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ORIGINAL ARTICLE

Storability at room temperature of *Steinernema yirgalemense* (Rhabditida: Steinernematidae) in diatomaceous earth and the effect of antifungal agents

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Abstract
The commercial use of entomopathogenic nematodes after mass production requires the development of formulation techniques that extend nematode survival and prevent virulence loss during storage. In this study, the room temperature viability of *Steinernema yirgalemense*, in diatomaceous earth (DE), was investigated. The shelf life was maintained above 70% mean survival rate by week 4. To avoid microbial contamination, the direct effect of antifungal agents, peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin on the efficacy of the infective juveniles (IJs) was preliminarily investigated. Short-term exposure of PAA lowered the efficacy of IJs, yet low concentrations of TCA and nipagin had no effect. Finally, the combined effect of DE and nipagin on IJ efficacy was assessed. High concentrations of nipagin in DE killed all the IJs in the first week of storage. Lower concentrations of nipagin in DE have potential to be incorporated in EPN products.

Keywords Formulation · Nipagin · Peroxyacetic acid · Entomopathogenic · Trans-cinnamic acid · Toxicity

Introduction

Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* Poinar, 1975 and *Steinernema* Travassos, 1927 and their associated mutualistic bacteria are biocontrol agents for the management of soilborne insect pests, occurring in soil and cryptic environments worldwide (Akhurst et al. 1996). For both the steinernematid and heterorhabditid, the dauer or infective juvenile (IJ) stage is the only free-living stage. The IJs on their entry into the target pest insect release their mutualistic bacteria, where they multiply, resulting in the death of the insect (Ciche et al. 2006; Lu et al. 2017). The insect usually dies within 24–48 h after the invasion.

The successful commercialization of EPNs after mass production requires the development of storage and formulation techniques that curtail nematode mortality, as well as their loss of virulence and pathogenicity. However, their short shelf life is a drawback, especially in terms of large-scale commercial use (Grewal 2000a, b). Yet, maintenance of high-quality EPNs at all levels from the bioreactor, liquid storage and storage in formulation, until the product reaches the farmers' field (Grewal and Peters 2005; Kagimu et al. 2017), is of importance. Such maintenance is necessary because EPNs are affected by both biotic and abiotic factors, especially when in formulation, concerning which the temperature, aeration, moisture content and water loss, as well as the contamination and toxicity of antimicrobial agents in terms of IJs, are of foremost importance (Grewal and Peters 2005; Kagimu et al. 2017).

Storage of formulated local South African species of EPNs is recommended at temperatures ranging between 14 and 25 °C, due to the relatively high survival and virulence rates attained at such temperatures (Kagimu and Malan 2019). Besides, the comparatively low density of 50,000 IJs/g of diatomaceous earth (DE), which was investigated by Kagimu and Malan (2019), coincidentally depicted the relatively improved shelf life of IJs, as observed at relatively high ideal densities of 3,700,000 IJs/g (Kagimu 2018). Thus, the need to provide a comparatively high density of IJs for a realistic and improved DE formulation was of interest to the current study.

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Storage of nematode formulations at room temperature, with high moisture content, is especially prone to contamination with microbial organisms (Grewal 2002). The reason for the above is that microbes tend to compete for existing oxygen supplies, decrease the usability of formulations and initiate the clogging of spray nozzles, thereby decreasing the suitability of the formulated product. Notably, some antimicrobial agents such as Proxel may also decrease the extent of different nematode species survival in the formulations (Grewal 2002).

Peroxyacetic/peracetic acid (PAA) is a strong oxidant and disinfectant, with a wide spectrum of antimicrobial activity, which is commercially available as a mixture containing acetic acid, hydrogen peroxide and water. Various industries have demonstrated its effectiveness as an antibacterial, antiviral and antifungal agent (Kitis 2004; Ayoub et al. 2017). Peracetic acid (CH_3CO_3H) is registered by the United States Environmental Protection Agency (US EPA 1993) for its utilization in agriculture and food processing, as well as in medical facilities, as an antimicrobial disinfectant. Furthermore, in Europe, PAA, which is approved for use in veterinary medicine, is one of the few compounds approved for use as a disinfectant in aquaculture (Straus et al. 2012).

Equally important, cinnamic acid, which is an organic acid that naturally occurs in plants, has low toxicity and a broad spectrum of biological activities. Cinnamic acid has antimicrobial activity and its derivatives, when isolated from the plant material and synthesized, have antibacterial, antiviral and antifungal properties (Nascimento et al. 2000; Sova 2012). Cinnamic acid mainly exists in cis and trans isomers. Besides, trans-cinnamic (TCA) has been isolated as a secondary metabolite of the EPN-associated bacteria *Photorhabdus luminescens* and reported to be having an immense antifungal capability (Bock et al. 2014; Hazir et al. 2016, 2017, 2018).

Last but not least, nipagin M (methyl 4-hydroxybenzoate), or methylparaben, methyl *p*-hydroxybenzoate, or methyl parahydroxybenzoate is the methyl ester of *p*-hydroxybenzoic acid. The compound, which has been used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years is stable and nonvolatile (Mahuzier et al. 2001; Soni et al. 2002; Błędzka et al. 2014). Nipagin, which is biodegradable, since it is readily metabolized by common soil bacteria, is often used as an antimicrobial agent in many insect food diets (Rohlfs et al. 2005; Quesada-Moraga et al. 2006; Garrido-Jurado et al. 2011).

The present study aimed to test a locally isolated EPN at a relatively high density in DE, for the improvement of its shelf life. Furthermore, the study considered the likelihood of bacterial and fungal infections being present in the sample at a comparatively high-water activity, at which such microbial as fungi tend to thrive in formulation, due to the increased amount of water in the formulation. The study determined the short-term survival and loss of pathogenicity of IJs directly exposed to three antifungal agents and the long-term effect in DE formulation containing nipagin.

Materials and methods

Source of insect hosts and nematodes

Steinernema yirgalemense Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005, which is endemic to South Africa, was used in the current study. Wax moth larvae *Galleria mellonella* L. (Lepidoptera: Pyralidae) and yellow mealworm larvae *Tenebrio molitor* L. (Lepidoptera: Pyralidae) were cultured according to Van Zyl and Malan (2015), on an artificial diet at 25 °C in a growth chamber. The *Galleria* larvae were inoculated with IJs in 9-mm-diameter Petri dishes, lined with moist filter paper. Freshly harvested IJs were cultured in vivo, using last-instar larvae of *G. mellonella*, kept at 25 °C in growth chambers. Modified White traps (Kaya and Stock 1997) were used to harvest the emerged EPNs. Harvested IJs were stored in distilled water at 14 °C, in a walk-in temperature-controlled room and collected in 5-L Erlenmeyer flasks that were constantly stirred using a 70 × 10 mm cylindrical magnetic stirring bar on an AGE magnetic stirrer (VELP® Scientifica). For the final formulation with the addition of an antifungal agent, a high concentration of IJ was used. These nematodes were cultured in vitro according to the technique of Dunn et al. (2020).

Effect of room temperature on nematodes formulated and stored in DE

Nematodes were concentrated into a paste using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). Forty million IJs of *S. yirgalemense* were formulated in DE (Celite 209—Imerys Refractory Minerals SA (Pty) Ltd). The proportions of the ingredients used in the formulation were employed according to Kagimu and Malan (2019) (Table 1). The water activity of 0.970 determined using the AquaLab Pawkit water activity meter (Decagon Devices Inc, Pullman,

Table 1 Ingredients used together with diatomaceous earth for the formulation

Item	Weight (g)
Nematodes (40 000 000 IJs)	10.81
Tixosol 38 A (anti-caking agent)	17.88
PEG 600 for adjusting water activity—Aw	− 6.38
Diatomaceous earth (Celite 209)	131.12
Water	40.19
Total	200.00

Washington, USA) was used in the formulation and induced the IJs into partial anhydrobiosis and slow desiccation (Grewal 2000a, b). The nematode paste was hand-mixed with all the ingredients, attaining a final density of 200 000 IJs/g of formulation (Kagimu and Malan 2019). The formulated nematodes weighing 10 g were placed in lidded containers (Mambo's Plastics) ($n=20$). Ten containers were further placed into larger covered containers, lined with moistened paper towels to maintain humidity at 100%. The containers were stored for 4 weeks at 14 °C and 25 °C. The experiment was conducted twice on different test dates, using different batches of nematodes, and stored under the same conditions as those that are described above.

The survival of the formulated nematodes was determined by dilution, according to a modification of the method of Peters (2004). One gram of the DE nematode formulation (200,000 IJs/g) was added to 10 ml of distilled water. Air from an aquarium pump (Second Nature Whisper™ 1000) was used to bring the nematodes in suspension. From these, 100-µl was pipetted into 5 ml distilled water, while 1 ml of the suspension was further diluted with 2 ml of distilled water. The number of alive and dead IJs was counted under a binocular microscope, weekly for 4 weeks and the survival percentage determined, at the respective temperatures of 14 °C and 25 °C.

Antifungal toxicity screening

Different doses/concentrations of three antifungal agents, PAA, TCA and nipagin, were tested for their short-term toxic effects on the survival and pathogenicity *S. yirgalemense* IJ. Approximately 250 IJs were suspended in 1 ml liquid, in a 2-cm-diameter watch glass containing either PAA solution (36–40 wt% in acetic acid, Sigma-Aldrich), TCA (Kosher; natural ≥ 99%, FCC, FG-Sigma-Aldrich), or nipagin (methylparaben). The concentrations used for each of the respective antifungal agents in the study were: 0.01% and doubled up to 10%. The concentrations were percentages of the corresponding antifungal agents in the 1 ml containing 250 IJs in a watch glass. The control involved 250 IJs suspended in distilled water. Five watch glasses per concentration, including the control in water only, were placed in a large glass Petri dish, with a piece of moist tissue paper being placed between them to maintain 100% humidity. They were incubated at 25 °C for 24 h, after which all 250 IJs were counted onto a Petri dish to determine the number of live and dead IJs. The experiment was repeated on a different test date, using a fresh batch of nematodes.

Effect of three antifungal agents on nematode pathogenicity

After exposing the EPN to the antifungal agents for 24 h, they were tested for pathogenicity against the last-instar larvae of *G. mellonella,* using 24-well bioassay plates (flat-bottom, Nunc™, Cat. No. 144530). Each bioassay plate contained 10 larvae, placed alternately in the wells, fitted with a piece of filter paper. Each of the wells was inoculated with 50 IJs in 100 µl of the mixture of IJs/antifungal agents, while IJs suspended in distilled water were used for the controls. The lid of each well was fitted with a piece of glass of the same shape as the lid, to prevent the *G. mellonella* larvae from escaping. Five 24-well-plates, with 10 wells in each ($n=50$), were used for each treatment/concentration. The plates were placed in a plastic container that was lined with wet paper towels, thus creating 100% humidity. They were then kept in a growth chamber at 25 °C for a period of 48 h. Mortality was confirmed through the visual observation of the color of the cadavers of the wax moth larvae, which turned yellowish for *S. yirgalemense*. The experiment was repeated once on a different test date, using another batch of nematodes.

Effect of DE formulation with nipagin on nematode survival

Due to the low short-term effect on the survival of *S. yirgalemense* IJs obtained in the above toxicity preliminary screening, nipagin was selected for its long-term effect in the formulation. A high concentration of in vitro-cultured IJs was formulated in DE with nipagin. Concentrations of 2.5%, 5% and 10% of nipagin powder were added to the formulation and a control without any nipagin, and these were chosen as treatments. A final density of 600,000 IJs/g of the formulation was attained. Containers of 10 g ($n=3$) per treatment were stored for four weeks at 14 °C. The IJs were counted weekly, using a binocular microscope to determine the survival percentage in each of the containers.

Effect of DE with nipagin on nematode pathogenicity

After 4 weeks, DE formulated IJs with the different concentrations of nipagin were tested for pathogenicity against mealworms. The test area used was 24-well bioassay plates, and it was handled in the same way as described when using DE without nipagin. However, in this case, the indicator for pathogenicity was mealworm. All experiments were conducted twice, at different test dates, using different batches of nematodes.

Statistical analyses

All the experiments were repeated on different test dates, with the results being combined for analysis should no significant difference be found. Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc).

Data on the negative effect of antifungal agents on the survival percentage were arcsine transformed before analysis. All the results on antifungal agents were analyzed using general linear models (GLM), with the mean being separated employing the Games–Howell post hoc test. The results on the DE were analyzed through a two-way repeated-measures ANOVA, and the means were separated using Fisher's least significant difference (LSD) post hoc test.

Results

Effect of room temperature on nematodes formulated and stored in DE

No significant difference occurred between the data from two repeated experiments with the main effects of treatment and date in the two-way ANOVA; thus, the data from the repeated experiments were pooled and analyzed using a one-way ANOVA. The analysis of the data from weeks 2 and 4 showed a significant difference (repeated-measures two-way ANOVA: ($F_{(1,38)} = 0.86115$, $p < 0.05$) between the treatments with regard to survival (Fig. 1). A high rate of survival was attained during the observation period. Besides the above, the mean percentage of survival was significantly ($p < 0.05$) higher in week 2 than it was in week 4. At week 2, the survival of *S. yirgalemense* in DE did not significantly differ at both 14 °C and 25 °C. The mean percentage survival rate obtained at 14 °C was 85.60% ± 0.84% and at 25 °C it was 85.16% ± 0.84%. Moreover, at week 4, the survival of *S. yirgalemense* in DE also did not differ significantly at both 14 °C and 25 °C. Unlike at week 2, the mean percentage survival rate of 80.67% ± 0.71% was obtained at 25 °C and 80.67% ± 0.71% at 14 °C.

Antifungal toxicity screening

As no significant differences were obtained between the two batches in terms of the main effects of time and treatment with the different antifungal agents, the data from the two repeated experiments were pooled then analyzed. There is a significant difference ($p < 0.001$) in the effect of the antifungal agents on the percentage survival of *S. yirgalemense* IJs after 24-h exposure (Fig. 2). Both nipagin and TCA acid differed significantly ($p < 0.01$) from PAA; however, there was no significant difference between them and the untreated control, in terms of nematode survival. There was no mortality in the control. Of the antifungal agents, nipagin had the highest mean percentage survival value of 99.89% ± 0.87%, followed by TCA acid 98.78% ± 0.87%, and then by PAA, with and 29.24% ± 0.87%, respectively.

Effect of three antifungal agents on nematode pathogenicity

No significant differences were obtained between the two batches with the main effects of mortality and the antifungal agents, and the data from the two repeated experiments were pooled before analysis. There is a significant difference ($p < 0.001$) in the percentage mortality of *G. mellonella* larvae by *S. yirgalemense* IJs after 24-h exposure to the respective antifungal agents (Fig. 3). The peracetic acid differed significantly from the TCA ($p < 0.001$) and the nipagin ($p < 0.001$), which did not differ significantly from

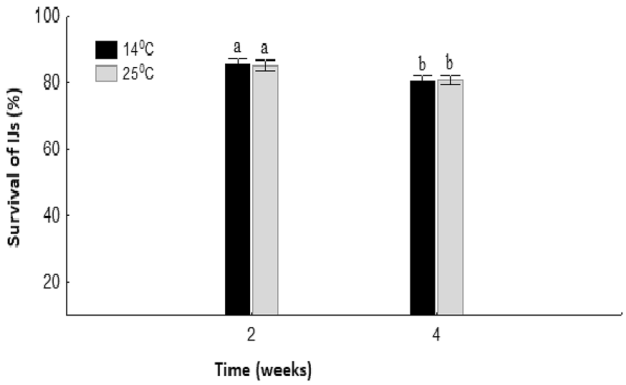

Fig. 1 Mean percentage survival (95% confidence level, i.e., error bars of the plot) of *Steinernema yirgalemense* infective juveniles (IJ) in diatomaceous earth at different temperatures during the 4 weeks. Different letters above the bars indicate significant differences between time in weeks and the percentage survival of IJs nematodes according to Fisher's least significant difference (LSD) post hoc test ($p < 0.05$)

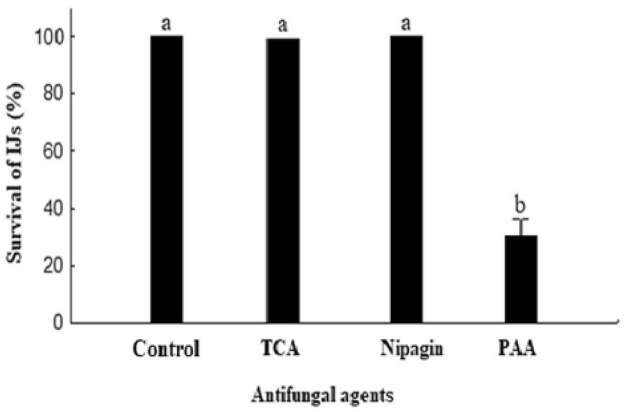

Fig. 2 Mean percentage survival (95% confidence level, i.e., error bars of the plot) of *Steinernema yirgalemense* infective juveniles (IJs) after 24 h in the antifungal agents peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin. Different letters above the bars indicate significant differences between antifungal agents and percentage survival of IJs nematodes according to the Games–Howell post hoc test ($p < 0.05$)

each other and the untreated control. The *S. yirgalemense* IJs in the untreated control and those that were treated with TCA caused the highest mean percentage mortality value of *G. mellonella* larvae of 100% ± 0.91%, followed by nipagin, and then by PAA, with 99.97% ± 0.91% and 36.70% ± 0.91%, respectively.

Effect of DE formulation with nipagin on nematode survival

Results indicated a significant difference ($F_{(3,36)} = 71.525$, $p < 0.001$) between the treatments with regard to the survival of IJs in nipagin during the long-term storage evaluation in DE. During the short-term toxicity screening for the direct effect of nipagin on 250 IJs per treatment in the 24 h of exposure, the highest dosage of 10% nipagin caused the highest mortality of *S. yirgalemense* with only 32.80% ± 2.77% survival (Fig. 4). In the long term for the combined effect of nipagin and diatomaceous earth on 600,000 IJs/g of formulation per treatment (Fig. 5), a gradual decline in the survival rate of the DE formulations was attained with increasing dosage of nipagin as compared to the control during the observation period. Though the nematode density in DE formulation was increased from 200,000 to 600,000 IJs/g to increase their survival rate, all IJs died in the first week of storage in the formulation containing 10% nipagin. For this reason, these data were excluded in the final analysis of the pathogenicity tests. Likewise, no significant difference between the other treatments with respect to mortality caused to the formulated IJs in nipagin and DE was noticed. The overall survival percentage after the fourth week in storage in the formulation was above 70%. While at the same time, the nematodes in water suspension were close to a 95% mean survival rate (results not reported). Furthermore, a

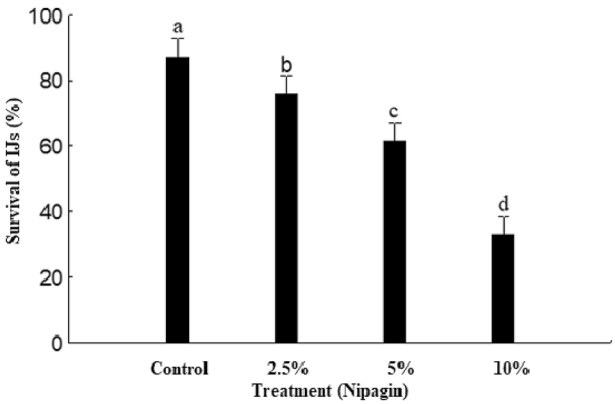

Fig. 4 Mean percentage survival (95% confidence level, i.e., error bars of the plot) of *Steinernema yirgalemense* infective juveniles (IJs) after 24-h exposure in the antifungal agents. Different letters above the bars indicate significant differences between the Nipagin treatment and the IJ percentage survival of IJs nematodes according to Fisher's least significant difference (LSD) post hoc test ($p < 0.05$)

significant difference ($F_{(3,60)} = 9.8470$, $p < 0.\ 0.001$) between the storage times with regard to the survival of IJs in DE formulation containing nipagin was obtained. Overall, the average number of IJ mortalities in formulation increased with each increasing week of storage (Fig. 5).

Effect of DE with nipagin on nematode pathogenicity

The analysis on pathogenicity revealed a significant difference ($F_{(2,27)} = 95.387$, $p < 0.\ 0.001$) between the treatments with regard to mortality of *T. molitor* larvae by *S. yirgalemense* IJs after four weeks of storage in DE formulation containing nipagin (Fig. 6). The control differed significantly

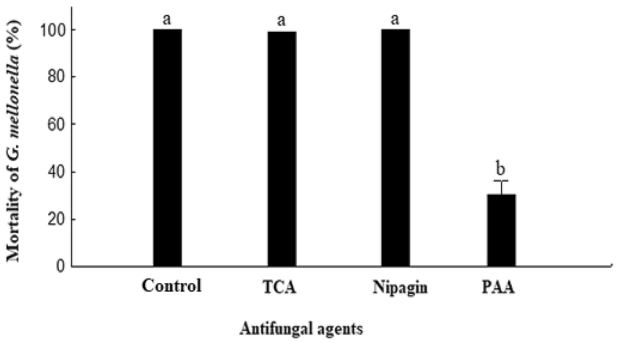

Fig. 3 Mean percentage mortality (95% confidence level, i.e., error bars of the plot) of *Galleria mellonella* larvae inoculated with *Steinernema yirgalemense*, after 24-h exposure to the respective antifungal agents, peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin. Different letters above the bars indicate significant differences between the antifungal agent and the mortality of *Galleria* larvae according to the Games–Howell post hoc test ($p < 0.05$)

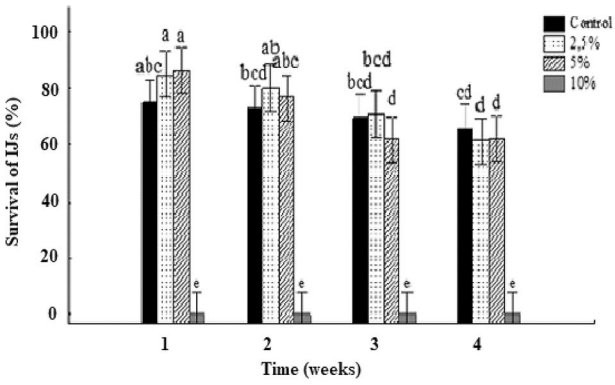

Fig. 5 Mean percentage survival (95% confidence level, i.e., error bars of the plot) of *Steinernema yirgalemense* infective juveniles (IJ) in diatomaceous earth formulation with nipagin at 14 °C. Different letters above the bars indicate significant differences between weeks and the percentage survival of IJs nematodes according to Fisher's least significant difference (LSD) post hoc test ($p < 0.05$)

from the dose 2.5% ($p = 0.004$) and the dose 5% ($p < 0.001$), which also differed significantly ($p < 0.001$) from each other. The control caused the highest mean percentage mortality value of *T. molitor* larvae of 98.33% ± 0.03%, followed treatment with 2.5% nipagin, and then by treatment with 5% nipagin, with 86.67% ± 0.03% and 49.17% ± 0.03%, respectively.

Discussion

Research showed an improvement in the shelf life of IJs stored in DE formulation alone and DE formulation containing nipagin, as compared to a previous study in the formulation of *S. yirgalemense* (Kagimu and Malan 2019). A notable result, in this case, is the high survival rate (80% and 70%) of *S. yirgalemense* in week 4 at 25 °C in the respective formulations. The above finding is reassuring in terms of the implications that it holds for the much-desired room temperature storage of IJs in DE formulation. The improvement in the number of nematodes in DE formulation has undoubtedly influenced the survival of EPNs in terms of the current research (Silver et al. 1995; Hiltpold et al. 2012; Matadamas-Ortiz et al. 2014).

The study reports on the loss of survival and pathogenicity of *S. yirgalemense* due to PAA. Despite PAA being a strong disinfectant, with a wide spectrum of antimicrobial activity, it is not suited to protect EPN formulation against contamination. Peroxyacetic acid has previously been used as a nematicide (Jagdale and Grewal 2002; Krishnayyaand and Grewal 2002; An et al. 2017), with the previously obtained results agreeing with the findings made in the current study. For example, An et al. (2017) report

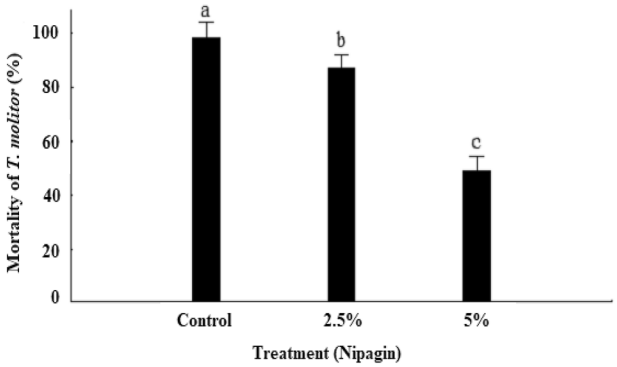

Fig. 6 Mean percentage mortality (95% confidence level, i.e., error bars of the plot) of *Tenebrio molitor* larvae by *Steinernema yirgalemense* infective juveniles (IJs) after four weeks of storage in DE formulation containing nipagin. Different letters above the bars indicate significant differences between the percentage the mortality of *T. molitor* and Treatment according to Fisher's least significant difference (LSD) post hoc test ($p < 0.05$)

that ZeroTol (BioSafe Systems) caused 100% mortality of *Aphelenchoides fragariae* (Ritzema Bos 1891) Christie, 1932 (Aphelenchida: Aphelenchoididae) in aqueous suspension at 20-fold (low) dilution. ZeroTol further reduced, by over 85% and 75%, *A. fragariae* population in soil 7 and 42 days after treatment, respectively, in drench application. An et al. (2017) deduced that spray application of ZeroTol could reduce > 70% of the *A. fragariae* population in leaf disks, and that it, thus, has great potential to manage foliar nematodes in floriculture. The active ingredient of ZeroTol is PAA (270 g/l). The above-mentioned results support the results obtained in the current study, in terms of which nematodes lost their survival and virulence in 24 h. Similarly, Krishnayyaand and Grewal (2002) reported that the hydrogen dioxide/PAA mixture (ZeroTol) was incompatible with the IJs of *Steinernema feltiae* (Filipjev 1934) Wouts, Mráček, Gerding and Bedding 1982, as it caused 100% mortality after 120 h of incubation, and, hence, could not be tank-mixed during application.

Results from the study showed that TCA does not affect the viability and virulence of the IJs of *S. yirgalemense* at all the tested concentrations, up to 24 h of exposure. Transcinnamic acid has been isolated as a secondary metabolite of the mutualistic bacteria, *Photorhabdus luminescens* (Bock et al. 2014; Hazir et al. 2016, 2017, 2018). However, the acid's effect on EPN IJs, to our current knowledge, has not previously been tested, with this being the first report showing its compatibility with EPNs. Overall, several other antimicrobials have been isolated from the metabolites of the *Photorhabdus* and *Xenorhabdus* species (Boemare and Akhurst 2006; Bode, 2009; Shapiro-Ilan et al. 2009, 2014; Bock et al. 2014).

Though the bioactive metabolite of *Xenorhabdus indica* Somvanshi, Lang, Ganguly, Swiderski, Saxena and Stackebrandt 2009 associated with *S. yirgalemense* (Ferreira et al. 2016) is unknown and has not yet been isolated, the notion that it is compatible with TCA probably holds. Incidentally, the results of the current study have revealed the bioactive secondary metabolites of the bacteria associated with EPN as being potent antimicrobials in the formulation.

Furthermore, the results of the present study also show that nipagin, likewise, does not affect the viability and virulence of the IJs of *S. yirgalemense* at all the tested concentrations, up to 24 h of exposure. By contrast, at higher concentration and long-term exposure, nipagin greatly affects the viability of *S. yirgalemense* IJs in DE formulation. This agrees with Kermarrec and Mauléon (1989) who reported that nipagin lowered the pathogenicity of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin and Bedding, 1982 to the caterpillars of two Pyralidae moths (*Diatraea saccharalis* and *Galleria mellonella*) by 200 times (all instars), ranging from 50 to 250 times with larval aging from the third to the fifth instar.

The above result of DE formulation is following the findings of Matadamas-Ortiz et al. (2014), who encapsulated *Steinernema glaseri* IJ with different proportions of diatomaceous earth and attapulgite clay, and reported the best survival times in proportions with diatomaceous earth only, comparable to those of Silver et al. (1995) at room temperature. The present results obtained are also similar to those of Ziaee et al. (2016), who reported the increased mortality of the adults of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), with the increasing exposure intervals and concentration levels of DE proving to be key to the function of serving as the grain protectants of stored product pests. Preliminary results (not shown here) of *S. jeffreyense* in DE at a density of 4.0×10^6 IJs/g of formulation have given 100% survival and pathogenicity for three months at 14 °C. Equally important, though not tested, and would require further tests for validation, the observations in this study have coincidentally revealed an inverse relationship between the number of IJs in the DE formulation and the presence of water, whereby the higher the number of IJs, the lower is the amount of DE and water that is required per gram of the formulation. The use of such a formula leads to improved DE formulation. At the point where the DE becomes saturated with IJs, so much so that it forms a paste, no additional water is required, as adding more at this stage would serve to expedite microbial growth.

To be concluded, for any safety and environmental concerns including residuals of the respective ingredients, we recommend EPN producers to follow each of the manufacturers' product sheet safety guidelines for the ingredients (PAA, nipagin, TCA and DE) before they are incorporated in any EPN formulation. Nevertheless, to date EPNs have had no recorded residual effects to the handlers. Due to low IJ efficacy treated with PAA, it is not recommended for use as an antimicrobial in the formulation of EPNs. The present study reports on the high survival and virulent abilities of *S. yirgalemense* to long-term exposure of low concentrations of nipagin in DE. Future studies are recommended to include the bioactive metabolites of the mutually associated *Photorhabdus* and *Xenorhabdus* as antimicrobials in the EPN formulations are concerned. Although good results were obtained during the study using the DE formulation described, still higher densities of nematodes are recommended for use in improving the survival rate and virulence of IJs in DE formulations. Farmers are advised to only order for EPNs when they are ready to spray. This is because they deteriorate easily on shelves unlike inorganic chemicals.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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