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Harnessing environmental microbiological interventions with micro- and macroorganisms for assessing cattle tick management

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***Rhipicephalus microplus*, commonly known as the cattle tick, is responsible for causing severe economic losses in livestock production in several countries. The utilization of entomopathogens in infested pastures may represent a sustainable and eco-friendly alternative for tick control. This study evaluated the effectiveness of combining entomopathogenic fungi (EPF, *Metarhizium* spp.) and entomopathogenic nematodes (EPN, *Heterorhabditis bacteriophora*) for controlling *R. microplus*. Laboratory assays tested sub-doses of *M. robertsii* IP 146 conidia in combination with *H. bacteriophora* HP88 infective juveniles against engorged females of *R. microplus*, whereas field trials assessed a granular formulation containing *M. robertsii* microsclerotia applied with *H. bacteriophora* infective juveniles in infested field plots to target the tick's non-parasitic phase during rainy and dry seasons. In laboratory experiments, the co-application of fungal sub-doses and nematodes demonstrated synergistic effects, significantly enhancing tick control. Field applications during the rainy season achieved tick population reductions of 54.09% (*M. robertsii*), 38.11% (*H. bacteriophora*), and 46.72% (combination). During the dry season, only the fungal formulation significantly reduced tick populations, with 26.27% efficacy. These findings underscore the potential of EPF and EPN, either singly or in combination, as complementary tools to traditional chemical methods for sustainable cattle tick management.**

Keywords Entomopathogenic fungi, Entomopathogenic nematodes, Field trial, Biological control, Microsclerotia, Granular formulation

Entomopathogenic fungi (EPF) and nematodes (EPN) are prominent among the micro- and macroorganisms, respectively, employed in pest biocontrol worldwide, as they pose minimal risk to non-target organisms and are pivotal in integrated pest management programs to arthropods of agricultural economic importance[1]. Species of the genus *Metarhizium* are pathogenic to a wide variety of arthropods[2]. They are also the most studied entomopathogenic fungus due to their broad host spectrum, genetic diversity, and lifestyles spanning saprobes, plant endophytes, and entomopathogens[3]. *Metarhizium* spp. can produce different types of propagules, such as conidia and blastospores[4,5], and some species may produce, by liquid fermentation, structures formed by aggregates of dark-pigmented and compact hyphal threads known as microsclerotia. Notably, microsclerotia are intrinsically more resistant to desiccation, heat stress, and UV-B radiation than aerial conidia, mycelial pellets, and blastospores[6,7], but they cannot directly infect the arthropod hosts; as such, when exposed to favorable climatic conditions, they produce conidia through sporogenesis that can initiate the infectious process[8].

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EPN within the genus *Heterorhabditis* (Poinar, 1975) (Rhabditida: Heterorhabditidae) are free-living, rounded worm-like macroorganisms used in the biological control of different arthropod pests[9,10], with marked virulence to ticks[11–14]. The infectivity of *Heterorhabditis* spp. occurs by the penetration of infective juveniles (IJs) through the natural openings of their hosts; after reaching the hemocoel, infective juveniles (IJs) release symbiotic bacteria that kill the arthropod by septicemia[10]. Both agents, fungi and nematodes, are produced on a commercial scale and extensively used for pest control worldwide[15,16]. However, biotic or abiotic variables such as adverse environmental conditions[17–19], microbial competitors, and host ecology may limit the success of entomopathogens in biological pest control programs[20]. Over the years, many formulations containing entomopathogens as active ingredients have been developed to enhance their efficacy against arthropod pests inhabiting different environments, above or below the ground[21], with appropriate formulations that may afford protection to entomopathogens when exposed to environmental constraints[22–25]. Moreover, efforts have optimized and fine-tuned processes for the mass production of entomopathogens aiming to support their field application strategies through innovative and cost-effective methods[15,26,27].

Engorged females of *Rhipicephalus microplus* (Canestrini, 1888) (Acari, Ixodidae), which is an important ectoparasite of cattle[28], drop off their host at the end of their parasitic phase and seek for microenvironments with mild temperatures and high humidity to lay their eggs on the soil, then start their non-parasitic phase in the pasture. These microenvironments are similarly conducive to the survival of both EPF and EPN[23,24,29], which suggests that these entomopathogens may be promising agents for tackling *R. microplus* in infested pastures. Cattle in tropical countries often have high levels of tick infestations, and these individuals do not represent the entire population of *R. microplus* in the area. Estimates indicate that only 5% of ticks are found parasitizing the cattle, whereas the remaining 95% of the tick population is dispersed in the pastures during the non-parasitic phase[30,31]. According to this principle, it is important to design strategies employing biocontrol agents against different stages of *R. microplus*[13,23,24,29].

The combined use of multiple entomopathogens with different mechanisms of action against a target host has been investigated previously, especially against Coleoptera and Lepidoptera[22,32–35]. Few studies investigated the combination of EPF and EPN for tick control[36,37]. The joint application of biocontrol agents may offer a smart, unconventional strategy to increase their effectiveness while reducing their inoculum doses[22]. In this study, we evaluated the efficacy of the combination between EPF and EPN against engorged females of *R. microplus*. In addition, we tested several granular formulations and selected one containing microsclerotia of *Metarhizium robertsii* IP 146 that afforded fungal sporulation in non-sterilized soil samples. Additionally, our study sought to evaluate the field effectiveness from the combined application between microsclerotial granular formulation of *M. robertsii* IP 146 and the IJs of *H. bacteriophora* HP88 against engorged females of *R. microplus* during their non-parasitic phase in artificially infested pastures. Lastly, we conducted investigations to assess the field persistence of nematodes and fungi in the soil.

Material and Methods

Origin of ticks

Engorged females of *R. microplus* (Gyn strain) were obtained from artificially infested cattle held at the Federal University of Goiás. This strain is resistant to synthetic pyrethroids, formamidines, organophosphates, and phenylpyrazoles[38]. The engorged females were selected according to their integrity, mobility, and engorgement. They were then weighed and distributed by weight as homogeneously as possible among treatments and control groups.

Entomopathogenic fungi and nematodes

The EPN, namely *Heterorhabditis bacteriophora* HP88, tested in the present study, was selected due to its high virulence against *R. microplus* engorged females[12,14]. This isolate is originated from infected June beetle larvae (*Phyllophaga* sp.)[39] and has been held in the laboratory in distilled water in 40-mL cell culture flasks, and IJs are routinely produced in larvae of *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae) at room temperature (22 ± 2 °C)[40,41]. The nematodes suspended in distilled water were counted using a light microscope[42]; the mean number of IJs was calculated, and the desired inoculum concentration was further adjusted as indicated for the bioassays.

Metarhizium spp. isolates (Table 1) were cultured on potato dextrose agar medium (Difco Laboratories, Maryland, NY, USA) supplemented with 0.01% (w/v) yeast extract (Difco Laboratories, Maryland, NY, USA) (PDAY) in polystyrene Petri dishes (90 × 15 mm; Cralplast®, São Paulo, SP, Brazil), incubated at 27 ± 1 °C and relative humidity (RH) > 90% for 15 days with 12 h photoperiod. Conidia were harvested from the surface of the culture medium with a spatula and suspended in 10 mL 0.01% (v/v) Tween 80® solution (Labsynth Prod. Lab. Ltda., Diadema, SP, Brazil). Suspensions were vortexed, filtered through sterile gauze, quantified, and adjusted to 1×10^7 conidia mL^{-1}. Conidial suspensions were used only if conidial viability was deemed to be > 98% in all experiments.

Microsclerotia were produced by submerged liquid fermentation with medium consisting of a carbon–to-nitrogen ratio (C:N) of 30:1, according to Mascarin et al.[27]. *Metarhizium robertsii* IP 146 was cultured in non-baffled conical Erlenmeyer glass flasks (250 mL) containing 1.5 g yeast extract, 53.5 mL basal medium, 36.5 mL glucose solution (7.3% w/v, Labsynth Prod. Lab. Ltda., Diadema, SP, Brazil) and inoculated with 10 mL of conidial suspension (1×10^6 conidia mL^{-1}), in a total volume of 100 mL per flask[27]. The flasks were sealed with hydrophobic cotton plugs and incubated at 250 rpm in an orbital shaker for 4 days at 27 ± 1 °C. The biomass was quantified and adjusted to 10^4 microsclerotia mL^{-1}.

Strains*	Substrate; Origin	Equivalent strains**
Metarhizium anisopliae s.l.		
IP 363	Soil; Terezópolis de Goiás, Goiás, Brazil	-
Metarhizium anisopliae s.str.		
IP 119	Soil; Niquelândia, Goiás, Brazil	CG 764; BRM 003099
Metarhizium humberii		
IP 46	Soil; Ema national park, Goiás, Brazil	CG 620; ARSEF 12874
Metarhizium robertsii		
IP 125	Soil; Pirenópolis, Goiás, Brazil	CG 770; ARSEF 12886
IP 146	Soil; Silvânia, Goiás, Brazil	CG 780; ARSEF 12889

Table 1. Strains of entomopathogenic fungi investigated in this study and its origin. (*) IP: Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás (Goiânia, Goiás, Brazil). (**) Equivalent strains in other Culture Collections: CG: Invertebrate Fungal Collection at Embrapa Genetic Resources and Biotechnology (Brasília, Federal District, Brazil); BRM: Embrapa Microbial Collections (Brasília, Federal District, Brazil); ARSEF: Agricultural Research Service—Entomopathogenic Fungus Collection, United States Department of Agriculture (Ithaca, New York, USA).

Combined effect of *Metarhizium* spp. and *H. bacteriophora* against *R. microplus* engorged females under laboratory conditions

The assays were composed of 12 groups: a control group treated with only 0.01% Tween 80® solution, five groups treated with conidial suspensions of *Metarhizium* spp. (IP 46, IP 119, IP 125, IP 146, or IP 363), a group treated with *H. bacteriophora* HP88, and five groups treated with the combination of *Metarhizium* spp. with *H. bacteriophora* HP88 (HP88 + IP 46, HP88 + IP 119, HP88 + IP 125, HP88 + IP 146, or HP88 + IP 363). Each treatment or control (mock) group had 10 *R. microplus* engorged females with the same mean body weight ($p < 0.05$). Each female from the same group was individually conditioned in wells of a 12-well tissue culture plate (Kasvi®, São José do Pinhais, Paraná, PR, Brazil) containing two sheets of filter paper at the bottom. One well of each plate was filled with a cotton plug that received 1 mL of sterile distilled water. The cotton plug was checked daily and kept moistened during the entire experiment to guarantee high humidity inside the plate.

In the groups of females treated with *H. bacteriophora*, a 200-µL aliquot of the nematode suspension (50 IJs) was applied to the filter paper at the bottom of each well. In the groups treated with fungi, 10 mL of conidial suspension (1×10^7 mL^{-1} conidia/mL) were equally distributed into 10 of 1.5-mL microcentrifuge tubes, and the females were individually immersed for 3 min and then transferred to the wells. In the groups treated with combined fungi and nematodes, the females were immersed in conidial suspension and then exposed to the nematodes on the treated filter papers, as described above. The females from the control group were immersed in 1 mL of 0.01% Tween 80® solution for 3 min and then transferred to the wells. After treatment, all ticks placed in tissue culture plates were incubated at 27 ± 1 °C and RH ≥ 90%. The egg mass of each female was harvested daily and transferred to a glass test tube (16 × 125 mm) sealed with a hydrophilic cotton plug and held at 27 ± 1 °C and RH ≥ 90%.

The following response variables were recorded: engorged female initial weight (before oviposition) (mg), egg mass weight (mg), female residual weight (mg) (after oviposition), and larval hatch (%). The eggs were observed daily, and the larval hatching in each replicate tube was visually estimated by microscopic examination. The values were assigned in percentages ranging from 0 to 100% by intervals of 5% in relation to the total mass of eggs[4]. Then, the following reproductive parameters were calculated: egg production index [EPI = weight of egg mass (g) / initial weight of engorged female (g) × 100][43], nutrient index [NI = weight of egg mass (g) / initial weight of engorged female (g) – final weight of female (g) × 100][43], estimate reproduction [ER = weight of egg mass (g) / initial weight of engorged female (g) × percentage of larval hatch × 20,000 (constant which refers to the number of larvae per gram of *R. microplus* egg mass)], and control efficacy [CE = (mean ER of the control group – mean ER of the treated group) / mean ER of the control group × 100][44]. The experiments were performed in three independent trials using new batches of fungi and nematodes to ensure data reproducibility.

Combined effect of low concentrations of *M. robertsii* and *H. bacteriophora* against *R. microplus* engorged females under laboratory conditions

The effect of low concentrations of *M. robertsii* IP 146 (10^6 and 10^7 conidia mL^{-1}) combined or not with low concentrations of *H. bacteriophora* HP88 (15 or 25 IJs/female) against *R. microplus* engorged females was investigated. The suspensions of conidia or nematodes were prepared, and the engorged females were treated as mentioned above; however, each treatment or control group had 20 engorged females with the same mean body weight. The experiments were performed in two independent trials.

Preparation of fungal granular formulations with different excipients

Various combinations of excipients or co-formulants (Table 2) were assembled based on the studies reported by Fernandes et al.[45] and Santos et al.[25] to prepare nine granular formulations. The excipients were weighed and mixed with microsclerotia of *M. robertsii* IP 146 (10^4 microsclerotia mL^{-1}), which were produced as reported above. The ratio of excipients was optimized to create uniform mixtures capable of passing through a 1-mm mesh to form granules by mechanical extrusion. After extrusion, different formulations were individually subjected to

Formulations	Description *
1	16 g cellulose + 4 g diatomaceous earth + 20 mL biomass suspensed in water
2	8 g cellulose + 8 g vermiculite + 1 g silicon dioxide + 20 mL biomass suspended in water
3	16 g cellulose + 4 g diatomaceous earth + 2 g silicon dioxide + 25 mL biomass suspended in culture medium
4	8 g cellulose + 8 g vermiculite + 1 g silicon dioxide + 1 g *Psyllium* + 25 mL biomass suspended in water
5	8 g cellulose + 8 g vermiculite + 2 g diatomaceous earth + 1 g *Psyllium* + 20 mL biomass suspended in culture medium
6	6 g silicon dioxide + 6 g *Psyllium* + 20 mL biomass suspended in water
7	16 g cellulose + 2 g *Psyllium* + 20 mL biomass suspended in culture medium
8	16 g vermiculite + 25 mL biomass suspended in culture medium
9	20 mL biomass suspended in culture medium

Table 2. Granular formulations containing different combinations of excipient and biomass (with or without culture medium) of *Metarhizium robertsii* IP 146 (10^4 microsclerotia mL^{-1}). (*) Origin of reagents: Cellulose: Mingtai Chemical Co., Lt., Taoyuan City, Taiwan; Vermiculite: D'Agro, Produtos Agropecuários, Itapetininga, São Paulo, Brazil; Diatomaceous earth: Agrosoil, Insumos Agrícolas, Jaguariúna, São Paulo, Brazil; Silicon dioxide: Labsynth, Produtos Para Laboratórios LTDA., Diadema, São Paulo, Brazil; *Psyllium*: Natural do Norte, Brazil. Husk comes from the crushed seeds of the *Plantago ovata* plant, a herb native from parts of Asia, the Mediterranean, and North Africa (Goble et al. 2016).

fluid-bed drying (Labmaq®, São Paulo, SP, Brazil) set to 35 ± 5 °C and airflow rate of 1.8 m^3 min^{-1} for 25 min or until obtaining final moisture of approximately 5% (w/w).

The efficacy of each formulation for producing viable conidia was assessed on water-agar medium and non-sterilized soil samples according to the methods reported by Jackson and Jaronski[8] and Paixão et al.[46], with modifications as indicated below. The tests were conducted in triplicates, with polystyrene Petri dishes (60 × 15 mm) containing water-agar medium (2 g of agar in 100 mL of distilled water) or 5 g of non-sterilized soil. The soil samples were previously collected from the Campus of the Federal University of Goiás (16°35′37.0" S 49°16′53.2" W) and stored in plastic bags in the laboratory at room temperature. The soil collected was classified as sandy clay loam soil, containing 30% clay, 12% silt, and 58% sand. In the test performed with water-agar medium, 30 mg (10 microsclerotia mg^{-1}) of each formulation were distributed on the medium surface, and the dishes were incubated at 27 ± 1 °C and RH ≥ 90% for 10 days. In tests carried out on non-sterilized soil, 7 mg (10 microsclerotia mg^{-1}) of the formulations were distributed on the surface of soil samples in dishes incubated at the same conditions indicated above. The experiments were performed in three independent trials.

After the tenth day, the granules were collected by harvesting conidia from the medium surface with a spatula and transferred into 50-mL test tubes containing 10 mL of sterile 0.01% Tween 80® solution, and spore suspensions were vortexed for 1 min before counting. The conidia produced from the different granular formulations were quantified using a hemocytometer. In contrast, the conidial viability was assessed by applying 20-µL aliquots of an adjusted conidial suspension (1×10^6 conidia mL^{-1}) onto the center of Petri dishes (55 × 15 mm) containing 8 mL PDAY medium supplemented with 0.002% (w/v) benomyl[47,48]. Fungal cultures were incubated at 27 ± 1 °C for 24 h. After this period, germinated and non-germinated conidia were quantified, and the relative germination was calculated[48].

The soil samples inoculated with fungal granules were also transferred to test tubes containing 10 mL 0.01% Tween 80®. After stirring for 1 min, 100 µL aliquot of each tube containing a different granular formulation was pipetted into 900 µL of distilled water in 1.5-mL microcentrifuge tubes. Thus, 50 µL aliquots were pipetted into the center of CTC medium[49] in Petri dishes (90 × 15 mm). The aliquot was spread evenly on the selective medium with bent glass rods, and the plates were further incubated at 27 ± 1 °C for 7 days. Then, the colony-forming units (CFU) on the CTC medium were quantified and expressed in CFU g^{-1} of soil.

Combined use of *M. robertsii* and *H. bacteriophora* to control *R. microplus* under field conditions

Two tests under field conditions were performed: one from January 5 and April 9, 2021 (rainy season) and one from July 6 to October 21, 2021 (dry period). The tests were composed of four groups: 1) the group treated with an aqueous suspension of the nematode *H. bacteriophora* HP88; 2) the group treated with the microsclerotial granular formulation of *M. robertsii* IP 146; 3) the group treated with both nematode suspension and fungal granules (HP88 + IP 146); and 4) a control group that received no treatment.

Preparation of nematode suspension and granular fungal formulation

EPN were produced in 80 T. *molitor* larvae in Petri dishes (150 × 25 mm) with the bottom covered with two layers of filter paper (J Prolab, São José dos Pinhais, Paraná, PR, Brazil). Five milliliters of the nematode stock suspension (ca. 200 nematodes) were applied to the filter papers. The dishes were sealed with Parafilm® and incubated at 27 ± 1 °C and RH ≥ 90% for 4 days. Then, *T. molitor* larvae with signs of infection (reddish color) were transferred to new Petri dishes (150 × 25 mm) with two layers of filter paper at the bottom, with no water source, and incubated again under the same conditions for 4 days. For EPN mass production, *T. molitor* infected larvae were distributed above 30-cm PVC pipes (halved, sagittal plane) placed (concave side down) at the bottom of plastic trays filled with distilled water at room temperature for 9 days. This process allowed the emerging IJs to move into the water phase for accumulation and subsequent collection. The suspension containing the IJs

was adjusted to 5×10^3 IJs/mL according to the method mentioned above. The nematode suspension was then distributed to 1 L-plastic hand sprayer to be dispersed in the field plots.

The fungal granules (formulation #5; see Table 2) prepared for the field trials were selected based on the results of previous laboratory tests. A total of 160 g of fungal granules with 3.75×10^2 microsclerotia g^{-1} were produced according to the method in section "Preparation of fungal granular formulations with different excipients" and stored at 5 ± 1 °C.

Preparation of the field plots

The experiments were carried out in an area of Embrapa Arroz e Feijão – Goiânia, Goiás, Brazil, located at 16°30′50.1" S and 49°16′33.8" W. One month before starting the experiments, thirty-two plots containing *Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs (formerly, *Panicum maximum*) (Panicoideae: Paniceae) 'BRS Quênia' were cut at 30 cm in height from the soil surface to the apex of the leaves. Eight plots were used for each treatment or control group. The plots were arranged in an area with direct incidence of sun and rain, with similar exposure to environmental conditions. Three weeks before the test started, the plots were randomly distributed as part of control or treatment groups. Each plot was delimited using a measuring tape, iron piles, and string, with an area of 1 m × 1 m (length × width) (Fig. 1). Three soil samples were collected at 10 cm depth from different points of the studied area, vigorously mixed, stored in plastic bags, identified, and sent to a soil analysis laboratory (Soil & Company Laboratory, Goiânia, GO, Brazil) for granulometry analysis. The temperature and relative humidity in the center of one of the plots randomly chosen were collected daily using a HOBO® data logger U12 (Onset Computer Corporation, Bourne, MA, USA) throughout the experiment. The probe sensor was placed over the ground in the center of the selected plot. Additionally, environmental conditions were recorded daily by the weather station located at 16°28′00"S and 49°17′00"W (approximately 1 km away from the experimentation site).

Treatment of the field plots

Ten grams of the fungal granules containing microsclerotia of *M. robertsii* IP 146 were applied to each treatment plot, distributed as homogeneously as possible on the soil surface, ten days prior to transferring the engorged tick females (day -10). One liter of aqueous suspension with *H. bacteriophora* HP88 was applied to each treatment plot at 50 IJs/cm^2 two days before transferring the engorged females (day -2). Five *R. microplus* engorged females were distributed to each plot from the control or treatment group at day zero. The recovery of larvae from every plot was performed weekly between day + 35 to + 85 during the rainy season and day + 42 to + 98 during the dry season. The collections were carried out by depositing a white flannel (1.0 × 1.0 m) on each plot for 15 min. Then, the flannels were removed, individually placed in plastic bags, identified, and stored at -20 °C for later counting. The larvae from each flannel were quantified using a surgical fluid aspirator (Aspiramax®, Kyoto, Japan), and the numbers were recorded. The efficacy of the treatments to control *R. microplus* in the field was calculated by using the following equation: Efficacy (%) = [(total number of larvae recovered from the control group – total number of larvae recovered from the treatment group) / (total number of larvae recovered from the control group)] × 100[50]. The Fig. 2 provides an overview of this method.

Persistence of M. robertsii and nematodes in field plots

The number of viable fungal propagules in the plots treated with the microsclerotial granular formulation was accessed during the experiment. Soil samples from the plots treated with the fungus were collected from three distinct points with 5–10 cm depth every two weeks (day + 10, + 23, + 38, + 52, + 66, and + 88) and stored in 50-mL conical centrifuge tubes adequately identified. Five grams of each soil sample were mixed with 10 mL of 0.01% (v/v) Tween 80® solution in 50-mL centrifuge tubes, vortexed for 1 min, and diluted to 1:10; then, 50 µL aliquots were pipetted onto individual Petri dishes (90 × 15 mm) containing CTC selective culture medium[49], and the inocula were spread evenly on the selective media with bent glass rods and the plates incubated at 27 ± 1 °C and RH ≥ 90% for 7 days. The colonies were quantified, and the presence of *M. robertsii* was confirmed by microscopic analysis[51].

Fig. 1. The area containing 1 m^2 plots of *Megathyrsus maximus* BRS Quênia used in the field test at Embrapa Arroz e Feijão, Santo Antônio de Goiás—Goiás, Brazil. Location: 16°30′50.1" S and 49°16′33.8" W. A) test performed in the rainy season and B) in the dry season.

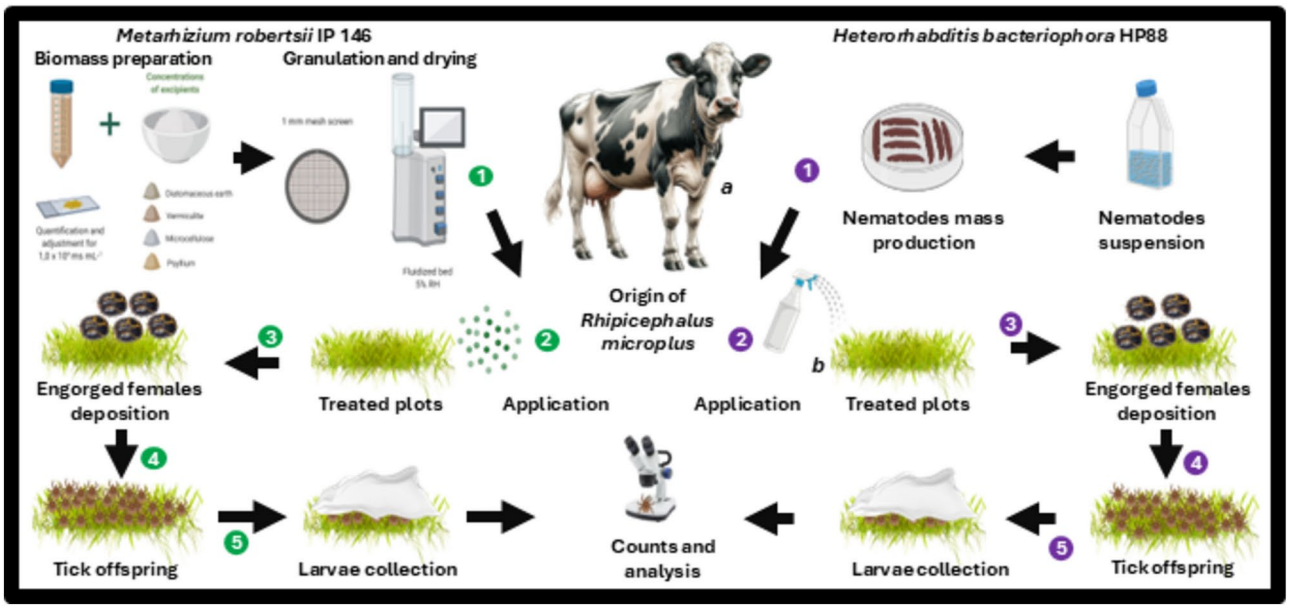

Fig. 2. The fungus *Metarhizium robertsii* IP 146 and the nematode *Heterorhabditis bacteriophora* HP 88 applied to field plots under challenging environmental conditions show promise as biological agents for controlling the non-parasitic phase of the tick *Rhipicephalus microplus* in cattle pastures: 1) Production of fungal granules or nematode suspension; 2) Application to field plots; 3) Deposition of engorged females to treated plots; 4) Larval hatching; 5) Larvae collection, counts and analysis. The images in ***a*** and ***b*** were sourced from free stock media websites (pixabay.com and designi.com.br, respectively), while the additional schematics were created using BioRender.com.

Soil samples from the nematode-treated plots were collected and stored as mentioned above. The isolation of nematodes from each sample used *T. molitor* as bait. Ten *T. molitor* larvae were applied over 5 g of soil deposited on Petri dishes (60 × 15 mm) and incubated at 27 ± 1 °C and RH ≥ 90% for 7 days. Dead larvae with signs of infection were superficially disinfected with 0.1% sodium hypochlorite solution, placed in Petri dishes (150 × 25 mm) with two layers of filter paper at the bottom, and incubated in a moist chamber at 27 ± 1 °C and RH ≥ 90%. After three days, the insects were transferred to White's traps[52] to isolate the IJs. The presence or absence of IJs was confirmed by pipetting aliquots of the suspension on slides and subsequent observation using a light microscope.

Statistical analysis

The analyses conducted in the present study were performed in the R software version 4.1.0[53], using the 'easyanova' package[54]. All data sets were previously checked for normality and homoscedasticity using Shapiro–Wilk's and Levene's tests. The data with normal distribution were analyzed using the Analysis of Variance (ANOVA) test followed by the Student–Newman–Keuls (SNK) means comparison test. In the bioassays, only the initial weight of engorged females and the viability of fungal formulations showed normal distribution. The data with non-normal distribution were analyzed by the Kruskal–Wallis test followed by the means comparison test using False Discovery Rate (FDR)[55]. The weight of egg mass, larval hatch, egg production index, nutrient index, and the number of colony-forming units (CFU) on non-sterilized soil (controlled conditions) showed a non-normal distribution. To determine the type of interaction between subdoses of nematode combined with doses of the fungus, the control efficacy (CE) of each single and combined treatments was calculated based on the variable ER (estimate reproduction) with a methodology adapted from Kaiser et al.[56]. ER data were fitted to a linear mixed model with normal distribution where the predictor included a fixed term for fungus and nematode interaction and a random effect attributed to the 40 female ticks tested in each treatment. According to this model, predicted values for ER from each single treatment of fungus and nematode were used to calculate the expected additive effect on control efficacy ($CE_{additive}$) using the following formula: $CE_{additive} = CE_{fungus} + CE_{nematode}$ x $(1 - CE_{fungus})$, where CE_{fungus} is the expected control efficacy attributed only to the fungus and $CE_{nematode}$ is the expected control efficacy due only to the nematode. To compute the difference between $CE_{additive}$ and the observed control efficacy from the combined treatments ($CE_{combined}$), we used the following: $\Delta CE = CE_{additive} - CE_{combined}$, where $CE_{combined}$ refers to the observed control efficacy (%) based on the variable ER from the mixture of both biocontrol agents. In that way, the interaction between these two biocontrol agents was classified as synergistic when $\Delta CE > 0$ was significant at $p < 0.05$ according to the *t*-ratio test.

The CFU (field trials) were adjusted to a mixed generalized linear model with $\log_{10}(x+1)$ transformation for data normalization, with the time factor as a random variable. Regarding the field tests, the same model was used to analyze the possible interactions of the climatic conditions (maximum and minimum temperature and relative humidity), climatic season (rainy or dry season), and applied treatment (EPF, EPN or their combination) on the

persistence of fungi in the soil. Data on nematode persistence in field plots expressed as proportion of parasitized mealworm larvae were fitted to a generalized linear mixed model with random effect for the observational level and fixed effects attributed to treatment, evaluation time, and their interaction term. The number of larvae recovered from the field plots was fitted with a generalized linear model (GLM) following a quasi-Poisson distribution (log link function), including the fixed effects of block, climatic season, time of evaluation after the application of bioagents, treatments with biological agents (alone or combined), and the possible interactions of these factors. The analyses were performed separately within each climatic season (rainy or dry season) to assess the effect of treatments over time on the larvae population in the field plots. The treatment means were compared using the Tukey HSD test at 5% significance using the 'emmeans' package[57].

Results

Combined effect of *Metarhizium* spp. and *H. bacteriophora* against *R. microplus* engorged females under laboratory conditions

The mean initial weight of engorged females did not differ significantly among control and treatment groups, demonstrating that the females were homogeneously distributed by weight ($F_{11,328} = 0.626$; $p = 0.806$) (Table 3). Females from all treated groups laid fewer eggs than the females from the control group ($F_{11,327} = 59.23$; $p < 0.001$). The mean egg mass weight ranged from 5.23 mg (in the group treated with HP88) to 97.08 mg (in the group treated with IP 125), whereas in the control group, the mean egg mass weight was 127.35 mg ($\chi2_{11} = 213.68$; $p < 0.001$). Mean larval hatching was significantly lower in the treatment groups [varying from 24.54% (HP88 + IP 146) to 79.80% (IP 119)] than in the control group (92.76%) ($\chi2_{11} = 87.21$; $p < 0.001$).

Also, the egg production index (EPI) and the nutrient index (NI) were lower in all treatment groups than in the control group ($\chi2_{11} = 216.11$; $p < {}^{0.001;}\chi^2_{11} = 73.667$; $p < 0.001$, respectively). The control efficacy of ticks treated with fungi ranged from 42.74% (IP 125) to 58.19% (IP 146), whereas the control efficacy of ticks treated with nematodes was 97.34% (HP88). The control efficacy of ticks treated with fungi and nematodes combined ranged between 94.51% (HP88 + IP 46) and 98.88% (HP88 + IP 146) (Table 3).

Combined effect of low concentrations of *M. robertsii* and *H. bacteriophora* against *R. microplus* engorged females under laboratory conditions

The mean initial weight of engorged females did not differ significantly among treatment and control groups ($F_{8,351} = 0.096$; $p = 0.999$) (Table 4). The mean weight of egg mass was low in the groups of females treated with fungi (10^6 or 10^7 conidia mL^{-1}), with values of 95.67 mg or 73.29 mg, respectively, or in their respective combinations with different concentrations of IJs (15 or 25), with values ranging from 42.35 mg (10^7 conidia mL^{-1} + 25 IJs) to 57.42 mg (10^7 conidia mL^{-1} + 15 IJs). The control group, however, had a mean weight of egg mass of 124.12 mg ($\chi2_8 = 123.78$; $p < 0.001$). A reduced larval hatch was detected in the group treated with fungi at 10^7 conidia mL^{-1} (81.15%) or with 15 IJs (87.10%), or in all combinations of fungi and nematodes with values

Treatment	Weight of engorged females (mg)	Weight of egg mass (mg)	Larval hatch (%)	EPI*	NI**	ER†	Control Efficacy ($n = 30$)‡
Control	234.73 ± 7.80^{a}	127.35 ± 5.37^{a}	92.76 ± 0.79^{a}	53.55 ± 1.36^{a}	82.22 ± 1.14^{a}	994,138.26	-
IP 46	232.75 ± 7.84^{a}	63.44 ± 8.76^{c}	75.18 ± 4.12^{b}	28.16 ± 3.72^{c}	46.07 ± 6.65^{bc}	457,653.81	53.96
IP 119	235.36 ± 8.90^{a}	66.5 ± 7.84^{c}	79.80 ± 2.67^{b}	29.33 ± 3.66^{c}	58.76 ± 9.20^{bc}	465,954.91	53.13
IP 125	253.22 ± 7.43^{a}	97.08 ± 8.49^{ab}	73.50 ± 3.63^{bc}	38.06 ± 2.49^{b}	64.54 ± 3.03^{cde}	569,193.63	42.74
IP 146	235.31 ± 7.52^{a}	75.7 ± 6.85^{bc}	66.54 ± 4.39^{bd}	33.01 ± 2.89^{bc}	63.02 ± 3.11^{b}	415,629.76	58.19
IP 363	235.73 ± 7.66^{a}	76.76 ± 5.39^{bc}	74.83 ± 4.72^{b}	33.38 ± 2.35^{bc}	52.46 ± 5.12^{bd}	533,902.23	46.29
HP88	234.89 ± 7.67^{a}	5.23 ± 2.36^{d}	38.64 ± 7.95^{de}	2.18 ± 0.94^{d}	23.97 ± 6.50^{e}	26,463.26	97.34
HP88 + IP 46	235.82 ± 7.54^{a}	11.50 ± 2.81^{d}	45.62 ± 7.05^{de}	4.71 ± 1.15^{d}	44.13 ± 8.22^{bd}	54,549.51	94.51
HP88 + IP 119	234.70 ± 7.72^{a}	8.77 ± 3.48^{d}	33.08 ± 6.92^{e}	3.73 ± 1.53^{d}	31.23 ± 11.28^{e}	30,173.11	96.96
HP88 + IP 125	253.78 ± 8.62^{a}	17.04 ± 4.94^{d}	25.83 ± 6.25^{e}	6.76 ± 1.94^{d}	43.60 ± 9.20^{be}	47,055.24	95.26
HP88 + IP 146	234.78 ± 7.92^{a}	5.14 ± 2.02^{d}	24.54 ± 5.20^{e}	2.45 ± 0.98^{d}	29.64 ± 9.64^{de}	11,132.72	98.88
HP88 + IP 363	236.62 ± 7.79^{a}	8.59 ± 2.70^{d}	51.43 ± 6.43^{cde}	3.52 ± 1.07^{d}	29.80 ± 6.72^{de}	29,546.02	97.03

Table 3. Mean weight of engorged females, weight of egg mass, larval hatch, egg production index (EPI), nutrient index (NI), estimate reproduction (ER), and control efficacy of *Rhipicephalus microplus* engorged females treated with *Metarhizium* spp. (1×10^7 conidia mL^{-1}) combined or not with *Heterorhabditis bacteriophora* HP88 (50 IJs/female), or treated with distilled water (control). Experiments were conducted at constant optimal temperature (27 °C) and relative humidity ≥ 90%. (*) EPI: Egg Production Index = weight of egg mass (g) / initial weight of engorged female (g) × 100 (Bennett, 1974). (**) NI: Nutrient index: weight of egg mass (g) / initial weight of engorged female (g) – final weight of female (g) × 100 (Bennett, 1974). (†) ER: Estimate Reproduction = weight of egg mass (g) / initial weight of engorged female (g) × percentage of larval hatch × 20,000 (constant which refers to the number of larvae per gram of *R. microplus* egg mass) (Drummond et al. 1971). (‡) Control Efficacy = (mean ER of the control group – mean ER of the treated group) / mean ER of the control group × 100 (Drummond et al. 1971). Means (± SE) followed by the same letter in the same column do not differ significantly ($p \geq 0.05$).

Treatment	Weight of engorged females (mg)	Weight of egg mass (mg)	Larval hatch (%)	EPI*	NI**	ER†	Control Efficacy (%, $n=40$)‡	$CE_{additive}$ (%) §§	Interaction (%, ΔE) §§
Control	225.87 ± 6.47^{a}	124.12 ± 4.63^{a}	92.87 ± 2.42^{a}	54.78 ± 1.31^{a}	76.57 ± 1.57^{a}	1,019,867.702	-	-	-
10^6	222.44 ± 4.89^{a}	95.67 ± 6.25^{b}	85.79 ± 3.05^{ab}	40.49 ± 2.95^{b}	59.74 ± 3.82^{b}	699,919.6378	31.37	-	-
10^7	226.87 ± 5.27^{a}	73.29 ± 5.63^{c}	81.15 ± 4.04^{b}	32.95 ± 2.51^{bc}	48.51 ± 3.08^{c}	574,527.9412	43.66	-	-
15 IJs	228.72 ± 6.03^{a}	118.34 ± 6.89^{a}	87.10 ± 2.96^{b}	52.11 ± 2.67^{a}	72.59 ± 3.29^{a}	933,277.9323	8.49	-	-
25 IJs	228.13 ± 6.16^{a}	117.06 ± 5.58^{a}	89.35 ± 2.32^{ab}	52.08 ± 2.35^{a}	72.64 ± 2.59^{a}	946,854.2951	7.16	-	-
10^6 + 15 IJs	227.06 ± 6.01^{a}	57.34 ± 8.50^{cd}	74.14 ± 4.95^{b}	25.33 ± 3.73^{cd}	37.46 ± 4.89^{cd}	430,713.2302	57.77	37.19 ($t=6.29$, $p<0.0001$)	Synergism (20.57)
10^7 + 15 IJs	228.10 ± 6.06^{a}	57.42 ± 7.94^{cd}	75.69 ± 4.98^{b}	23 ± 3.43^{cd}	37.34 ± 4.84^{cd}	379,261.4986	62.81	48.45 ($t=6.94$, $p<0.0001$)	Synergism (14.36)
10^6 + 25 IJs	226.94 ± 7.02^{a}	44.72 ± 9.10^{cd}	72.29 ± 5.35^{b}	19.24 ± 3.68^{cd}	31.65 ± 5.21^{cd}	328,472.9939	67.79	36.28 ($t=7.75$, $p<0.0001$)	Synergism (31.51)
10^7 + 25 IJs	227.47 ± 5.80^{a}	42.35 ± 8.48^{d}	84.31 ± 3.31^{b}	18.14 ± 3.47^{d}	26.66 ± 4.58^{d}	287,188.6975	71.84	47.67 ($t=8.27$, $p<0.0001$)	Synergism (24.14)

Table 4. Mean weight of engorged females, weight of egg mass, larval hatch, egg production index (EPI), nutrient index (NI), estimate reproduction (ER), and control efficacy of *Rhipicephalus microplus* engorged females treated with *Metarhizium robertsii* IP 146 (10^6 or 10^7 conidia mL^{-1}) combined or not combined with *Heterorhabditis bacteriophora* HP88 (15 or 25 IJs/female), or treated with distilled water (control). Experiments were conducted at constant optimal temperature (27 °C) and relative humidity ≥ 90%. (*) EPI: Egg Production Index = weight of egg mass (g) / initial weight of engorged female (g) × 100 (Bennett, 1974). (**) NI: Nutrient index: weight of egg mass (g) / initial weight of engorged female (g) – final weight of female (g) × 100 (Bennett, 1974). (†) ER: Estimate Reproduction = weight of egg mass (g) / initial weight of engorged female (g) × percentage of larval hatch × 20,000 (constant which refers to the number of larvae per gram of *R. microplus* egg mass) (Drummond et al. 1971). (‡) CE: Control Efficacy = (mean ER of the control group – mean ER of the treated group) / mean ER of the control group × 100 (Drummond et al. 1971). Means (± SE) followed by the same letter in the same column do not differ significantly ($p > 0.05$). (§) Additive effect ($CE_{additive}$) expected between the fungus and the nematode estimated by the linear mixed model: $CE_{additive} = CE_{fungus} + CE_{nematode}$ x $(1 - CE_{fungus})$, where CE_{fungus} is the expected control efficacy attributed only to the fungus and $CE_{nematode}$ is the expected control efficacy due only to the nematode. (§§) Interaction is deemed synergistic when $\Delta CE > 0$ at $p < 0.05$ according to the *t*-ratio test. $\Delta CE = CE_{additive} - CE_{combined}$, where $CE_{combined}$ refers to the observed control efficacy (%) based on the variable ER from the mixture of both biocontrol agents.

ranging from 72.29% (10^6 conidia mL^{-1} + 25 IJs) to 84.31% (10^7 conidia mL^{-1} + 25 IJs), compared to the control group (92.87%) ($\chi 2_8 = 30.60$; $p = 0.0002$) (Table 4).

The egg production index also was lower in the groups treated with fungi (40.49% at 10^6 conidia mL^{-1} or 32.9% at 10^7 conidia mL^{-1}) or in their combinations with nematodes (15 or 25 IJs), with values ranging from 18.14% (10^7 conidia mL^{-1} + 25 IJs) to 25.33% (10^6 conidia mL^{-1} + 15 IJs), than in the control group (54.78%) ($\chi 2_8 = 150.23$; $p < 0.001$). Lower nutrient indexes were registered in the groups treated with fungi (10^6 or 10^7 conidia mL^{-1}) with values of 59.74% or 48.51%, respectively, or in the combinations with nematodes (15 or 25 IJs), with values ranging from 26.66 (10^7 conidia mL^{-1} + 25 IJs) to 37.46 (10^6 conidia mL^{-1} + 15 IJs), than in the control group ($\chi 2_8 = 157.25$; $p < 0.001$) (Table 4). Moreover, the control efficacy of the groups treated with fungi (10^6 or 10^7 conidia mL^{-1}) was 31.37% and 43.66%, respectively, compared to the low control efficacy of ticks treated with nematodes alone: 8.49% (15 IJs) or 7.16% (25 IJs). A combined effect of fungi and nematodes was detected with a control efficacy of ticks ranging from 57.77% (10^6 conidia mL^{-1} + 15 IJs) to 71.84% (10^7 conidia mL^{-1} + 25 IJs) (Table 4). These findings revealed that the combination of fungi and nematodes (e.g., 10^6 conidia mL^{-1} + 25 IJs or 10^7 conidia mL^{-1} + 25 IJs) was more effective than either the fungus (10^6 conidia mL^{-1} or 10^7 conidia mL^{-1}) or nematodes (25 IJs) applied separately, regarding the mean weight of egg mass, egg production index, nutrient index, and control efficacy computed based on the estimate reproduction (CE). According to the expected additive effect in contrast to the observed control efficacy attributed to the combined treatments, the interaction of these two biocontrol agents, regardless of their concentrations tested, was found to be synergistic against this tick species (Table 4).

The efficacy of different fungal granular formulations in producing viable conidia

The mean conidial production of the nine granular formulations applied on water-agar medium did not differ significantly among them ($\chi^2_8 = 5.65$; $p = 0.687$) (Fig. 3A). Additionally, the conidia produced by the fungal granules showed high viability (> 85%), with no significant differences among the nine formulations tested ($F_{8,17} = 1.54$; $p = 0.216$) (data not shown). On the other hand, the remarkable conidial production by the granular formulations #1 and #5 in non-sterilized soil yielded a pronounced number of CFU retrieved on selective medium compared to most of the formulations tested ($\chi^2_8 = 34.10$; $p < 0.001$), resulting in 1.09×10^4 and 1.75×10^4 CFU per gram of soil, respectively (Fig. 3B).

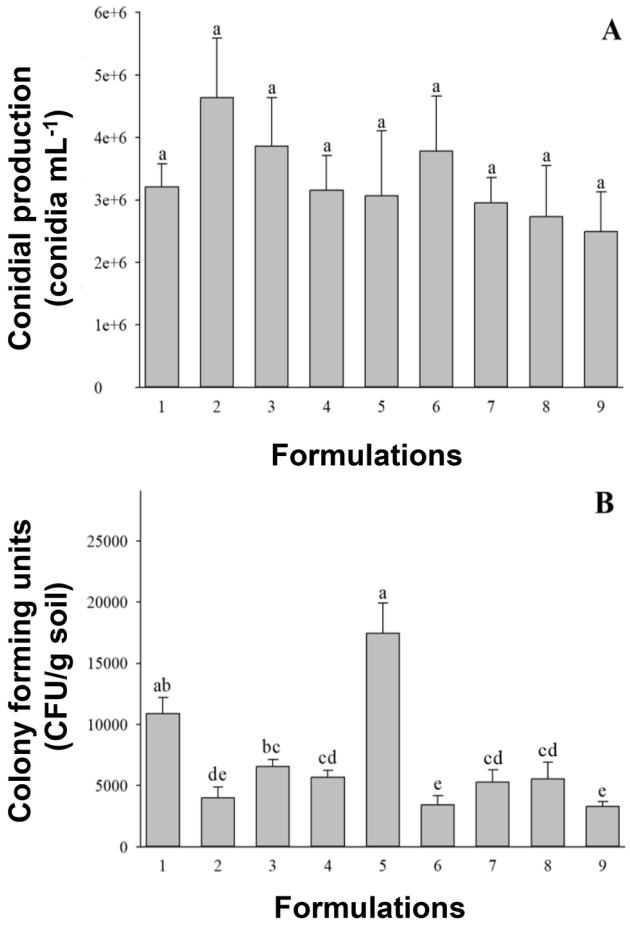

Fig. 3. Fungal development of different groups of granular formulations of *Metarhizium robertsii* IP 146 in water-agar medium or non-sterilized soil. A) Mean conidial production (± SE) of different granular formulations sporulated on water-agar medium and suspended in 10 mL 0.01% Tween 80; B) Colony forming units per gram of soil (± standard error) of different granular formulations sporulated on non-sterilized soil. Bars (± SE) followed by different letters differ significantly from each other ($p < 0.05$).

Combined use of *M. robertsii* and *H. bacteriophora* to control *R. microplus* under field conditions

The area used for the field tests presented the soil with a clayey texture, classified as clay soil containing 62% clay, 10% silt, and 28% sand. During the rainy season, from day -10 to + 84, the weekly averages (± standard deviation) of daily temperature (Supporting Information, Fig. S1A) and relative humidity (Supporting Information, Fig. S1B) of the interior of the field plot were 23.88 ± 4.08 °C and 86.09 ± 25.47%, respectively. Regarding the environmental climate, the weekly averages of daily temperature (maximum and minimum) (Supporting Information, Fig. S1C), precipitation (Supporting Information, Fig. S1D), and global solar radiation (Supporting Information, Figure S1E) were 29.39 ± 2.15 °C and 19.09 ± 1.15 °C, 5.39 ± 9.81 mm/day, 18.71 ± 5.06 MJ/m2, respectively.

The different treatments performed in the rainy season (entomopathogenic fungus or nematode alone and their combination) were effective in reducing the number of *R. microplus* larvae in the second and third week of evaluation, except for the treatment with nematodes alone, which showed a significant reduction of recovered larvae only in the second week (Fig. 4A). The analysis of the accumulated data (total tick larvae recovered in all evaluation weeks) showed that all treatments reduced the number of *R. microplus* larvae in the field plots, resulting in control efficacy of 54.09% (fungus), 38.11% (nematodes) and 46.72% (fungus and nematodes combined) (Fig. 4B).

During the dry season, from the day -10 to + 98, the weekly averages (± standard deviation) of daily temperature (Supporting Information, Fig. S2A) and relative humidity (Supporting Information, Fig. S2B) of the interior of the plot were 25.34 ± 11.35 °C and 64.38 ± 24.47%, respectively. Regarding the environment climate, the weekly averages (± standard deviation) of daily temperature (maximum and minimum) (Supporting Information, Fig. S2C), precipitation (Supporting Information, Fig. S2D), and global solar radiation (Supporting Information, Fig. S2E) were 32.44 ± 3.04 °C and 16.74 ± 3.70 °C, 1.45 ± 5.41 mm/day, and 17.21 ± 5.3 MJ/m2, respectively.

The treatments during the dry season did not bring about a significant reduction of recovered larvae in any of the sampling weeks (Fig. 5A). However, the accumulated number of larvae recovered overall sampling weeks

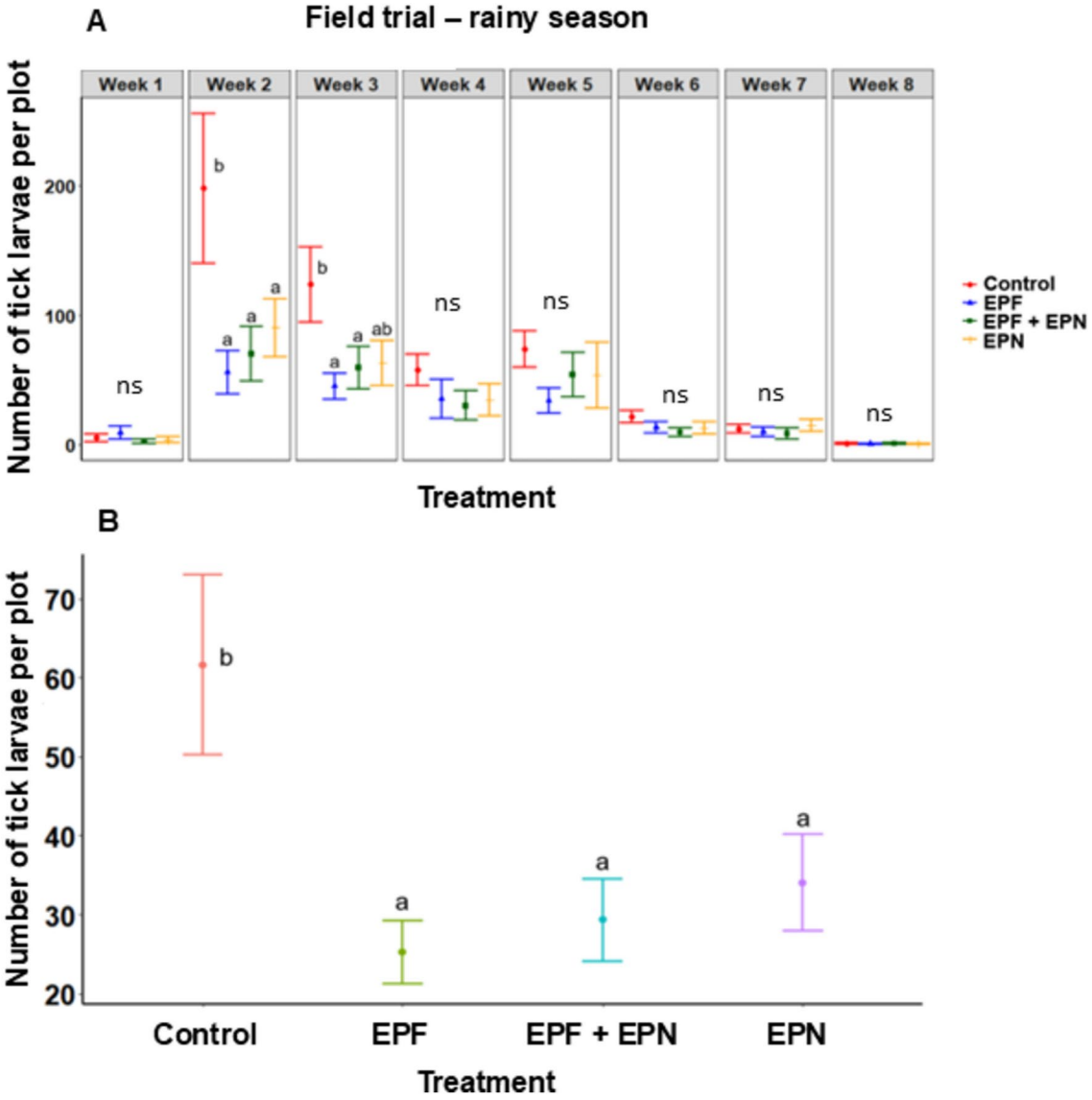

Fig. 4. Impact of treatment performed in the rainy season with entomopathogenic fungus (EPF) and nematode (EPN) applied separately or in combination in the population of tick larvae recovered from pastures. A) Segplots represent the mean (± SE, n = 8) for each evaluation week. Means followed by different letters within the same week differ significantly from each other ($p < 0.05$) based on the Tukey HSD test. B) Segplots represent the mean (± SE, n = 64) of ticks recovered for each treatment over all weeks of evaluation. Means followed by different letters differ significantly ($p < 0.05$) based on the Tukey HSD test. No significant differences between means are indicated by "ns".

revealed an effective suppression of larvae in the field plots treated with granules of *M. robertsii* microsclerotia, which resulted in an efficacy of 26.27% (Fig. 5B).

The persistence of *M. robertsii* IP 146 in the soil was not influenced by the presence of the applied nematodes, but it was greatly affected by the season ($F_{1,291} = 7.06$; $p = 0.008$), notably by the rainy season (summer), when a higher number of fungal CFU was recovered in comparison to the experiment carried out in the dry season (winter). Irrespective of the season, the fungus alone or applied jointly with the nematodes suffered a drastic decrease in persistence in the soil over the sampling time ($F_{1,3} = 80.65$; $p < 0.0001$) (Fig. 6). It is noteworthy the initial inoculum of the fungus in the soil, measured after 10 days of application, was ~ 13-fold and ~ 44-fold higher in the humid season than in the dry season, when the fungus was applied singly or applied together with the nematodes, respectively. In soil where the fungus was applied alone, its initial population dropped to 50% at 66 and 59 days post application, while when applied together with the nematodes the initial fungus population declined to 50% at 73 and 61 days post application during the humid and dry seasons, respectively. Similarly, the prevalence of *H. bacteriophora* HP88 infective juveniles significantly decreased over time (Fig. 7). A significant difference in the percentage of infection of *T. molitor* larvae with *H. bacteriophora* HP88 was observed only 24 days post-application of the nematode in the field plots ($\chi^2_4 = 12.28$; $p = 0.015$), with a higher number of

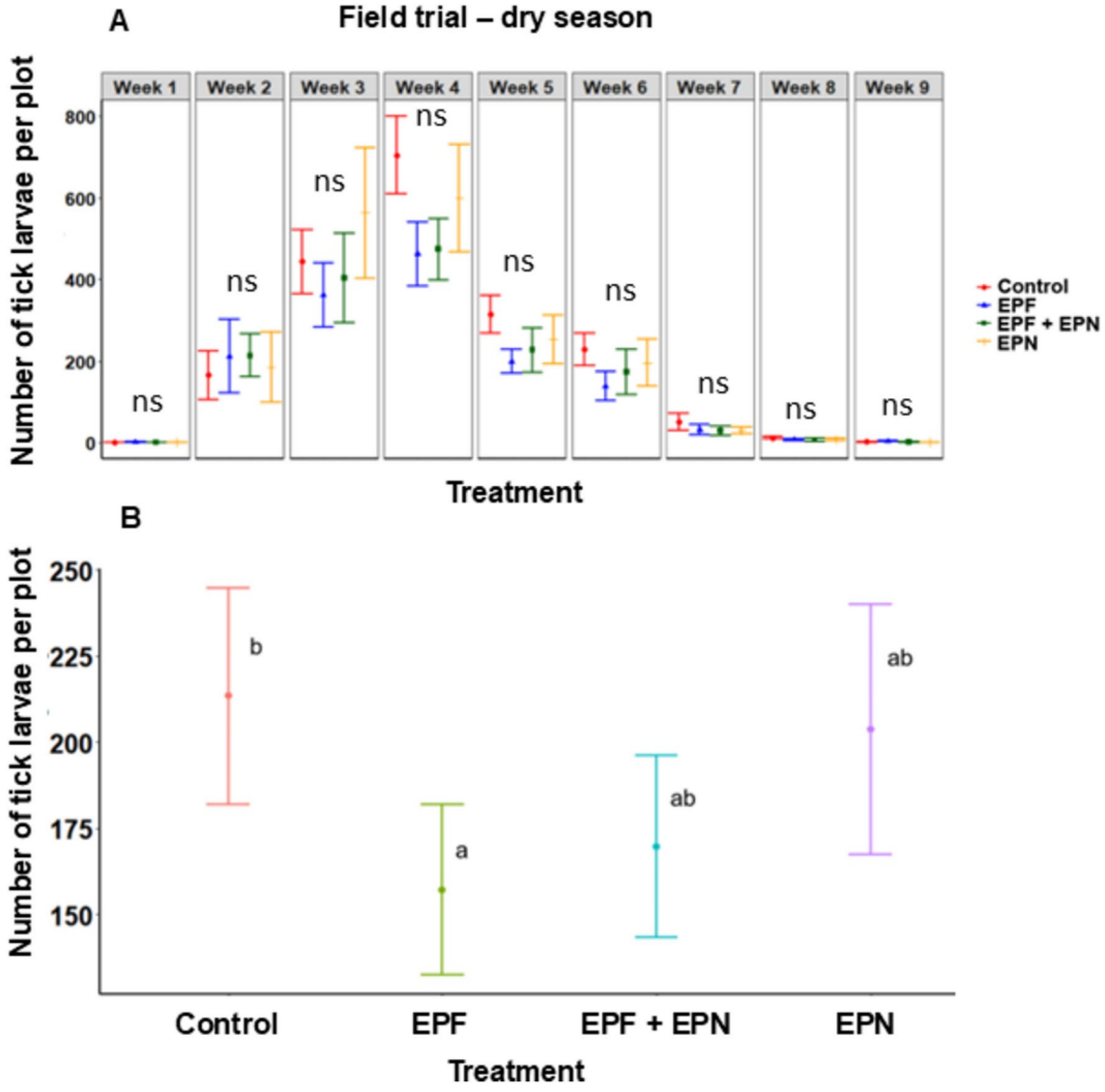

Fig. 5. Impact of treatment performed in the dry season with entomopathogenic fungus (EPF) and nematode (EPN) applied separately or in combination in the population of tick larvae recovered from pastures. A) Segplots represent the mean (± SE, n = 8) for each evaluation week. Means within the same week did not differ significantly ($p \geq 0.05$) based on the Tukey HSD test. B) Segplots represent the mean (± SE, n = 72) of ticks recovered for each treatment over all weeks of evaluation. Means followed by different letters differ significantly ($p < 0.05$) based on the Tukey HSD test. No significant differences between means are indicated by "ns".

infected larvae in the combined treatment (EPN + EPF) than in the group treated with nematodes singly (EPN) (Fig. 7). The nematode persistence in field plots, regardless of whether applied singly or combined with the fungus, significantly decreased over time ($\chi^2_6 = 43.92$; $p < 0.0001$), with periods where no mealworm larvae were found parasitized by the nematode, such as at 66 and 68 days post application.

Discussion

This study represents the pioneering effort to assess, under field conditions, the joint action of entomopathogenic fungi and nematodes with the aim at controlling the non-parasitic phase of *R. microplus* in infested pastures under tropical conditions in Brazil. Prior to the field experiment, we evaluated the overall control of tick females by combining the fungus and the nematode at different inoculum loads to provide important background information about their interactions. Indeed, the combined use of entomopathogenic fungi and nematodes against ticks has been a relatively unexplored area, with only a handful of studies addressing this subject under laboratory conditions[36,37]. On the other hand, the interest in combining entomopathogenic nematodes and fungi against agricultural pests has increased in recent years, evidenced by a rise in publications featuring investigations conducted under laboratory, greenhouse, and field conditions[58–60]. Our study underscores the notable virulence of *M. robertsii* IP 146 against engorged females of *R. microplus*, a finding consistent with the previous study of Bernardo et al.[5]. Furthermore, our investigation reveals that *H. bacteriophora* HP88 also significantly diminishes

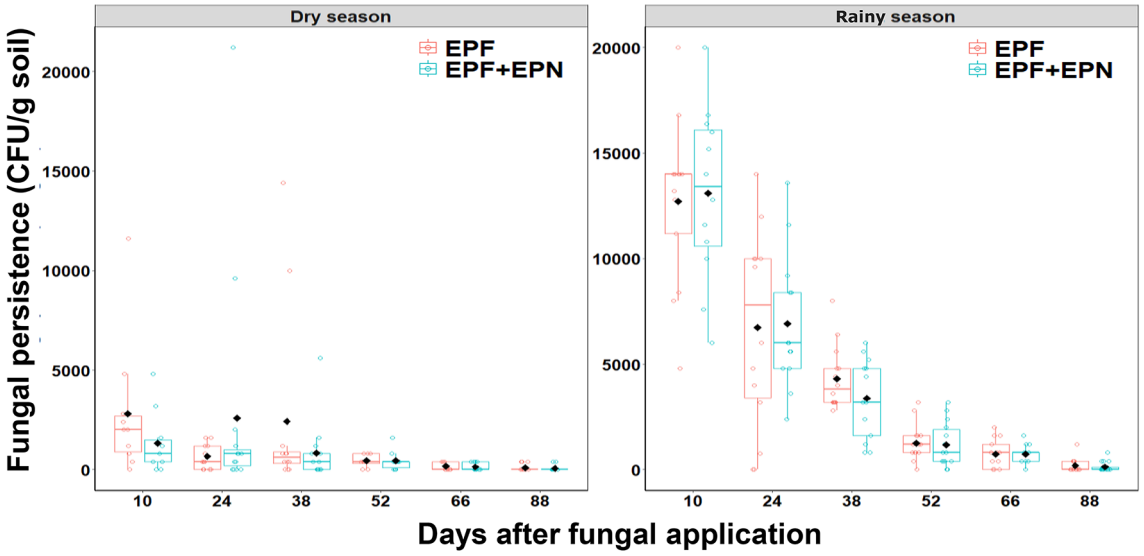

Fig. 6. Fungal persistence in the soil from field plots treated with granular formulation of *Metarhizium robertsii* IP 146 (EPF) or combined with the nematode *Heterorhabditis bacteriophora* HP88 (EPN) in the two seasons (dry or rainy). Box plot of colony forming units (CFU) of *M. robertsii* IP 146 per gram of soil obtained after fungal application under field conditions. The boxes show the median, 25th, and 75th percentiles, while the error bars show the 10th and 90th percentiles and the means (n = 8) are the black diamonds.

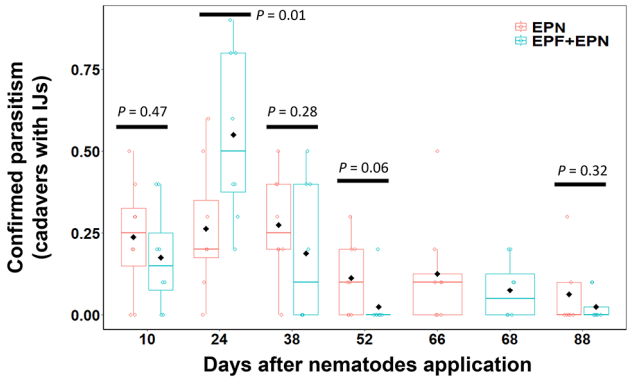

Fig. 7. Persistence of nematodes in the soil from treated field plots. Persistence was evaluated by assessing the percentage of larvae of *Tenebrio molitor* infected by *H. bacteriophora* HP88 (n = 10) exposed to the soil from plots (n = 8) treated with infective juveniles of *H. bacteriophora* HP88 (EPN) or its combination with granular formulation of *M. robertsii* IP 146 (EPF) in the dry season. Means (black diamonds) followed by $p < 0.05$ on the same assessment day differ significantly. The boxes show the median, 25th, and 75th percentiles, while the error bars show the 10th and 90th percentiles.

the biotic potential of *R. microplus* engorged females. This aligns with existing literature, which has consistently identified *H. bacteriophora* as a highly virulent nematode against *R. microplus* engorged females[12,14,36,61].

In an initial assessment conducted under laboratory conditions, no synergistic interaction between *Metarhizium* spp. and *H. bacteriophora* was observed in their combined action against engorged females of *R. microplus*. These outcomes could be attributed to the already substantial efficacy exerted by the nematode applied singly. This finding corroborates previous research conducted by Monteiro et al.[36], as they found that *H. bacteriophora* HP88, whether tested alone or in conjunction with *M. anisopliae* s.l. IBCB 116 or *Beauveria bassiana* s.l. ESALQ 986, achieved a control efficacy over 90% against engorged *R. microplus* ticks under laboratory conditions. Accordingly, in the present study, a follow-up experiment was conducted with reduced concentrations of EPF and EPN to better explore their complementary actions; these entomopathogens, when applied jointly, enhanced their effectiveness against *R. microplus* compared to their single counterparts. These results demonstrate a synergistic effect when applying together entomopathogenic fungi and nematodes for tick control at low inoculum loads, thus supporting the initial hypothesis of combining entomopathogens with different modes of action to enhance the overall biocontrol efficacy against the target host.

Positive (additive or synergistic) effects of combining EPF and EPN have been demonstrated against *Hoplia philanthus* (Coleoptera: Rutelidae) [34], *Hylobius abietis* (Coleoptera: Curculionidae)[62], and *Curculio caryae* (Coleoptera: Curculionidae)[63]. In addition, the combined use of *M. anisopliae* CLO 53 and *Heterorhabditis megidis* or *Steinernema glaseri* has proven effective against *H. philanthus* under laboratory, greenhouse, and field settings[34,35]. While the precise mechanisms underlying these synergistic interactions between entomopathogenic fungi and nematodes remain somewhat enigmatic, a hypothesis suggests that the fungus acts as a stressor. This stressor disrupts the host's homeostasis and weakens its defenses, rendering the arthropod more susceptible to nematode parasitism[34,63]. Additionally, it is noteworthy that fungus-infected hosts emit increased amounts of carbon dioxide (CO_2), which, intriguingly, nematodes exploit as a chemical cue to locate their hosts[64].

The various granular formulations rehydrated on water-agar medium exhibited similar conidial production and viability, suggesting no significant effect due to the formulation composition on microsclerotial sporogenesis under suitable temperature and moisture. Conversely, when these granular formulations were applied to non-sterilized soil samples, there was a notable divergence in conidial production among our test formulations. Formulation #5, which comprises microcrystalline cellulose (MC), diatomaceous earth (DT), vermiculite (VMC), and the plant-based fiber known as *Psyllium*, attained a significantly higher conidial yield compared to the remaining formulations. This outcome may be attributed to the exceptional water-retaining capacity of these components present in this formulation, which creates an optimal moisture microenvironment conducive to fungal development. The well-documented hygroscopic properties of MC[65], DT[66], and VMC[67], in combination with *Psyllium*, most likely played a crucial role in creating and maintaining favorable conditions for fungal sporulation on non-sterilized soil despite the potential soil fungistasis due to resident soil microbes. Moisture is indispensable for optimal fungal development, and the moisture-retaining abilities of those formulation components may have ensured the essential moisture levels required for fungal growth and sporulation[19,68,69]. Indeed, the efficacy of granules containing microsclerotia, when applied to specific microenvironments, can face various environmental stressors, with low relative humidity being one of them. In situations characterized by low humidity levels, the role of enhancers or additives within well-formulated granules becomes pivotal to overcome such abiotic hurdles. These additives can facilitate free water absorption from the air or soil, thereby enabling fungal sporulation to thrive even in adverse environmental scenarios[19].

During the field trials, we observed that all treatments with the biological agents significantly reduced the population of *R. microplus* larvae retrieved from the pastures during the rainy season in the Cerrado biome. On the other hand, during the dry season in the Cerrado biome, only the field plots treated with a single application of the granular formulation of *M. robertsii* IP 146 resulted in a decreased number of tick larvae. This observation is substantiated by the fact that this particular fungal strain possesses a natural and remarkable tolerance to high temperatures, reinforced by its microsclerotia with natural tolerance to heat and UV-B. Moreover, it appears that the components incorporated into the granular formulation acted as a protective shield, safeguarding the fungus against the adverse effects of heat and low moisture during the dry season[19,46].

In a previous study[14], we found that the application of *H. bacteriophora* HP88, delivered within insect cadavers, significantly reduced the non-parasitic phase of *R. microplus* by 73.1%. However, it is worth noting that this nematode-based treatment proved effective in reducing *R. microplus* larvae during the rainy season but showed a weaker efficacy of only 38.11% during the harsh dry season when applied just once. We attribute this difference to variations in the formulations employed in these two studies. In our pursuit of optimizing the production scale of EPN and minimizing application costs, our current research explored nematodes in aqueous suspension. Although the insect cadaver method offers advantages such as heightened infectivity, survival rates, and effectiveness, it remains challenging for extensive application and making difficult for use in field experiments[70,71]. In contrast, using aqueous formulations for tick control in infested pastures requires enhancements, such as adding adjuvants to shield EPN from environmental conditions or developing improved strategies for sequential applications, although aqueous nematode suspension can be easily sprayed on the field by using conventional spray equipment.

Although a positive effect was observed in the combination of sub-doses of *H. bacteriophora* with *M. robertsii* under laboratory conditions, a similar response was not observed under field conditions. This reduced efficacy of the biocontrol agents under field conditions was not entirely unexpected. Given the complexity of environmental factors that can influence their interactions [i.e., temperature fluctuations, humidity levels, and microbial interactions in the soil], suboptimal environmental conditions may hinder fungal sporulation or nematode infectivity, thereby attenuating their combined efficacy. Our results, however, diverge from previous studies[34,72,73] conducted under field conditions with combinations of EPN and EPF. Two aspects may have influenced the divergence of results: 1) the time between the application of EPN and EPF in the field; 2) the different virulence of fungal and nematode isolates tested. In the current study, the entomopathogens were applied in the field 8 days apart, and in many greenhouse and field studies, the best results (additive or synergistic effect) with the combination of EPN and EPF were retrieved when the application gap among them occur in we eks[22,34,35,58,74]. This is an approach that deserves future investigation. In addition, EPN is generally considered to be more virulent to engorged females, whereas EPF is more virulent to larvae[11]. In the present study, a single application of the two isolates tested in the field (*H. bacteriophora* HP88 and *M. robertsii* IP146) showed high virulence to *R. microplus*[5,13,23]. Studies have demonstrated, however, that with the combined application of EPN and EPF at short intervals in multiple applications, the best results were obtained with a moderately virulent fungal isolate and a highly virulent nematode isolate[75]. In order to improve the efficacy of this integrated biocontrol strategy, it would be worth applying these biocontrol agents multiple times in the pasture rather than relying on their erratic and short persistence in the soil after a single application. This approach would require an economic analysis to evaluate the cost–benefit of multiple applications of these biocontrol agents compared with their single application in the field. In addition, continued research on innovative formulations is essential to enhance biocontrol efficacy while minimizing application costs. Developing cost-effective production

methods for fungal spores and nematodes, optimizing formulation systems to ensure agent viability, and refining application techniques are critical for scalability and for achieving consistently satisfactory results. Exploring low-cost substrates for fungal culture and automated systems for nematode production are promising avenues for reducing costs. Large-scale field trials are also necessary to assess the practical applicability of these methods under diverse environmental conditions. Such trials will provide valuable insights into balancing cost and efficacy, which are pivotal for broader implementation. By addressing these factors, biocontrol strategies such as the combination of *M. robertsii* and *H. bacteriophora* could become more accessible and viable for extensive cattle pastures. Furthermore, despite the widespread use of *Metarhizium* spp. as biological control agents, studies specifically addressing their safety for non-target organisms remain scarce. Although some research has demonstrated minimal impacts on non-target organisms[76], there is a lack of comprehensive field studies on their effects on beneficial arthropods, soil organisms, and broader ecosystem dynamics. This gap underscores the necessity for further investigations to ensure the ecological compatibility of these fungi before their extensive application in integrated tick management systems.

The persistence of *M. robertsii* in the field plots suffered a significant decline over time, with great variation between dry and rainy seasons. This decline was anticipated, aligning with the outcomes of prior studies[17]. However, a more pronounced reduction in fungal persistence in the soil was evident during the dry period, typically characterized by higher temperatures and reduced relative humidity. Furthermore, the absence of rainfall and the intensified solar radiation are prevalent factors during the dry season in this region, which likely played a significant role in diminishing the persistence of *M. robertsii* IP 146 in the field plots[17–19]. During the rainy season (summer), it is hypothesized that the fungus benefitted from the favorable climatic conditions, particularly the high relative humidity, warm temperatures, and regular precipitation. These abiotic factors likely supported the development of the fungus on the microsclerotial granules. Moreover, the rainy season's climatic conditions also promoted lush vegetation growth, which was abundant and potentially shielded the fungal propagules from sunlight damage by covering the soil with pastures. In contrast, vegetation coverage notably declined during the dry season, as no artificial irrigation was employed in these field plots.

The persistence of IJs applied to the field plots during the dry season also experienced a substantial decrease over time, similar to what happened with the fungus persistence. The survival and development of IJs are directly influenced by biotic and abiotic factors, including soil moisture, soil texture, and particularly temperature[77,78]. The harsh climatic conditions encountered during the dry season, coupled with the clayey soil that offers limited aeration, as observed at the experimental site, likely hindered the movement and survival of the juvenile nematodes[79,80]. These factors may have created a harmful impact on the effectiveness of IJs in the field, thus limiting their ability to quest and penetrate *R. microplus* females in the pastures. While the persistence of IJs was not specifically assessed during the rainy season, there was a notable reduction in tick larvae numbers within the treated field plots. This reduction could be attributed to the favorable micro-environmental conditions created by the combination of high humidity and conducive temperatures, thanks to the shelter provided by the dense pasture canopy. This sheltering effect of the dense vegetation canopy likely shielded the nematodes from dehydration and the harsh effects of solar radiation. Consequently, it may have facilitated enhanced locomotion and improved IJ survival, ultimately contributing to their successful activity against ticks.

The results obtained from field tests conducted during both rainy and dry seasons bear significant relevance as they pertain to controlling the non-parasitic phase of *R. microplus* in pastures, potentially eradicating thousands of active larvae in subsequent generations. Our present study concludes that *M. robertsii* IP 146 and *H. bacteriophora* HP88 might exhibit additive or synergistic effects against *R. microplus* ticks, at least under controlled environmental conditions. Furthermore, our research indicates that a microsclerotial granular formulation comprising microcrystalline cellulose, vermiculite, diatomaceous earth, and *Psyllium* effectively yields viable conidia when applied to non-sterilized soil samples in the laboratory.

In summary, we demonstrate that *M. robertsii* IP 146 and *H. bacteriophora* HP88, when applied to field plots under challenging environmental conditions, show promise as biological agents for controlling the non-parasitic phase of *R. microplus*. Future studies exploring fungal and nematode formulations with additional protective components or novel application strategies with repeated applications may further enhance the results obtained in this current study, especially during harsh environmental conditions such as the dry season in the Central-West region of Brazil. The application of fungi and nematodes, either in combination or singly, in extensive cattle pastures holds the potential as a supplemental method to traditional chemical control strategies within an integrated cattle tick management framework. This approach can sustainably contribute to mitigating or postponing the acaricide resistance of this tick. Future research should integrate cost analyses and scalable production techniques to ensure the adoption of these biocontrol agents in commercial settings.

Data availability

Data are provided within the manuscript or supplementary information files.

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References

1. Lacey, L. A. et al. Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* **132**, 1–41. https://doi.org/10.1016/j.jip.2015.07.009 (2015).
2. Bischoff, J. F., Rehner, S. A. & Humber, R. A. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* **101**, 512–530. https://doi.org/10.3852/07-202 (2009).
3. Jackson, M. A., Dunlap, C. A. & Jaronski, S. T. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. *BioControl* **55**, 129–145. https://doi.org/10.1007/s10526-009-9240-y (2010).

4. Fernandes, É. K. K. & Bittencourt, V. R. E. P. Entomopathogenic fungi against South American tick species. *Exp. Appl. Acarol.* **46**, 71–93. https://doi.org/10.1007/s10493-008-9161-y (2008).
5. Bernardo, C. C. et al. Conidia and blastospores of *Metarhizium* spp. and *Beauveria bassiana* s.l.: Their development during the infection process and virulence against the tick *Rhipicephalus microplus*. *Ticks Tick Borne Dis.* **9**, 1334–1342. https://doi.org/10.1016/j.ttbdis.2018.06.001 (2018).
6. Paixão, F. R. S. et al. Tolerance to abiotic factors of microsclerotia and mycelial pellets from *Metarhizium robertsii*, and molecular and ultrastructural changes during microsclerotial differentiation. *Front. Fungal Biol.* **2**, 654–737. https://doi.org/10.3389/ffunb.2021.654737 (2021).
7. García Riaño, J. L., Barrera, G. P., Hernández, L. C. & Villamizar, L. F. Microsclerotia from *Metarhizium robertsii*: Production, ultrastructural analysis, robustness, and insecticidal activity. *Fungal Biol.* **128**, 1643–1656. https://doi.org/10.1016/j.funbio.2024.01.006 (2024).
8. Jaronski, S. T. & Jackson, M. A. Efficacy of *Metarhizium anisopliae* microsclerotial granules. *Biocontrol Sci. Technol.* **18**, 849–863. https://doi.org/10.1080/09583150802381144 (2008).
9. Grewal, P. S., De Nardo, E. A. B. & Aguillera, M. M. Entomopathogenic nematodes: Potential for exploration and use in South America. *Neotrop. Entomol.* **30**, 191–205. https://doi.org/10.1590/S1519-566X2001000200001 (2001).
10. Dolinski, C. Uso de nematoides entomopatogênicos para o controle de pragas, p 261–289. in *Tecnologias Alternativas para o Controle Alternativo de Pragas e Doenças* (eds. Venzon, M., Paula, T. J. & Pallini, A.) 261–289. Viçosa UFV Brasil. (2006).
11. Samish, M., Ginsberg, H. & Glazer, I. Anti-tick biological control agents: assessment and future perspectives. In *Ticks: Biology, Disease and Control*. (eds. Bowman, A. S. & Nuttall, P. A), 447–469 (Cambridge University Press, Cambridge UK, 2008).
12. Monteiro, C. M. O. et al. Evaluation of the action of *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) isolate HP88 on the biology of engorged females of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). *Vet. Parasitol.* **170**, 355–358. https://doi.org/10.1016/j.vetpar.2010.02.021 (2010).
13. Monteiro, C. et al. Entomopathogenic nematodes in insect cadaver formulations for the control of *Rhipicephalus microplus* (Acari: Ixodidae). *Vet. Parasitol.* **203**, 310–317. https://doi.org/10.1016/j.vetpar.2014.04.003 (2014).
14. Filgueiras, M. D. G. et al. From the laboratory to the field: Efficacy of entomopathogenic nematodes to control the cattle tick. *Pest Manag. Sci.* **79**, 216–225. https://doi.org/10.1002/ps.7190 (2023).
15. Mascarin, G. M. et al. Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. *J. Invertebr. Pathol.* **165**, 46–53. https://doi.org/10.1016/j.jip.2018.01.001 (2019).
16. San-Blas, E. et al. Entomopathogenic nematology in Latin America: A brief history, current research, and future prospects. *J. Invertebr. Pathol.* **165**, 22–45. https://doi.org/10.1016/j.jip.2019.03.010 (2019).
17. Scheepmaker, J. W. A. & Butt, T. M. Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. *Biocontrol Sci. Technol.* **20**, 503–552. https://doi.org/10.1080/09583150903545035 (2010).
18. Fernandes, É. K. K., Rangel, D. E. N., Braga, G. U. L. & Roberts, D. W. Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation. *Curr. Genet.* **61**, 427–440. https://doi.org/10.1007/s00294-015-0492-z (2015).
19. Rodrigues, J. et al. Relative humidity impacts development and activity against *Aedes aegypti* adults by granular formulations of *Metarhizium humberi* microsclerotia. *Appl. Microbiol. Biotechnol.* **105**, 2725–2736. https://doi.org/10.1007/s00253-021-11157-6 (2021).
20. Vega, F. E. et al. Fungal entomopathogens: new insights on their ecology. *Fungal Ecol.* **2**, 149–159. https://doi.org/10.1016/j.funeco.2009.05.001 (2009).
21. Vemmer, M. & Patel, A. V. Review of encapsulation methods suitable for microbial biological control agents. *Biol. Control* **67**, 380–389. https://doi.org/10.1016/j.biocontrol.2013.09.003 (2013).
22. Ansari, M. A., Tirry, L. & Moens, M. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. *Biol. Control* **31**, 172–180. https://doi.org/10.1016/j.biocontrol.2004.04.002 (2004).
23. Monteiro, C. et al. Efficacy of entomopathogenic nematodes in insect cadaver formulation against engorged females of *Rhipicephalus microplus* (Acari: Ixodidae) in semi-field conditions. *Ticks Tick Borne Dis.* **11**, 101313. https://doi.org/10.1016/j.ttbdis.2019.101313 (2020).
24. Marciano, A. F. et al. Innovative granular formulation of *Metarhizium robertsii* microsclerotia and blastospores for cattle tick control. *Sci. Rep.* **11**, 1–11. https://doi.org/10.1038/s41598-021-84142-8 (2021).
25. Santos, T. R. et al. Inorganic pellets containing microsclerotia of *Metarhizium anisopliae*: a new technological platform for the biological control of the cattle tick *Rhipicephalus microplus*. *Appl. Microbiol. Biotechnol.* **105**, 5001–5012. https://doi.org/10.1007/s00253-021-11372-1 (2021).
26. Mascarin, G. M. et al. Production of microsclerotia by Brazilian strains of *Metarhizium* spp. using submerged liquid culture fermentation. *World J. Microbiol. Biotechnol.* **30**, 1583–1590. https://doi.org/10.1007/s11274-013-1581-0 (2014).
27. Mascarin, G. M., Jackson, M. A., Kobori, N. N., Behle, R. W. & Delalibera, I. Liquid culture fermentation for rapid production of desiccation tolerant blastospores of *Beauveria bassiana* and *Isaria fumosorosea* strains. *J. Invertebr. Pathol.* **127**, 11–20. https://doi.org/10.1016/j.jip.2014.12.001 (2015).
28. Grisi, L. et al. Reassessment of the potential economic impact of cattle parasites in Brazil. *Ver. Bras. Parasitol. Vet.* **23**, 150–156. https://doi.org/10.1590/S1984-29612014042 (2014).
29. Mastropaolo, M., Mangold, A. J., Guglielmone, A. A. & Nava, S. Non-parasitic life cycle of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* in *Panicum maximum* pastures in northern Argentina. *Res. Vet. Sci.* **115**, 138–145. https://doi.org/10.1016/j.rvsc.2017.03.009 (2017).
30. Powell, R. T. & Reid, T. J. Project tick control. Queensland. *Agric. J.* **108**, 279–300 (1982).
31. Klafke, G. M. Resistência de *Rhipicephalus* (*Boophilus*) *microplus* aos carrapaticidas. In: *Rhipicephalus* (*Boophilus*) *microplus: Biologia, Controle e Resistência* (eds. Pereira, M. C., Labruna, M. B., Szabo, M. P. J. & Klafke, G. M.), 65–80 (São Paulo: MEDVET, São Paulo, 2008).
32. Barbercheck, M. E. & Kaya, H. K. Interactions between *Beauveria bassiana* and the entomogenous nematodes *Steirnernema feltiae* and *Heterorhabditis heliothidis*. *J. Invertebr. Pathol.* **55**, 225–234. https://doi.org/10.1016/0022-2011(90)90058-E (1990).
33. Ansari, M. A., Tirry, L. & Moens, M. Antagonism between entomopathogenic fungi and bacterial symbionts of entomopathogenic nematodes. *BioControl* **50**, 465–475. https://doi.org/10.1007/s10526-004-5524-4 (2005).
34. Ansari, M. A., Shah, F. A., Tirry, L. & Moens, M. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. *Biol. Control.* **39**, 453–459. https://doi.org/10.1016/j.biocontrol.2006.07.004 (2006).
35. Ansari, M. A., Shah, F. A. & Butt, T. M. Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil *Otiorhynchus sulcatus* control. *Entomol. Exp. Appl.* **129**, 340–347. https://doi.org/10.1111/j.1570-7458.2008.00783.x (2008).
36. Monteiro, C. M. et al. Association between entomopathogenic nematodes and fungi for control of *Rhipicephalus microplus* (Acari: Ixodidae). *Parasitol. Res.* **112**, 3645–3651. https://doi.org/10.1007/s00436-013-3552-7 (2013).
37. Golo, P. S. et al. Lab-on-a-chip and SDS-PAGE analysis of hemolymph protein profile from *Rhipicephalus microplus* (Acari: Ixodidae) infected with entomopathogenic nematode and fungus. *Parasitol. Res.* **115**, 3459–3468. https://doi.org/10.1007/s00436-016-5109-z (2016).

38. Nicaretta, J. E. et al. Selective versus strategic control against *Rhipicephalus microplus* in cattle: a comparative analysis of efficacy, animal health, productivity, cost and resistance management. *Vet. Parasitol.* **321**, 109999. https://doi.org/10.1016/j.vetpar.2023.109999 (2023).
39. Poinar, G. O. J. & Georgis, R. Characterization and field application of *Heterorhabditis bacteriophora* strain HP88 (Heterorhabditidae: Rhabditida). *Revue. Nématol.* **14**, 387–393 (1990).
40. Lindegren, J. E., Valero, K. A. & Mackey, B. E. Simple in vivo production and storage methods for *Steinernema carpocapsae* infective juveniles. *J. Nematol.* **25**, 193–197 (1993).
41. Kaya, H. K. & Stock, P. S. Techniques in insect nematology. In *Manual of Techniques in Invertebrate Pathology* (ed. Lacey, L. A.) 281–324 (Academic Press, London UK, 1997).
42. Monteiro, C. M. O. et al. First report of pathogenicity of entomopathogenic nematodes of the genus *Heterorhabditis* on partially engorged females of *Dermacentor nitens* (Acari: Ixodidae). *Biol. Control.* **69**, 78–81. https://doi.org/10.1016/j.biocontrol.2013.11.003 (2014).
43. Bennett, G. F. Oviposition of *Boophilus microplus* (Acarida: Ixodidae) I. Influence of tick size on egg production. *Acarologia* **16**, 52–61 (1974).
44. Drummond, R. O., Gladney, W. J., Whetstone, T. M. & Ernst, S. E. Laboratory testing of insecticides for control of the winter tick. *J. Econ. Entomo.* **64**, 686–688 (1971).
45. Fernandes, É. K. K. et al. Composição e processo de preparação multiparticulada de natureza sólida contendo microescleródios de *Metarhizium anisopliae* para controle de artrópodes-praga de importância na saúde humana veterinária e na agropecuária e seus usos. BR1020170040615 (2017).
46. Paixão, F. R. S. et al. Microsclerotial pellets of *Metarhizium* spp.: thermotolerance and bioefficacy against the cattle tick. *Appl. Microbiol. Biotechnol.* **107**, 2263–2275. https://doi.org/10.1007/s00253-023-12467-7 (2023).
47. Milner, R. J., Huppatz, R. J. & Swaris, S. C. A new method for assessment of germination of *Metarhizium* conidia. *J. Invertebr. Pathol.* **57**, 121–123. https://doi.org/10.1016/0022-2011(91)90048-U (1991).
48. Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J. & Roberts, D. W. Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitudes from 61°N to 54°S. *J. Invertebr. Pathol.* **78**, 98–108. https://doi.org/10.1006/jipa.2001.5048 (2001).
49. Fernandes, É. K. K., Keyser, C. A., Rangel, D. E. N., Foster, R. N. & Roberts, D. W. CTC medium: A novel dodine-free selective medium for isolating entomopathogenic fungi especially *Metarhizium acridum* from soil. *Biol. Control* **54**, 197–205. https://doi.org/10.1016/j.biocontrol.2010.05.009 (2010).
50. Bittencourt, V. R. E. P., Bahiense, T. C., Fernandes, É. K. K. & Souza, E. J. Avaliação da ação in vivo de *Metarhizium anisopliae* (Metschnikoff 1879) Sorokin 1883 aplicado sobre *Brachiaria decumbens* infestada com larvas de *Boophilus microplus* (Canestrini 1887) (Acari: Ixodidae). *Rev. Bras. Parasitol. Vet.* **12**, 38–42 (2003).
51. Humber, R. A. Identification of entomopathogenic fungi. In *Manual of Techniques in Invertebrate Pathology* (ed. Lacey, L. A.) 151–187 (Academic Press, London UK, 2012)
52. White, G. F. A method for obtaining infective nematode larvae from cultures. *Science* **30**, 302–303. https://doi.org/10.1126/science.66.1709.302-a (1927).
53. R Core Team. R: a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria. 2023).
54. Arnhold, E. Package in the R environment for analysis of variance and complementary analyses. *Braz. J. Vet. Res. Anim. Sci.* https://doi.org/10.11606/issn.1678-4456.v50i6p488-492 (2013).
55. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. Royal Stat. Soc. Ser. B.* **57**, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x (1995).
56. Kaiser, D., Handschin, S., Rohr, R. P., Bacher, S. & Grabenweger, G. Co-formulation of *Beauveria bassiana* with natural substances to control pollen beetles – Synergy between fungal spores and colza oil. *Biol. Control* **140**, 104106. https://doi.org/10.1016/j.biocontrol.2019.104106 (2020).
57. Lenth, R., Singmann, H. & Love, J. Emmeans: Estimated Marginal Means Aka Least-Squares Means. *R pkg. vers.* **1**(4), 7. https://doi.org/10.1080/00031305.1980.10483031(2020) (2020).
58. Wakil, W., Yasin, M. & Shapiro-Ilan, D. Effects of single and combined applications of entomopathogenic fungi and nematodes against *Rhynchophorus ferrugineus* (Olivier). *Sci. Rep.* **7**, 5971. https://doi.org/10.1038/s41598-017-05615-3 (2017).
59. Puza, V. & Tarasco, E. Interactions between entomopathogenic fungi and entomopathogenic nematodes. *Microorganisms* **11**, 163. https://doi.org/10.3390/microorganisms11010163 (2023).
60. Tabima, L., Gómez, E. S. & Gaigl, A. Susceptibility of *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) to *Heterorhabditis* sp. (Poinar) *Steinernema feltiae* (Filipjev) and isolates of entomopathogenic fungi. *Egypt. J. Biol. Pest Control* https://doi.org/10.1186/s41938-023-00662-7 (2023).
61. Singh, N. K. et al. Efficacy evaluation of six entomopathogenic nematode species against engorged females of southern cattle fever tick *Rhipicephalus* (*Boophilus*) *microplus*. *Southwest. Entomol.* **43**, 1–17. https://doi.org/10.3958/059.043.0119 (2018).
62. McNamara, L. et al. Efficacy of entomopathogenic fungi against large pine weevil *Hylobius abietis* and their additive effects when combined with entomopathogenic nematodes. *J. Pest. Sci.* **91**, 1407–1419. https://doi.org/10.1007/s10340-018-0994-9 (2018).
63. Shapiro-Ilan, D. I., Jackson, M., Reilly, C. C. & Hotchkiss, M. W. Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). *Biol. Control* **30**, 119–126. https://doi.org/10.1016/j.biocontrol.2003.09.014 (2004).
64. Malan, A. P., von Diest, J. I., Moore, S. D. & Addison, P. Control options for false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in South Africa with emphasis on the potential use of entomopathogenic nematodes and fungi. *African Entomol.* **26**, 14–29. https://doi.org/10.4001/003.026.0014 (2018).
65. Ek, R. & Newton, J. M. Microcrystalline cellulose as a sponge as an alternative concept to the crystallite-gel model for extrusion and spheronization. *Pharm. Res.* **15**, 509–512. https://doi.org/10.1023/A:1011905222168 (1998).
66. Mewis, I. & Ulrichs, C. Action of amorphous diatomaceous earth against different stages of the stored product pests *Tribolium confusum*, *Tenebrio molitor*, *Sitophilus granarius* and *Plodia interpunctella*. *J. Stored. Prod. Res.* **37**, 153–164. https://doi.org/10.1016/S0022-474X(00)00016-3 (2001).
67. Silva, L. P. C. et al. Viability of *Strongyloides venezuelensis* eggs and larvae in vermiculite containing the fungus *Duddingtonia flagrans*. *Parasitol. Res.* https://doi.org/10.1007/s00436-017-5502-2 (2017).
68. Ignoffo, C. M. Environmental factors affecting persistence of entomopathogens. *Fla. Entomol.* **75**, 516–525 (1992).
69. Catão, A. M. L. et al. Optimization of granular formulations of *Metarhizium humberi* microsclerotia with humectants. *J. Basic. Microbiol.* **61**, 808–813. https://doi.org/10.1002/jobm.202100171 (2021).
70. Shapiro-Ilan, D. I., Lewis, E. E. & Tedders, W. L. Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. *J. Invertebr. Pathol.* **83**, 270–272. https://doi.org/10.1016/s0022-2011(03)00101-0 (2003).
71. Shapiro-Ilan, D. I., Han, R. & Dolinski, C. Entomopathogenic nematode production and application technology. *J. Nematol.* **44**, 206–217 (2012).
72. Sáenz-Aponte, A., Correa-Cuadros, J. & Rodríguez-Bocanegra, M. Foliar application of entomopathogenic nematodes and fungi for the management of the diamond back moth in greenhouse and field. *Biol. Control* **142**, 104163. https://doi.org/10.1016/j.biocontrol.2019.104163 (2020).

73. Wakil, W. et al. Combined application of entomopathogenic nematodes and fungi against fruit flies, *Bactrocera zonata* and *B. dorsalis* (Diptera: Tephritidae): Laboratory cups to field study. *Pest Manag. Sci.* **78**, 2779–2791. https://doi.org/10.1002/ps.6899 (2022).
74. Anbesse, S. A., Adge, B. J. & Gebru, W. M. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. *Nematology* **10**, 701–709. https://doi.org/10.1163/156854108785787217 (2008).
75. Acevedo, J. P. M., Samuels, R. I., Machado, I. R. & Dolinski, C. Interactions between isolates of the entomopathogenic fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Heterorhabditis bacteriophora* JPM4 during infection of the sugar cane borer *Diatraea saccharalis* (Lepidoptera: Pyralidae). *J. Invertebr. Pathol.* **96**, 187–192. https://doi.org/10.1016/j.jip.2007.04.003 (2007).
76. Zimmermann, G. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Technol.* **17**, 879–920. https://doi.org/10.1080/09583150701593963 (2007).
77. Grewal, P. S. Formulation and application technology. In *Entomopathogenic Nematology,* Wallingford UK (ed. Gaugler), 265 – 287 (CABI publishing, 2002).
78. Stuart, R. J., Barbercheck, M. E. & Grewal, P. S. Entomopathogenic nematodes in the soil environment: distributions, interactions, and the influence of biotic and abiotic factors. In *Nematode Pathogenesis of Insects and Other Pests: Ecology and Applied Technologies for Sustainable Plant and Crop Protection* (ed. Campos-Herrera, R.) 97–137 (Springer, Cham., 2015) https://doi.org/10.1007/978-3-319-18266-7_4.
79. Molyneux, A. S. & Bedding, R. A. Influence of soil texture and moisture on the infectivity of *Heterorhabditis* sp. D1 and *Steinernema glaseri* for larvae of the sheep blowfly *Lucilia cuprina*. *Nematologica* **30**, 358–365. https://doi.org/10.1163/187529284X00266 (1984).
80. Kung, S. P., Gaugler, R. & Kaya, H. K. Soil type and entomopathogenic nematode persistence. *J. Invertebr. Pathol.* **55**, 401–406. https://doi.org/10.1016/0022-2011(90)90084-J (1990).
81. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412. https://doi.org/10.1371/journal.pbio.1000412 (2010).

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Author contributions

M.D.G.F., É.K.K.F., C.M. and F.E.F.S conceived the experiments; M.D.G.F., V.H.L., L.P.B., C.S.R-S and S.M.N.P. conducted the experiments; F.E.F.S and P.V.R. assisted field tests technically; M.D.G.F and G.M.M. analyzed the results; M.D.G.F., V.H.L., É.K.K.F., C.M., G.M.M. prepared a manuscript draft; M.D.G.F., V.H.L., É.K.K.F., C.M., G.M.M., F.E.F.S, P.V.R., F.M.C.F edited the manuscript and revised the manuscript for technical and scientific accuracy; É.K.K.F. and C.M. acquired funding and supervised the project. All authors approved the manuscript.

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Declaration

Competing interests

The authors declare no potential conflicts of interest concerning the research, authorship, and publication of this article.

Ethics approval

The studies with *R. microplus* were conducted following the regulations of the Ethics Committee on the Use of Animals of the Federal University of Goiás (*Universidade Federal de Goiás*, UFG, protocol #032/17), and reported in accordance with ARRIVE guidelines[81]. Access to Brazilian genetic heritage was approved by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge of Brazil (Sisgen, protocol #A420934).

Additional information

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