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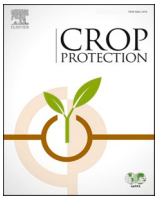

Screening five local entomopathogenic nematode species for their virulence against pupae of the Eucalyptus snout beetle, *Gonipterus* sp. n. 2, under laboratory conditions

Innocent L. Rakubu *, Agil Katumanyane, Brett P. Hurley

Department of Zoology and Entomology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

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ABSTRACT

The Eucalyptus snout beetle, *Gonipterus* sp. n. 2, is an important pest of eucalypt trees in South Africa and other countries. The pest is partially controlled with a mymarid egg parasitoid, *Anaphes nitens*. Identifying additional biological control agents that target other developmental stages of *Gonipterus* sp. n. 2 is necessary to improve control. Entomopathogenic nematodes (EPNs) are used as biological control agents for numerous soil pests and thus have the potential to be used against the pupal stage of *Gonipterus* sp. n. 2, which occurs in the soil. In this study, five South African EPN species were screened for their virulence on uncased pupae of *Gonipterus* sp. n. 2. At a concentration of 200 IJs/pupa in 50 µl of sterile water, *Steinernema jeffreyense*, *S. fabii*, *Heterorhabditis noenieputensis* and *H. safricana* caused pupal mortality of less than 40 %, while *S. yirgalemense* caused the highest pupal mortality of 100 % two days post-inoculation. *Steinernema yirgalemense* was selected and applied at different concentrations, namely 0, 12, 25, 50, 100, 200, and 400 IJs/pupa to determine its lethal concentration. Probit analysis indicated that the LC_{50} and LC_{90} of *S. yirgalemense* were 48.29 and 260.63 IJs/pupa, respectively. The study also showed that the pupal cases do not affect the efficacy of *S. yirgalemense* as it caused more than 90 % mortality of cased pupae of *Gonipterus* sp. n. 2 in soil bioassays. In addition, pupal age did not affect the efficacy of *S. yirgalemense*. Based on our findings, *S. yirgalemense* is a potential candidate for the biological control of pupae of *Gonipterus* sp. n. 2.

1. Introduction

The increased invasion of non-native insect pests threatens the global eucalypt plantation forest industry (Hurley et al., 2016). The Eucalyptus snout beetle, *Gonipterus* spp. (Coleoptera; Curculionidae), native to Australia, is an invasive insect pests causing damage to *Eucalyptus* spp. grown in non-native areas. The beetle is now considered to be a species complex consisting of 10 cryptic species (Mapondera et al., 2012). One of these cryptic species, *Gonipterus* sp. n. 2, was detected in South Africa in 1916 where it is now an established and important pest of *Eucalyptus* spp. (Mapondera et al., 2012; Schröder et al., 2020). The adult and larval stages of this pest cause damage to the host plant by feeding on new leaves, shoots and buds (Mally, 1924; Tooke, 1955). In South Africa, the different growth stages of *Gonipterus* sp. n. 2, namely adult, egg, larval and pupal stages, overlap and occur throughout the year (Tooke, 1955). Populations peak in September during spring when the host trees begin to produce new leaves (Tooke, 1955).

Biological control with the egg parasitoid wasp, *Anaphes nitens* Girault, has been one of the main methods used to control populations of *Gonipterus* sp. n. 2 in South Africa and elsewhere in the world (Garnas et al., 2012; Schröder et al., 2020). *Anaphes nitens* was originally imported from Australia to South Africa in 1926 (Tooke, 1955). However, the efficacy of *A. nitens* to suppress the population of *Gonipterus* spp. has not been consistent over the years (Reis et al., 2012; Schröder et al., 2020). For example, Loch (2008) reported that *A. nitens* was not as effective in cold highland areas in southwestern Australia and South Africa. In Portugal, *A. nitens* failed to control *Gonipterus platensis* Marelli in areas with altitudes higher than 400 m (Valente et al., 2017). Given the failure of *A. nitens* to provide consistent and widespread control of *Gonipterus* spp., exploring other biological control measures is necessary. Potential alternative biological control agents include entomopathogenic nematodes (EPNs), which are virulent to a wide range of insect pests (Kerry and Hominick, 2002; Katumanyane et al., 2018).

Entomopathogenic nematodes are soft-bodied non-segmented

* Corresponding author.
E-mail address: Innocent.rakubu@up.ac.za (I.L. Rakubu).

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roundworms, naturally occurring obligate parasites of many soil-dwelling insects (Vashisth et al., 2013). They belong to the two families Steinernematidae and Heterorhabditidae. The steinernematid and heterorhabditid nematodes have a mutual relationship with bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively (Poinar Jr, 1990). The free-living stage of these nematodes called infective juveniles (IJs), is responsible for locating and infecting insect hosts. The IJs infect the insect host by penetrating through the insect's natural openings (mouth, anus, and spiracles) to gain access to the hemocoel, where they release their symbiont bacteria. The nematode-bacterium complex results in the death of the insect host within 24 h post-inoculation (PI) or after a few days depending on the EPN species and the insect host (Adams and Nguyen, 2002; Adams et al., 2006; Dillman et al., 2012). The IJs grow and reproduce inside the insect cadaver and exit when they have depleted the food resources. Usually, they produce 2–3 generations before exiting the insect cadaver and searching for new hosts (Shapiro-Ilan et al., 2012; Heriberto et al., 2017).

Entomopathogenic nematodes are environmentally friendly as they do not pose any known negative effects on non-target vertebrate organisms and human health (Bathon, 1996). They can be easily mass-produced using in vivo culture method for small-scale applications and *in vitro* (solid and liquid culture) methods for large-scale applications (Inman et al., 2012, Shapiro-Ilan et al., 2012). EPN formulation techniques are used to facilitate long-term storage, transportation, and handling (Grewal, 2002; Heriberto et al., 2017; Nxitywa and Malan, 2021). In addition, EPNs can easily be applied using agrichemical spray equipment and irrigation systems (Georgis, 1990; Wright et al., 2005; Shapiro-Ilan et al., 2006).

The larvae of *Gonipterus* sp. n. 2 spend most of their developmental time on the tree canopy feeding on leaves, where the application of EPNs is difficult as the EPNs will be exposed to detrimental factors such as ultraviolet radiation and desiccation (Begley, 1990; Glazer et al., 1992; Shapiro-Ilan et al., 2006). However, the fully-grown larvae of *Gonipterus* sp. n. 2 fall to the ground and form pupal cases with soil in which they pupate (Tooke, 1955). While in the soil, the pupal stage lasts 15–17 days and adult beetles emerge after 12–15 days post-pupation (Tooke, 1955). The duration that the pupae spend in the soil presents an ideal opportunity to apply EPNs.

To date, the virulence of EPNs on *Gonipterus* sp. n. 2 has not been investigated. The current study aimed to test the virulence of five local EPN species, namely *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler, and Adams; *Steinernema jeffreyense* Malan, Knoetze, and Tiedt; *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers, and Hurley; *Heterorhabditis noenieputensis* Malan, Knoetze, and Tiedt; and *Heterorhabditis safricana* Malan, Nguyen, De Waal, and Tiedt, on pupae of *Gonipterus* sp. n. 2. We hypothesized that: (1) the five EPN species will show varying virulence on uncased pupae of *Gonipterus* sp. n. 2; (2) the LC_{50} and LC_{90} values of a particular EPN species will vary with insect host and its developmental stage; (3) the pupal age will have no effect on the efficacy of the most effective EPN species selected from the screening experiment. The pupal casing is a potential barrier to invading antagonist organisms, such as EPNs, so the initial assays as well as the probit test targeted the uncased pupae to study their susceptibility without the influence of the pupal casing. The soil bioassays and pupal age experiment used cased pupae to test the efficacy of EPNs on pupae with their potential protective casing and within their natural habitat.

2. Materials and methods

2.1. Source of EPNs

EPN species used in the study were sourced from the FABI EPN collection. The EPN species used are listed in Table 1. The EPNs were cultured in vivo using fourth-instar larvae of *Galleria mellonella*. This was done by using a 9 cm diameter Petri dish lined with filter paper. Each EPN species was inoculated in its respective Petri dish at a concentration of 1000 IJs in 800 μl of sterile water. Ten larvae of *G. mellonella* were immediately introduced per Petri dish. The Petri dishes were sealed with their lids, wrapped with a parafilm, and then transferred into 2 L capacity rectangular plastic boxes lined with wet paper towels. The boxes were incubated in darkness in an incubator set at 23 ± 2 °C for 48 h. After 48 h, the dead larvae from each Petri dish were rinsed with sterile water, using a wash bottle and 8 cm sieve to wash off nematodes on the surface, and transferred into new Petri dishes. The Petri dishes were sealed as described above and incubated for six more days, after which the cadavers were transferred to White traps. The IJs of each EPN species caught in the White trap were harvested by transferring the White trap contents into 500 ml capacity culture flasks. The concentration in each flask was adjusted to 1000 IJs/ml and the flasks were stored horizontally in an incubator at 12 °C. The culture flasks were gently shaken periodically to allow ventilation.

Table 1
The local EPN species used in the study, their associated bacteria, place of origin and GenBank accession number.

EPN species	Associated bacteria	Origin (province/ town)	GenBank accession no.	Reference
Steinernema yirgalemense	*Xenorhabdus indica*	Nelspruit, Mpumalanga	EU625295	Malan et al. (2011)
S. jeffreyense	*X. khoisanae*	Jeffreys Bay, Eastern Cape	KC897093	(Malan et al., 2016)
S. fabii	*X. khoisanae*	Mpumalanga	KR527216	Abate et al. (2016)
Heterorhabditis safricana	*Photorhabdus luminescens* subsp. *laumondii*	Western Cape	EF488006	Malan et al. (2008)
H. noenieputensis	*P. luminescens* subsp. *noenieputensis*	Northern Cape	JN620538	Malan et al. (2014)

2.2. Source of insect and pupation soil

The larvae of *Gonipterus* sp. n. 2 were collected at Mondi's Mistley plantation (GPS coordinates: 29.218415 S and 30.679624 E), in KwaZulu-Natal province, South Africa. The soil for pupation was a mineral soil collected from the same plantation and was autoclaved before use for larval pupation. Larvae were placed in pillowcases with eucalypt leaves and transported to the insect rearing laboratory at the Biological Control and Insect Rearing facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, for further rearing until pupation. The larvae were reared in 1.2 l plastic containers lined with a paper towel containing eucalypt leaves as food for the larvae, which were replaced every second day. Fully-grown larvae were selected for the experiments.

2.3. Screening EPNs

Five local EPN species were screened for their virulence on uncased pupae of *Gonipterus* sp. n. 2. The field collected soil was autoclaved and allowed to cool overnight before use. The soil was then poured into 500 ml micro box containers and moistened with 10 ml of sterile water. Fully-grown larvae were transferred into the micro box containers (20 larvae per container), which were then incubated at 25 °C for 18 days to allow the larvae to pupate. After 18 days, pupae of *Gonipterus* sp. n. 2. were carefully removed from the pupal cases and used in the screening bioassays. The 12-well bioassay plates were used as the screening arena. Ten of the 12-wells were used in each plate. Each of the 10-wells, lined with 23 mm diameter filter paper, received one pupa of *Gonipterus* sp. n. 2. Each EPN species (treatment) was inoculated on three bioassay plates

at a concentration of 200 IJs per pupae in 50 μl of water using a pipette. Each bioassay plate was treated as a replicate. The three control plates received 50 μl of distilled water. The plates were covered with a damp paper towel and closed with their lids. The plates were then transferred into 2 l plastic boxes lined with a wet paper towel (100 % moisture). These plastic boxes were then closed with their lids and stored for 48 h in an incubator set at 25 ± 2 °C.

After 48 h, pupae that showed color change and/or did not respond with a movement when poked with a soft paintbrush were considered dead. The dead pupae were removed from the bioassay plates, rinsed with sterile distilled water, and transferred into 9 cm diameter Petri dishes lined with filter paper to incubate for 48 h. Dead pupae from the same bioassay plate were transferred into one Petri dish. Mortality caused by the EPNs was further confirmed by dissecting the dead pupae 48 h PI and examining the presence of nematodes under a stereomicroscope. The experiment was repeated on a different date using a fresh batch of nematodes.

2.4. Probit test

The lethal concentrations (LC_{50} and LC_{90}) of *S. yirgalemense* towards uncased pupae of *Gonipterus* sp. n. 2 was determined. Similar methods as explained for the screening bioassay were used. *Steinernema yirgalemense* was selected as it caused the highest pupal mortality during the screening bioassay. *Steinernema yirgalemense* was applied at different concentrations, namely 12, 25, 50, 100, 200, and 400 IJs/pupa. Each concentration was inoculated on three bioassay plates. Each plate, consisting of ten pupae, was treated as a replicate. The experiment was repeated on a different date using fresh IJs of *S. yirgalemense* and pupae of *Gonipterus* sp. n. 2.

2.5. The soil bioassay

The ability of *S. yirgalemense* to penetrate the pupal cases and infect the pupae of *Gonipterus* sp. n. 2 was tested in the soil bioassay. The soil was collected from Mondi's Mistley plantation in KwaZulu-Natal and treated as mentioned above. 90 ml vials each consisting of 40 g of soil moistened with 2 ml of sterile water were used as the infection arena. One larva was transferred into each vial. Larvae that had not burrowed into the soil after 24 h were replaced with new ones. The larvae were then incubated at 25 °C for 18 days to allow the larvae to pupate. After 18 days when the larvae had pupated, *S. yirgalemense* was administered per vial at concentrations of 12, 25, 50, 100, 200, and 400 IJs/pupa in 2 ml of sterile distilled water and was followed by an additional 1 ml of water to wash the IJs into the soil. The different concentrations were used to determine effective concentration for soil application. Each concentration was inoculated on ten vials and replicated three times to provide a total of thirty vials. On the 7th-day PI, the number of dead and live pupae and adult beetles (as some of the pupae had molted to adults) was recorded. Mortality caused by *S. yirgalemense* was confirmed by dissecting the dead individuals to check for the presence of nematodes with the aid of the stereomicroscope. Even though some larvae died due to the infection of *S. yirgalemense,* larval mortality was not recorded as the focus of this experiment was on pupal mortality. The mortality was determined by combining the number of dead pupae and adult beetles. The experiment was repeated on a different date using a fresh batch of nematodes and pupae of *Gonipterus* sp. n. 2.

2.6. Pupal age experiment

The effect of pupal age on the efficacy of *S. yirgalemense* was tested. A similar method as explained in the soil bioassay experiment was used for the pupal age experiment. 90 ml vials were used as the infection arena, each consisting of 40 g of soil moistened with 3 ml of sterile water. One larva of *Gonipterus* sp. n. 2 was transferred into each vial and larvae that had not burrowed into the soil after 24 h were replaced with new ones. For this experiment, we inoculated *S. yirgalemense* on *Gonipterus* sp. n. 2 cased pupae of two age groups, namely 1–3 and 9-12 days-old. Inoculation of the cased early age (1–3 days old) and late age (9–12 days) pupae was done on the 18th and 25th days post introduction of the larvae into the soil, respectively. The same batch of *S. yirgalemense* was administered for both age groups, at a concentration of 200 IJs/pupa in 2 ml of sterilized distilled water per vial. An additional 1 ml of water was added to wash the IJs into the soil. The control was prepared in the same way but received 2 ml of sterilized distilled water without nematodes. Each pupal age group consisted of ten vials that were replicated three times for a total of thirty vials. The inoculated vials were incubated in the dark at 25 °C for 7 days. On the 7th-day PI, the number of dead and live pupae as well as adult beetles were recorded. Mortality caused by *S. yirgalemense* was confirmed by dissecting the dead individuals to check for the presence of IJs under a stereomicroscope. The experiment was repeated on a different date using a fresh batch of nematodes and pupae of *Gonipterus* sp. n. 2.

2.7. Data analysis

The pupal mortality recorded in the screening experiment was subjected to two-way ANOVA (test date and EPN species as factors) for analysis using the RStudio statistical program (RStudio Team, 2020). In the absence of a significant difference between the test dates and replicates of the same treatment, the data were pooled and analyzed with one-way ANOVA. One-way ANOVA was used to test the significant difference between mean pupal mortalities provided by EPN species. The mean pupal mortalities of EPN species were then separated with the Tukey post hoc test if there was a significant difference detected. Pupal mortalities for each EPN concentration were subjected to probit analysis in SPSS to determine the lethal concentrations of *S. yirgalemense* (IBM Corp, 2021). The correlation between EPN concentration and pupal mortality was determined with RStudio. Data from the pupal age experiment were subjected to a *t*-test in RStudio to determine the effects of pupal age on the efficacy of *S. yirgalemense* (RStudio Team, 2020).

3. Results

3.1. Screening EPNs

All five local EPN species were able to kill uncased pupae of *Gonipterus* sp. n. 2 (Fig. 1). Analysis using one-way ANOVA showed significant effects of the EPN treatments on pupal mortality ($F_{5,12} = 29.92$, $p < 0.0001$). After 48h, mortality of uncased pupae of *Gonipterus* sp. n. 2 caused by *S. yirgalemense* was significantly higher than that caused by

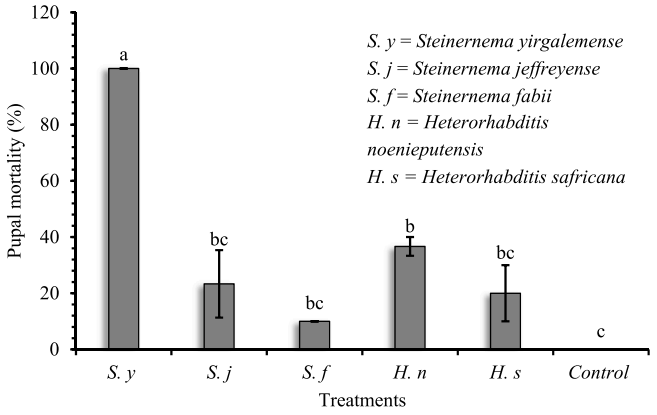

Fig. 1. The mean (±SE) mortality of uncased pupae of *Gonipterus* sp. n. 2 after 48 h exposure to five EPN species at a concentration of 200 IJs/pupa in 12-well bioassay plates. Different letters above the bars indicate significant differences ($p < 0.05$).

H. noenieputensis, S. jeffreyense, H. safricana, *S. fabii*, and the control. Although *H. noenieputensis* provided the second-highest pupal mortality, it was not significantly different from the other three EPN species. The percentage mortality of the control did not differ from mortality obtained from *S. jeffreyense, H. safricana* and *S. fabii*.

3.2. Probit test

There was a significant effect of the concentration of *S. yirgalemense* on mortality of uncased pupae of *Gonipterus* sp. n. 2, 48 h PI ($F_{5,12} = 31.43$, $p < 0.001$, Table 2). The mortality of uncased pupae at 400 IJs/pupa did not differ significantly from mortality at 200 IJs/pupa but was significantly higher than pupal mortality at 100 IJs/pupa, 50 IJs/pupa, 25 IJs/pupa and 12 IJs/pupa. A positive correlation was observed between the EPN concentration and pupal mortality after 48 h PI ($y = 54.63x - 41.987$; $r = 0.99$) (Fig. 2). The LC_{50} and LC_{90} values of *S. yirgalemense* against uncased pupae of *Gonipterus* sp. n. 2, calculated using the regression line formula, were 48.29 and 260.63 IJs/pupa, respectively.

3.3. The soil bioassay

There was a significant effect of the concentration of *S. yirgalemense* on mortality of cased pupae of *Gonipterus* sp. n. 2, seven days PI ($F_{6,14} = 12.67$, $P < 0.001$, Table 2). On the 7th-day PI, the mortality of the cased pupae at the concentration of 400, 200, 100, 50, and 25 IJs/pupa was more than 90 % and there was no significant difference between the concentrations (Tukey multiple comparisons of means; 95 % confidence level). The concentration of 12 IJs/pupa caused the lowest pupal mortality of 41.67 % and was not significantly different from the control with 0 % mortality. A moderate positive correlation ($r = 0.5$) was observed between the concentrations and mortality ($t = 2.4988$, $df = 19$, p-value $= 0.0218$).

3.4. Pupal age experiment

Pupal age did not affect the efficacy of *S. yirgalemense*, as mortality recorded from both age groups did not differ significantly ($t = 0$, $df = 4$, $p = 1$, Fig. 3). The mortality caused by *S. yirgalemense* on cased pupae of *Gonipterus* sp. n. 2 from the early and late age groups was 95.38 % and 100 %, respectively. Mortality of cased pupae for both age groups was significantly higher than in the control, i.e. natural mortality ($F_{4,6} = 35,20$, $p = 0.00048$).

4. Discussion

The local EPN species used in this study (*S. yirgalemense, S. jeffreyense, S. fabii, H. noenieputensis*, and *H. safricana*) showed varied virulence on uncased pupae of *Gonipterus* sp. n. 2. Of the five EPN species tested, *S. yirgalemense* caused the highest mortality of uncased pupae and was selected for further experiments. The result of probit analysis showed that the LC_{50} and LC_{90} of *S. yirgalemense* are 48.29 and 260.63 IJs/pupa, respectively. Infective juveniles of *S. yirgalemense* at different concentrations, namely 12, 25, 50, 100, 200 and 400 IJs/pupa, penetrated the pupal cases of *Gonipterus* sp. n. 2 and caused mortality ranging from 41.67 % to 100 %. The virulence of *S. yirgalemense* was not affected by the pupal age of cased pupae of *Gonipterus* sp. n. 2.

Table 2
Mean (±SE) pupal mortality of *Gonipterus* sp. n. 2 at different concentrations of *S. yirgalemense*. Different superscript letters on mortality values indicate significant differences.

Concentration (%)	Mortality (%) of uncased pupae 48 h PI in bioassay	Mortality (%) of cased pupae 7 days PI in soil
400	100 ± 0^{a}	100 ± 0^{a}
200	76.67 ± 6.67^{ab}	95.24 ± 4.76^{a}
100	73.33 ± 3.33^{b}	95.24 ± 4.76^{a}
50	56.67 ± 6.67^{bc}	94.44 ± 5.56^{a}
25	33.33 ± 6.67^{cd}	90.48 ± 9.52^{a}
12	13.33 ± 6.67^{d}	41.67 ± 25.34^{b}
0 (control)	0^{d}	0^{b}

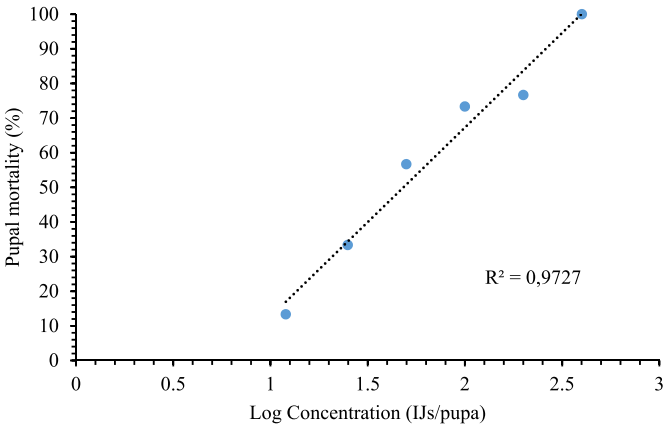

Fig. 2. The probit mortality of uncased pupae of *Gonipterus* sp. n. 2 by *S. yirgalemense* at different concentrations (Log): 12, 25, 50, 100, 200, 400 IJs/pupa, after 48 h PI in 12-well bioassay plates.

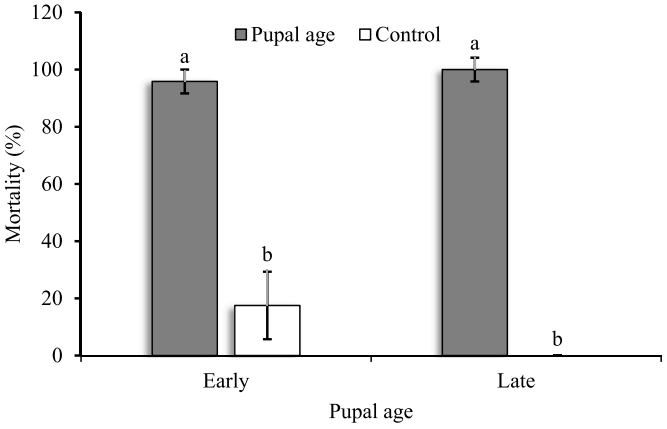

Fig. 3. Mean (±SE) mortality caused by *S. yirgalemense* on the 7th-day PI on cased pupae of *Gonipterus* sp. n. 2 from two age groups, early and late age, in soil. Early and late pupal age refers to pupae that are 1–3 days old and 8–12 days old, respectively ($P < 0.001$).

The current study showed that uncased pupae of *Gonipterus* sp. n. 2 are susceptible to local EPNs. This is in alignment with studies by Damascena et al. (2020) and Brida et al. (2021), which showed that pupae and pre-pupae of *G. platensis* (a species closely related to *Gonipterus* sp. n. 2) were susceptible to *Steinernema diaprepesi* Nguyen and Duncan and *Steinernema brazilense* Nguyen, Ginarte, Leite, dos Santos, and Harakava, respectively. In the current study, *S. yirgalemense* caused the highest mortality of the uncased pupae (100 ± 0 %) 48 h PI and it was significantly different from other EPN species. Other EPN species (*S. jeffreyense, S. fabii, H. noenieputensis,* and *H. safricana*) used in the study caused mortality ranging from 10 % to 36.67 % and their effects on mortality of uncased pupae did not differ significantly. The mortality recorded included dead adult beetles because it is likely that pupae managed to molt into adult beetles post-infection, then succumbed to the infection as teneral adult beetles.

In South Africa, several studies have reported varied mortality caused by *S. yirgalemense* and *H. noenieputensis* on different insect pests (Malan et al., 2011; James et al., 2018; Katumanyane et al., 2018; Platt et al., 2018; Dlamini et al., 2019). In the highlighted studies, both EPN

species caused mortality ranging from 55 % to 94 %. *Heterorhabditis safricana* and *S. jeffreyense* at a concentration of 100 IJs/insect caused mortality of less than 5 % against female adults of *Planococcus ficus* Signoret and larvae of *Bradysia impatiens* Johannsen, respectively (Vieux and Malan, 2013; Katumanyane et al., 2018). The current study also recorded mortality of less than 20 % caused by *H. safricana* and *S. jeffreyense* at a concentration of 200 IJs/pupa 48 h PI against uncased pupae *Gonipterus* sp. n. 2. Based on the results of the current study and the above-mentioned studies, we suggest that *Gonipterus* sp. n. 2 is not a suitable host for *S. jeffreyense*, *S. fabii, H. noenieputensis* and *H. safricana*, but is a good host for *S. yirgalemense*, which have shown an acceptable virulence against a wide range of important insect pests in South Africa.

The current study determined the LC_{50} (48.29 IJs/pupa) and LC_{90} (260.631 IJs/pupa) of *S. yirgalemense* against uncased pupae of *Gonipterus* sp. n. 2. Katumanyane et al. (2018) determined the LC_{50} (8 IJs/pupa) and LC_{90} (65 IJs/pupa) of *S. yirgalemense* against *B. impatiens*. The LC_{50} and LC_{90} of *S. yirgalemense* against larvae of *Lobesia vanillana* De Joannis were 7.335 IJs/larva and 62.761 IJs/larva, respectively (du Preez et al., 2021). Prinsloo et al. (2022) also determined the LC_{50} (4.38 IJs/larva) and LC_{90} (46.9 IJs/larva) of *S. yirgalemense* against the fifth instar larvae of *Thaumatotibia leucotreta* Meyrick. Similar methodology was used in the above-mentioned studies and the current study, thus the different LC_{50} and LC_{90} values are likely influenced by the host insect, demonstrating the variation in the virulence of the same EPN species across different insect pests and their developmental stages (Malan et al., 2011; Dlamini et al., 2020)

In most cases, the pupae of insect pests are less susceptible than other developmental stages to EPNs due to closed natural openings and barriers such as cocoons, which in turn reduces the infection success of EPNs (Abbas et al., 2001; Langford et al., 2014; Garriga et al., 2018). However, the current study has shown that, at concentrations of 25, 50, 100, 200, and 400 IJs/pupa, *S. yirgalemense* caused pupal mortality of more than 90 % against *Gonipterus* sp. n. 2. The pupae were inoculated whilst in their pupal cases made of soil, and mortality was recorded on the 7th-day PI. Malan et al. (2011) also reported that *S. yirgalemense* at 800 IJs/ml concentration caused 93 % mortality of *T. leucotreta* pupae, 14 days PI in sand bioassays.

The current study showed that pupal age of *Gonipterus* sp. n. 2 does not affect the efficacy of EPNs. On the 7th-day PI at a concentration of 200 IJs/pupa, *S. yirgalemense* caused mortality of 95.83 % and 100 % of cased pupae of *Gonipterus* sp. n. 2 that were 1–3 and 8–12 days old, respectively. The mortality observed among the age groups did not differ significantly. This differs to what has been observed for the effect of EPNs on the larval stage of insects, where the larval instars can significantly affect the efficacy of EPNs (Shapiro et al., 1999; Ebssa and Koppenhöfer, 2012; Acharya et al., 2020).

This is the first study in South Africa to report the potential of local EPNs to control *Gonipterus* sp. n. 2. Specifically, *S. yirgalemense* should be considered as a potential biological control agent for the pupal stage of *Gonipterus* sp. n. 2. In addition, more native EPN species should be tested for their virulence against *Gonipterus* sp. n. 2. Promoting the use of native EPN species is important as this can help to avoid costs and regulations imposed on the exotic EPN species (Abate et al., 2017).

Despite the promising results from this study on the potential of *S. yirgalemense* as a biological control agent for *Gonipterus* sp. n. 2, there are several challenges to the successful use of EPNs in the field for management of *Gonipterus* sp. n. 2. The pupal stage is cryptic within the soil (in small pupal cases made from soil particles) and this makes it difficult to determine the prevalence of pupae to inform decisions on whether management, such as application of EPNs, would be required. For the same reason, post-application monitoring of the EPNs efficacy in the field, i.e. percentage pupal mortality, will also be difficult. In addition, the methods used to apply EPNs have different impacts on the efficacy, dispersal, and survival of EPNs (Shapiro-Ilan and Glazer, 1996; Perez et al., 2003; Shapiro-Ilan et al., 2003). Therefore, research to investigate the timing of application and application methods that induce better dispersal of EPNs into the soil is necessary for successful field applications.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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