



Susceptibility of white grubs from forestry and sugarcane plantations in South Africa to entomopathogenic nematodes

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Abstract The control of white grub (Coleoptera: Scarabaeidae) pests of sugarcane and forest plantations is difficult due to their cryptic nature and resistance to chemicals. This study evaluated the potential use of entomopathogenic nematodes (EPNs) as an alternative control method. Laboratory bioassays were performed with 12 locally isolated EPN species to determine the susceptibility of third instar larvae of the white grubs *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Monochelus* sp. and *Maladera* sp. 4. Concentration trials to determine lethal dosages for three of the white grub species were performed using *Heterorhabditis zealandica* Poinar MJ2C. Bioassays were performed to determine whether nematodes could develop inside the cadavers of *S. affinis* and *P. sommeri* as these had shown the lowest

susceptibility to EPNs. The mortality percentage of the white grubs, although varying significantly, was found to be low for most of the EPN species, except *H. zealandica*. The highest percentage mortality of white grubs was observed at four weeks post inoculation with the rate of mortality being highest in the first week. *Schizonycha affinis* had the lowest LD₅₀ of 38 Infective juveniles (IJs) per larva after 28 days, compared to *Maladera* sp. 4, with 284 IJs per larva, and *P. sommeri*, with 1035 IJs per larva. The dissection of insect cadavers revealed possible limiting factors for low susceptibility, due to the nematodes and their associated symbiotic bacteria's inability to infect the insect haemocoel, with no EPNs being found inside some of the cadavers.

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Introduction

White grubs (Coleoptera: Scarabaeidae) are the root-feeding larvae of Scarabid beetles which are sporadic pests of various crops (Ritcher 1966; Jackson and Klein 2006). These larvae feed on plant roots, resulting in significant root damage. The adults tend to feed on plant leaves or to bore into underground stems (Jackson and Klein 2006). In South Africa, white grubs are the main insect pests of sugarcane and

important establishment pests in plantation forestry (Echeverri-Molina and Govender 2016a, b; Sivparsad et al. 2018). Govender (2007, 2014) reported that white grubs were responsible for 13% of the damage caused in black wattle plantations in the KwaZulu-Natal (KZN) province, while McArthur and Leslie (2004) reported an average of 23–55% reduction in sugarcane yield in the KZN Midlands North area. Forestry and sugarcane plantations are often in proximity and an overlap of white grub species affecting these two crops has been observed (Sivparsad et al. 2018).

The control of white grubs is difficult mainly because of their soil-dwelling nature, their resistance to chemical insecticides (Grewal et al. 2005) and the nocturnal lifestyle of the adults (Jackson and Klein 2006). While white grub control efforts have been dominated using chemical insecticides, the use of entomopathogenic nematodes (EPNs) offers a relatively safe and efficient control option (Grewal et al. 2005; Koppenhöfer et al. 2020). EPNs (Rhabditida: Steinernematidae and Heterorhabditidae) are known to infect various below- and above-ground insects (Grewal et al. 2005; Lacey and Georgis 2012). The two EPN families are used worldwide in the biological control of various insect pests (Shapiro-Ilan et al. 2010; Lacey and Georgis 2012). EPNs kill their insect hosts using symbiotic bacteria, which they carry in their digestive system and in specialised bacterial chambers (Nobuyoshi 2002). Death of the insect normally occurs within 48 h after infection (Kaya et al. 1993; Nobuyoshi 2002).

South Africa has a diversity of native EPNs, with a total of 17 *Steinernema* and seven *Heterorhabditis* species reported by 2017 (Malan and Ferreira 2017). A recent description of *Steinernema bertusi* Katumanyane, Tiedt, Malan & Hurley brought the total of *Steinernema* species described from South Africa to 12, constituting 12% of the described species of this genus in the world (Katumanyane et al. 2020). Despite the diversity of native EPNs in South Africa and their potential as biological control agents for white grubs, so far only one study has been undertaken to evaluate the potential use of EPNs to control white grubs in South Africa. Abate et al. (2019) evaluated the efficacy of both native and non-native EPNs to control the white grub, *Heteronychus licas* Klug in sugarcane plantations. There is need to evaluate the

use of EPNs on more white grub pests in sugarcane and plantation forests in South Africa.

In the current study, we screened locally isolated EPNs for their biocontrol potential against native white grubs from forestry and sugarcane plantations in South Africa. More specifically, we (1) screened twelve locally isolated EPN species for their biocontrol potential against four most dominant white grub species, (2) determined whether (and how) mortality rate of white grub species varied with infected EPN species and over time, (3) tested different concentrations of EPN to determine the lethal doses needed, and (4) examined the potential for different EPNs to infect, kill and reproduce in two of the most resistant white grub species.

Materials and methods

Source of white grubs

Four white grub species, namely *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Maladera* sp. 4 and a *Monochelus* sp. were used in this study. Insect identification was done by means of a LUCID key, previously developed for white grubs in sugarcane plantations in southern Africa: https://keys.lucidcentral.org/keys/v3/sugarcane_white_grubs/sugarcane_white_grubs.html. The white grubs were collected from wattle and sugarcane plantations in the KwaZulu-Natal province of South Africa. Each white grub was placed in a 30 ml plastic vial, filled with moist autoclaved peat moss (Hygrotech sustainable solutions) and provided with a fresh carrot disc to feed. The vials containing white grubs were placed in cooler boxes for transport and later rearing at the Forestry and Agricultural Biotechnology Institute (FABI) Biocontrol Centre at the University of Pretoria, South Africa. Rearing was done at 23 °C and 60–70% RH. The autoclaved peat and carrot discs were replaced weekly. Only third instar larvae were used in the experiments. The grubs were only used three weeks onwards after collection to eliminate those that could have come infected from the field.

Source of EPNs

Twelve EPN species namely *Heterorhabditis bacteriophora* Poinar strain SF351, *Heterorhabditis baujardi*

Phan, Subbotin, Nguyen & Moens strain BA, *Heterorhabditis indica* Poinar, Karunakar & David strain SGS, *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt strain SF669, *Heterorhabditis safricana* Malan, Nguyen, de Waal & Tiedt strain SF281, *Heterorhabditis zealandica* Poinar strain MJ2C (green), *H. zealandica* strain SF41 (blue), *Oscheius myriophila* Poinar strain AK30, *S. bertusi* Katumanyane, Tiedt, Malan & Hurley strain AK27, *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley strain ML15, *S. fabii* strain SCH10, *Steinernema jeffreyense* Malan, Knoetze & Tiedt strain J194, *Steinernema sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan strain DUK and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams strain 157-C were used in this study. The nematode isolates were obtained from the nematode collections at the FABI Biocontrol Centre, University of Pretoria and the Department of Conservation Ecology and Entomology at Stellenbosch University, Stellenbosch, South Africa. All nematodes used were reared using the third instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). Infective juveniles (IJs) were harvested on modified White's traps and stored in distilled water in horizontally placed culture flasks at 12 °C. The EPNs were shaken periodically for ventilation and used within three weeks after harvesting. A fresh batch of nematodes was used for each repetition of the experiment.

Screening bioassays

Soil bioassays

The methods used by Koppenhöfer and Fuzy (2003), An et al. (2012) and Wu et al. (2014) were modified and used in the present experiment. The EPNs *H. bacteriophora*, *H. zealandica*-MJ2C, *S. fabii*, *S. jeffreyense* and *S. yirgalemense* were separately tested for their potential to kill the third instar larvae of *P. sommeri*, *Monochelus* sp., *Maladera* sp. 4. Two additional species, namely *S. sacchari* and a strain of *S. fabii*-SCH10, were further tested against the larvae of *S. affinis*.

For the setup, 30 ml plastic cups were filled with 30 g autoclaved sandy loam soil. A white grub was then placed on top and allowed to dig itself into the soil, and the soil was inoculated with the EPNs. From a nematode suspension, 400 IJs were sprayed onto the

surface of the soil in each cup and the soil moisture was adjusted to 18% v/w. A carrot disc was provided for white grub feeding. The vials were then placed in a plastic container lined with moist tissue paper (100% moisture) and stored at 25 °C in a dark room. The experiment included five treatments (EPN species), and each was tested on ten white grubs. This was replicated three times. Each replicate was undertaken on a different test date using a different batch of nematodes. The cumulative percentage mortality for each treatment was recorded over a period of one month, at weekly intervals. For *S. affinis*, two additional EPN species were tested.

12-well laboratory bioassays

Based on the preliminary results of the soil bioassays, the most resistant white grubs, namely *S. affinis* and *P. sommeri*, were subjected to further tests in 12-well bioassay plates. The aim was to investigate any changes that occurred in the susceptibility of the white grub species, when they were directly exposed to the EPNs. The susceptibility of the two white grub species to nine EPNs, namely *H. bacteriophora*, *H. baujardi*, *H. indica*, *H. noenieputensis*, *H. safricana*, *H. zealandica*-MJ2C, *H. zealandica*-SF41, *O. myriophila* and *S. fabii*-SCH10, was tested. Additionally, the susceptibility of *S. affinis* to *S. bertusi* was also tested.

For the experimental setup, a single white grub was placed in a cell of a 12-well bioassay plate lined with a filter paper disc. It was inoculated with 200 IJs suspended in 50 µl distilled water. Ten grubs of each white grub species were used for each of the treatments plus the control. The controls received water only. The plates were placed in a plastic container lined with wet paper towels (at 100% moisture level), closed with the lid, and kept in a growth chamber, adjusted at 25 °C. Mortality reading was taken daily for up to seven days for *S. affinis* and for up to ten days for *P. sommeri*, as the mortality for the latter was observed to take longer than for the former. Any dead larvae were removed, rinsed with distilled water and placed in a new 12-well plate lined with moist filter paper. Dead larvae were returned to the growth chamber to allow any EPNs, if present, to develop. All dissections of the cadavers, aimed at confirming the presence of EPNs, were performed on the 10th day post-inoculation (PI). Only those cadavers that were

observed to have EPNs present were counted as part of the mortality (equal infection) data. The experiment was replicated three times, on a different test date, for each nematode species, and using a fresh batch of nematodes.

Concentration trial

Based on the preliminary results obtained with both the soil and the 12-well bioassay disk (Figs. 1, 2, 3), *H. zealandica* MJ2C was selected for the probit test to determine its lethal dosages (LD₅₀) on the third instar larvae of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. *Monochelus* sp. was not included in the experiment, as its larvae were not available in sufficient numbers. Similar methods to those that were used in the soil bioassay were employed in this experiment. However, the treatments included different concentrations of

H. zealandica MJ2C, namely 0, 100, 200, 400, 800 and 1600 IJs per vial. Ten white grubs were used in each treatment (EPN concentration × ten white grub species), with the experiment being replicated three times, and with each replicate being undertaken on a different test date, using a different batch of nematodes. Cumulative percentage mortality was recorded at weekly intervals over a period of one month.

Examining EPN survival and development inside insect cadavers

The ability of EPNs to develop in the insect cadavers of *S. affinis* and *P. sommeri* was tested using *H. safricana*, *H. noenieputensis*, *H. indica*, *H. zealandica*-MJ2C, *H. baujardi*, *S. fabii*-SCH10, *H. bacteriophora*, *H. zealandica*-SF41, *O. myriophila* and *S. bertusi*. The aim was to test the survival and development

Fig. 1 Effects of different entomopathogenic nematode treatments and time (7–28 days) on the mortality rate of the white grub, *Schizonycha affinis*, placed in vials containing soil (a) and 12-well bioassay disks mortality recorded after seven days (b). Error bars on the graph represent SE. The probability values presented are derived from Kruskal–Wallis statistic testing the difference in the treatments for each period

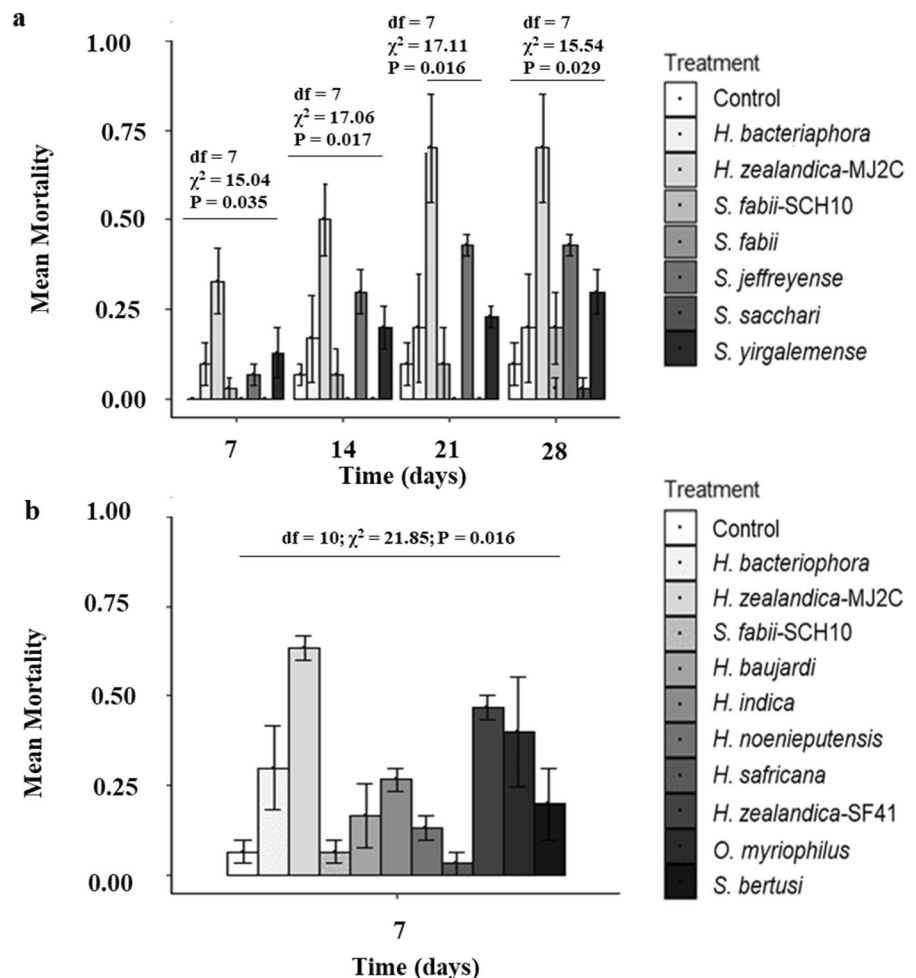
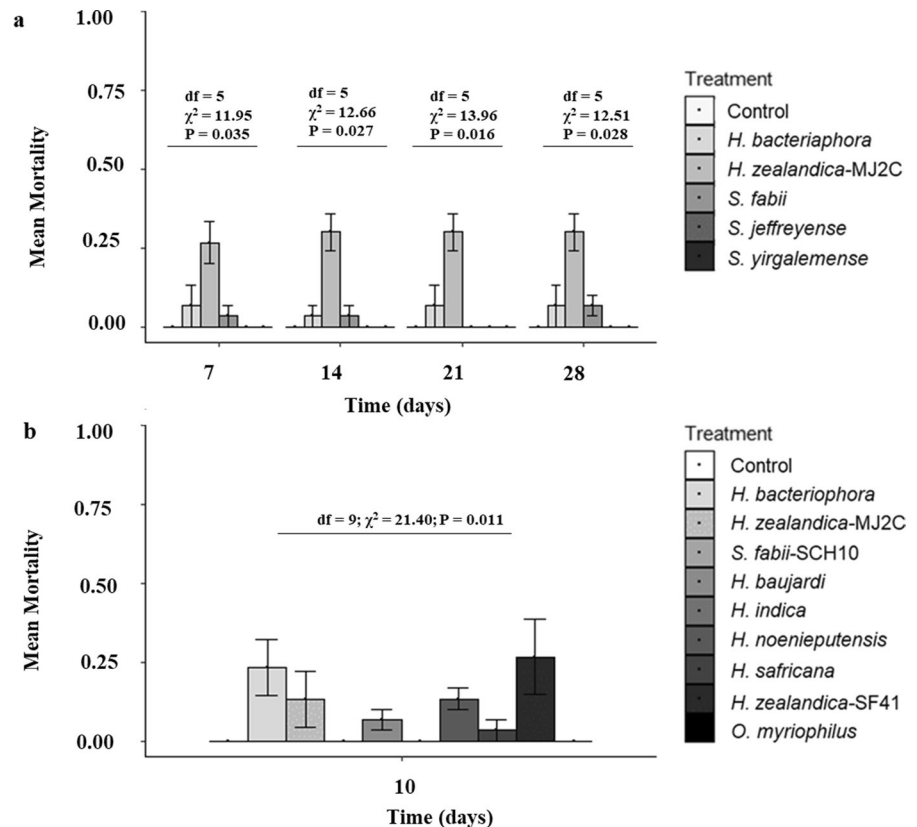


Fig. 2 Individual effects of different EPN treatments and time (7–28 days) on the mortality rate of the white grub, *Pegylis sommeri*, placed in vials containing soil (a) and 12-well bioassay disks mortality recorded after ten days (b). Error bars on the graph represent SE. The probability values presented are derived from Kruskal–Wallis statistic testing the difference in the treatments for each period



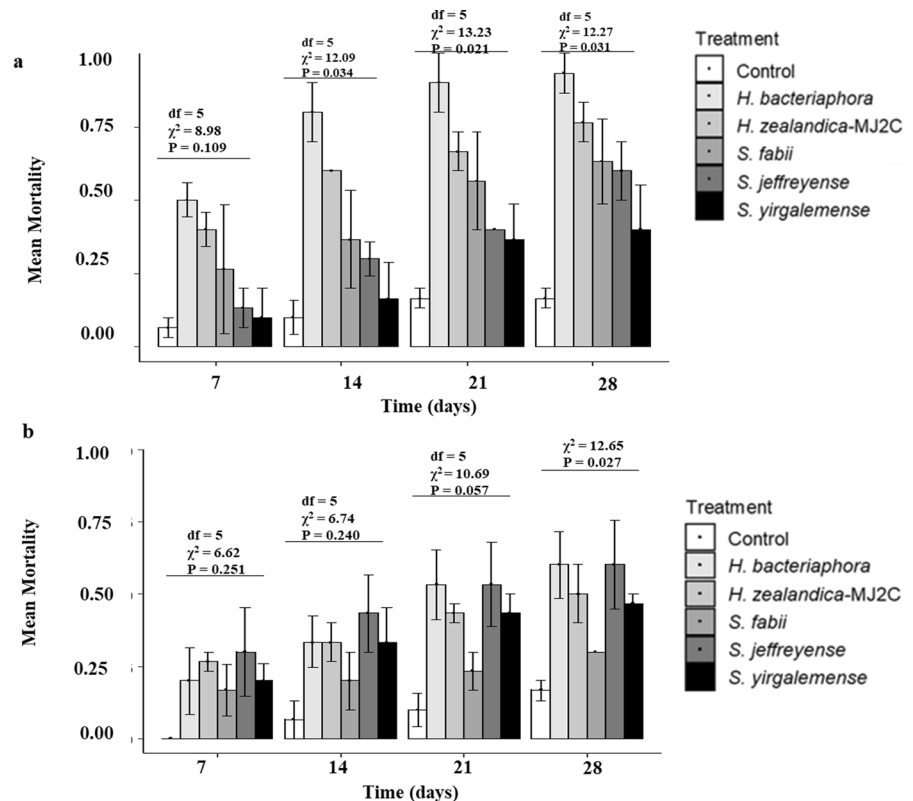
of the nematodes to gain improved understanding of the mechanisms of resistance employed by the two most resistant grub species, namely *S. affinis* and *P. sommeri*, as observed in the soil and 12-well bioassays. Similar methods to those used in the 12-well bioassays were employed in the experiment. Grubs were individually placed in 90 mm diam. Petri dishes and inoculated with 200 IJs of the nematodes. Ten grubs each of *S. affinis* and *P. sommeri* were used per EPN treatment. The inoculated insects were incubated at 25 °C, in containers lined with moist tissue paper. Insect mortality was recorded daily for a period of ten days. Irrespective of the day of insect death, all dissections of dead insects were performed on the 10th day PI to be able to observe and record the status of the EPNs developing inside the haemocoel. The presence or absence of the EPNs and the symbiotic bacteria, the development stage of the EPNs, and the general state of the cadaver were recorded. The EPN symbiotic bacteria colonisation was visually observed in the haemolymph for characteristics typical to a successful entomopathogenic symbiotic

bacteria (ESB) colonisation which included gumminess of the bacterial body mass for *Photorhabdus* bacteria, colour changes, absence of a putrid smell. The limitation with visual observation for ESB could lie in the inability to observe these characters in the presence of a suppressed ESB colonization.

Statistical analysis

All statistical analyses were performed using the R statistical software, version 4.00 (R Development Core Team 2020). To determine whether mortality rates of white grub species (percentage of white grubs that died during the experiment) varied with infected EPN species and over time, we used generalised linear models (GLM) with a binomial distribution and a logit link function (Zuur et al. 2009), which assessed the main and interaction effects of time and EPN species. In order to better depict the trends in these effects, the variation in mortality among treatments (EPN species) was analysed and represented using bar plot (with error bars representing SE) for

Fig. 3 Individual effects of treatments (EPN species) and time (7–28 days) on the mortality rate of the white grub **a** *Maladera* sp. 4 and **b** *Monochelus* sp. Error bars on the graph represent SE. The probability values presented are derived from Kruskal–Wallis statistic testing the difference in the treatments for each period



each time interval (7, 14, 21 and 28 days). Due to the limited number of observations for each time interval, the Kruskal–Wallis non-parametric tests were performed to compare treatments (EPN species) for each time interval.

To test for the effect of time and different concentrations of EPN on mortality of white grubs, and determine the lethal doses needed, the log-logistic dose–response model (Eq. 1) was fitted with the ‘drm’ function of the package drc (Ritz et al. 2015) in R:

$$M(t) = \frac{1}{1 + \exp(b \cdot \log(t) - \log(\text{LD}_{50}))} \quad (1)$$

where $M(t)$ is the expected mortality rate at time t (number of weeks); b is proportional to the slope of M , at time t ; and LD_{50} is the concentration dose by which 50% of the white grubs would have died.

Results

Screening bioassays

Schizonycha affinis

The results of the soil bioassays showed that the mortality rate of *S. affinis* varied significantly among different EPN species ($\chi^2 = 167.48$; $df = 7$; $p < 0.001$) and over time ($\chi^2 = 29.42$; $df = 3$; $p < 0.001$). However, we found no interaction effects between EPN species and time ($\chi^2 = 8.73$; $df = 21$; $p = 0.991$). *Schizonycha affinis* mortality increased over time for all treatments (Fig. 1a). Its highest mortality rate was constantly observed with *H. zealandica* MJ2C (Fig. 1a). The maximum percentage mortality of $70.0\% \pm 1.2\%$, which was obtained after 21 days, was significantly different from the next closest efficient species, *S. jeffreyense*, at $43.0\% \pm 1.2\%$, and *S. yirgalemense*, at $30.0\% \pm 1.2\%$. Overall, *H. bacteriophora*, *S. fabii* (BA), *S. sacchari* and an isolate of *S. fabii* (SCH10) showed very low mortality rates of less than 20%

after 28 days. For all the tested nematodes, the mortality graph levelled off after 21 days (Fig. 1a). In keeping with the results obtained in the soil bioassays, the highest percentage mortality for the 12-well laboratory bioassays was obtained from *H. zealandica* MJ2C at $63.0\% \pm 2.2\%$, seven days PI. *Heterorhabditis zealandica* SF41 gave the second highest mean mortality at $46.7\% \pm 3.3\%$, followed by *Oscheius myriophila* Poinar, at $40.0\% \pm 1.5\%$. The rest of the tested nematodes resulted in less than 30% mortality, seven days PI (Fig. 1b).

Pegylis sommeri

The results of the soil bioassay showed that mortality percentage varied significantly among the different EPN species investigated ($\chi^2=214.42$; $df = 5$; $p < 0.001$), but not over time ($\chi^2=0.66$; $df = 3$; $p=0.883$). Furthermore, there was no significant interaction effect between time and EPN species ($\chi^2=6.19$; $df = 15$; $p=0.976$). These results suggest that the mortality did not increase over time. At 28 days, the mortality rates of *P. sommeri* were $<5\%$ across all EPNs, except for in the case of *H. zealandica* MJ2C (Fig. 2a). *Heterorhabditis zealandica* MJ2C showed the highest percentage mortality, although it was only $30.0\% \pm 5.8\%$. *Steinernema jeffreyense* and *S. yirgalemense* caused no mortality. In the 12-well bioassay disks, *P. sommeri* still maintained a very high resistance towards the EPNs tested (Fig. 2b). The highest mortality of $26.7\% \pm 1.2\%$, was obtained using *H. zealandica* SF41.

Maladera sp. 4

Mortality rates of *Maladera* sp. varied significantly among the different EPN species ($\chi^2=90.74$; $df = 5$; $p < 0.001$), and over time for all EPN species ($\chi^2=35.23$; $df = 3$; $p < 0.001$). However, there was no interaction effects between EPN species and time ($\chi^2=2.96$; $df = 15$; $p=0.999$). At 28 days PI, *H. bacteriophora* exhibited the highest percentages of mortality, $93.0\% \pm 6.7\%$, followed by *H. zealandica* MJ2C, with a percentage mortality of $76.7\% \pm 6.7\%$ (Fig. 3a). *Steinernema fabii* and *S. jeffreyense* resulted in $63\% \pm 15\%$ and $60\% \pm 10\%$ percentage mortality, respectively, while the lowest mortality

rate ($40\% \pm 15\%$) was recorded for *S. yirgalemense* (Fig. 3a).

Monochelus sp.

The results of the soil bioassays for *Monochelus* sp. showed that its mortality also varied significantly among the different EPN species ($\chi^2=50.71$; $df = 5$; $p < 0.001$), and over time for all EPN species ($\chi^2=25.55$; $df = 3$; $p < 0.001$), but there was no significant interaction effect between time and EPN species ($\chi^2=4.17$; $df = 15$; $p=0.997$). More specifically, we found that mortality rate increased over time but varied considerably among treatments (Fig. 3b). The highest mortality after 28 days of inoculation was recorded from *H. bacteriophora* ($60.0\% \pm 11.5\%$) and *S. jeffreyense* ($60.0\% \pm 15.3\%$) treated grubs. The average mortality of $50\% \pm 10\%$, caused by *H. zealandica* MJ2C, was closely followed by the average mortality caused by *S. yirgalemense*, at $47\% \pm 3\%$. The lowest level of mortality was caused by *S. fabii*, which attained a $30\% \pm 0\%$ mean mortality on day 28 PI.

Concentration trial

Heterorhabditis zealandica MJ2C was used in the trial because it was the most efficient EPN for three of the white grub species, namely *S. affinis*, *P. sommeri* and *Monochelus* sp., in the screening bioassays. According to the results of the study, the LD_{50} was found to be generally lowest for *S. affinis*, at 38 IJs per grub, intermediate for *Maladera* sp. 4, at 284 IJs per grub, and highest for *Pegylis sommeri*, at 1035 IJs per grub, at 28 days PI (Table 1).

Examining EPN development inside the insect cadaver

Heterorhabditis zealandica MJ2C showed a high mortality potential for *S. affinis*, causing the highest number of infections (19 out of the 30 white grubs tested), and was able to complete its life cycle inside the haemocoel of the grubs (Table 2). It was also observed to be the only nematode species able to protect its cadavers from attack by unidentified mite species. The cadavers were observed to remain intact, not smelly, or semi-decomposed, typical of EPN infected cadavers. *Oscheius myriophila* showed a

Table 1 Parameter estimates of the log-logistic dose response model showing the LD₅₀ obtained for different white grubs, after inoculation with different concentrations of *Heterorhabditis zealandica* MJ2C: 0, 100, 200, 400, 800 and 1600 IJs

	<i>Schizonychia affinis</i>				<i>Pegylis sommeri</i>				<i>Maladera sp. 4</i>			
	Est	SE	P		Est	SE	P		Est	SE	P	
	<i>Slope</i>	-0.65	0.19	<0.001		-0.99	0.26	<0.001		-0.86	0.21	<0.001
7 days	-0.38	0.17	0.029		-1.21	0.26	<0.001		-1.10	0.21	<0.001	
14 days	-0.39	0.18	0.031		-1.23	0.25	<0.001		-1.04	0.20	<0.001	
21 days	-0.53	0.21	0.011		-1.30	0.25	<0.001		-1.29	0.23	<0.001	
28 days												
<i>LD₅₀</i>	762	235.39	0.001		2226	857.17	0.009		1152	329.65	<0.001	
7 days	238	117.98	0.044		1369	315.44	<0.001		497	84.58	<0.001	
14 days	74	63.47	0.241		1136	231.46	<0.001		319	56.91	<0.001	
21 days	38	34.08	0.269		1035	191.72	<0.001		284	43.23	<0.001	
28 days												

Estimate slope refers to the parameter *b* in the log-logistic function; and LD₅₀ is the dosage by which 50% of the white grub population would have died

Est estimate; P probability value testing the significance of the estimates

comparatively high mortality for *S. affinis*, killing, on average, four out of ten individuals and being able to complete its life cycle within a week (Table 2). However, *O. myriophila* was unable to protect any of its cadavers, resulting in most of them being putrid and full of mites. Whenever *H. indica* colonised and killed *S. affinis*, the EPNs were unable to develop inside the cadavers, with only bacteria being observable at the end of the experiment (Table 2). For *H. bacteriophora* and *H. zealandica* SF41, mostly entomopathogenic nematode symbiotic bacterial (ESB) colonisation was observed, with no surviving EPNs appearing to remain in the cadavers. *Steinernema bertusi* showed a low percentage mortality towards *S. affinis*, with all the infections recorded being successful but was unable to keep the cadavers clean. The rest of the EPN species tested showed very low mortality rates. For *H. safricana*, *H. noenieputensis* and *S. fabii* SCH10, no ESBs/EPNs were observed in the dead cadavers (Table 2).

Heterorhabditis bacteriophora were found to have killed 20% of the *P. sommeri* grubs (Table 3) and were observed to have kept the cadavers clean and free of mites. In the cadavers, the red colour of the symbiotic bacteria was observed, although no EPN development of *H. bacteriophora* was observed. In two of the grubs, the symbiotic bacteria of *H. bacteriophora* were observed to have colonised only the upper body of *P. sommeri*. However, the rest of the EPNs used were found mostly to be unable to infect *P. sommeri*. *Heterorhabditis zealandica* SF41 was also found to have infected about 20% of the grubs involved (Table 3), but the EPNs were unable to complete their life cycle. Instead, stunted and dead nematodes were observed.

Most of the EPN-associated bacteria were generally observed to be unable to protect the *P. sommeri* and *S. affinis* cadavers from colonisation by other bacteria, possibly due to interference from the insects' own gut bacteria. The above could be seen in terms of the colour changes occurring in the cadaver, from colours typical of the symbiotic bacteria concerned to darkened colours only hours after infection, coupled with the emission of a foul odour after a few days had elapsed.

Table 2 Dissection observations for *Schizonycha affinis*

EPN species	Days PI and cumulative number of dead larvae					Notes
	1	2	3	4	5, 6, 7, 8, 9, 10	
<i>Heterorhabditis bacteriophora</i>	0	7	8	9	9	Mostly ESB present but no successful EPNs
<i>H. baujardi</i>	0	0	1	4	5	Three successful EPN cycles. No ESB/EPNs observed for the rest of the dead grubs
<i>H. indica</i>	0	4	5	6	8	All life cycles, except for one, unsuccessful. ESB unable to develop in the haemocoel
<i>H. noenieputensis</i>	0	1	3	4	4	No ESB/EPNs observed in any of the dead grubs
<i>H. safricana</i>	0	0	0	1	1	No ESB/EPNs observed in any of the dead grubs
<i>H. zealandica</i> MJ2C	0	9	16	19	19	All colonisations successful and cadavers kept clean
<i>H. zealandica</i> SF41	0	5	7	12	14	Mostly ESB colonisations and dead EPNs, or no EPNs at all. Only two EPNs completed their life cycle. ESB was unable to keep a cadaver clean. Cadavers semi-decomposed and full of mites
<i>Oscheius myriophila</i>	5	10	11	12	12	Mostly successful colonisations but could not keep cadavers clean. The life cycle was completed within a short period of time, being less than seven days
<i>Steinernema bertusi</i>	0	5	6	6	6	All infections successful. However, ESB unable to keep a cadaver clean. Cadavers semi decomposed and full of mites
<i>S. fabii</i> -SCH10	0	0	1	2	2	No ESB/EPNs observed for any of the dead grubs
Control	0	0	0	0	0	No mortality recorded

The table shows the cumulative number of dead white grubs out of 30 white grubs inoculated per EPN species, the status of the entomopathogenic nematodes (EPNs) and of the symbiotic bacteria (ESB) inside the insect cadaver, as well as the status of the insect cadaver over a period of ten days post-infection, PI. No new mortalities recorded after five days PI. The nematode species are listed alphabetically

Discussion

The results of the current study showed varying degrees of susceptibility of the white grubs *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and *Monochelus* sp. to South African EPNs. Generally, *Maladera* sp. 4 was found to be the most susceptible, while *P. sommeri* was found to be the least susceptible. *Schizonycha affinis* and *Monochelus* sp. were found to be moderately susceptible. Other than *P. sommeri*, all the white grubs tested were found to be moderately susceptible to *H. zealandica* MJ2C. From the probit analysis, the LD₅₀ values obtained using *H. zealandica* MJ2C were found to be relatively high for *P. sommeri* (1035 IJs per larva), followed by the values obtained for *Maladera* sp. 4 (284 IJs per larva) and, lastly, the values obtained for *S. affinis* (38 IJs per larva), at 28 days PI.

The varying degrees of susceptibility of white grubs for the different EPN species or isolates in the current study aligns well with what has already been shown for white grub susceptibility to EPNs elsewhere (Grewal et al. 2005; An et al. 2012; Abate et al. 2019). Consensus exists that the efficacy of EPNs against white grubs can be difficult to predict, due to the control efficacy recorded from previous experiments being inconsistent (Grewal et al. 2005). Such inconsistency can be explained by the presence of various biotic differences, including differences in the attractiveness of hosts, EPN species, their dispersal rates and ability to penetrate the grubs involved through their cuticle/gut wall (Georgis and Gaugler 1991; Koppenhöfer et al. 2007). In addition, environmental factors, like moisture, temperature and the physical properties of soil (Kaya et al. 1993), can also be responsible for the inconsistencies. In our experiments, only *H. zealandica* MJ2C was able to provide

Table 3 Dissection observations for *Pegylis sommeri*

EPN species	Days PI and cumulative number of dead larvae										Notes	
	1	2	3	4	5	6	7	8	9	10		
<i>Heterorhabditis bacteriophora</i>	0	1	1	4	5	6	6	6	6	6	6	EPNs mostly able to complete life cycle in cadavers. However, ESB present in some, although adult EPNs dead or absent. ESB able to maintain a clean cadaver
<i>H. baujardi</i>	0	0	0	0	1	1	2	2	2	2	2	No ESP/EPNs observed in cadavers
<i>H. indica</i>	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded
<i>H. noenieputensis</i>	0	0	0	1	2	2	4	4	4	4	4	Mostly ESB present, but dead or stunted adult EPNs observed. No ESB/EPNs present in the cadavers that died after seven day
<i>H. zealandica</i> MJ2C	0	1	2	2	3	3	3	3	3	3	3	Life cycle completed in two of the cadavers. ESB was unable to keep a cadaver clean. Cadavers semi-decomposed and full of mites. ESB present in one cadaver, but no EPNs present
<i>H. zealandica</i> SF41	0	0	1	5	6	7	8	8	9	9	9	Mostly ESB present, but dead or stunted adult EPNs observed. ESB unable to protect some of the cadavers
<i>H. safricana</i>	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded
<i>Oscheius myriophila</i>	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded
<i>Steinernema bertusi</i>	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded
<i>S. fabii</i> -SCH10	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded
Control	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded

The table shows the number of white grubs that were found dead over a period of ten days post-infection, PI, the status of entomopathogenic nematodes (EPNs) and of symbiotic bacteria (ESB) inside the haemolymph, as well as the status of the insect cadaver. The nematode species are listed alphabetically

consistency in terms of mortality potential across the white grub species tested. Even then, the percentage mortality varied with repetitions and *H. zealandica* MJ2C proved unable to kill off the third instar larvae of *P. sommeri* effectively.

The obtained LD₅₀ values were found to gradually decrease over time, in alignment with the increasing rate of mortality of the white grubs over time for all the EPN species apart from *P. sommeri*, for which the mortality, in the vial experiments, remained constant after seven days PI. At 28 days PI, a LD₅₀ of 1035 IJs per larva was obtained for *P. sommeri*, of 284 IJs per larva for *Maladera* sp. 4 and of 38 IJs per larva for *S. affinis*. Although the obtained LD₅₀ is high, it is nevertheless comparable to what has previously been obtained in other studies conducted on white grubs, further emphasising the high resistance of some white grubs to EPNs. For example, Sankaranarayanan et al. (2019), in using 24-well plates to test different strains of *H. indica* on the third instars of the white grub, *Holotrichia serrata* F. (Coleoptera: Scarabaeidae), obtained mean lethal dosage in the range 2015 IJs per grub to 7359 IJs per grub. However, Pokhrel et al. (2018) obtained rather lower LD₅₀ after exposing second instar larvae of *Chiloloba acuta* (Coleoptera: Scarabaeidae) to *S. abbasi* and *S. siamkayai* in 40 g of silt loam soil. The resulting LD₅₀ values were 44.9 IJs ml⁻¹ and 50 98.1 IJs ml⁻¹, respectively, 14 days PI. These LD₅₀ values were very low compared to the values obtained during the probit experiment 14 days PI, namely 238, 497 and 1369 IJs per larva for *S. affinis*, *Maladera* sp. 4 and *P. sommeri*, respectively. However, the low LD₅₀ of Pokhrel et al. (2018) was obtained with second instar larvae, which are generally known to be less resistant than are third instars (Alvandi et al. 2017).

Observing the EPN development occurring in the haemocoel of *S. affinis* and *P. sommeri* revealed several factors that might cause the low susceptibility of the two white grubs to EPNs. Such factors might include the inability of the EPNs to penetrate the insect's cuticle and the gut cavity, as the haemocoel of some of the grubs dissected was found to contain no EPNs. Additionally, the inability of the EPN symbiotic bacteria to multiply and protect the EPNs and the cadaver concerned might have hindered the spreading of otherwise successful infection.

Stokwe and Malan (2017) observed that ESB were unable to multiply on the haemolymph of

the woolly apple aphid, *Eriosoma lanigerum*, and that the nematodes involved were unable to effect successful infection. For white grubs, possible interference with symbiotic bacteria multiplication can include antagonistic effects brought about through the action of the white grubs' gut bacteria (Skowronek et al. 2020). For example, *H. zealandica* MJ2C was observed to be able to protect its cadaver from the inroads of mites, with the cadavers not developing a foul smell. The fact that *H. zealandica* SF41 did not yield similar results suggests that *H. zealandica* MJ2C's unique characteristic may be attributed to its different symbiotic bacteria. Booyzen (2022) reported the existence of three different symbiotic bacteria associated with South African *H. zealandica*, turning early infected *Galleria mellonella* larvae 'blue', 'green' and 'red'. The *H. zealandica* MJ2C (green) and isolate SF41 (blue) used in the current study are associated with *P. thracensis* and *P. heterorhabditis* subsp. *Heterorhabditis*, respectively. Of key importance is the fact that the *H. zealandica* (MJ2C) associated with *P. thracensis* was found to be the most pathogenic EPN, which can probably be ascribed to the nature of the associated bacteria. The observation of the red colour in the *H. bacteriophora* infected cadaver and yet no EPNs in the dissected cadavers indicate that the bacteria of *H. bacteriophora* is able to multiply in the haemolymph of the white grubs.

Previously, Skowronek et al. (2020) found that bacteria from the midgut of the common white grub, *Melolontha melolontha* L. larvae, exhibited antagonistic activity against ESB. The possibility that the symbiotic bacteria of *H. zealandica* MJ2C can counteract the white grubs' antagonistic bacteria requires further investigation. Similar to the observations made in the current study are the observations made in the study conducted by Karagoz et al. (2007). These authors observed that when the white grubs investigated were killed by EPNs the associated *Sancassania* sp. (Acari: Acaridae) mites moulted to the adult stage and began feeding on the host tissues and/or microbes associated with the cadavers, as well as on the IJs. Such a finding could serve to emphasise still further *H. zealandica* MJ2C's use of its symbiotic bacteria to protect its cadaver.

The results of the current study confirm the results obtained in previous studies, showing that white grubs have varying degrees of susceptibility to EPNs.

Variations in susceptibility cut across different EPN species and strains of the same EPN species. The implication of the occurrence of such a pattern for the biological control of white grubs, through the effective utilisation of EPNs, is that EPN screening is required for each white grub species. Furthermore, different strains of the same EPN species might need to be tested for their utilisation in different geographical regions, as inconsistency in efficacy has been shown in such regard.

For South African white grub species, the current study shows that high mortality can be achieved using *H. zealandica* MJ2C. However, *H. zealandica* MJ2C showed limited capacity to control *P. sommeri*. Further investigation is therefore required to test for the resistance mechanisms of *P. sommeri*. Most of the EPNs tested were found to lack the ability to develop within the haemocoel of the two most resistant grubs investigated, namely *P. sommeri* and *S. affinis*. This finding implies that such EPNs, even when they prove capable of penetrating the white grubs involved, tend to lack the biological compatibility that they require to kill the white grubs. Such incompatibility might be present because of the immunological defences possessed by the white grubs. Future studies should focus on identifying the specific mechanisms used by the white grubs to defend themselves against the EPNs investigated, while further screening studies can be conducted to identify the comparatively efficient EPN species.

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