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Virulence and survival of native entomopathogenic nematodes for the management of white grubs in South Africa

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ABSTRACT

Entomopathogenic nematodes (EPNs) provide a potential alternative to chemical insecticides to control root-feeding pests of agricultural and forestry crops. One of the challenges in using EPNs in these environments is low post-application survival. We evaluated the survival of infective juveniles (IJs) of four EPN species (*Heterorhabditis bacteriophora*, *H. baujardi*, *Steinernema sacchari* and *S. yirgalemense*) in three different substrates for application. Survival of IJs was assessed directly by counting living IJs and indirectly by baiting with *Galleria mellonella* larvae. The virulence of different native and non-native EPNs against the third-instar larvae of *Heteronychus licas,* an important soil-insect pest of agricultural crops in South Africa, was also tested. Nematode survival was highest in a gel medium, followed by a gel-soil mixture, and lowest in soil. Survival of *Steinernema* species was higher than *Heterorhabditis* species when considering direct nematode counts, but these results were not consistent when using wax moth mortality as a proxy for survival. The South African strain of *H. bacteriophora* gave the highest and most rapid levels of mortality in *H. licas* grubs, followed by a commercially applied strain of *H. bacteriophora.* The results suggest that native EPNs could provide an option for soil-insect management in South Africa and provide insights into effective means of application.

1. Introduction

White grubs, the root-feeding larvae of scarab beetles (Coleoptera: Scarabaeidae), cause significant damage to many important forestry and agricultural crops (Anithaa et al., 2006; Oyafuso et al., 2002; Potter, 1998; Vittum et al., 1999), including in Africa (Harrison and Wingfield, 2016). In most cases the larvae are the most damaging life stage, but adult beetles can also be important pests. Species that also cause damage as adults include *Heteronychus licas,* which feeds on shoots of sugarcane (Cackett, 1992), and *Pegylis sommeri* (previously known as *Hypopholis sommeri*), a defoliator of fruit and plantation trees (Carnegie, 1988; Govender, 2007; Harrison, 2014).

In South Africa, white grubs are amongst the most important and widespread pests in both forestry plantations (Govender, 2014, 2007) and sugarcane fields (Way, 1997). Here, mortality of non-native *Eucalyptus* spp., *Pinus* spp. and *Acacia mearnsii* (wattle) seedlings due to white grub damage poses a major challenge to plantation establishment (Roux et al., 2012). For example, surveys in wattle plantations have shown that, depending on plantation residue management practices, seedling mortality was as high as 59%, mostly because of damage by white grubs (Govender, 2014, 2007). Similarly, between 23 and 55 tons cane/ha is lost in sugarcane due to white grubs, depending on the variety and season (McArthur and Leslie, 2004).

White grub management has been strongly dependent on chemical insecticides (Cackett, 1992; Govender, 2007), but entomopathogenic nematodes (EPNs) provide a possible alternative (Grewal et al., 2005). Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae, together with their symbiotic bacteria in the genera *Xenorhabdus* and *Photorhabdus,* respectively, have been used successfully in agriculture and forestry for the biological control of numerous insect pests (Kaya et al., 2006). Given the coexistence of white grubs and EPNs in the soil, it is not surprising that white grubs can be

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parasitized by a large number of EPN species (Peters, 1996; Stock and Koppenhöfer, 2003). Even though EPNs have shown a great potential for the control of various soil insect pests, their application has mostly been restricted to high value crops. However, EPNs are becoming an important and desirable means to control several pest problems in forestry (Torr et al., 2005).

Poor post-application survival remains one of the major challenges for the use of EPNs as biological control agents. This is mainly due to abiotic factors such as ultraviolet radiation, soil type, pH, temperature, relative humidity (RH) and moisture content, with the latter three being especially important (Koppenhöfer and Fuzy, 2007; Shapiro-Ilan et al., 2012; Shapiro-Ilan et al., 2006). Such unfavourable environmental conditions cause dehydration/desiccation and depletion of energy, and these factors are thus important determinants for the movement and the survival or mortality of EPNs (Smits, 1996).

Due to the sensitivity of EPNs to low soil moisture, it is necessary to manage moisture before, during and after application. Pre- and post-irrigation application can increase EPN efficacy and persistence, but restricts its application to high value crops (Wright et al., 2005; Georgis et al., 2006). Entomopathogenic nematodes can be applied using ground or aerial spraying equipment depending on the cropping system in the targeted area (Shapiro-Ilan et al., 2012; Shapiro-Ilan et al., 2006). In crops where irrigation is not present, such as plantation forestry, spot application is recommended to reduce the number of nematodes and/or the volume of water applied to a given area (Torr et al., 2005). For example, Dillon et al. (2006) used spot applications where EPNs were applied in a water suspension onto the soil around the stumps to control large pine weevil, *Hylobius abietis* in pine plantations. Specific formulations of EPNs may also influence post-application survival, as well as shelf-life and survival during transport. This includes using an active carrier, such as sponge, vermiculite, liquid, wettable powder, water-dispersible granule and gel (Grewal and Peters, 2005).

Most applications of EPNs involve non-native EPN species, because these are typically the only species that are available as commercial products (Kaya et al., 2006; Lacey et al., 2015). Some authors have argued for the use of native EPNs due to possible non-target effects of non-native EPN species (Abate et al., 2017; Millar and Barbercheck, 2001; Rojht et al., 2009). The effects of native EPNs against several insect pests have been evaluated in many countries, with promising results. For example, a recent report which included Cuba, Czech Republic, Iran, Italy, South Africa and Venezuela showed that native EPN species have been effectively used as management options against various important insect pests (Campos-Herrera, 2015). In addition, Duncan et al. (2003) reported that native EPNs significantly reduced populations of *Diaprepes abbreviatus* L., an important citrus orchard insect pest in central Florida. Similarly, Dillon et al. (2006) showed that application of the native *Heterorhabditis downesi* in Ireland was more effective against *Hylobius abietis* compared to commercial EPNs.

The aim of this study was to evaluate the survival and virulence of native EPN species recently isolated from plantation and natural forests in South Africa (Abate et al., 2016, 2018) and compare these with non-native commercially available EPN species. The survival of the EPNs was tested on three different substrates that represent possible media for their application in the field. The best performing of these media was then used to test the virulence of the EPNs against the white grub, *H. licas*.

2. Materials and methods

2.1. Survival on different media

The survival of four EPNs (two species from each *Steinernema* and *Heterorhabditis*) was tested on the three different media. Three of these were native EPN species, including *Heterorhabditis baujardi* and *Steinernema sacchari* recently isolated from plantation and natural forests in South Africa (Abate et al., 2018), and *S. yirgalemenese,* a previously isolated native species (Malan et al., 2011). We also included the non-native commercially available EPN species, *Heterorhabditis bacteriophora* Poinar, obtained from Dr. Antoinette Malan (Stellenbosch University, South Africa). All nematode species were reared at 25 °C in the last instar of the greater wax moth *Galleria mellonella* L. following the methods described by Kaya and Stock (1997). After storage at 14 °C for one week, the nematodes were acclimatized at 23 ± 2 °C for 24 h before being subjected to the various assays.

Water retaining polymer (polyacrylamide gel, STOCKOSORB®, Agro-Serve (Pty) Ltd. Bryanston, South Africa) is widely used in South African plantation forestry to enhance survival and growth of seedlings during transplanting (Viero and Little, 2006). This polymer (STOCKOSORB®) is commercially available in the form of powder where and the gel was prepared following the manufacturer's instructions by mixing 5 g of powder with 500 ml of sterilized water. The gel is commonly applied around the root zones of seedlings, where it retains moisture. It thus provides a potential medium for the application of EPNs in plantation forestry and other environments. This is specifically because it would provide a suitably moist environment to increase post-application survival of the EPNs. Another option for application would be to apply the EPNs directly to the soil in water or to apply the nematodes in a soil-gel mixture. Consequently, we compared the survival of EPNs on these media i.e gel, soil and an 80/20 gel-soil mixture. We chose the 80/20 mixture considering a possible ratio that would be applied in the field.

The different media were placed into a 86 mm × 128 mm dimension 12-multiple-well cell culture plates (Thermo Fisher Scientific, Rochester, USA), using only eight cells for each plate. Four replicates (plates) were used for each combination of media and EPN species (one 12-multiple-well cell culture plate representing a single replication). For the soil treatment, 4 g of sterilized mineral sand soil, particle size 0.05–0.5 mm (autoclaved at 121 °C) was used per cell, and 0.6 ml of sterilized water per cell was added to the sand to reach 15% moisture w/w (Caroli et al., 1996). A similar volume of water was added every week depending on moisture content of the soil. The gel-soil mixture treatment was made by mixing 5 g of STOCKOSORB® powder with 100 g of soil and 400 ml sterilized water, to obtain an 80/20 gel-soil mixture. Half the volume (*ca*. 3 ml) of each cell was filled with either gel or gel-soil mixture. Approximately 200 IJs were mixed into the media for each cell. All treatments were maintained in the dark at room temperature (24.8 ± 0.8 °C).

Two approaches were used to evaluate the survival of the IJs on the three different media. Of the eight cells per plate, two of the cells were used to directly count the IJs. The media was dissolved in sterilized water and the nematodes in the suspensions were counted under a dissecting microscope. Counting nematodes from the soil was complicated and thus a small portion (*ca*. 25 mg) of the 400 mg soil per cell was sampled at a time, by doing so all 400 mg was processed. For the remaining six cells per plate, EPN survival was tested by placing one wax moth larva on the surface of the medium in each cell and determining mortality rate of the larvae per plate. The larvae were added at different time periods (2, 15, 30 and 42 days) post-inoculation to determine mortality rate over time (n = 24 wax moth larvae per media-EPN combination at each time point). Mortality of larvae was recorded 48 and 72 h after they had been placed on the medium.

2.2. Virulence against white grubs

A total of seven EPN species/strains were used to evaluate their virulence against the third-instar larvae of *H. licas*. This included four EPN species recently isolated from plantations and natural forests in South Africa i.e. *Steinernema fabii*, *S. sacchari*, *H. bacteriophora* (SA strain) and *H. baujardi* (Abate et al., 2016, 2018). In addition, *H. bacteriophora* and *S. feltiae* (non-native commercial product) and *S. yirgalemense* (native to South Africa) were obtained from Dr. Antoinette Malan (Stellenbosch University, South Africa). All nematodes were

cultured *in vivo* at 23 ± 2 °C, in last instar *G. mellonella* following the procedures described in Kaya and Stock (1997). The nematodes were stored in culture flasks at 14 °C for less than two weeks prior to their being utilized. Third-instar larvae of the scarab *H. licas* were collected from sugarcane plantations in KwaZulu-Natal (S 29°12′35″; E 31°28′06″), South Africa. Individual grubs were placed in a vial containing sterilized peat and transported to the laboratory. The grubs were kept at 23 ± 2 °C in moist peat and fed with carrot pieces.

Heteronychus licas was used in this study because this white grub was available in large numbers from sugarcane plantations, and because it is an important pest in South Africa. The species of the collected white grubs was confirmed by the most commonly used morphological characterization method, mainly based on the raster pattern that was located ventrally on the last abdominal segment.

The experiments were conducted at 24.8 ± 0.8 °C in 12-multiple-well cell culture plates, using the two media from the survival experiments that resulted in the highest survival, namely the gel and the 80/20 gel-soil mixture. The same amount of medium was applied per cell as for the experiment testing the survival of the EPNs. Two separate experiments were conducted with different batches of EPNs, but both using the same collection of white grubs and data from the two experiments were combined for analysis. In the first experiment all the above-mentioned EPNs and a control were evaluated using eight grubs per plate and replicated 10 times, thus 80 grubs per medium/EPN species combination. In the second experiment only four EPNs, namely *S. fabii*, *H. bacteriophora* (SA strain), *H. bacteriophora* (commercial), *H. baujardi* and a control were evaluated using seven grubs per plate and replicated 10 times, thus 70 grubs per medium/EPN species combination. The cells were filled with the respective media and 200 IJs were inoculated in each cell. One white grub larva was placed in each cell, on the top of the medium. The control cells received only water. Mortality of grubs was recorded every two days for a period of 12 days.

2.3. Data analyses

The estimates and 95% confidence intervals (CIs) for survival percentage of nematodes were presented by media (gel, soil and gel- soil mixture), day and the associated differences between nematode species. Survival percentage of IJs over time was analyzed based on the 800 IJs (i.e. four replication with 200 each) initially applied and assuming 100% survival at time of application. Similarly, the estimates and 95% CIs for white grub mortality percentages were presented by media (gel and gel-soil mixture), nematode species and time (day), and the associated differences between nematode species. The median times (the time of 50% white grub mortality) and associated ratios versus nematode species were analyzed through parametric survival modelling. The parametric survival model assumed a log-logistic distribution was assumed for the time to death of grubs.

3. Results

3.1. Survival on different media

3.1.1. Direct nematode count

Survival of IJs for all EPN species was highest in the gel, followed by gel-soil mixture, and with the lowest survival in the soil (Fig. 1). These differences were significant for the survival percentages at the end of the experiment i. e day 42 (Table 1), as well as for the majority of time points evaluated.

There were significant differences in survival of EPN species on gel at the different time points (Fig. 1A; Table 2). After two days, the survival percentage was 78.6% for *S. yirgalemense*, 75.5% for *S. sacchari*, 67.9% for *H. baujardi* and 60.5% for *H. bacteriophora*. At this time point, estimated difference between species was significantly different except between the two *Steinernema* species (estimated difference between *S. yirgalemense* and *S. sacchari* = −3.1, (95% CI: [-1.0; 7.2])).

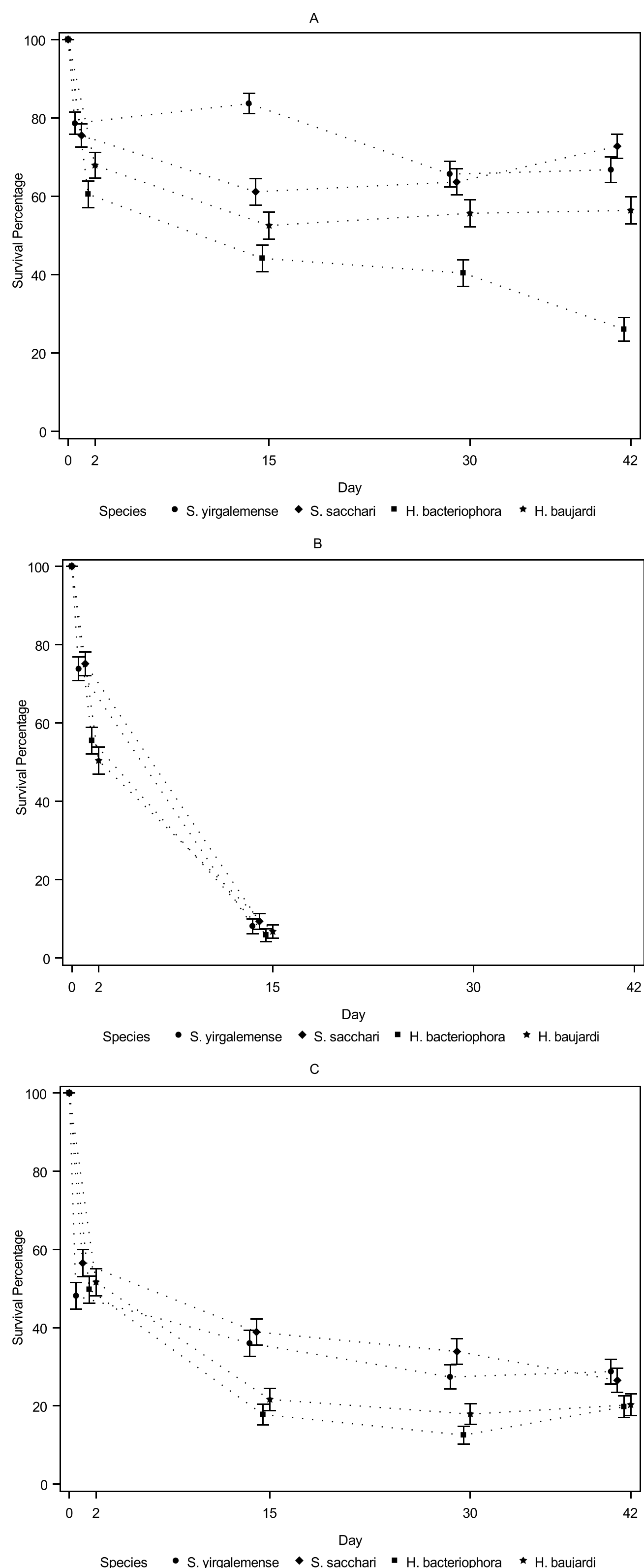

Fig. 1. Percentage survival of different entomopathogenic nematode species over time, on (A) gel, (B) soil and (C) gel-soil mixture. Bars represent 95% confidence intervals.

After 15 days, *Steinernema* species had a higher survival percentage (61.1–83.6%) than *Heterorhadditis* species (44.1–52.5%). A similar trend was observed after 30 days and 42 days (Fig. 1A). The decline of IJs after 15 days was gradual and more than 50% of IJs survived after

Table 1
Estimates and 95% confidence intervals (CIs) for survival percentage of nematodes by species at day 42: Comparison between treatments (*indicates statistically significant difference, when the difference in percentage of 95% CIs do not include zero).

			Percentage		Difference in Percentage	
Species	Day	Treatment	Estimate	95% CI	Estimate	95% CI
S. yirgalemense	42	Gel	66.8	[63.5; 70.0]		
		Gel-Soil	28.8	[25.6; 31.9]		
		Gel Vs Gel-Soil			38.0	[33.5; 42.5]*
S. sacchari	42	Gel	72.8	[69.7; 75.8]		
		Gel-Soil	26.5	[23.4; 29.6]		
		Gel Vs Gel-Soil			46.3	[41.9; 50.6]*
H. bacteriophora	42	Gel	26.0	[23.0; 29.0]		
		Gel-Soil	19.8	[17.0; 22.5]		
		Gel Vs Gel-Soil			6.3	[2.1; 10.4]*
H. baujardi	42	Gel	56.4	[49.3; 63.5]		
		Gel-Soil	20.2	[15.5; 25.8]		
		Gel Vs Gel-Soil			36.3	[27.2; 44.7]*

42 days, with the exception of *H. bacteriophora* with only 26% survival.

When IJs were applied onto the soil, their survival was generally very low (Fig. 1B). After two days the survival of *S. yirgalemense* and *S. sacchari* was 73.9 and 75.1%, respectively, and thus similar to the results for the gel. Survival of *H. baujardi* and *H. bacteriophora* at this time point was 50.4 and 55.5%, respectively, which was substantially lower than that for the gel. However, after 15 days, survival was less than 10% for all the EPN species and there was no survival of the tested species after 30 and 42 days (Fig. 1B). Survival of IJs on the gel-soil mixture showed a rapid decline for all EPN species within the first two days, resulting in 66, 64, 56 and 40% survival for *S. yirgalemense*, *S. sacchari*, *H. baujardi* and *H. bacteriophora,* respectively (Fig. 1C). However, the rate of decline after 15 days was gradual for all species. Similar to the gel, the survival of *Steinernema* species on the gel-soil mixture was higher than for the *Heterorhabditis* species, although it was substantially lower than in the gel (Fig. 1C; Table 2).

3.1.2. Mortality of greater wax moth larvae

Survival of EPN species on the different media was evaluated based on the number of EPN-infected greater wax moth larvae (Fig. 2). The results from this experiment were not consistent with those of the previously discussed survival tests. In the gel, *H. bacteriophora, H. baujardi* and *S. yirgalemense* resulted in high levels of mortality (71–100%) of greater wax moth larvae throughout the study period, with *S. sacchari* showing variable and generally low virulence (Fig. 2A). A similar trend was observed on the gel-soil mixture, where high mortality (88–100%) of wax moth larvae was recorded by *H. bacteriophora, H. baujardi* and *S. yirgalemense,* even when the larvae were placed on the cells 42 days after the EPN application (Fig. 2C), but with lower morality caused by *S. sacchari.* For the soil treatment, high mortality (92–100%) of greater wax moth larvae from all four EPN species was recorded when larvae were placed on the media in cells two days after the EPN application (Fig. 2B). The percentage mortality of the larvae declined when the larvae were placed on the cells later, namely 15 days (52–75% mortality) and 30 days (8–54% mortality). No mortality of wax moth larvae was recorded when larvae were placed on the cells after 42 days for the soil treatment.

3.2. Virulence against white grubs

Mortality of the white grub, *H. licas*, on the gel medium was significantly different between the different EPN species (Fig. 3; Table 3).

Table 2
Estimates and 95% confidence intervals (CIs) for survival percentage of nematodes by treatment at day 42: Comparison between species (*indicates statistically significant difference, when the difference in percentage of 95% CIs do not include zero).

			Percentage		Difference in Percentage	
Treatment	Day	Species	Estimate	95% CI	Estimate	95% CI
Gel	42	*S. yirgalemense*	66.8	[63.5; 70.0]		
		S. sacchari	72.8	[69.7; 75.8]		
		H. bacteriophora	26.0	[23.0; 29.0]		
		H. baujardi	56.4	[52.9; 59.8]		
		S. yirgalemense Versus *S. sacchari*			−6.0	[-10.5; −1.5]*
		S. yirgalemense Versus *H. bacteriophora*			40.8	[36.3; 45.2]*
		S. yirgalemense Versus *H. baujardi*			10.4	[5.6; 15.1]*
		S. sacchari Versus *H. bacteriophora*			46.8	[42.4; 51.1]*
		S. sacchari Versus *H. baujardi*			16.4	[11.8; 21.0]*
		H. bacteriophora Versus *H. baujardi*			−30.4	[-35.0; −25.8]*
Gel-Soil	42	*S. yirgalemense*	28.8	[25.6; 31.9]		
		S. sacchari	26.5	[23.4; 29.6]		
		H. bacteriophora	19.8	[17.0; 22.5]		
		H. baujardi	20.3	[17.5; 23.0]		
		S. yirgalemense Versus *S. sacchari*			2.2	[-2.1; 6.6]
		S. yirgalemense Versus *H. bacteriophora*			9.0	[4.8; 13.2]*
		S. yirgalemense Versus *H. baujardi*			8.5	[4.3; 12.7]*
		S. sacchari Versus *H. bacteriophora*			6.8	[2.6; 10.9]*
		S. sacchari Versus *H. baujardi*			6.3	[2.1; 10.4]*
		H. bacteriophora Versus *H. baujardi*			−0.5	[-4.4; 3.4]

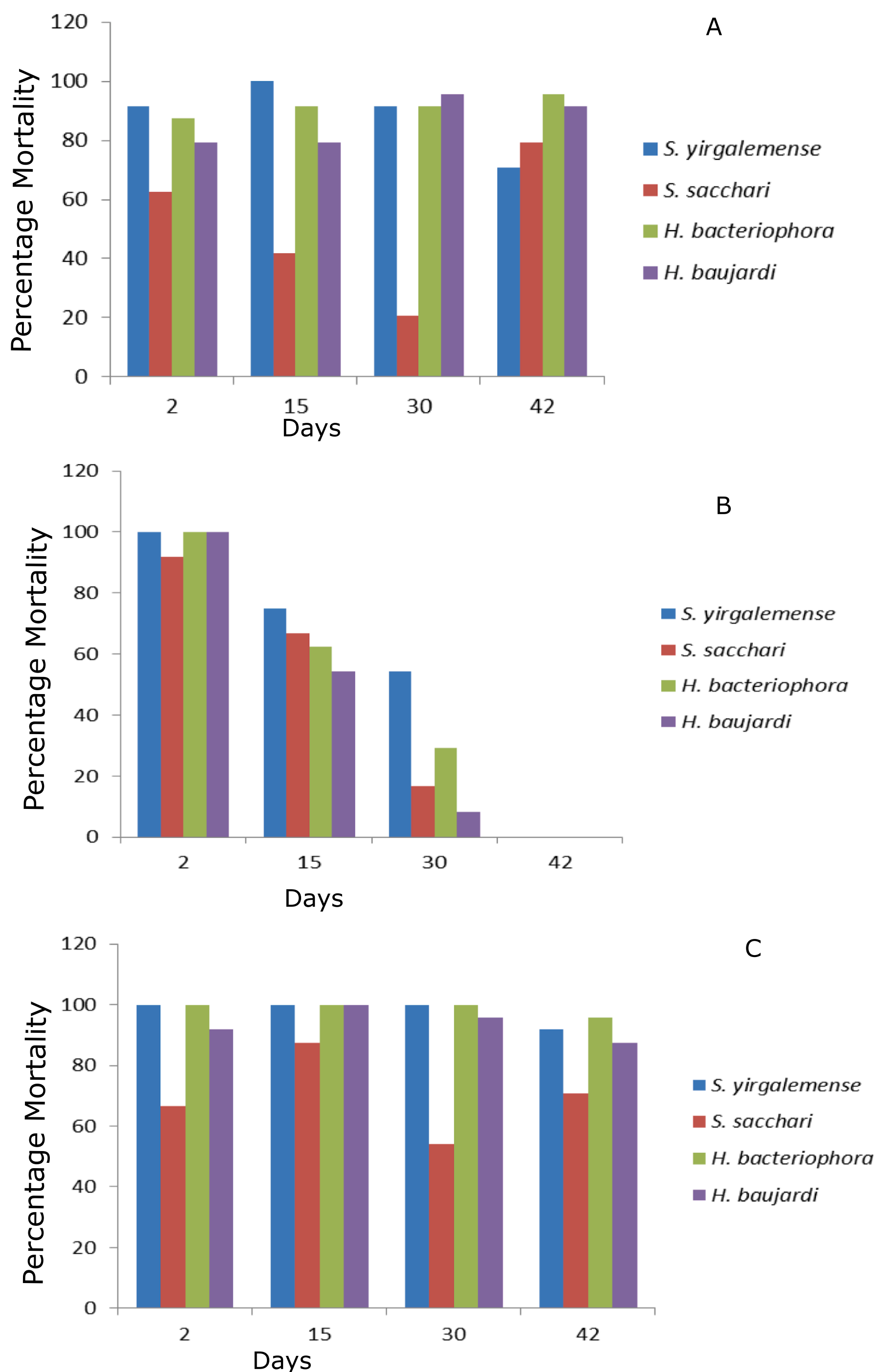

Fig. 2. Percentage mortality of Galleria melonella larvae infected by different entomopathogenic nematode species using (A) gel, (B) soil, (C) soil-gel mixture.

After two days, mortality was generally low, varying between 1.3 and 11.3 %. However, after day four, mortality increased substantially for some EPN species, including *H. baujardi* (28%), *H. bacteriophora* commercial strain (24.7%), *H. bacteriophora* SA strain (23.3%) and *S. fabii* (22.7%). There were no significant differences in white grub mortality exposed to these four species after day four, but all four species resulted in significantly higher mortality than *S. feltiae* (8.8%), *S. sacchari* (1,3%), *S. yirgalemense* (10%) and the control (4.7%). Mortality due to the South African *H. bacteriophora* strain increased substantially over time (up to 92% mortality after 12 days) and gave significantly higher mortality of white grubs compared to all the other EPN species from day eight. The commercially available *H. bacteriophora* gave the next highest level of white grub mortality, with 68% after 12 days. *Heterorhabditis baujardi* and *S. fabii* showed moderate virulence with 52% and 46% mortality of white grubs after 12 days, respectively. *Steinernema feltiae*, *S. sacchari* and *S. yirgalemense* were the least effective species, with only 20% mortality by both *S. feltiae* and *S. yirgalemense* and only 2.5% by *S. sacchari* after 12 days (Table 3).

Patterns of white grub mortality between EPN species on the gel-soil mixture were similar to the results from the gel medium (Fig. 4). Percentage mortality of white grubs was very low for all EPN species (3.3–11.3%) after two days. From day four to ten, the South African *H. bacteriophora* strain resulted in significantly higher mortality than the other EPNs, reaching up to 93.3% by day 12. Following *H. bacteriophora*

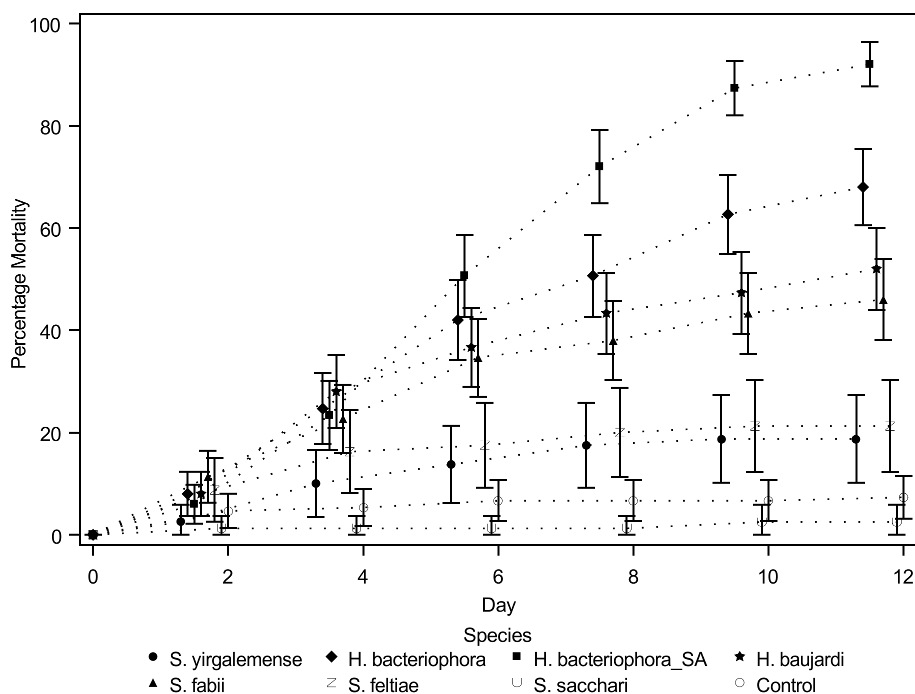

Fig. 3. Percentage mortality over time of third-instar larvae of Heteronychus licas infected by seven EPN species/strains, on gel. Bars represent 95% confidence intervals.

strain SA, the commercial *H. bacteriophora* caused the greatest white grub mortality, with 87.3% by day 12. *Heterorhabditis baujardi* and *S. fabii* showed moderate virulence, both resulting in 44.7% mortality after 12 days. *Steinernema yirgalemense*, *S. feltiae* and *S. sacchari* were the least effective species against the tested white grub, with only 25%, 18.8% and 2.5% mortality after 12 days, respectively. After 12 days in gel and gel-soil mixture, the mortality from *S. sacchari* was lower than that obtained in the control.

Percentage mortality of white grubs both in gel and gel-soil mixtures increased significantly over time by some species of EPNs (Table 4). The median times (estimated days for 50% grub mortality) were significantly different between EPN species in both the gel and gel-soil mixture (Table 5). The South African *H. bacteriophora* strain had the shortest estimated median time (6.9 days for gel and 4.7 days for gel-soil mixture), followed by *H. bacteriophora* commercial strain, *H. baujardi* and *S. fabii* (Table 5). The estimated time to achieve 50% mortality of the white grubs was much longer for *S. feltiae* and *S. yirgalemense* and the longest for *S. sacchari* (70.8 days on gel and 71.2 days on gel-soil).

4. Discussion

Results of this study showed that EPN survival was very high in a gel medium, illustrating the potential to use this medium for EPN spot applications. There were significant differences in survival between EPN species, but these results were not consistent between the experiments where nematode numbers were counted over time as a direct measure of survival, and the experiment where mortality of wax moth larvae was used as a proxy of nematode survival. Mortality of white grubs was the greatest with *Heterorhabditis* species, with the native South African strain of *H. bacteriophora* resulting in the highest percentage and most rapid mortality of white grubs.

There were indications that survival of IJs on gel was generally higher than in soil and the gel-soil mixture. This suggests that long-term survival of IJs was not severely affected by the low relative humidity (41 ± 0.9% RH) coupled with a temperature of 24.8 ± 0.8 °C in the gel treatment. This is likely because the high water holding capacity of gel can enhance the long term survival of IJs by providing the moisture to conteract dessication, which constitutes an important element in maintaining nematode viability and infectivity (Nelsen & Mannion, 1986; Shapiro-Ilan et al., 2010). Although the results of this study indicate that gel could be a suitable medium in which to apply EPNs, field evaluation under natural conditions will be required. In addition, EPNs exhibit various host-finding behaviors including “ambusher”, “cruisers” and “sprinters” (Bal et al., 2014; Griffin, 2015; Lewis et al., 2006) and it is unclear whether mixing EPNs with gel will impact their host- finding strategies or abilities.

Survival differed significantly between the nematode species in the six week study period. *Steinernema* species generally showed higher survival than *Heterorhabditis* species when counting nematode survival over time, both in gel and gel-soil mixture. Molyneux (1985) reported similar results, where *Steinernama* species displayed better survival than *Heterorhabditis* species on sand. However, these results were not supported when using mortality of wax moth larvae as a proxy for nematode survival. Mortality of wax moth larvae on the gel and gel-soil mixture was greater than 90% even after 42 days, with the exception of *S. sacchari* where mortality was low on the gel and gel-soil mixture. The high mortality level of wax moth larvae caused by *Heterorhabditis* species despite their low survival could have been due to the fact that wax moth larvae are highly susceptible to EPNs, where a single EPN is known to be able to kill a larva (Converse and Miller, 1999; Ricci et al., 1996).

Survival of IJs showed a rapid decline after two days on all three media tested. This is in agreement with previous studies by Smits (1996) and Griffin (2015), where post-application of EPNs was characterized by a rapid decline in nematode numbers after the first few days, followed by a moderate decline over the next two to six weeks. Extreme environmental conditions such as high temperature and low relative humidity are among the critical factors influencing nematode survival (Glazer, 1996). These environmental factors have a significant impact on long-term survival of IJs influencing the amount of lipids, a major stored energy reserve, in their bodies (Glazer, 2002), and the rate at which this energy reserve is depleted (Grewal, 2000). At lower temperatures and high humidities, the physiological activity of the IJs

Table 3
Estimates and 95% confidence intervals (CIs) for mortality percentage of *H. licas* larvae by treatment at day 12: Comparison between nematode species (*indicates statistically significant difference, when the difference in percentage of 95% CIs do not include zero).

Treatment	Day	Species	Percentage		Difference in Percentage	
			Estimate	95% CI	Estimate	95% CI
Gel	12	*S. yirgalemense*	18.8	[10.2; 27.3]		
		H. bacteriophora	68.0	[60.5; 75.5]		
		*H. bacteriophora*_SA	92.0	[87.7; 96.3]		
		H. baujardi	52.0	[44.0; 60.0]		
		S. fabii	46.0	[38.0; 54.0]		
		S. feltiae	21.3	[12.3; 30.2]		
		S. sacchari	2.5	[0.0; 5.9]		
		Control	7.3	[3.2; 11.5]		
		S. yirgalemense Versus *H. bacteriophora*			−49.3	[−60.6; −37.9]*
		S. yirgalemense Versus *H. bacteriophora_SA*			−73.3	[-82.8; −63.7]*
		S. yirgalemense Versus *H. baujardi*			–33.3	[−45.0; −21.5]*
		S. yirgalemense Versus *S. fabii*			−27.3	[−38.9; −15.6]*
		S. yirgalemense Versus *S. feltiae*			−2.5	[−14.9; 9.9]
		S. yirgalemense Versus *S. sacchari*			16.3	[7.0; 25.5]*
		S. yirgalemense Versus Control			11.4	[1.9; 20.9]*
		H. bacteriophora Versus *H. bacteriophora*_SA			−24.0	[–32.6; −15.4]*
		H. bacteriophora Versus *H. baujardi*			16.0	[5.1; 26.9]*
		H. bacteriophora Versus *S. fabii*			22.0	[11.1; 32.9]*
		H. bacteriophora Versus *S. feltiae*			46.8	[35.1; 58.4]*
		H. bacteriophora Versus *S. sacchari*			65.5	[57.3; 73.7]*
		H. bacteriophora Versus Control			60.7	[52.1; 69.2]*
		*H. bacteriophora*_SA Versus *H. baujardi*			40.0	[30.9; 49.1]*
		*H. bacteriophora*_SA Versus *S. fabii*			46.0	[36.9; 55.1]*
		*H. bacteriophora*_SA Versus *S. feltiae*			70.8	[60.8; 80.7]*
		*H. bacteriophora*_SA Versus *S. sacchari*			89.5	[84.0; 95.0]*
		*H. bacteriophora*_SA Versus Control			84.7	[78.6; 90.7]*
		H. baujardi Versus *S. fabii*			6.0	[−5.3; 17.3]
		H. baujardi Versus *S. feltiae*			30.8	[18.7; 42.8]*
		H. baujardi Versus *S. sacchari*			49.5	[40.8; 58.2]*
		H. baujardi Versus Control			44.7	[35.6; 53.7]*
		S. fabii Versus *S. feltiae*			24.8	[12.8; 36.7]*
		S. fabii Versus *S. sacchari*			43.5	[34.8; 52.2]*
		S. fabii Versus Control			38.7	[29.7; 47.7]*
		S. feltiae Versus *S. sacchari*			18.8	[9.2; 28.3]*
		S. feltiae Versus Control			13.9	[4.0; 23.8]*
		S. sacchari Versus Control			−4.8	[−10.2; 0.6]
Gel-Soil	12	*S. yirgalemense*	25.0	[15.5; 34.5]		
		H. bacteriophora	87.3	[82.0; 92.7]		
		*H. bacteriophora*_SA	93.3	[89.3; 97.3]		
		H. baujardi	44.7	[36.7; 52.6]		
		S. fabii	44.7	[36.7; 52.6]		
		S. feltiae	18.8	[10.2; 27.3]		
		S. sacchari	2.5	[0.0; 5.9]		
		Control	7.3	[3.2; 11.5]		
		S. yirgalemense Versus *H. bacteriophora*			−62.3	[−73.2; −51.5]*
		S. yirgalemense Versus *H. bacteriophora_SA*			−68.3	[−78.6; −58.0]*
		S. yirgalemense Versus *H. baujardi*			−19.7	[–32.0; −7.3]*
		S. yirgalemense Versus *S. fabii*			−19.7	[–32.0; −7.3]*
		S. yirgalemense Versus *S. feltiae*			6.3	[−6.5; 19.0]
		S. yirgalemense Versus *S. sacchari*			22.5	[12.4; 32.6]*
		S. yirgalemense Versus Control			17.7	[7.3; 28.0]*
		H. bacteriophora Versus *H. bacteriophora*_SA			−6.0	[-12.7; 0.7]
		H. bacteriophora Versus *H. baujardi*			42.7	[33.1; 52.2]*
		H. bacteriophora Versus *S. fabii*			42.7	[33.1; 52.2]*
		H. bacteriophora Versus *S. feltiae*			68.6	[58.5; 78.7]*
		H. bacteriophora Versus *S. sacchari*			84.8	[78.5; 91.2]*
		H. bacteriophora Versus Control			80.0	[73.2; 86.8]*
		*H. bacteriophora*_SA Versus *H. baujardi*			48.7	[39.8; 57.6]*
		*H. bacteriophora*_SA Versus *S. fabii*			48.7	[39.8; 57.6]*
		*H. bacteriophora*_SA Versus *S. feltiae*			74.6	[65.1; 84.0]*
		*H. bacteriophora*_SA Versus *S. sacchari*			90.8	[85.6; 96.1]*
		*H. bacteriophora*_SA Versus Control			86.0	[80.2; 91.8]*
		H. baujardi Versus *S. fabii*			0.0	[−11.3; 11.3]
		H. baujardi Versus *S. feltiae*			25.9	[14.2; 37.6]*
		H. baujardi Versus *S. sacchari*			42.2	[33.5; 50.8]*
		H. baujardi Versus Control			37.3	[28.4; 46.3]*
		S. fabii Versus *S. feltiae*			25.9	[14.2; 37.6]*
		S. fabii Versus *S. sacchari*			42.2	[33.5; 50.8]*
		S. fabii Versus Control			37.3	[28.4; 46.3]*
		S. feltiae Versus *S. sacchari*			16.3	[7.0; 25.5]*
		S. feltiae Versus Control			11.4	[1.9; 20.9]*
		S. sacchari Versus Control			−4.8	[−10.2; 0.6]

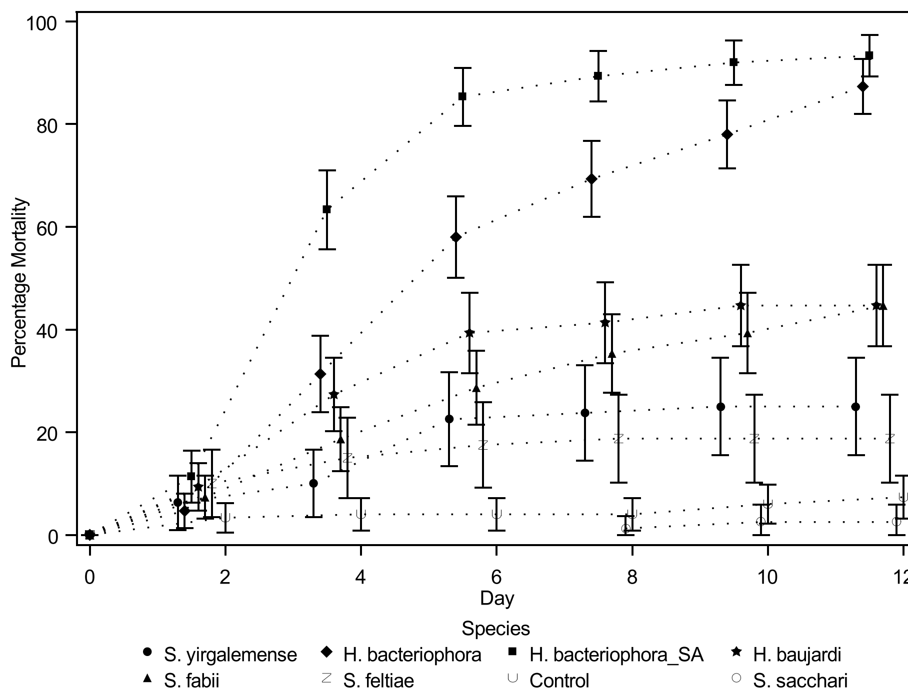

Fig. 4. Percentage mortality over time of third-instar larvae of *Heteronychus licas* infected by seven EPN species/strains, on gel-soil mixture. Bars represent 95% confidence intervals.

is decreased, resulting in lower motility and decreased food reserves utilization, thus ensuring long-term survival.

Nematode survival remained relatively consistent in our study after two days, despite the relatively high temperature and low relative humidity. This suggests that the gel provides the nematodes with a buffer from the ambient conditions. Although different EPN species and populations exhibit various temperature tolerances related to their thermal adaptation (Grewal et al., 1994; Hill et al., 2015), higher temperature and lower relative humidity is generally lethal. For example, in a study by Glazer (1992), application of *S. carpocapsae* at 45% RH resulted in high mortality within two hours. As such, an effective method that enhances EPN long term survival is required.

Application of EPNs in a gel medium during transplanting could provide an effective option for the management of establishment insect pests, such as white grubs, in plantation forestry. The most common method of EPN application is in aqueous suspensions in various irrigation systems and spraying equipment, with high numbers of nematodes (2.5×10^9 IJs/hectare) (Shapiro-Ilan et al., 2012). However, very few of these nematodes reach into the soil and a large portion (40–90%) die within hours or days of application (Griffin, 2015; Smits, 1996). This is mainly due to various abiotic factors, including UV radiation, desiccation and temperature extremes. Therefore, mixing EPNs with gel and applying the mixture during seedling transplantation would not only enhance survival of IJs, but also significantly reduce costs by reducing the number of applied nematodes, non-target areas and volume of water used (Brixey et al., 2006). However, further study is required

Table 4
Mortality of *H. licas larvae* over time at different media and entomopathogenic nematode species combinations. 'N' indicates the original number of white grubs at day 0; 'n' and '%' indicate the cumulative number and percentage, respectively, of white grubs that died at that time period.

Substrate	EPN species (No. of grubs)	Mortality of white grubs over time											
		Day 2		Day 4		Day 6		Day 8		Day 10		Day 12	
		n	%	n	%	n	%	n	%	n	%	n	%
GEL	*H. bacteriophora*_Com (N = 150)	12	8.0	37	24.7	63	42.0	76	50.7	94	62.7	102	68.0
	*H. bacteriophora*_SA (N = 150)	9	6.0	35	23.3	76	50.7	108	72.0	131	87.3	138	92.0
	H. baujardi (N = 150)	12	8.0	42	28.0	55	36.7	65	43.3	71	47.3	78	52.0
	S. fabii (N = 150)	17	11.3	34	22.7	52	34.7	57	38.0	65	43.3	69	46.0
	S. feltiae (N = 80)	7	8.8	13	16.3	14	17.5	16	20.0	17	21.3	17	21.3
	S. sacchari (N = 80)	1	1.3	1	1.3	1	1.3	1	1.3	2	2.5	2	2.5
	S. yirgalemense (N = 80)	2	2.5	8	10.0	11	13.8	14	17.5	15	18.8	15	18.8
	Control (N = 150)	7	4.7	8	5.3	10	6.7	10	6.7	10	6.7	11	7.3
GEL-SOIL	*H. bacteriophora*_Com (N = 150)	7	4.7	47	31.3	87	58.0	104	69.3	117	78.0	131	87.3
	*H. bacteriophora*_SA (N = 150)	17	11.3	95	63.3	128	85.3	134	89.3	138	92.0	140	93.3
	H. baujardi (N = 150)	14	9.3	41	27.3	59	39.3	62	41.3	67	44.7	67	44.7
	S. fabii (N = 150)	11	7.3	28	18.7	43	28.7	53	35.3	59	39.3	67	44.7
	S. feltiae (N = 80)	8	10.0	12	15.0	14	17.5	15	18.8	15	18.8	15	18.8
	S. sacchari (N = 80)	0	0.0	0	0.0	0	0.0	1	1.3	2	2.5	2	2.5
	S. yirgalemense (N = 80)	5	6.3	8	10.0	18	22.5	19	23.8	20	25.0	20	25.0
	Control (N = 150)	5	3.3	6	4.0	6	4.0	6	4.0	9	6.0	11	7.3

Table 5
Estimates and 95% confidence intervals (CI) for median time to mortality of *H. licas* larvae at different combinations of media and entomopathogenic nematode species. Statistically significant difference are indicated by '*', with 'x' indicating no significant difference. *Hb*_Com: *H. bacteriophora*_commercial strain; *Hb*_SA: *H. bacteriophora*_South African strain; *Sy*: *S. yirgalemense*.

Treatment	Species	Median Time (Days)	Level of significant difference between species based on ratio of median time								
		Estimate	95% CI	*Hb*_Com	*Hb*_SA	*H. baujardi*	*S. fabii*	*S. feltiae*	*S. sacchari*	*Sy*	Control
GEL	*Hb*_Com	8.6	[7.5; 9.8]	–							
	*Hb*_SA	6.9	[6.1; 7.7]	*	–						
	H. baujardi	10.3	[8.8; 11.9]	x	*	–					
	S. fabii	11.4	[9.8; 13.3]	*	*	x	–				
	S. feltiae	20.7	[15.9; 27]	*	*	*	*	–			
	S. sacchari	70.8	[35.6; 141.1]	*	*	*	*	*	–		
	Sy	23.1	[17.5; 30.5]	*	*	*	*	x	*	–	
	Control	40.1 l	[29.5; 54.4]	*	*	*	*	*	*	x	–
GEL-SOIL	*Hb*_Com	6.7	[5.9; 7.6]	–							
	*Hb*_SA	4.7	[4.1; 5.3]	*	–						
	H. baujardi	10.9	[9.3; 12.8]	*	*	–					
	S. fabii	12.4	[10.7; 14.5]	*	*	*	–				
	S. feltiae	22.3	[16.9; 29.5]	*	*	x	*	–			
	S. sacchari	71.2	[35.7; 141.8]	*	*	*	*	*	–		
	Sy	18.7	[14.6; 24]	*	*	*	*	x	*	–	
	Control	40.8	[30.0; 55.4]	*	*	*	*	*	x	*	–

to assess the effect of gel on EPN movement and host finding ability.

This study has shown that EPNs are capable of efficiently killing a high percentage of 3rd instar *H. licas*. Despite being an important pest in many agricultural crops in Africa, no effective biological control agent has been developed for the management of *H. licas*, and to the best of our knowledge, this is the first study to consider the use of EPNs against this species. Among the nematode species applied against white grubs, the South African strain of *H. bacteriophora* and the commercially produced *H. bacteriophora* provided the best control both on the gel and gel-soil mixtures. Therefore, these two EPN species represent potentially attractive biological control agents for *H. licas* larvae in South Africa.

An encouraging aspect of this study are the results suggesting that native EPN species have the potential to be used for the management of important white grub pests, such as *H. licas*, in South Africa. However, further studies are required including tests of different nematode doses (rates) and exposure times on *H. licas*, and similar studies completed on other common white grub pests such as *P. sommeri* and other species. For example, only one rate of nematode concentration (200 IJs/grub) was compared in the present study. It is possible that a higher nematode concentration of less effective species may be as effective as *H. bacteriophora* against the tested white grub species. It will also be important to determine the LD50 of the chosen EPN species in order to apply the appropriate EPN dose during application. Furthermore, evaluations of the different nematode species against the early instars (1st and 2nd) of target white grubs are required, because susceptibility of the different life stage of the grubs varies significantly within the same nematode species, as do different white grub species themselves (Koppenhöfer and Fuzy, 2004).

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