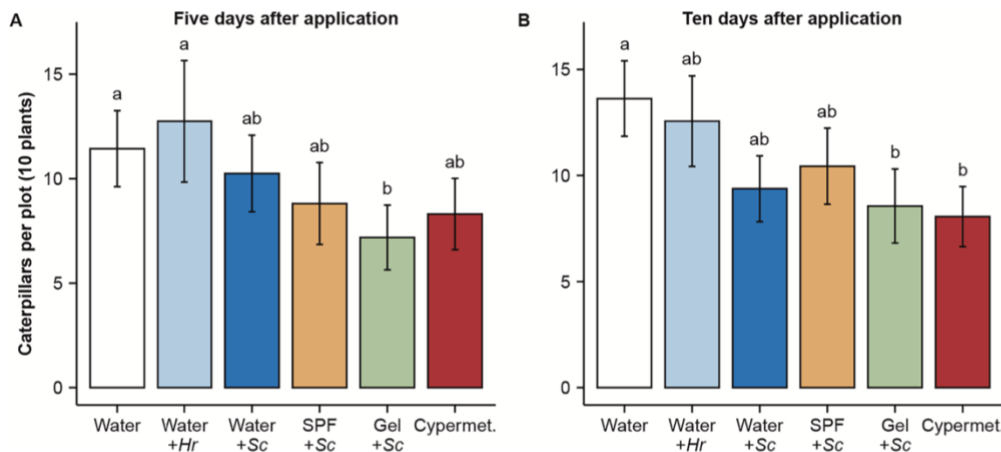


Fig. 5. Average number of fall armyworms per plot of ten plants five (A) and ten days (B) after applying differently formulated *Steinerma carpocapsae* RW14-G-R3a-2 (Sc) or *Heterorhabditis ruandica* Rw18_M-Hr1a (Hr) into leaf whorls of maize, as compared to application of water and a commonly used insecticide, 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 *Steinerma 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 *Steinerma* sp. in water into the whorl of artificially infested maize. Using a similar approach, [Negrisoni et al. \(2010\)](#) achieved low efficacy with *Steinerma carpocapsae* and *Heterorhabditis indica*. We also did not

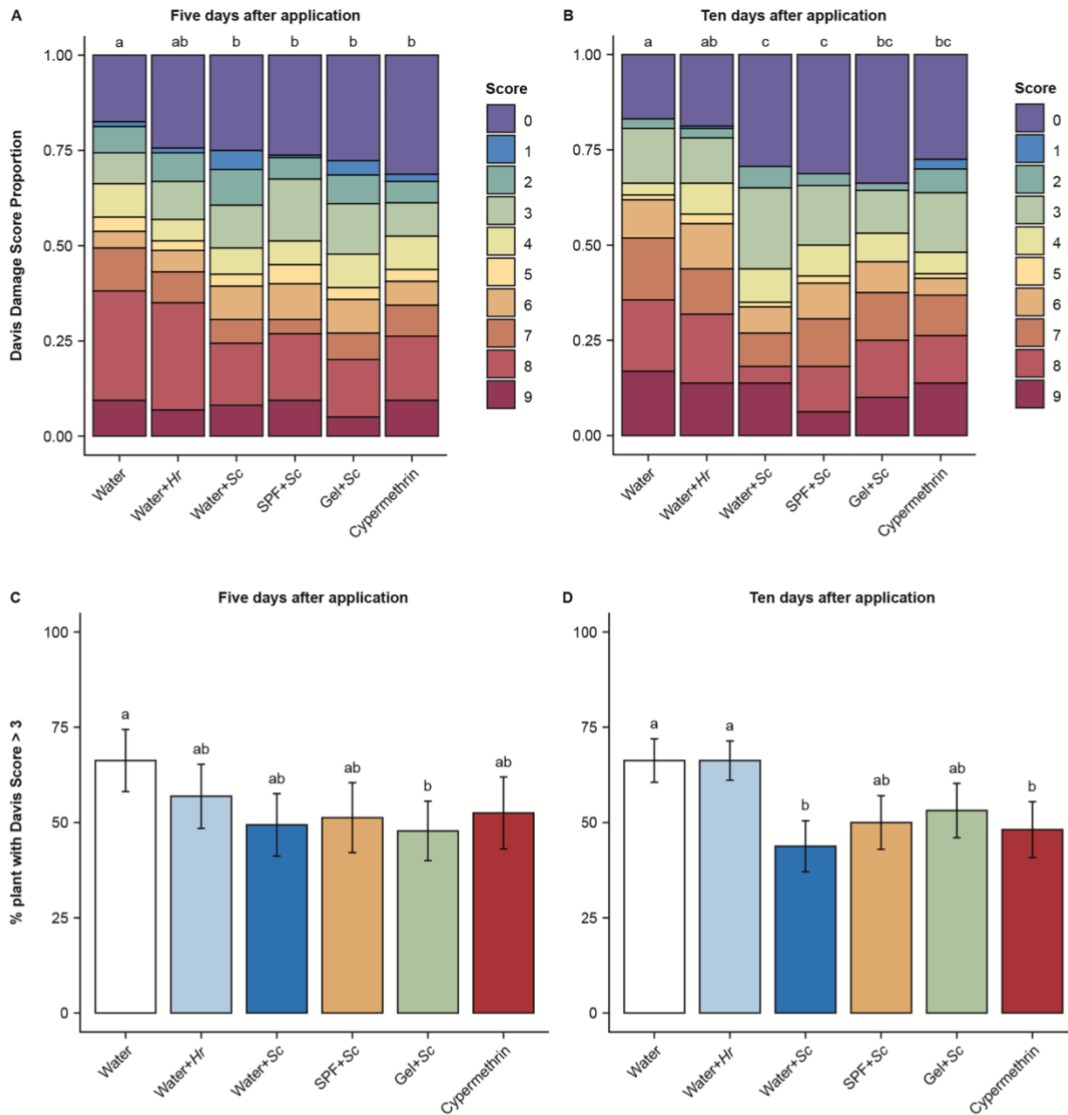


Fig. 6. Leaf damage at five (A and C) and ten (B and D) days after applying differently formulated *Steinerema carpocapsae* RW14-G-R3a-2 (Sc) or *Heterorhabditis ruandica* Rw18_M-Hr1a (Hr) into leaf whorls, as compared to the application of water, as a control, and a commonly used insecticide cypermethrin. 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 *Steinerema* 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

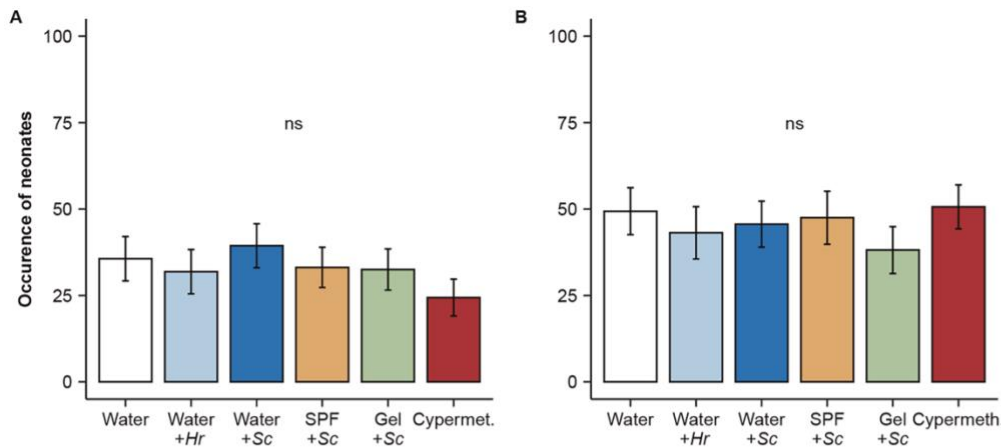


Fig. 7. Proportion of plant re-infested with fall armyworm neonates (A) five and (B) ten days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 (Sc) or *Heterorhabditis ruandica* Rw18_M-Hr1a (Hr) into leaf whorls, and in comparison to the application of just water and a commonly 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 (Hiltbold, 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 (Hiltbold 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; Hiltbold 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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Supplementary Material

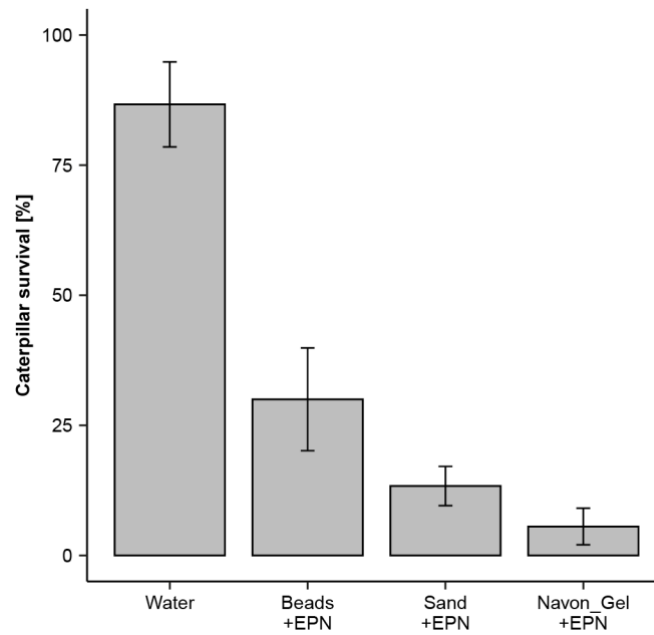


Figure S1: Caterpillar survival (mean±se) six days after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 1 ml of formulation per plant. Five cages (each containing two plants) per treatment were used in one experiment (n = 5 cages; 10 plants per treatment).

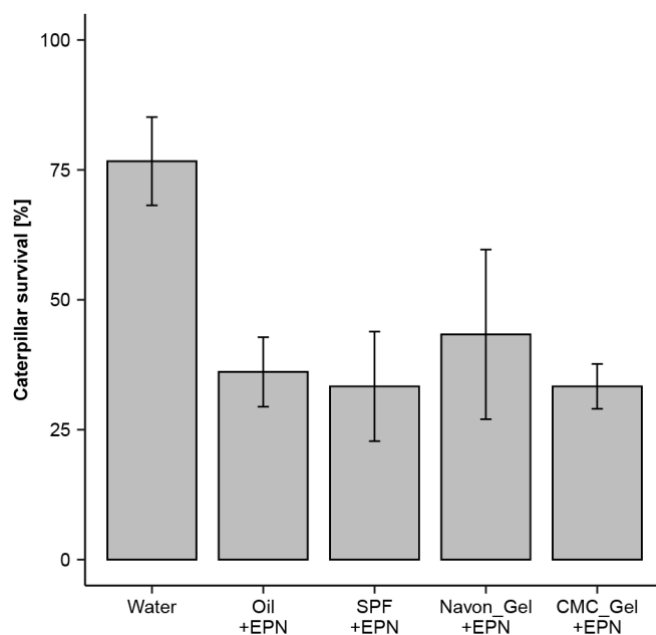


Figure S2: Caterpillar survival (mean±se) six days after applying differently formulated *Heterorhabditis ruandica* Rw18_M-Hr1a into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 1 ml of formulation per plant. Six cages (each containing two plants) per treatment were used in one experiment (n = 6 cages; 12 plants per treatment).

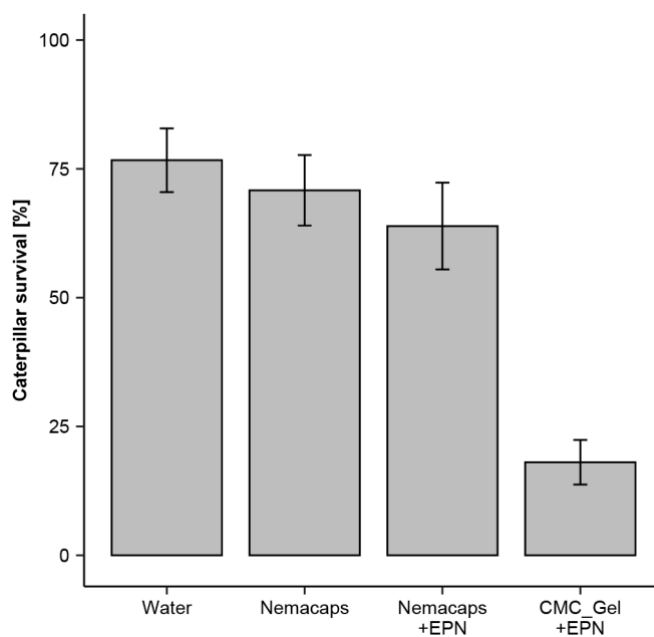


Figure S3: Caterpillar survival (mean \pm se) six days after applying differently formulated *Heterorhabditis ruandica* Rw18_M-Hr1a into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 1 ml of formulation per plant. Six cages (each containing two plants) per treatment were used in each of two independent experiments (n = 12 cages; 24 plants per treatment).

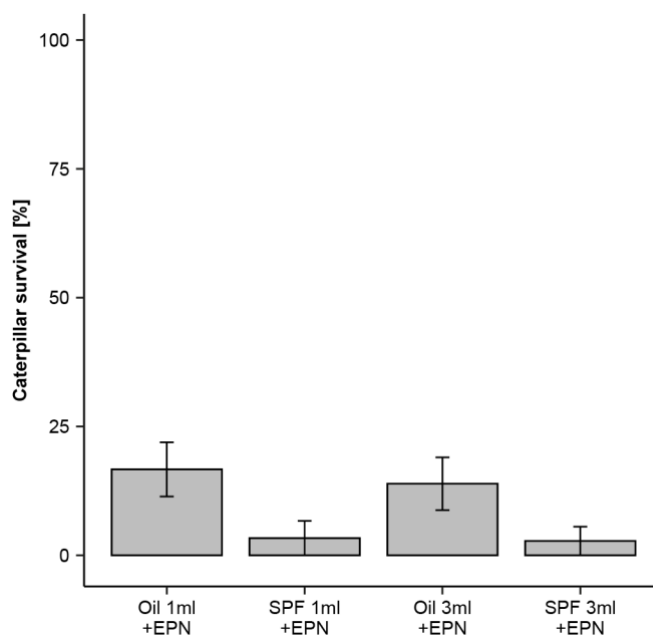


Figure S4: Caterpillar survival (mean \pm se) six days after applying differently formulated *Heterorhabditis ruandica* Rw18_M-Hr1a into the whorl of maize plants. Plants were infested with three third-instar fall armyworms per plant. About 3000 infective juvenile nematodes were applied in 1 or 3 ml of formulation per plant. Five cages (each containing two plants) per treatment were used in one experiment (n = 5 cages; 10 plants per treatment).

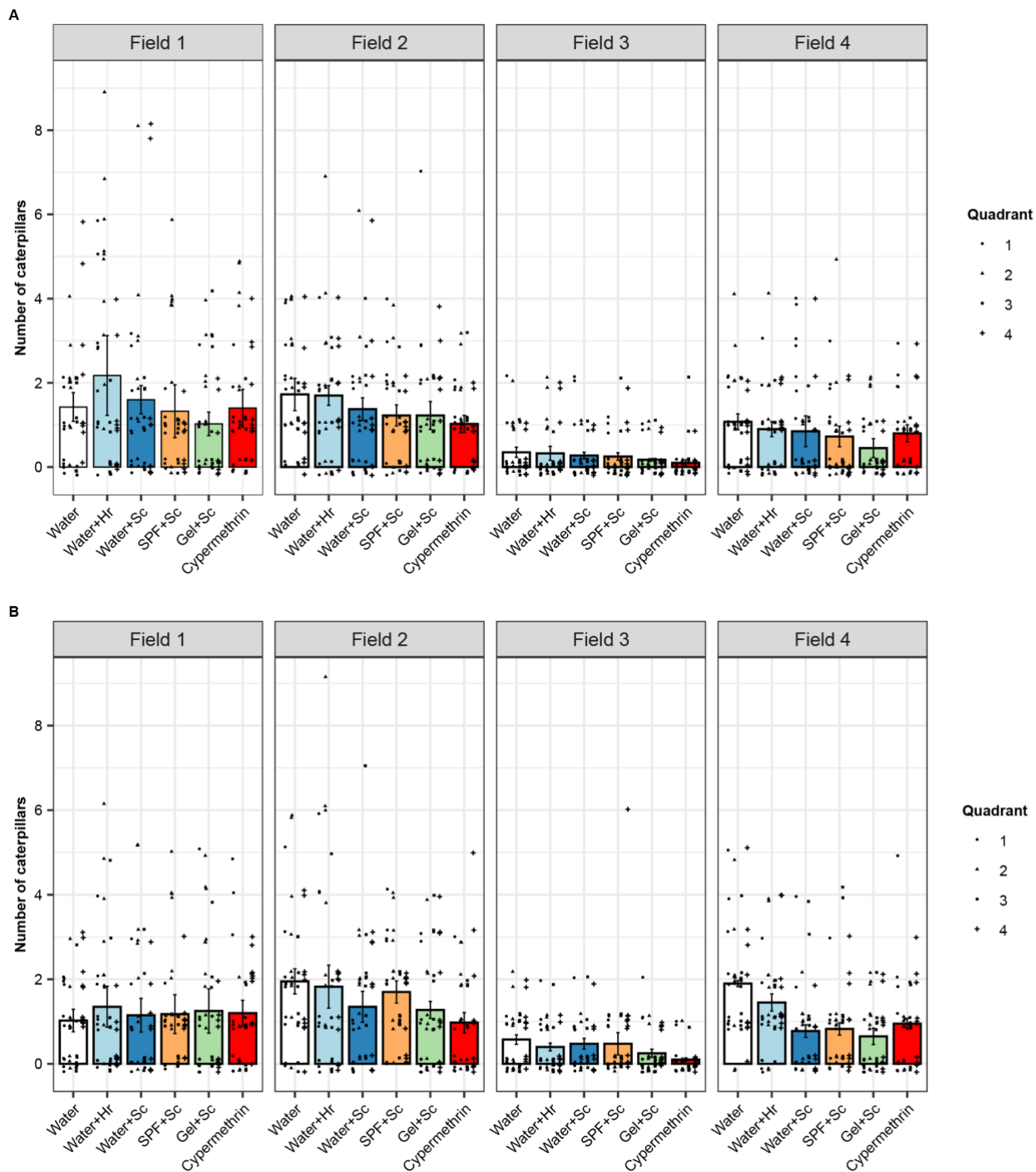


Figure S5: Number of fall armyworm caterpillars per treatments within each field, five (A) and ten days (B) after applying differently formulated *Steinernema carpocapsae* RW14-G-R3a-2 (*Sc*) or *Heterorhabditis ruandica* Rw18_M-Hr1a (*Hr*) into leaf whorls of maize, as compared to a commonly used insecticide, cypermethrin (Cypermethtin). Bars represents the average number caterpillars per treatments. Dots represents the number of caterpillars in each plot. Four experiments (= maize fields) with natural infestation of fall armyworms 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. Treatment consisted of about 3000 infective juvenile nematodes 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).

Equations

With the laboratory data, the average efficacy of each formulation in killing FAW was estimated as follows:

$$(Eq. B1) \quad \bar{E}_a = \frac{1}{15} \sum_{i=1}^{15} \left(100 - \frac{\alpha_i}{\bar{\beta}} * 100 \right)$$

where, α_i is the number alive caterpillars in the treated cage i , and

$$(Eq. B1.1) \quad \bar{\beta} = \frac{1}{15} \sum_{i=1}^{15} \beta_i$$

is the average number of alive caterpillars in the control cages, with β_i is the number of caterpillars in the control cage i .

With the laboratory data, the average efficacy of each formulation in preventing medium to heavy damage was estimated as follows:

$$(Eq. B2) \quad \bar{E}_b = 100 - \frac{\gamma}{\delta} * 100$$

where, γ is the number of damaged plants in the treatment, and δ is the number of damaged plants in the control.

With the field data, the average efficacy of each treatment in reducing FAW infestation was estimated as follows:

$$(Eq. B3) \quad \bar{E}_c = \frac{1}{16} \sum_{y=1}^4 \sum_{i=1}^4 \left(100 - \frac{\varepsilon_{iy}}{\bar{\zeta}_y} * 100 \right)$$

where, ε_{iy} is the number of caterpillars in the treated plot i in field y , and

$$(Eq. B3.1) \quad \bar{\zeta}_y = \frac{1}{4} \sum_{i=1}^4 \zeta_{iy}$$

is the average number of caterpillars in the control plots of field y , with ζ_{iy} is the number of caterpillars in the control plot i in field y .

Chapter 4

Entomopathogenic nematodes as an effective and sustainable
alternative to control the fall armyworm in Africa

Abstract

The invasion of the fall armyworm into Africa and Asia has massively increased the use of insecticides in maize cultivation. The hazards posed by these toxins call for safer and more sustainable control strategies. Entomopathogenic nematodes are highly lethal to the armyworms, but their application aboveground has been challenging. We evaluated the efficacy of nematodes formulated in an innocuous biodegradable gel made from carboxymethyl cellulose. Field trials in Rwanda revealed that repeated treatments of maize plants with the nematode-gel achieved superior control of armyworms compared to nematodes in a commercial surfactant and to cypermethrin, a commonly applied insecticide. While all three treatments reduced leaf damage, only the gel formulation decreased caterpillars infestation by about 50% and yielded an additional ton of maize per hectare compared to untreated plots. The simple and affordable gel-based formulation can be a safe and highly effective alternative to insecticides for fall armyworm control.

Key words: biological control; Integrated Pest Management; sustainable agriculture; food security; invasive pest

Introduction

The fall armyworm (FAW; *Spodoptera frugiperda*, Smith; Lepidoptera: Noctuidae) is a major pest of maize (corn, *Zea mays*). Originating from the Americas, it has recently spread into Africa and Asia (Luginbill, 1928; Goergen et al., 2016; Sharanabasappa et al., 2018), causing severe plant damage and tremendous economic and yield losses (Day et al., 2017; Baudron et al., 2019; Kenis et al., 2022). Its invasion threatens the livelihoods of millions of farmers (Day et al., 2017; Rwomushana et al., 2018; Babendreier et al., 2020) and has led to an excessive reliance on chemical insecticides (Yang et al., 2021; Kenis et al., 2022). The environmental and health concerns associated with pesticides have prompted the urgent need for safer and more sustainable control measures (Day et al., 2017; Kenis et al., 2022). Several biocontrol agents, including entomopathogenic nematodes (EPN) have emerged as alternatives to chemical insecticides (Guo et al., 2020; Hussain et al., 2021; Kenis et al., 2022).

EPN are minute soil-dwelling roundworms found worldwide (Hominick et al., 2002). They exclusively parasitize insects, which they locate with the help of chemical cues (Turlings et al., 2012) and enter via natural orifices (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). EPN carry symbiotic bacteria in their guts that are released inside their host (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). These bacteria, as well as the EPN, exude toxins that rapidly kill their insect host (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). EPN can kill a large variety of insects and can be easily mass-produced (Ehlers, 2001), also in Africa (Holmes et al., 2015). They are safe to humans and the environment (Ehlers and Hokkanen, 1996)

and have been successfully used as biocontrol agents against belowground insect pests (Georgis et al., 2006). Although EPN are sensitive to UV radiation and desiccation (Kaya and Gaugler, 1993; Lacey and Georgis, 2012; Kagimu et al., 2017), the protective location of the feeding FAW caterpillars, mostly deep within the maize whorl (Luginbill, 1928; Buntin, 1986; Labatte, 1993), makes the maize-FAW system a suitable candidate for EPN application. Moreover, EPN exhibit exceptional virulence against FAW with the ability to kill young FAW caterpillars within twenty-four hours (Richter and Fuxa, 1990; Andaló et al., 2010; Acharya et al., 2020; Fallet et al., 2022a), which is considerably faster than entomopathogenic fungi (Guo et al., 2020; Montecalvo and Navasero, 2021), viruses (Behle and Popham, 2012; Simón et al., 2012; Popham et al., 2021; Hussain et al., 2021) or parasitoids (Hoballah et al., 2004). These high levels of virulence can be attributed to the absence of co-evolution between EPN and FAW, precluding FAW from developing any form of resistance to EPN. This makes it possible to apply far lower dosage of EPN than those normally applied against belowground pests. Furthermore, the isolation of highly virulent EPN strains from local soils eliminates the need for introducing non-native organisms (Fallet et al., 2020; 2022a), thereby avoiding potential regulatory barriers.

In a recent laboratory study and pilot field experiment, we demonstrated the potential of EPN formulated in a cost-effective gel designed to specifically target FAW caterpillars feeding on maize (Fallet et al., 2022b). The preliminary trial with a single application of the EPN-gel formulation suggested it can reduce FAW infestation and plant damage as effectively as cypermethrin, a commonly used contact insecticide (Uzayisenga et al., 2020). Here, we report on the successful application of the EPN-gel throughout a complete maize growing season in Rwanda, encompassing differing climatic conditions and infestation rates. We compared FAW presence and damage as well as maize yield in control plots and plots that were treated with either our novel EPN-gel formulation, a commercial liquid EPN formulation, or cypermethrin. These findings demonstrate that EPN formulated in a biodegradable gel represent a safe and effective alternative to insecticides for FAW control, particularly in smallholder farms.

Material and Methods

Nematodes and formulations

We used the Rwandan EPN *Steinernema carpocapsae* (strain RW14-G-R3a-2) for this study (Yan et al., 2016), as we aim to only use local genotypes and this particular strain has been shown to be highly virulent against FAW (Fallet et al., 2022a). EPN were reared *in vivo* on last instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) as described by White (1927). Harvested nematodes were stored in the dark at cool temperature (12°C) and used within two weeks post-harvest.

Four plant treatments were compared in this study: (1) untreated control, (2) EPN in a commercially available liquid Surfactant-Polymer-Formulation (SPF; e-nema GmbH, Schwentimental, Germany), (3) EPN in a carboxymethyl cellulose (CMC) gel (Fallet et al., 2022b) and (4) a positive control with the pyrethroid contact insecticide cypermethrin.

The SPF and CMC were dissolved in sterile water to a final concentration of 0.11% (as recommended by the provider) and 3% (w/v), respectively. First, 42 g of CMC or 1.54 g of SPF were added to 1'300 ml of water and let to rest. The next day, solutions were vigorously mixed with a whisk until fully dissolved. Then, 2.1 million EPN concentrated in 100 ml of water were added to the formulations and gently mixed. Using a magnifier, we confirmed that the formulations contained about 1'500 EPN/ml (for an application of ~3'000 EPN per plant in a 2 ml spot-injection/spray).

The cypermethrin (Supra EC, ETG inputs ltd, India; 50 g a.i. per liter) was dissolved in water at 1.0 ml/l (~0.1 mg a.i. applied per plant in a 2 ml spot spray). The prepared formulations were kept in cool boxes and used within four hours.

Field sites

The experiments were carried out in six fields in Southern Rwanda at three locations. Two fields (Field I and Field II) were located in an agricultural landscape at the Rwanda Agriculture and Animal Resources Development Board station in Rubona in the district of Huye (Location "Station"; GPS: 2°29'00.6" S, 29°46'29.8" E; altitude 1660 m.a.s.l.). Two fields (Field III and Field IV) were located close to a natural forest edge in the district of Huye (Location "Forest"; GPS: 2°29'10.0" S, 29°46'57.3" E; altitude 1680 m.a.s.l.). The last two fields (Field V and Field VI) were located in an agricultural terrace landscape on a hillside in the district of Nyamagabe (Location "Hillside"; GPS: 2°30'46.0" S, 29°29'58.2" E; altitude 2140 m.a.s.l.). Through the duration of our experiment, the mean temperature during the day (6 am to 6 pm) at each location was 24.4±5.4, 24.1±5.6, 22.1±5.2 °C (mean±sd) and was 17.6±2.7, 18.2±2.4, 14.1±1.5 °C (mean±sd) at night (6 pm to 6 am), for "Station", "Forest" and "Hillside" respectively. In the same order, the average relative humidity recorded was 76.1±18.1, 73.1±18.1, 77.2±18.0% at these locations.

Fields at the "Station" and "Forest" locations measured approximately 16 by 42 m, while the fields at the "Hillside" location measured 5 by 110 m. Five to ten meters separated fields in the same location. They were planted either on the 17th or the 18th of February 2022. The Rwandan maize hybrid RHMN 1601 was sown every 30 cm in rows separated by 70 cm, representing about 47'000 plants per hectare. Fields were fertilized twice with NPK and urea at a rate of 300 kg/ha or 100 kg/ha respectively, once before sowing and a second time after four weeks, in accordance with local practices. Fields were left untreated against insects until the beginning of the experiment.

Experimental design

Fields were divided into 20 plots comprising six rows of 12 to 16 plants (approximately 72-96 plants per plot). Five plots per fields were attributed to one of the four treatments in a systematic block design (30 plots per treatment in total). The four treatments [(1) untreated, (2) EPN in SPF, (3) EPN in CMC gel, (4) cypermethrin] were prepared as described above. Treatments were applied as 2 ml spot-injections/sprays into the whorl of each plants using 20 ml plastic syringes for the gel, or hand sprayers for the SPF and cypermethrin. The first application occurred when at least 30% of the plants were showing FAW damage (upper recommended action threshold; McGrath et al., 2018) and was then repeated every two weeks until the maize flowers emerged. In fields I, II, III and IV (“Rubona” and “Forest” locations), the first application occurred 18-days post sowing (about 90% plants damaged), when plants carried 4-5 leaves. In total, four applications were performed in these fields (“Rubona” and “Forest” locations). In fields V and VI, which were higher in altitude and infested at a later stage, the first applications occurred 38-days post sowing (about 35% plants damaged), when plants carried 7-9 leaves. In total, three applications were done in the “Hillside” fields, before the maize started to flower. All plants within a plot were treated, but only the plants in the four central rows (and at least 1.5 m away from the next plot) were used to collect data.

Data collection

Seven- and fourteen-days after the first, second and third applications, plant damage was visually assessed for forty plants in the centre of each plot using the Davis whorl damage scale (Davis et al., 1992) as described in Toepfer et al. (2021). Briefly, the Davis scale is a top-view assessment, where only the damage to the youngest leaves of the whorl is evaluated, where FAW caterpillars are mostly feeding. Hence, the damage recorded is non-cumulative, as old damages located on lower leaves are not considered. The Davis whorl damage scale ranges from “0” to “9”, whereby a fully intact whorl is attributed a score of “0”, while an almost totally destroyed whorl is attributed a score of “9”. Minor damages are represented by scores ranging from 1-3, while moderate or severe damages by scores ranging from 4-6 or 7-9, respectively. In total, 40 plants from the four central rows of a plot were assessed at both seven- and fourteen-days post applications (200 plants per treatment and field). Plant damage was evaluated by two assessors per plot (each evaluating 20 plants in 20 plots per field). Plant damage was not assessed after the fourth-application (fields I, II, III and IV; only three applications in fields V and VI) as plants were too tall to visually inspect the whorl without damaging the plants.

Five days after the third application (second-to-last application in fields I, II, III and IV; last application in field V and VI), we also evaluated the presence of FAW caterpillars on the plants. For this, ten specific plants within the four central rows of each plot were systematically destroyed to inspect the plants for

caterpillars. The occurrence (yes/no) of young caterpillars (shorter than 0.5 cm) was determined as a proxy for re-infestation, whereas the number of older caterpillars (longer than 0.5 cm) was used as a proxy for treatment efficacy. The dissection of the plants and search for caterpillars was done by several assessors who had no knowledge of the treatment being evaluated, but each assessed plants of all treatments.

At harvest, the number of fully-developed and underdeveloped cobs from 30 plants in the centre of each plot was recorded. The fully-developed cobs were collected and husked to assess cob damage, which was evaluated visually using a “0” to “3” customized damage score. A score of “0” represents an intact cob, while scores of “1”, “2”, or “3” represent minor (<5% damaged kernels), moderate (5-20% damaged kernels) or severe (>20% damaged kernels) cob damage, respectively. The husked cobs from the thirty plants were weighed together to estimate fresh weight (FW) yield per plot and evaluate treatment efficacy. In each plot, the assessments were carried out by two assessors that had no knowledge of the treatment being evaluated.

Data analyses

Statistical analyses were performed using R version 4.2.1 (R Core Team, 2022). Plant damage was analysed using cumulative link mixed models (“ordinal” package; Christensen, 2019) followed by multiple comparisons (“emmeans” package; Lenth, 2022) corrected for false discovery using the Benjamini & Hochberg (1995) method. The damage score given to a plant was used as the response variable. Treatments, the application number (four applications) as well as assessment times (seven- or fourteen-days post treatment) were used as fixed factors, plus their triple interactions. Field number (six fields) and assessors were used as fixed factors, while plots and plants were used as random factors. The damage score probabilities reported were calculated using the function “rating.emmeans” (“RVAideMemoire” package; Hervé, 2022).

Cob damage was analysed using cumulative link mixed models (“ordinal” package; Christensen, 2019), similarly as plant damage. The damage score given to a cob was used as the response variable, while treatments and field number (six fields) were used as fixed factors. Plots were used as a random factor.

Maize yield was analysed using generalized linear mixed model using Template Model Builder (“glmmTMB” package; Brooks et al., 2017) with a gaussian error distribution. Yield per plot was used as the response variable, while treatments and field numbers (six fields) were used as fixed factors. The number of caterpillars (longer than 0.5 cm) per plot, the total number of all cobs (underdeveloped + developed) as well as the number of developed cobs only, were analysed similarly, but with a poisson error distribution. Plots were included here as a random factor to correct for overdispersion. The occurrence of young caterpillars (shorter than 0.5 cm) per plant was also analysed similarly, with a binomial error

distribution. Plots were used as a random factor to account for the repeated observations within a plot. The function “Effect” (“effects” package; Fox and Weisberg, 2019) was used to compute the model estimates presented in the figures and result section. A multiple comparison (“emmeans” package; Lenth, 2022) corrected for false discovery using the Benjamini & Hochberg (1995) method was performed to compare treatments when they had a significant effect.

Results

Plant damage

The overall comparison of the control methods revealed that the whorl damage of maize was different among treatments (Fig. 1 and S1; Treatments: $\chi^2_{(3)} = 36$, $p < 0.001$; triple interaction [Treatments*Application Number*Assessments]: $\chi^2_{(6)} = 35.85$, $p < 0.001$) and increased during the season (Fig. 1A; $\chi^2_{(2)} = 1037$, $p < 0.001$). Throughout the season, untreated plants were consistently more damaged than treated plants, regardless of the treatment (Fig. 1A: see capital letters; $p < 0.001$). Plants treated with the gel-formulated EPN were less damaged than plants treated with EPN formulated in the Surfactant-Polymer-Formulation (SPF) ($p < 0.01$) and untreated plants ($p < 0.001$). The effect of treatment with cypermethrin was intermediate (Fig. 1A). Overall, the probability that a plant suffered severe damage (Davis score > 6) was 14% for the EPN-gel-, 18% for the cypermethrin-, and 19% for the EPN-SPF-treated plants. The highest probability of severe damage was observed in untreated control plants (30%). Hence, the EPN-gel treatment reduced heavy damage by more than 50%.

At all six measured time points (covering six weeks starting when the plants were about three weeks-old), we found the plants treated with the EPN-gel less damaged than untreated plants (Fig. 1A). This was still evident after the third application (>28 days post first application), when plants treated with the EPN-gel were less damaged than all other treatments, including cypermethrin (Fig. 1A: see lowercase letters; at 35 days: gel vs cypermethrin, $p < 0.01$; gel vs SPF, $p < 0.001$; gel vs control, $p < 0.001$; at 42 days: gel vs cypermethrin, $p < 0.001$; gel vs SPF, $p = 0.04$; gel vs control, $p < 0.001$).

Damage was more severe two weeks after each treatment than after one week (Fig. 1B; $\chi^2_{(1)} = 119$, $p < 0.001$), suggesting that FAW reinfested the plants after each treatment and that a more frequent application of the treatments should provide even better protection against FAW.

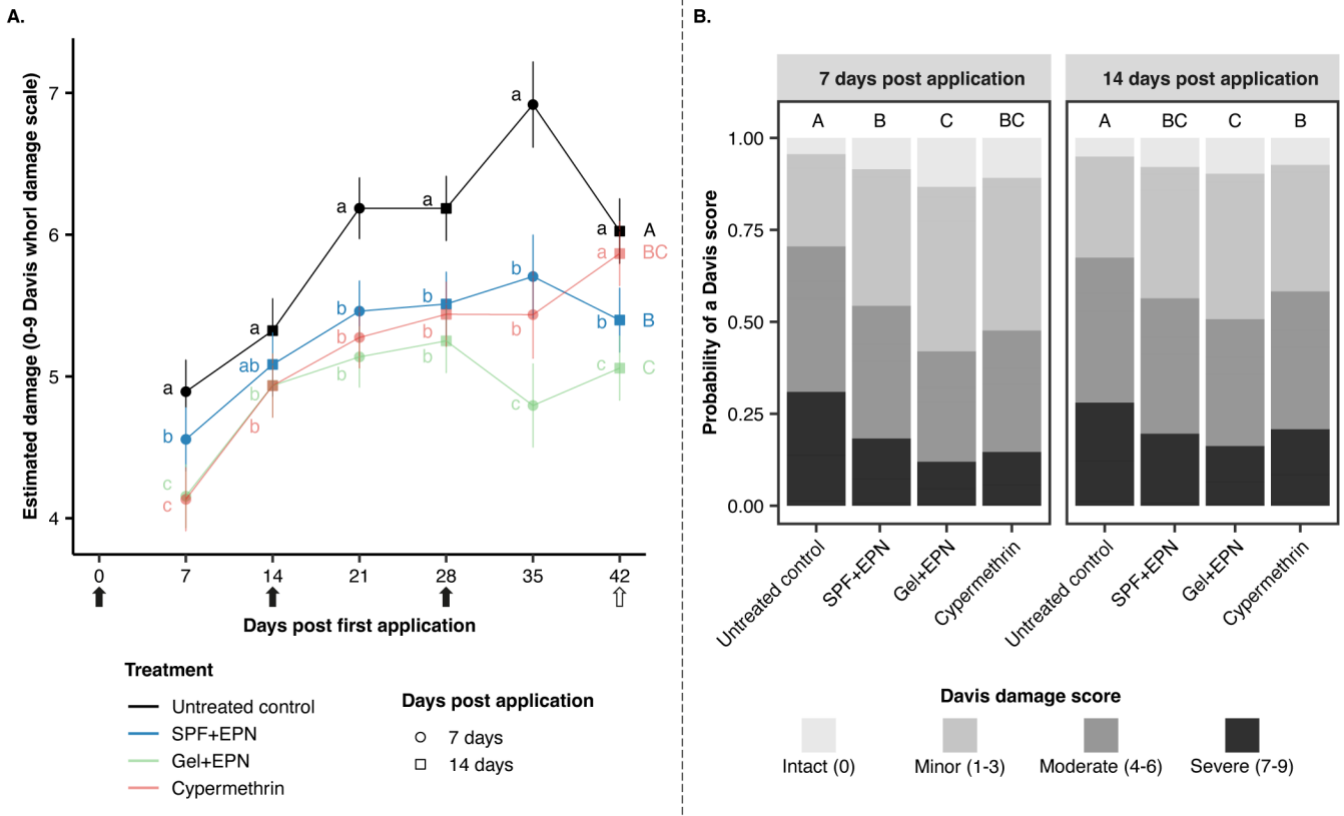


Fig. 6: Whorl damage. (A) Estimated marginal means \pm 95% CI of recorded whorl damage scores averaged for the six fields with each five plots per treatment. Arrows indicate the days of the treatments (black arrows: all fields treated; white arrow: only fields I, II, III and IV treated). (B) Probability of a given damage score within each treatment seven- or fourteen-days post applications averaged for six fields and three applications. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a Surfactant-Polymer-Formulation (SPF) or a carboxymethyl cellulose gel. About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Whorl damage was assessed seven- and fourteen-days after each application for 40 plants per plot (n = 30 plots per treatment) using the Davis whorl damage scale, where a “0” represents an intact whorl and a “9” represents an almost completely or completely destroyed whorl. Data were analysed using cumulative link mixed models. Letters indicate significant differences ($p < 0.05$) according to multiple comparisons corrected for false discovery using the Benjamini & Hochberg method (1995). Fig. 1A, small letters: differences among treatments at a given assessment; capital letters: differences among treatments throughout the field trials.

Fall armyworm infestation

After the third application of the treatments, we dissected a cohort of ten plants in the centre of each plot to evaluate FAW infestation levels. Overall, the number of caterpillars recovered was different among treatments (Fig. 2 and S2; $\chi^2_{(3)} = 22.5$, $p < 0.001$), but only plots treated with the EPN formulated in gel were less infested than untreated control plots (gel vs control: $p < 0.001$). More specifically, on average 13 ± 1.4 SE caterpillars were found in untreated control plots, 12 ± 1.3 in cypermethrin-, 10 ± 1.1 in EPN-SPF- and 6.5 ± 0.8 in EPN-gel-treated plots. Re-infestation by FAW was prevalent and not affected by any of the treatments, as indicated by the similar occurrence of early instar caterpillars (shorter than 0.5cm) on plants in all plots (Fig. S3; $\chi^2_{(3)} = 5.2$, $p = 0.16$).

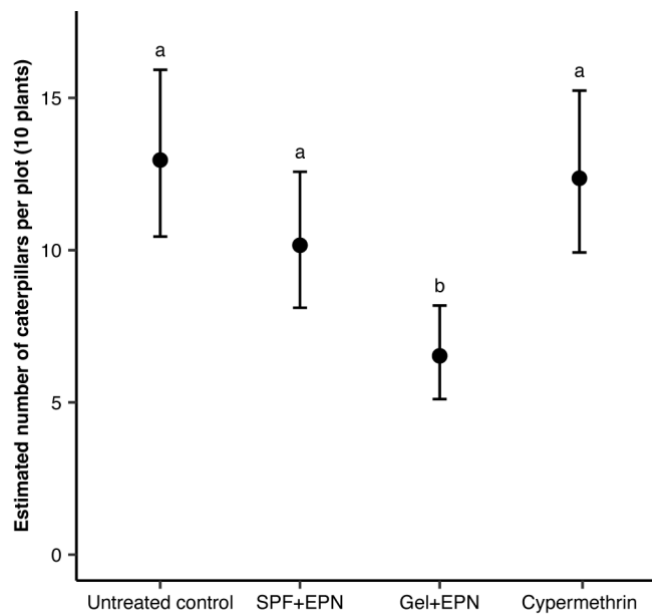


Fig. 7: Number of caterpillars per plot. Estimated marginal means \pm 95% CI of the number of caterpillars recovered per plot five-days after the third application, averaged for the six fields with each five plots per treatment. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a surfactant-polymer-formulation (SPF) or a carboxymethyl cellulose gel (Gel). About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Five-days after the third application of the treatments, ten specific plants per plot ($n = 30$ plots per treatment) were destroyed to inspect for caterpillars. The number of caterpillars (bigger than 0.5 cm) was recorded as a proxy for treatment efficacy. Data were analysed with generalized linear mixed model using Template Model Builder and a poisson error distribution. Letters indicate significant differences ($p < 0.05$) according to a multiple comparison corrected for false discovery using the Benjamini & Hochberg method (1995).

Maize yield

There was an overall treatment effect on yield (Fig. 3 and S4; fresh cob weight, $\chi^2_{(3)} = 8.3$, $p = 0.04$). At the end of the cropping season plots that were repeatedly treated with the EPN-gel produced almost one additional ton of cobs (fresh weight) per hectare as compared to untreated control plots (6.6 ± 0.2 SE $t \cdot ha^{-1}$ versus 5.7 ± 0.2 $t \cdot ha^{-1}$; $p = 0.03$). Cypermethrin-treated plots yielded 6.1 ± 0.2 $t \cdot ha^{-1}$, and plots treated with EPN formulated in SPF produced 6.0 ± 0.2 $t \cdot ha^{-1}$, neither one being significantly different from the control plots, nor the plots treated with the EPN-gel.

The cobs themselves had little FAW damage (~60% intact; ~35% little damage [<5% damaged kernels]; <4% medium to heavy damage [>5% damaged kernels]), regardless whether plants had been treated or not (Fig. S5). Cob damage as well as the number of cobs produced were similar in all treatments (Fig. S5 and S6; cob damage: $\chi^2_{(3)} = 4.5$, $p = 0.21$; number of cobs: $\chi^2_{(3)} = 4.5$, $p = 0.21$; number of fully developed cobs only: $\chi^2_{(3)} = 1.7$, $p = 0.65$).

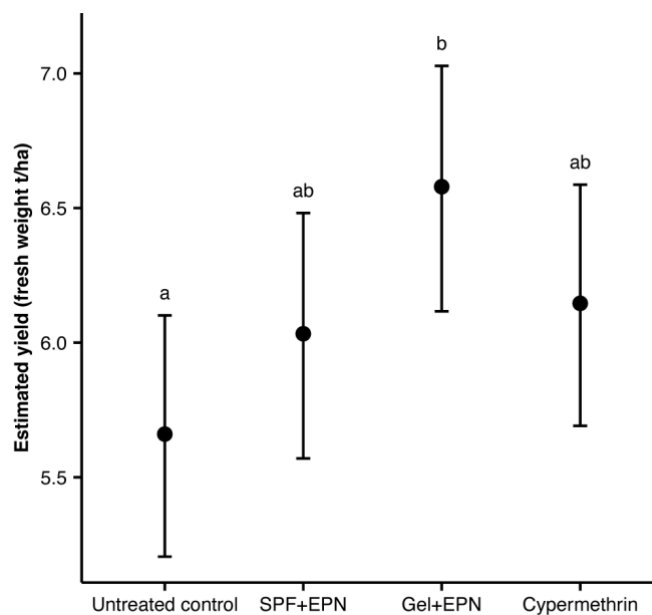


Fig. 8: Maize yield. Estimated marginal means \pm 95% CI of fresh cob weight averaged from six fields with each five plots per treatment. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a surfactant-polymer-formulation (SPF) or a carboxymethyl cellulose gel (Gel). About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. At harvest, developed cobs from 30 plants in the centre of each plot ($n = 30$ plots per treatment) were weighed. Data were analysed with generalized linear mixed model using Template Model Builder and gaussian error distribution. Letters indicate significant differences ($p < 0.05$) according to a multiple comparison corrected for false discovery using the Benjamini & Hochberg method (1995).

Discussion

Our study demonstrates that EPN represent a promising alternative to insecticides towards a more sustainable control of FAW. EPN pose no harm to farmers, consumers or life stock, and are safe to the environment (Ehlers and Hokkanen, 1996). Given the complex nature of the insecticidal compounds released by EPN and their symbiotic bacteria in an infected insect, it is highly unlikely that FAW is able to develop resistance against EPN (Georghiou., 2005; Lewis and Clarke., 2012; Lu et al., 2017). In contrast, several populations of FAW have readily developed resistance to a variety of chemical insecticides (Wan et al., 2021) as well as to *Bacillus thuringiensis* (*Bt*) toxins expressed in transgenic maize (Storer et al., 2010; Farias et al., 2014; Blanco et al., 2016). Another advantage of EPN is that highly virulent strains can be locally isolated and produced around the world (Holmes et al., 2015; Fallet et al., 2020), reducing the registration hurdles and providing the opportunity to use native biological control agents without having to rely on external sources.

Importantly, the EPN-gel application is expected to be cost effective, since we used doses of EPN across the whole season that were at least three-fold lower than what is commonly used to control below ground pests (0.4-0.6 vs 2-4 billion EPN per Ha; Georgis, 1990; Toepfer et al., 2010). Based on our estimations, the amount of gel-formulated EPN used in this study over the season would cost a farmer approximately 54 or 72 USD (representing three or four applications). Considering an average retail sale price of 354 USD per ton of maize grain in Rwanda (Adong et al., 2022), as well as the additional ton of cobs produced in plots treated with the EPN-gel formulation as compared to control plots, the net benefit of our approach could be of at least 70 USD (we estimated an additional ton of cobs to represent about 0.4 ton of grains).

We showed that repeated application of EPN during the entire vegetative growing season of maize can consistently reduce plant damage. The gel formulation as well as the surfactant formulation were both as effective as the insecticide cypermethrin. The three treatments showed a similar efficacy throughout the first four weeks of the field trials (Fig. 1A; plants with up to ten leaves). After the fifth week, however, the EPN applied in the gel provided considerably better protection, indicating that it was the most effective treatment on older plants (>6 weeks-old plants; >12 leaves). In contrast, the cypermethrin was found to be less effective after the fourth week and failed to prevent plant damage at six weeks (Fig. 1A). This lack of efficacy of the insecticide could be because of heavy rains that followed the last two applications, which may have washed away or diluted the insecticide, whereas the more viscous gel or the commercial liquid EPN-formulation containing surfactants may have remained better on the plants. That the insecticide did not work at the end of the season may also have been a matter of dose. We consistently applied each treatment as a 2 ml spot-spray into the whorl of the plants. This volume of insecticide proved effective for younger plants during the first four weeks of the experiments, but it may have been less effective on older

plants (>6 weeks-old plants). The application covered the entire whorl area (2-3 youngest leaves), but not the lower leaves, which may have harboured some caterpillars in case of the older plants. These limitations may also explain the relatively high number of caterpillars recovered from older plants treated with the insecticide. In contrast, the gel treatment, which was applied similarly to the insecticide, consistently prevented damage and significantly reduced FAW infestation, even on older plants (Fig. 1 and 2). The lower number of caterpillars recovered on gel-treated plants may indicate that EPN migrated on lower leaves of the plants, away from their application site, where they could infest additional caterpillars. Indeed, we found living EPN inside the shoot of maize plants two weeks after their application (pers. observation). During the field trials, the plants were subject to high FAW infestation (i.e., more than one caterpillar per plant, with about 75% infested plants at “Hillside” and 100% at “Station” and “Forest” locations, in untreated control plots). Plant damage was more severe two weeks after each treatment than after one week regardless of the treatment (Fig. 1B). This implies that FAW rapidly re-infested plants after each treatment. Hence, under such high pest pressures, a weekly application of the treatment may provide better protection. This may be different under other climatic and agronomic conditions. Nonetheless, our bi-weekly application of the EPN-gel treatment resulted in a significant increase in yield, as compared to untreated control plots (Fig. 3). Subsequently, the additional ton of maize produced readily compensates for the estimated cost of the EPN and should be a major incentive for African farmers to use this safer alternative instead of chemical insecticides.

Apart from a recent study (Patil et al., 2022), previous attempts in field conditions to control FAW with EPN applied with water or in combination with surfactants have failed, indicating that the gel is key to the success of EPN application against FAW (Richter and Fuxa., 1990; Garcia et al., 2008; Negrisoli et al., 2010; Fallet et al., 2022b). However, Patil et al., (2022) showed that EPN applied with water in combination with pheromone traps and releases of the egg parasitoid *Trichogramma chilonis* (Ishii) successfully suppressed FAW populations and increased yields. Although, treatment effects of the different control methods cannot be disentangled in that study, it further highlights the potential of using non-synthetic approaches in Integrated Pest Management strategies (IPM) against FAW.

Further effort should focus on fine-tuning the application frequency of EPN in accordance with local maize phenology, pest population dynamics and environmental conditions. In addition, we believe that the efficacy of EPN could be increased by recent developments with regards to their formulation. For instance, an innovative strategy has been developed to coat individual EPN with nanoparticles of titanium dioxide (Kotliarevski et al., 2022), which provides them with an increased tolerance to UV radiation (Wu et al., 2023).

We envision that local biocontrol companies could produce native EPN (Holmes et al., 2015) and incorporate the EPN-gel in large glue gun-like devices specifically designed for small holder-farmers, allowing them to rapidly treat their maize plots with a simple and reusable technology. Another approach would be to train farmers in the mass-production of EPN, for example using black soldier fly larvae (Manu et al., 2022), so that they could use their own, on-farm produced, biological control agents without relying on external sources. For large-scale farming, high-precision machinery could be developed that applies low-doses of EPN-containing gel on maize plants.

To conclude, our Rwandan field trials demonstrate that EPN represent an effective and safe alternative to synthetic insecticides in the fight against FAW. The targeted gel application allowed us to use across the season at least three-fold less EPN than what is normally applied to control belowground pest insects, which should make the approach economically feasible. Hence, the application of the gel-formulated EPN alone or in combination with other IPM practices hold great promise for sustainable maize production and food security in Africa and beyond.

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Supplementary Material

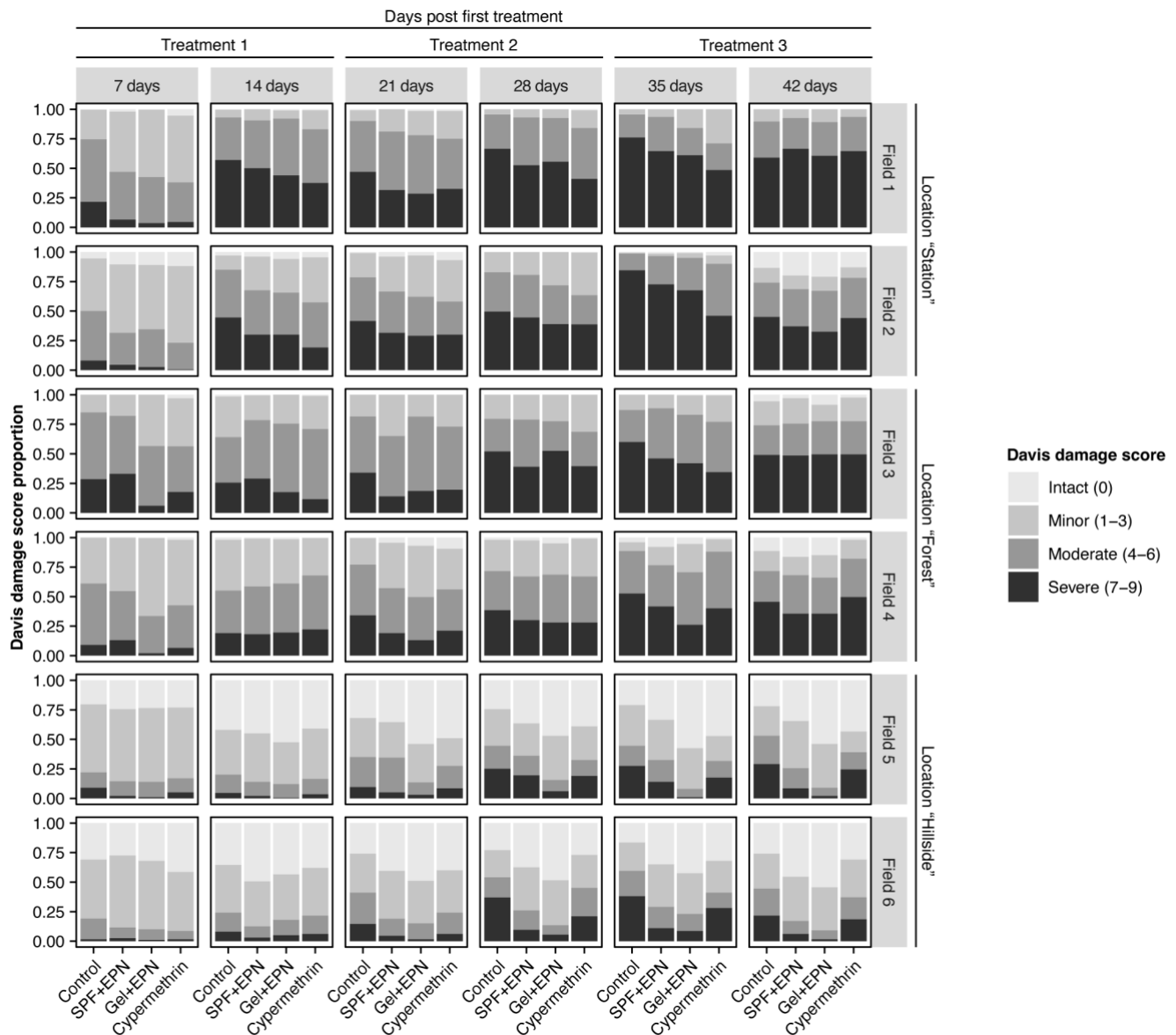


Figure S1: Recorded whorl damage at six assessments and in six fields. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a Surfactant-Polymer-Formulation (SPF) or a carboxymethyl cellulose gel. About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Whorl damage was assessed seven- and fourteen-days after each application for 40 plants per plot (n = 200 plants per treatment, assessment and field) using the Davis whorl damage scale, where a “0” represents an intact whorl and a “9” represents an almost completely or completely destroyed whorl.

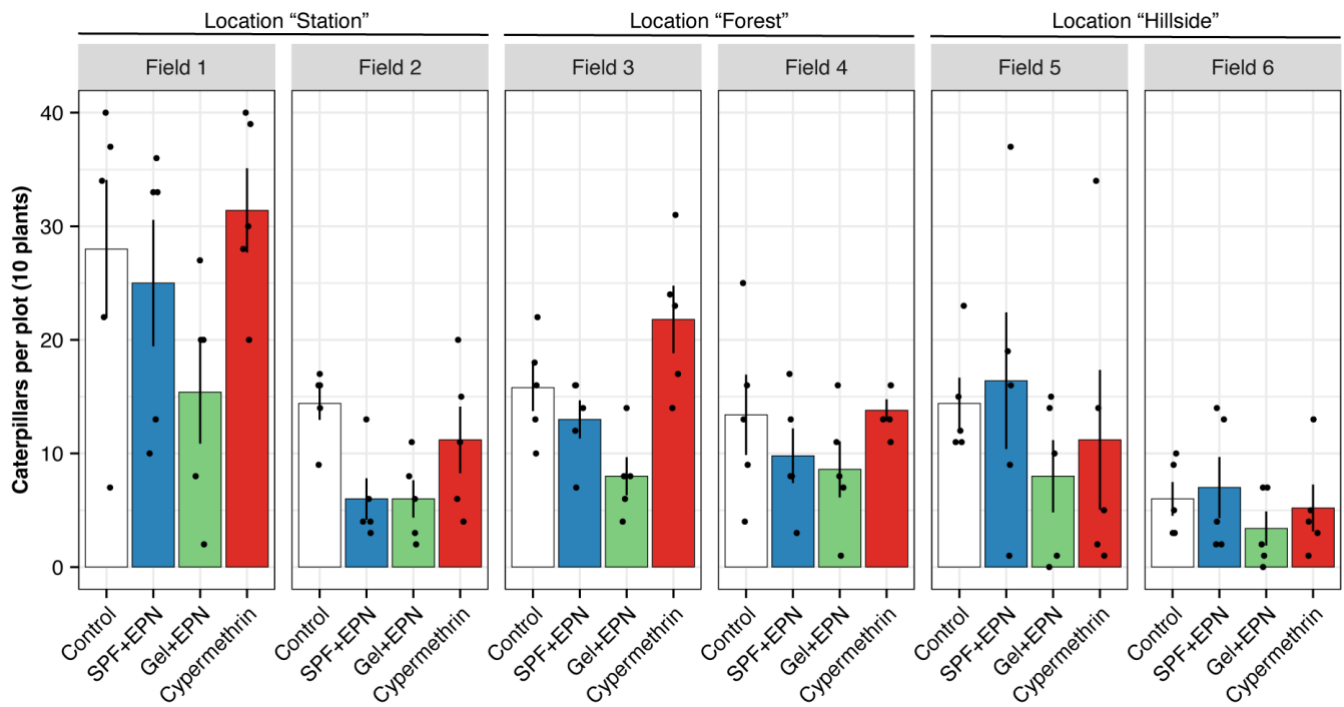


Figure S2: Average number of caterpillars (\pm SE) per treatment and field. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a surfactant-polymer-formulation (SPF) or a carboxymethyl cellulose gel (Gel). About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Five-days after the third application of the treatments, ten specific plants per plot ($n = 5$ plots per treatment and field) were destroyed to inspect for caterpillars. The number of caterpillars (bigger than 0.5 cm) was recorded as a proxy for treatment efficacy. Dots indicate the number of caterpillars recovered per plot.

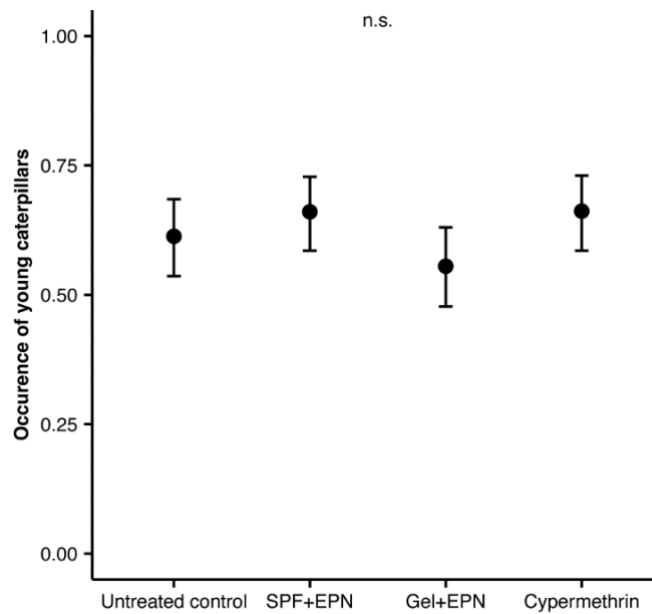


Fig. S3: Occurrence of young caterpillars on plants, indicating rapid re-infestation by the fall armyworm. Estimated least square means \pm 95% CI of the occurrence of young caterpillars (smaller than 0.5 cm) recorded per plant and averaged from five plots in each of six fields. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a Surfactant-Polymer-Formulation (SPF) or a carboxymethyl cellulose gel. About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Five-days after the third application of the treatments, ten specific plants per plot ($n = 30$ plots per treatment) were destroyed to search for caterpillars. The occurrence of young caterpillars (smaller than 0.5 cm) was recorded as a proxy for reinfestation. Data were analysed with generalized linear mixed model using Template Model Builder and a binomial error distribution. “n.s.” indicates non-significant differences ($p > 0.05$).

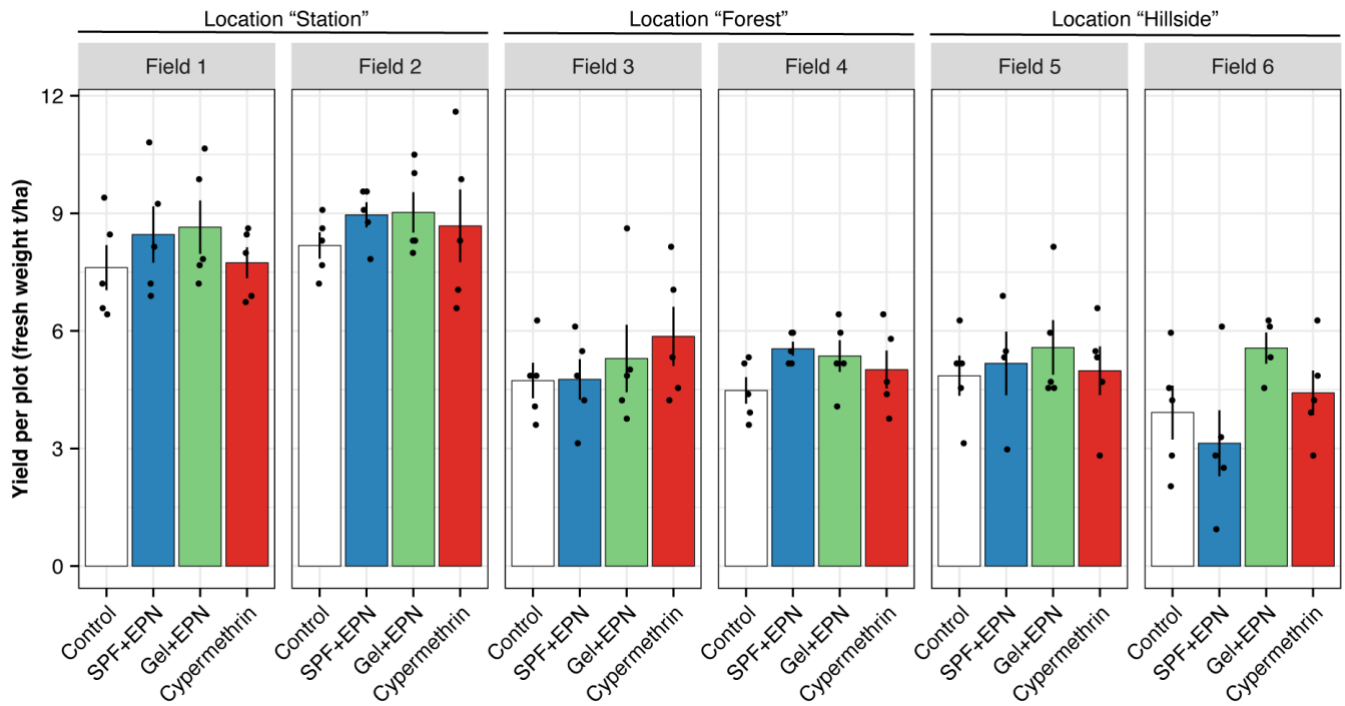


Figure S4: Average fresh weight cob yield (\pm SE) per treatment and field. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a surfactant-polymer-formulation (SPF) or a carboxymethyl cellulose gel (Gel). About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. At harvest, developed cobs from 30 plants in the centre of each plot ($n = 5$ plots per treatment and field) were weighed. Dots indicate yield per plot.

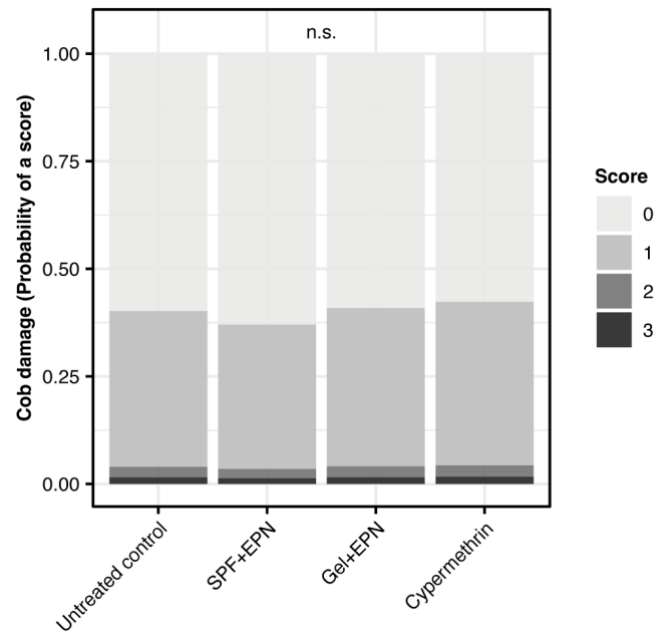


Fig. S5: Cob damage. Probability of a given damage score within each treatment averaged for six fields, with each five plots per treatment. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a Surfactant-Polymer-Formulation (SPF) or a carboxymethyl cellulose gel. About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. Cob damage was assessed on developed cobs from 30 plants in the centre of each plot ($n = 30$ plots). A score of “0” represent an intact cob, while scores of “1”, “2”, or “3” represent minor (<5% damaged kernels), moderate (5-20% damaged kernels) or severe (>20% damaged kernels) cob damage, respectively. Data were analysed using cumulative link mixed models. “n.s.” indicates non-significant differences ($p > 0.05$).

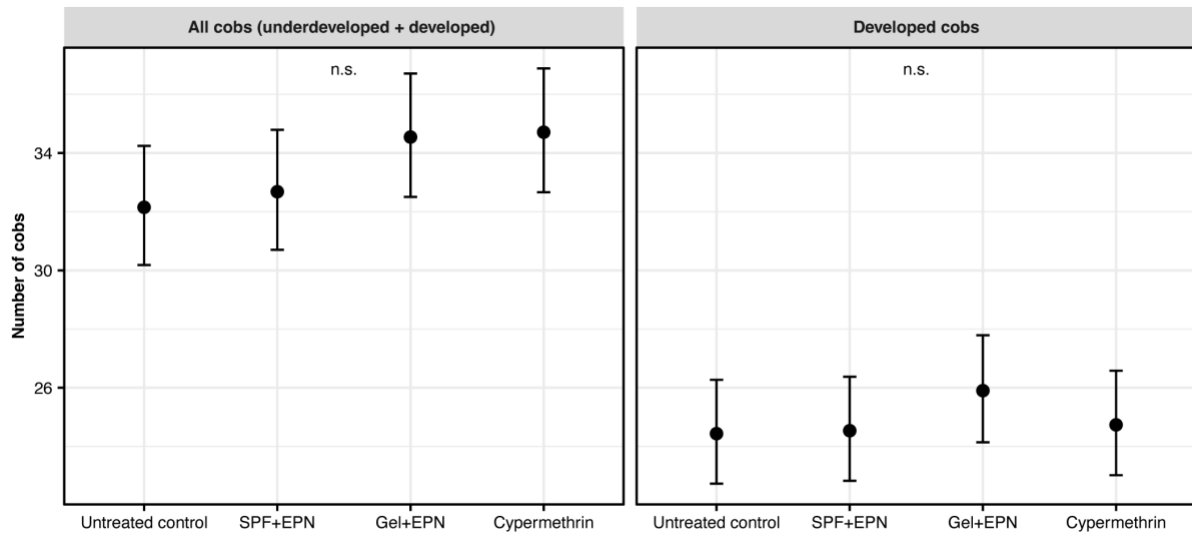


Fig. S6: Number of cobs per plot. Estimated least square means \pm 95% CI of the number of cobs recorded per plot and averaged from six fields, with each five plots per treatment. Maize plants were treated every two weeks with *Steinernema carpocapsae* RW14-G-R3a-2 applied either in a Surfactant-Polymer-Formulation (SPF) or a carboxymethyl cellulose gel. About 3000 infective juveniles were applied per plants. Control plots were left untreated, while the positive control plots were treated with the contact insecticide cypermethrin. Treatments were applied as a 2 ml spot injection/spray into the whorl of each plant. At harvest, underdeveloped as well as developed cobs from thirty plants in the centre of each plot ($n = 30$ plots per treatment) were counted. Data were analysed with generalized linear mixed model using Template Model Builder and poisson error distribution. “n.s.” indicates non-significant differences ($p > 0.05$).

Chapter 5

Protection of entomopathogenic nematodes against ultraviolet radiation with natural substances enhances their potential to control leaf-herbivores

Abstract

Ultraviolet (UV) radiation is particularly detrimental to entomopathogenic nematodes (EPN) and therefore limits the use of these biological control agents against aboveground pest insects. Recent efforts to apply EPN to control the fall armyworm (*Spodoptera frugiperda*, Smith), a highly destructive pest of maize plants, has seen considerable success with EPN formulated in a carboxymethyl cellulose (CMC) gel. The gel seems to protect the EPN from desiccation and prevents them from seeping out of the wrapped leaves of the whorl, where FAW mostly feeds. Further improvements of such EPN formulations can be expected with enhanced protection against UV radiation and desiccation. In the present study, we tested whether supplementing the CMC-gel with substances that are known to protect organisms from the detrimental effects of UV radiation would increase the longevity and maintain the infectiousness of the EPN *Steinernema carpocapsae* (Weiser; All strain) under artificial UV radiation conditions. Six substances were tested: titanium dioxide, zeolite, rapeseed oil, egg albumin, lignin and humic acid sodium salt. Toxicity tests showed that egg albumin, lignin and humic acid sodium salt were lethal to EPN. Therefore, only titanium dioxide, zeolite and rapeseed oil were used in a subsequent dose-response experiment evaluating the UV protection conferred by these substances. They were found to prevent the loss of virulence in EPN exposed to UV radiation for 2.5 hours when a sufficient dose of a substance was used. This was achieved at doses as low as 0.1, 5 and 5% for titanium dioxide, zeolite and rapeseed oil, respectively. A final experiment showed that supplementing CMC-gels with either 1% titanium dioxide or 10% rapeseed oil resulted in complete prevention of EPN' loss of virulence for at least twenty-four hours of UV exposure, the longest duration tested. These findings are expected to expand the use of EPN for aboveground applications and contributing to sustainable agricultural practices.

Key words: *Steinernema carpocapsae*, titanium dioxide, zeolite, rapeseed oil, biological control, sustainable agriculture

Introduction

Entomopathogenic nematodes are lethal parasites of insects that can be found in soils worldwide (Hominick et al., 2002). In the soil, EPN use chemical cues to locate their insect hosts and invade them via natural orifices (such as mouth, anus or spiracles; Kaya and Gaugler, 1993; Lewis and Clarke, 2012; Turlings et al., 2012). Inside the insect, EPN will release symbiotic bacteria that they carry inside their guts (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). Together, bacteria and EPN will produce toxins that will kill the insects generally within twenty-four hours (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). EPN can infest a wide range of insects in laboratory assays and are therefore considered generalist pathogens of

insects. However, their natural host range is expected to be much narrower, due to differing ecology between EPN species and their potential hosts (Shapiro-Ilan et al., 2018).

EPN have been commonly used as biological control agents for decades as they can be highly virulent, are found worldwide and can be mass produced (Ehlers, 2001; Georgis et al., 2006). Moreover, they are considered safe for the environment and are no threat to humans or domesticated animals (Ehlers and Hokkanen, 1996). For these reasons, they are considered as an effective and safe alternative to chemical insecticides (Kaya et al., 2006).

EPN are mainly used against belowground pests, but have also been considered for aboveground applications (Koppenhöfer et al., 2020). This has its limitations because EPN are particularly sensitive to aboveground abiotic factors, such as heat, desiccation and ultraviolet radiation (Kaya and Gaugler, 1993; Lacey and Georgis, 2012; Kagimu et al., 2017). Efforts to develop formulations to protect EPN under these environmental constraints and to facilitate their use outside their natural soil habitat have had some success (Shapiro-Ilan and Dolinski, 2015; Hiltbold, 2015; Koppenhöfer et al., 2020). For instance, gels have been used to protect EPN from desiccation, like a fire gel (Barricade[®]) that significantly reduced *Synanthedon pictipes* (Grote & Robinson) infestation in peaches, whereas EPN alone had no such effect (Shapiro-Ilan et al., 2010). In a recent study, EPN formulated in a carboxymethyl cellulose (CMC) gel effectively controlled the fall armyworm (*Spodoptera frugiperda*, Smith), a devastating pest of maize, whereas EPN in just water were only marginally effective (Fallet et al., 2022). Further improvements should be possible, such as by incorporating UV protectants into formulations. Notably, P-amino benzoic acid (PABA), octyl methoxycinnamate (OMC), Tinopal LPW, liquid starch and titanium dioxide (TiO₂) have shown promise as UV protectants (Nickle and Shapiro, 1992; Walia et al., 2008; Dito et al., 2016; Acar and Sipes, 2022; Wu et al., 2023a; 2023b).

In the here presented laboratory study, we explored the potential of six substances mixed in a CMC-gel to protect the EPN *Steinernema carpocapsae* (Weiser, All strain) from the detrimental effects of UV radiation. The tested substances were: (1) titanium dioxide, (2) zeolite, (3) rapeseed oil, (4) lignin, (5) egg albumin and (6) humic acid sodium salt (Table 1). These are either among the most effective additives to protect EPN (e.g., titanium dioxide) or substances that showed promise when tested with other organisms (e.g., egg albumin, humic acid sodium salt; Edgington et al., 2000; Kaiser et al., 2019). In a first step, we conducted toxicity experiments to ensure the compatibility of EPN with the tested substances. In a second step, we tested in dose-response experiments the efficacy of each substance in protecting EPN during 2.5 hours of UV exposure. Lastly, the protection provided by titanium dioxide (0.1 and 1%) as well as rapeseed oil (5 and 10%) was evaluated over twenty-four hours of UV exposure.

The results highlight the efficacy of inexpensive and readily available additives that can be incorporated into formulations and protect EPN from the detrimental effects of UV radiation. This should enhance the efficacy of EPN applications aboveground and contribute to sustainable agricultural practices.

Material and Methods

Entomopathogenic nematodes and substances

In this study, we used the commercially available EPN *Steinernema carpocapsae* (Weiser) strain All, provided by e-nema GmbH (Nemastar[®], Schwentinental, Germany). EPN were reared *in-vivo* on last-instar *Galleria mellonella* (L.; Lepidoptera: Pyralidae), as described by White (1927). Freshly emerged EPN, were collected in tap water and stored at 12°C in the dark. They were used within two weeks post emergence.

The substances tested for their protective effect against UV radiation are presented in table 1.

Table 1: Substances used in the study.

Substance	Source	Reference
Titanium dioxide (TiO ₂) CAS: 13463-67-7	Carl Roth GmbH, Karlsruhe, Germany	Walia et al., 2008; Dito et al., 2016; Acar and Sipes, 2022; Wu et al., 2023a
Zeolite - LithoFillBM [®] CAS: Unknown	Lithos Natural GmbH, Ennsdorf, Austria	Kaiser et al., 2019
Rapeseed oil CAS: NA	Denner AG, Zurich, Switzerland	Kaiser et al., 2019 (Authors tested colza and sesame oils)
Lignin CAS: 8068-05-1	Sigma-Aldrich, St. Louis, MO, USA	Kaiser et al., 2019
Egg albumin CAS: NA	My Protein, The Hut.com Limited, Manchester, UK	Edgington et al., 2000
Humic acid sodium salt CAS: 68131-04-4	Sigma-Aldrich, St. Louis, MO, USA	Kaiser et al., 2019
Tween [®] 80 CAS: 9005-65-6	Sigma-Aldrich, St. Louis, MO, USA	/
Carboxymethyl cellulose, high viscosity CAS: 9004-32-4	Sigma-Aldrich, St. Louis, MO, USA	Fallet et al., 2022

Toxicity of the substances to nematodes

To evaluate the compatibility between EPN and the selected substances, we first prepared stock solutions of each substance diluted in 0.2% Tween[®] 80. For this, we mixed 200 µl of Tween[®] 80 into 90 ml of tap water and then added 10 g (10% w/v) or 10 ml (10% v/v) of each substance. The solutions were homogeneously mixed on a magnetic stirrer. Fresh stock solutions were prepared before each experiment. Parallely, 500'000 EPN were incorporated into 500 ml of tap water (1'000 EPN/ml). The experiments were carried out in 30 ml plastic cups (Frontier Scientific Inc., Newark, DE, USA), in which we mixed 5 ml of a substance stock solution with 5 ml of the EPN solution. Hence, a cup contained 5'000 EPN and 5%

(v/v or w/v) of a substance in 10 ml of Tween[®] 80 0.1%. As control, EPN in just 0.1% Tween[®] 80 were used. Cups were then stored at $24 \pm 1^\circ\text{C}$ in the dark. After one, two or three weeks, the solution in the cups were sieved (25 μm mesh) and rinsed to collect the EPN, which were then laid to rest ($24 \pm 1^\circ\text{C}$ in the dark). After 24h of recovery, EPN' mortality was evaluated in each cup on about 100 individuals by poking them with a fine needle. Ten live (unpoked) EPN were then transferred with 400 μl of tap water onto a filter paper in a 5.5 cm Petri dish with five *G. mellonella*. The dishes were stored in the dark at $24 \pm 1^\circ\text{C}$. After six days, we recorded insect's mortality to evaluate EPN' virulence. The experiment was performed twice with each six or twelve cup per treatment and week ($n = 18$ cups in total per treatment and week).

Ultraviolet protection

In preliminary assays, we found that a short exposure to UV radiation (90-150 min) did not affect the survival of EPN, but strongly altered their virulence (Fig. S1). To conduct these preliminary assays, we placed 3.5 cm Petri dishes containing 2'000 EPN in 3 ml of tap water (666 EPN/ml) under artificial UV radiation. The artificial UV radiation exposure system consisted of seven 90 cm terrarium lamps (Reptile UVB200 T8, Exo Terra, Rolf C. Hagen Inc., Montreal, Canada) mounted in a cupboard. The Petri dishes were placed 58.5 cm below the lamps, where they were exposed to an erythema UV radiation intensity of $0.15 \text{ W} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, measured with a Solarmeter[®] model 7.5 (Solar Light Company, LLC, Glenside, PA, USA). The Petri dishes were recovered from the UV radiation exposure system after being exposed for 01:30, 01:45, 02:00, 02:15 and 02:30 (hh:mm). They were then stored for 24h in the dark at $24 \pm 1^\circ\text{C}$, as a recovering time for the EPN. EPN' mortality and virulence were evaluated as described above. Eight replicates per treatment were used in one experiment ($n = 8$ Petri dishes per treatment). Since only the virulence of the EPN was affected, we did not assess EPN' survival in the subsequent experiments.

To evaluate the UV protection conferred by the different substances, we incorporated them in a carboxymethyl cellulose (CMC) gel that can be used to apply EPN on maize plants against the fall armyworm (Fallet et al., 2022). Since, lignin, egg albumin and humic acid sodium salt were lethal to the EPN, only titanium dioxide, zeolite and rapeseed oil were used. The three substances were tested at doses of 0.1, 1 and 5% (v/v or w/v). Rapeseed oil was also tested at 10% (v/v). First, either 0.04 (0.1%), 0.4 (1%), 2 (5%) or 4 (10%) g (solid substances) or ml (liquid substances) was added to respectively 38.8, 38.4, 36.8 or 34.8 ml of tap water containing 80'000 EPN. Then, 1.2 g (3% w/v) of CMC was incorporated to the solutions and vigorously mixed until a homogenous gel was formed (about 2'000 EPN/ml in final gels). One millilitre of the prepared gels was then applied with a 1 ml plastic syringe into a 3.5 cm Petri dish. Dishes were then placed in the UV radiation exposure system for 2.5 hours. As negative control, EPN in a CMC gel without additional substance was used. As positive control, dishes containing EPN in the sole CMC gel were wrapped in aluminium foil and hence unexposed to UV radiation. After the irradiation

period, 5 ml of tap water was added to the Petri dishes to dilute the gel. They were then stored in the dark at $24 \pm 1^\circ\text{C}$. After 24h (EPN recovery), the diluted gels were sieved and rinsed to collect the EPN. EPN' virulence was then evaluated as described above. Ten replicates per treatment were used in each of two experiments ($n = 20$ Petri dishes in total per treatment).

Finally, we evaluated for how long 0.1 and 1% titanium dioxide as well as 5 and 10% rapeseed oil mixed in a CMC gel can protect EPN from the detrimental effects of UV radiation. For this, gels were prepared as described above, transferred as 4 ml droplets into 3.5 cm Petri dishes and placed in the UV radiation exposure system for 0, 8, 16 and 24h, corresponding to a cumulated erythemal UV radiation of 0, 4.3, 8.6 and $13 \text{ kW}\cdot\text{m}^{-2}$, respectively. As controls, gels without the addition of any substance were used. They were either irradiated or wrapped in aluminium foil (unexposed control). At the end of the UV exposure, about 1 ml of irradiated gel was transfer into a 5.5 cm Petri dish supplemented with 10 ml of tap water to dissolve the gel. After 24h of recovery, the gels were sieved to collect the EPN and their virulence was evaluated as described above. Eight Petri dishes per treatment were used in one experiment ($n = 8$ Petri dishes per treatment).

Data analyses

Statistical analyses were performed using R version 4.2.1 (R Core Team, 2022). Mortality and virulence of EPN were analysed using generalized linear mixed-effects models (“lme4” package; Bates et al., 2015), with a binomial error distribution. EPN' mortality or virulence were used as the response variable, while treatments and experimental blocks were used as fixed factors. Petri dishes were used as a random factor to account for the repeated observations (several nematodes or *Galleria mellonella* larvae) within a Petri dish. For the toxicity, each exposure period (weeks) was analysed independently. To evaluate the efficacy of the substances over twenty-four hours, treatment and exposition time were used as fixed factors with their interaction, while Petri dishes were used as a random factor. Multiple comparisons or many-to-one comparisons (“emmeans” package; Lenth, 2022), corrected for false discovery using the Benjamini & Hochberg (1995) method, were used to compare treatments when they had a significant effect. Data presented in the figures are models' estimates computed using the function “Effect” (“effects” package; Fox and Weisberg, 2019).

Results

In a first step, we investigated whether the selected substances were compatible with EPN. After being exposed to titanium dioxide, zeolite or rapeseed oil for three weeks, neither the survival nor the virulence of the EPN were affected (Fig. S2; $p > 0.05$). In contrast, lignin, egg albumin and humic acid sodium salt were lethal to the EPN (Fig. S2; $p < 0.001$). Therefore, only the three former substances were used in the subsequent experiments.

In a second step, we evaluated, in a dose-response experiment, the efficacy of the three selected substances in protecting EPN from the detrimental effect of UV radiation. In preliminary trials, we showed that the survival of EPN was not affected by short exposition to UV radiation (UV exposition time: $\chi^2_{(5)} = 8.9$, $p = 0.1$), whereas the EPN' virulence was rapidly altered (Fig. S1; UV exposition time: $\chi^2_{(5)} = 38.5$, $p < 0.001$). Therefore, we only assessed the virulence of the EPN when comparing the different protective substances.

After being exposed to UV radiation for 2.5 hours, EPN formulated in CMC only were significantly less virulent than unexposed EPN (Fig. 1; $p < 0.001$). However, the virulence of EPN formulated in CMC mixed with titanium dioxide did not differ from unexposed EPN at the three tested concentrations (Fig. 1; 0.1, 1 or 5% TiO₂+CMC; $p > 0.05$). Zeolite and rapeseed oil, could also significantly protect the EPN, but only at the highest doses tested (Fig. 1; $p < 0.05$: Zeolite: 5%; Rapeseed oil: 5 and 10%). These results indicate that EPN formulated in CMC-gels can be protected from the detrimental effects of UV radiation when a sufficient dose of a protective substance is incorporated to the gel (Fig. 1; i.e., TiO₂: min 0.1%; Zeolite: min 5%; Rapeseed oil: min 5%).

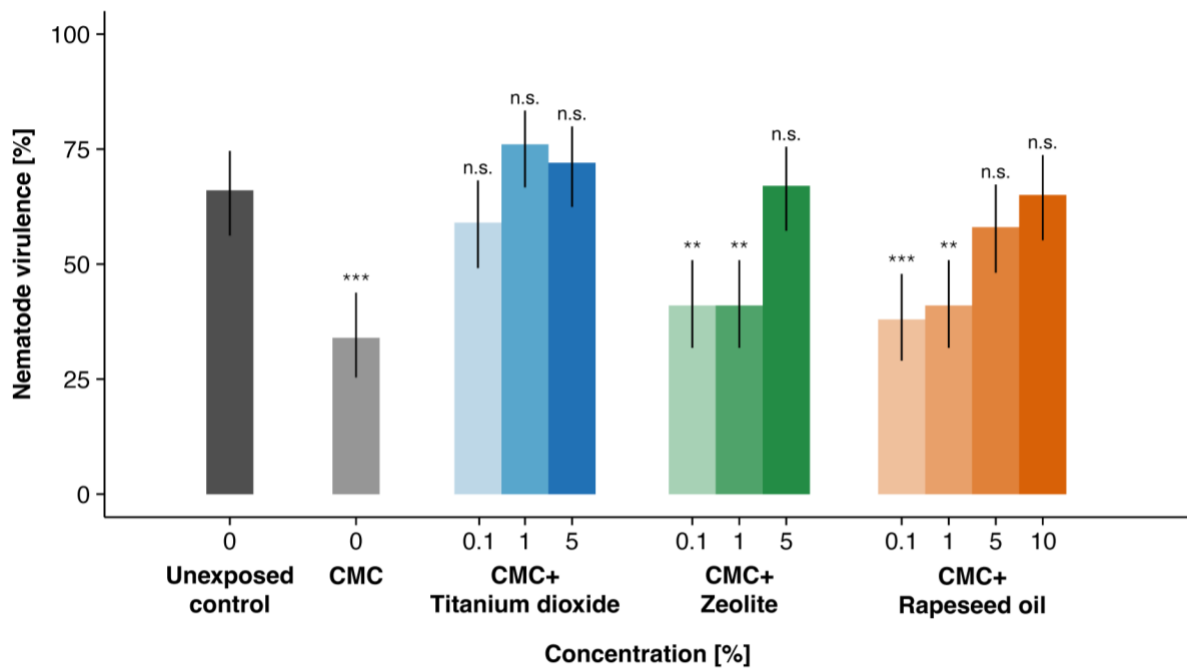


Figure 1: Entomopathogenic nematodes' virulence after exposure to UV radiation for 2.5 hours. Estimated least square means \pm 95% CI of the proportion of dead *Galleria mellonella* in Petri dishes inoculated for six days with EPN previously exposed to UV radiation. EPN were formulated in a 3% (w/v) carboxymethyl cellulose (CMC) gel with or without a protective substance at a dose of 0.1, 1, 5 or 10% (v/v or w/v). As unexposed control, EPN in a CMC gel without any additional substance were wrapped in aluminium foil. Ten Petri dishes per treatment and dose were used in each of two independent experiments ($n = 20$). Data were analysed with a generalized mixed-effect model with a binomial error distribution. Stars (*) indicate significant differences between the unexposed control and each treatment obtained from a many-to-one comparison corrected for false discovery using the Benjamini and Hochberg (1995) method (n.s. = non-significant, $p > 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

In a last experiment, we evaluated over 24 hours the protection conferred to the EPN by titanium dioxide at 0.1 and 1% as well as by rapeseed oil at 5 and 10% when mixed in the CMC-gel. Treatments had a significant effect on EPN's virulence (Fig. 2; Treatments: $\chi^2_{(5)} = 39$, $p < 0.001$; interaction [Treatments*Exposition]: $\chi^2_{(15)} = 59$, $p < 0.001$). After eight hours of exposure to radiation, the virulence of EPN formulated in the sole CMC gel was strongly impaired (Fig. 2; CMC vs unexposed control at 4h: $p < 0.001$), while longer exposures were lethal to unprotected EPN (Fig. 2). However, EPN formulated in either 1% of titanium dioxide or 10% of rapeseed oil remained as virulent as unexposed EPN until the end of the experiment after 24h of radiation ($p > 0.05$). At lower concentrations, the two substances had an intermediate efficacy. Indeed, EPN formulated in rapeseed oil at 5% or titanium dioxide at 0.1% suffered a loss in virulence after 8 or 16 hours, respectively ($p < 0.05$).

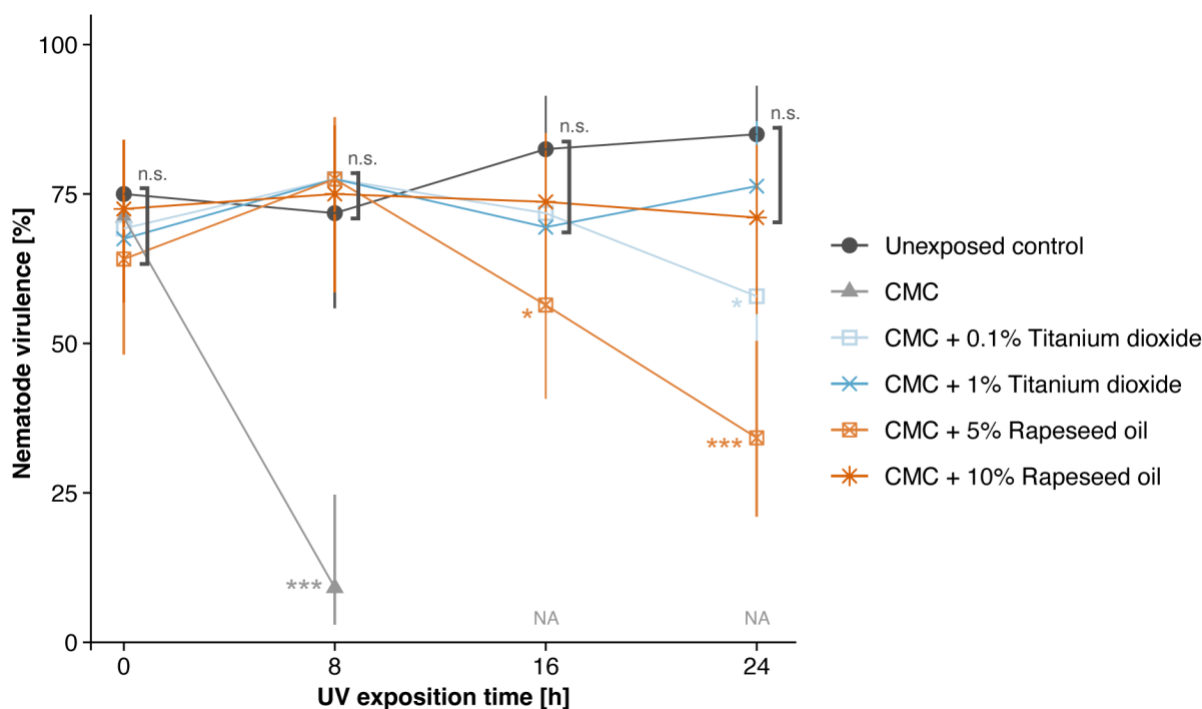


Figure 2: Entomopathogenic nematodes' virulence after being exposed to UV radiation for 0, 8, 16 or 24h. Estimated least square means \pm 95% CI of the proportion of dead *Galleria mellonella* in Petri dishes inoculated for six days with EPN previously exposed to UV radiation. EPN were formulated in a 3% (w/v) carboxymethyl supplemented with titanium dioxide or rapeseed oil tested at 0.1 and 1% (w/v), or at 5 and 10% (v/v), respectively. As controls, CMC-gels without any additional substance were either exposed (CMC; negative control) or wrapped in aluminium foil (Unexposed control). In total, eight Petri dishes per treatment and exposition were used in one experiment ($n = 8$). Data were analysed with a generalized mixed-effect model with a binomial error distribution. Stars (*) indicate significant differences between the unexposed control and each treatment obtained from a many-to-one comparison corrected for false discovery using the Benjamini and Hochberg (1995) method (n.s. = non-significant, $p > 0.05$; * = $p < 0.05$; *** = $p < 0.001$). "NA" indicates that the virulence could not be evaluated, as EPN's mortality was above 80% in these treatments.

Discussion

We have shown that short expositions (up to 2.5 hours) to UV radiation did not cause EPN' mortality within 24h post exposure (Fig. S1). However, such expositions are likely lethal to EPN, as mortality often occurs several days post exposure (Gaugler et al., 1992). In any case, and in line with previous studies (Gaugler and Bush, 1978; Gaugler et al., 1992), the virulence of EPN was strongly and rapidly affected by UV radiation. After an exposure of just 01:45 (hh:mm) the virulence of EPN were reduced by at least 50% and was completely inhibited after 02:15 (hh:mm; Fig. S1). Hence, it is evident that UV radiation strongly limits the potential of EPN for aboveground applications.

To overcome this limitation, the use of substances that could provide protection to the EPN is key to their successful application aboveground (Lacey and Georgis, 2012; Shapiro-Ilan and Dolinski, 2015; Hiltbold, 2015; Koppenhöfer et al., 2020). We explored the potential of six substances. The first, titanium dioxide,

is known to be highly effective in protecting EPN for the detrimental effects of UV radiation (e.g., Nickle and Shapiro, 1992; Walia et al., 2008; Dito et al., 2016; Acar and Sipes, 2022; Wu et al., 2023a). In addition to titanium dioxide, we included in this study substances that showed promise when used in combination with other organisms, including natural, biodegradable, readily available and affordable substances (Kaiser et al., 2019; Edgington et al., 2000). Unfortunately, three of the tested substances were lethal to EPN in toxicity tests (Fig. S2). Alkalinity, is likely responsible for the incompatibility of EPN with egg albumin and lignin. Indeed, solutions became highly alkaline ($\text{pH} > 10$) in just a few days, likely causing the death of the EPN (Khathwayo et al., 2021). Besides, it is not surprising that humic acid sodium salt was lethal to EPN, as it can be used to control plant parasitic nematodes (Kesba et al., 2008; 2012).

When mixed in a CMC-gel, the three non-lethal substances (titanium dioxide, zeolite and rapeseed oil) successfully protected EPN from a 2.5 hours exposure to UV radiation, whereas EPN in CMC-gel without additional substance were strongly less pathogenic to *G. mellonella* (Fig. 1). These results were concentration-dependent. In this line, titanium dioxide prevented the loss of EPN' virulence already at the lowest concentration tested (0.1%). However, to effectively protect EPN, a concentration of at least 5% was needed for zeolite and rapeseed oil. Considering the minimal dosage and the cost of each substance, it appears that zeolite might be too costly and therefore unrealistic, but titanium dioxide and rapeseed oil represent promising candidates.

At doses of 1 or 10% respectively, titanium dioxide or rapeseed oil prevented the loss of EPN' virulence for twenty-four hours, the longest exposure tested (Fig. 2), corresponding to a cumulative erythemal UV emission of $13 \text{ kW}\cdot\text{m}^{-2}$. As comparison, we measured the UV emission of natural sunlight around noon during sunny days in lowland Switzerland (Neuchâtel, Switzerland; GPS: $47^{\circ}00'00.9''\text{N } 6^{\circ}56'56.8''\text{E}$; 490m a.s.l.). The highest intensity recorded was of $0.18 \text{ W}\cdot\text{m}^{-2}\cdot\text{s}$ (August 10th 2023 at 1:30 pm), which was slightly, but not considerably, above the radiation emitted by our artificial system (i.e., $13 \text{ kW}\cdot\text{m}^{-2}$ corresponds to an exposition of 24h in our system, whereas this should correspond to approximately 20h of mid-day sunlight), indicating that the artificial system used may, to some extent, be representative of natural sunlight conditions. However, these comparisons should be considered with care. Firstly, if the radiation intensity appears similar in both natural sunlight and our system, we cannot exclude that a distinct radiation spectrum may impact EPN differently. Secondly, our estimation of EPN' virulence may be overestimated. To evaluate EPN' virulence, we transferred ten live EPN onto insect's larvae. By choosing the EPN (i.e., motile ones), we may collect particularly resistant individuals, less affected by the radiation and therefore more pathogenic than the rest of the population. Nonetheless, our results allow relative comparisons among treatments and should, regardless of the limitations, translate into a considerable increase in the duration at which EPN can be exposed to natural sunlight without losing their pathogenicity.

Therefore, the addition of protective substances should offer a prolonged window of opportunity for EPN to infect insects on foliage under realistic field conditions and result in an increased EPN' efficacy.

Powdery titanium dioxide or other UV blocking agents may not be the most suited substances for application onto an expanded leaf surface, as they may interfere with photosynthesis by substantially blocking light. Therefore, it might be more relevant in this context to apply oils or metals formulated as nanoparticles, such as described by Wu et al., (2023a). Nonetheless, the use of powdery titanium dioxide remains highly valuable in other contexts, such as for applications onto tree bark (Dito et al., 2016; Acar and Spies, 2022).

In a recent field study, it was shown that 2 ml of an EPN-CMC-gel applied in the whorl of maize plants could significantly reduce fall armyworm infestation, a devastating pest of maize (Fallet et al., 2022). In this context, we speculate that the use of either oils or powdery metals would be relevant and should not alter photosynthesis. Indeed, the substances applied into the whorl would likely be washed away by rain by the time the leaf would expand. In this context, the cost of supplementing the CMC formulation with a protective substance might be as low as 0.8, 8, 6, 11 \$/ha for titanium dioxide 0.1%, 1%, rapeseed oil 5%, 10%, respectively, depending on the providers and bulk purchases.

To conclude, our results contribute to expanding the use of EPN for aboveground applications. Formulations protecting EPN from both desiccation and UV radiation can substantially increase opportunities of applying EPN aboveground in different crop-pest systems, contributing to sustainable agricultural practices. Furthermore, we showed that rapeseed oil, as an affordable, readily available and biodegradable substance, represents a promising additive.

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Supplementary Material

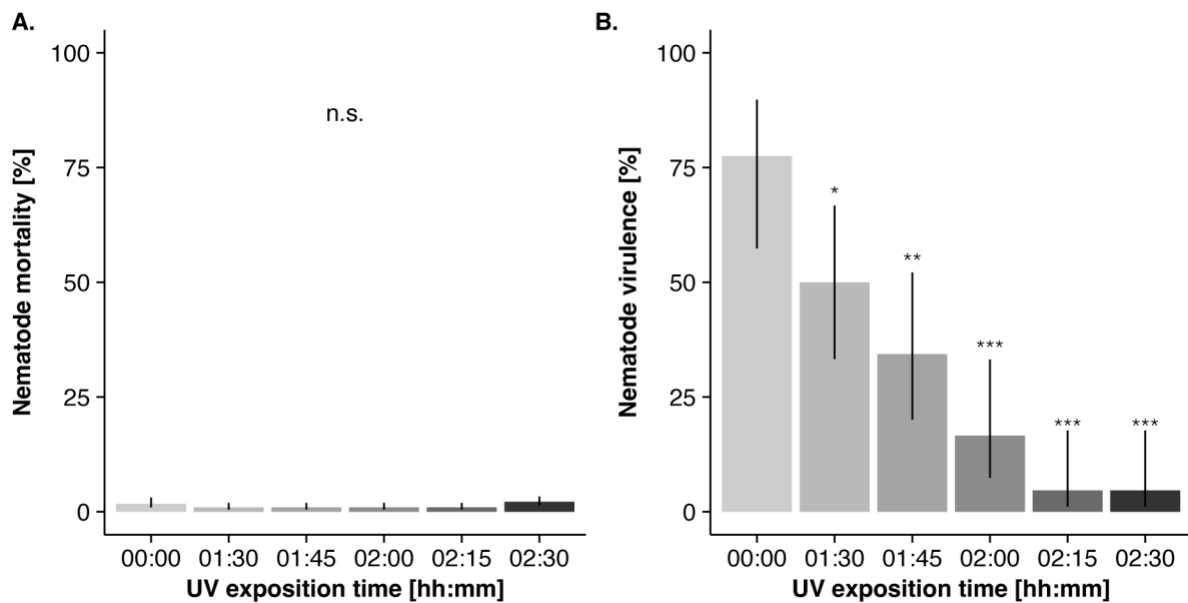


Figure S1: Mortality (A) and virulence (B) of EPN exposed to UV radiation for up to 2.5 hours. (A) Estimated least square means \pm 95% CI of the proportion of dead EPN in a Petri dish. (B) Estimated least square means \pm 95% CI of the proportion of dead *Galleria mellonella* in Petri dishes inoculated for six days with EPN previously exposed to UV radiation. EPN were tested in tap water without any additional substances. Eight Petri dishes per treatment and exposition were used in one experiment ($n = 8$). Data were analysed with generalized mixed-effect models with a binomial error distribution. Stars (*) indicate significant differences between the unexposed control (time 00:00) and each treatment obtained from many-to-one comparisons corrected for false discovery using the Benjamini and Hochberg (1995) method (n.s. = non-significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

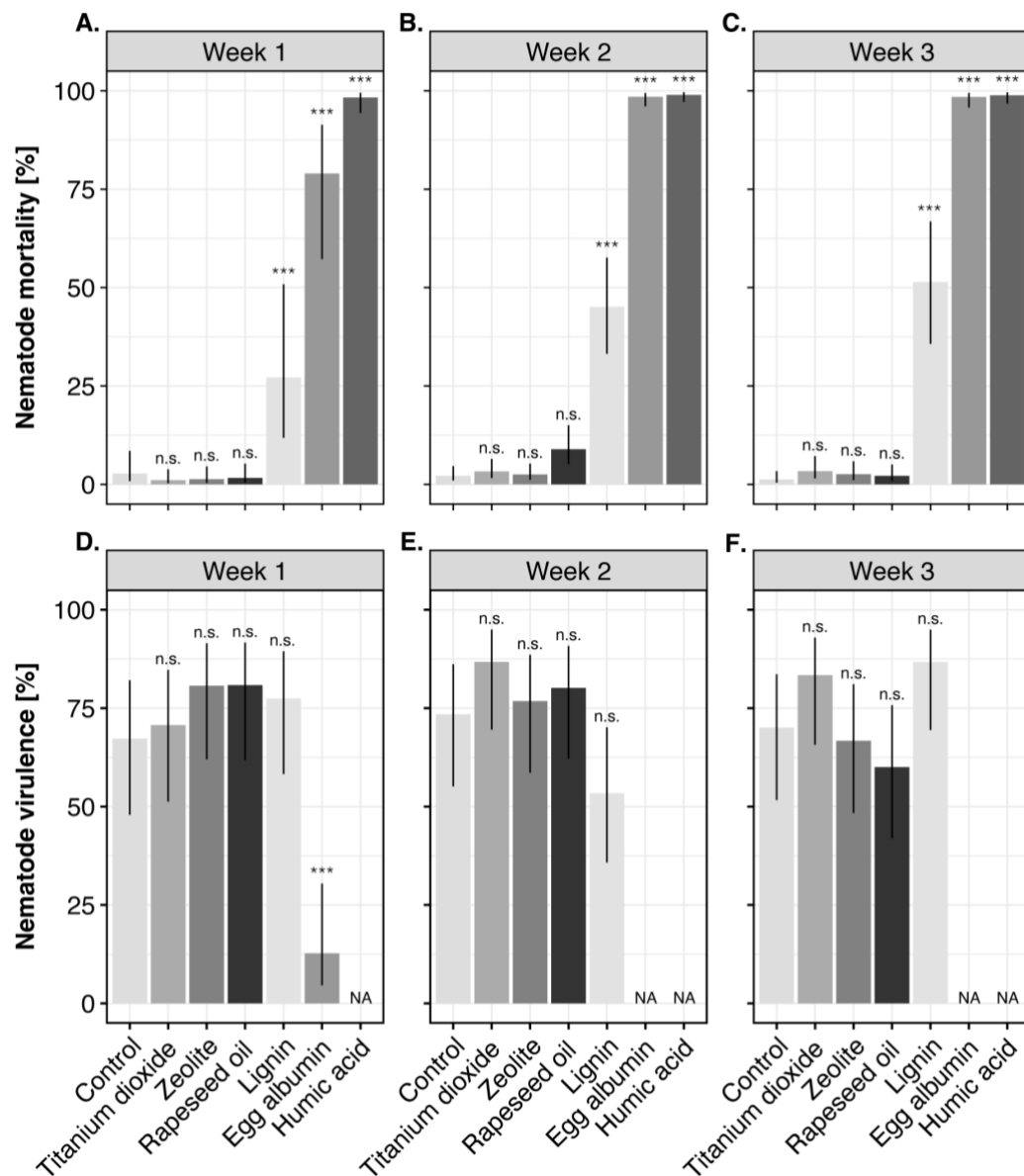


Figure S2: Toxicity of substances that can potentially be used to protect entomopathogenic nematodes against UV radiation. Mortality (A, B and C) and virulence (D, E and F) of EPN exposed to a substance for one (A and D) two (B and E) or three (C and F) weeks. (A, B and C) Estimated least square means \pm 95% CI of the proportion of dead EPN in Petri dishes. (D, E and F) Estimated least square means \pm 95% CI of the proportion of dead *Galleria mellonella* in Petri dishes inoculated for six days with EPN previously exposed to a substance. EPN were tested in solutions of 0.1% (v/v) Tween[®] 80 containing 5% (v/v or w/v) of a protective substance. As control, EPN were only exposed to the 0.1% (v/v) Tween[®] 80 solution. In total, 16 Petri dishes per treatment and week were used in two independent experiments (n = 16). Data were analysed with generalized mixed-effect models with a binomial error distribution. Stars (*) indicate significant differences between the control and each treatment obtained from many-to-one comparisons corrected for false discovery using the Benjamini and Hochberg (1995) method (n.s. = non-significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

General discussion and Outlooks

General discussion

We have shown that, when specially formulated, EPN represent highly promising alternatives to synthetic insecticides against FAW. Not only are EPN safe for farmers and the environment, they also were found to be particularly effective against the pest. The strong vulnerability of FAW caterpillars allowed the use of only small amounts of EPN in field trials – at least three-fold lower than what is normally applied to target belowground pests – making our approach economically realistic.

Chapter 1 confirms that it might be safer to use local EPN in a target country as opposed to importing commercially available strains from another region. Although we found that the strain isolated in Rwanda shared over 99% ITS sequence similarity with the *H. bacteriophora* reference strain (Fallet et al., 2020), further investigations revealed that it in fact represents a novel species, *H. ruandica* (Machado et al., 2021). This highlights the risk of misidentifying closely related species and the possibility of inadvertently introducing exotic organisms in a target country (by believing that they are already present, such as *H. bacteriophora* in our case). Furthermore, of the seven EPN strains isolated (and successfully maintained) from soils collected in Rwanda in 2014 and 2018 (Yan et al., 2016; Fallet et al., 2020), only one was a known species (Yan et al., 2016), whereas five were described as new species and one is still unidentified (Yan et al., 2016; Machado et al., 2021; Machado et al., 2022). This clearly indicates that a large proportion of the EPN species in Rwanda, and certainly throughout the world, are undescribed. Only a few companies produce EPN in Africa. It is important that these companies focus on producing native EPN. This requires the isolation of local EPN strains in most parts of the continent prior to using them to control insect pests.

Fortunately, we found in **Chapter 2** that locally isolated EPN can be as virulent as commercial ones and that most tested strains were highly lethal to FAW (Fallet et al., 2022a), which should facilitate the implementation of biological control programs based on native EPN. The use of local EPN not only mitigates the risks associated with introducing foreign species, but also offers the advantage of using strains that are well adapted to the local climatic conditions. Hence, these strains may exhibit prolonged longevity post-application – as they may be more tolerant to the prevailing environmental conditions – and thus higher performance compared to foreign strains unadapted to the local climate (Hiltpold, 2015; Shapiro-Ilan and Dolinski, 2015). In this line, and considering that most strains were highly pathogenic to FAW (Fallet et al., 2022a), it may be more beneficial to screen for strains that are tolerant to abiotic stresses, such as desiccation, heat and UV radiation, than exceptionally virulent.

In **Chapters 3 and 4**, we investigated how best to apply EPN on plants to ensure that they remain on plants post-application and that the conditions required for them to infect caterpillars are met. For this purpose, we tested three commercially available (alginate capsules and two liquid adjuvants) as well as two in-house

developed formulations (alginate beads and the CMC-based gel) and one published alginate-based formulation (Navon et al., 2002). We also tested the efficacy of EPN applied with just water or in sand. We demonstrated that formulation is key to the success of EPN against FAW. Most attempts to control FAW with EPN formulated in just water or in combination with adjuvants have been unsuccessful (Richter and Fuxa, 1990; Garcia et al., 2008; Negrisoli et al., 2010; Fallet et al., 2022b). It appears crucial to provide EPN with traits facilitating their search and infection of caterpillars (Garcia et al., 2008; Fallet et al., 2022b). In this context, the gel formulation proposed here prevents EPN from seeping out of the whorl and ensure that they remain on the plants after their application. Furthermore, it confers humidity for better EPN survival and a substrate through which EPN can orient towards and infect caterpillars. In contrast, when applied with liquid formulations, such as water or adjuvants, it is likely that most EPN will drop to the ground. The remaining EPN will accumulate deep inside the maize whorl, close to the growing point, and below the caterpillars, which usually feed a few centimetres above this point (Garcia et al., 2008). From the growing point, it is presumably very difficult for EPN to crawl up the whorl and infect caterpillars (Garcia et al., 2008).

Along with desiccation, UV radiation is one of the main factors limiting the use of EPN aboveground (Shapiro-Ilan and Dolinski, 2015). After being exposed to UV radiation, EPN rapidly lose their capacity to kill insects (Gaugler and Boush, 1978; Nickle and Shapiro, 1992; Shapiro-Ilan et al., 2015). In **Chapter 5**, we show that incorporating affordable and readily available natural substances into the gel formulation considerably improves the amount of UV radiation that EPN could withstand, and prolongs their survival without losing their pathogenicity. This should translate into enhanced efficacy under field conditions by providing a longer window of opportunity for EPN to infect FAW caterpillars. These findings contribute to improving the use of EPN in aboveground applications.

Within the framework of this thesis, we conducted two side projects that resulted in two MSc thesis. In the first project (Paupe, 2022), we explored whether selective breeding could enhance the tolerance of EPN to detrimental aboveground conditions, such as heat, desiccation and intense UV radiation. For this, we created ten lines of *S. carpocapsae* RW14-G-R3a-2. Five were reared over ten generations under laboratory conditions and were used as the control group. The other five lines were applied into maize whorls and exposed to field conditions for four days prior to bring them back to the laboratory for each rearing cycles. After ten generations, we tested the tolerance of the ten lines to extreme heat (35°C), desiccation (80% relative humidity) and UV radiation (2h exposition). Unfortunately, we observed no differences between the control and the exposed lines (Paupe, 2022). Other studies have shown that certain traits (e.g., desiccation tolerance or chemiotaxis) can be enhanced in EPN by breeding them selectively for just a few generations (Strauch et al., 2004; Hiltpold et al., 2010). We hypothesize that the selective pressure

(EPN were exposed only for four days to field conditions) was not strong enough to induce a change in the population. Alternatively, other traits may have been more important in the selection process.

In the second master's project (Chabloz, 2023), we tested whether FAW would actively feed on gels containing plant juices (different plant species, varieties and juice concentrations), plant juice extracts (different fractions of plant juices) or phagostimulant substances, such as sucrose. To this end, we placed neonates that had previously fed on maize in the centre of a petri-dish containing gels spiked with the different solutions. Although caterpillars occasionally fed on the gels, their choices were very inconsistent among experimental blocks (Chabloz, 2023). Neonates seemed to heavily prefer a treatment in one block, but then avoid it in the next, so that we were unable to discern any clear feeding preference. Yet, neonates have been shown to strongly discriminate among plants species and actively orient towards favoured hosts, while older caterpillar have shown a tendency to disperse randomly (Rojas et al., 2018). Regardless of the discrepancies in our results, it was clear that if neonates are given a choice, they will much prefer to feed on leaves than on any flavoured-gel tested.

Outlooks

To successfully apply EPN in FAW control, further research and developments are required. We feel that the focus should be on the following steps:

- **Strain selection:** Identifying EPN strains that are not only highly virulent against FAW, but importantly that are also tolerant to prevailing environmental conditions in target regions is crucial. This requires extensive screening and testing to determine the most tolerant and effective strains. Using local EPN will avoid the introduction of non-native organisms into the ecosystem, also limiting potentially cumbersome regulatory procedures. Moreover, the local strains can be expected to already be well adapted to the local conditions.
- **Formulation development:** Developing effective and practical formulations for EPN application, is essential to ensure their ease of use and optimal performance in the field. It is crucial that the nematodes remain on the plants after their application and that the conditions allowing them to infect caterpillars are met (sufficient humidity; ability to move towards their host). In this context, the here presented gel formulation was proven quite effective in the Rwandan field trials, but further improvement are envisioned (Fallet et al., 2022a). For instance, antidesiccants should contribute to the survival and persistence of EPN on plants. Furthermore, as shown, adjuvants that protect EPN from solar radiation might also increase EPN survival on exposed leaves. Finally, efforts should aim at facilitating the delivery of EPN on plants. The use of large caulking-guns may allow the

application of the gel formulation on large areas. Alternatively, developing sprayable formulations could enable the use of conventional farming equipment and machinery.

- **Integrated Pest Management (IPM):** Integrating EPN with other compatible pest management strategies, including cultural practices and other control agents, such as egg parasitoids and insect-killing microbes, could enhance the overall efficacy of FAW control. Further, the addition of repellent odours in the formulation may discourage moths and/or caterpillars to lay eggs or feed on the plants, providing dual beneficial effects.
- **Field trials and monitoring:** Evaluating the effectiveness of EPN-based control methods in diverse agroecosystems with varying pest pressure and temporal patterns would allow to fine-tune the frequency of treatment applications and ensure high efficacy. This should include regular monitoring and assessment of the impact on the target pest, as well as on non-target organisms and overall environmental safety.

From research to commercial use

Academic research alone will not lead to the adoption and use of any EPN-based control strategy. Raising awareness among farmers about the potential of EPN for FAW management as well as about the negative impacts of synthetic insecticides and providing educational resources and training programs is needed. Further, the involvement of industrial partners is key to the widespread distribution of any control strategy. In addition, limiting the cost of the EPN is primordial to ensure that farmers can afford their use. Subsidies or governmental programs may be needed. Alternatively, we envision that in low-income countries with subsistence farming, EPN could be produced on farms by farmers themselves if they were provided with specific training. To facilitate this, we could imagine micro family-businesses producing EPN and selling them to neighbours for FAW – or any other pest – control.

Conclusion

The application of EPN holds immense promise to sustainably manage FAW in its native as well as in the invasive range. The ecology of FAW, with its specific feeding behaviour, is particularly well suited to successfully apply EPN, while the unique biology of EPN makes them highly effective in rapidly killing FAW, with virtually no risk to the environment or to the development of insect resistance. The fully implementation of an EPN-based strategy requires further research to optimize their formulation and to ensure their integration into IPM approaches. By exploring next steps and by fostering collaboration among researchers, policymakers, and farmers, we should be able to harness the full potential of EPN to combat FAW and contribute to sustainable agriculture and food security, particularly in Africa and Asia.

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