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In vitro liquid culture and optimization of *Steinernema jeffreyense* using shake flasks

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Abstract Entomopathogenic nematodes (EPNs) of the families *Heterorhabditidae* and *Steinernematidae* are efficient biological control agents against important insect pests. In vitro liquid culture production technology is a key factor in the success of implementing EPNs as a biological control agent. One of the first steps of in vitro mass culture is to use shake flasks to obtain nematode inoculum for optimising and upscaling to desktop and industrial fermenters. This study was the first attempt on the in vitro liquid mass culture of a local South African isolate, *Steinernema jeffreyense,* in 250 ml Erlenmeyer flasks, together with their mutualistic bacteria, *Xenorhabdus khoisanae*. After the successful in vitro production of *S. jeffreyense*-inoculum, different parameters for optimizing infective juvenile (IJ) recovery (developmental step when the IJ moult to initiate the life cycle) and yield, were investigated. This includes the effect of the volume of liquid medium in the flasks, two different orbital shakers setups and the initial IJ inoculum density. With 30 ml of liquid medium the mean percentage recovery of IJ after six days was 86%, with a yield of 121,833 IJ ml^{-1} after 14 days, in comparison to 75% and 99,875 IJs ml^{-1} respectively when 50 ml of liquid medium was used. No significant difference was found between IJ recovery and yield, using different orbital shakers setups. Among the three inoculum concentrations tested (1000, 2000 and 3000 IJ ml^{-1}), the lowest concentration gave the highest IJ recovery and yield. Pathogenicity of IJs cultured in vitro was higher than those cultured in vivo.

Keywords Entomopathogenic nematode · In vitro culture · Pathogenicity · Shake flask · *Steinernema jeffreyense*

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Introduction

Entomopathogenic nematodes (EPNs) of the genus *Steinernema* (Rhabditida: Steinernematidae) have a symbiotic relationship with bacteria of the genus *Xenorhabdus* (Boemare et al. 1993; Ehlers et al. 1988). This bacto-helminthic complex is a safe biological control agent (Ehlers and Hokkanen 1996) and has been widely studied as a biological control agent against a wide range of insects (Kaya and Gaugler

1993; Shapiro-Ilan et al. 2014; Malan and Hatting 2015; Hatting and Malan 2017).

The infective juvenile (IJ) is morphologically distinct from the normal third stage larvae, which is an evolutionary adaptation to a depleted food supply, adverse environmental conditions and is well adapted to long-term survival in the soil (Poinar and Thomas 1996). In the haemolymph of the insect, an unknown chemical food signal activates the arrested juvenile stage to develop into a feeding stage within hours (Strauch and Ehlers 1998). The start of feeding of the IJ stage, which is referred to as "recovery" or "activation", results in the opening of the mouth, starting active feeding (Golden and Riddle 1984). Depending on the availability of food, the nematode will undergo one or more generations (Strauch et al. 1994). Following copulation, the females lay eggs, which hatch as J1, with the life cycle continuing to J2, J3, J4 and then to male or female adults. The aim of in vitro liquid culture is to produce a pure culture of IJ within the shortest period of time.

Mass culture of EPNs requires biotechnology advanced techniques and, to reach the commercial market, an effective and low-cost production protocol is required. EPNs are effective insect biocontrol agents, but the culture and formulation process requires scaling up and, moreover, requires the reduction in the cost of production to be acceptable for the market. However, even though EPNs have been cultured for nearly three decades, their successful implementation as a biological control agent in South Africa has been problematic due to the lack of a commercial product.

Mass production of EPNs became possible with the first attempts in the 1940s (Glaser and Tenbroeck 1940), with the methods of production having steadily improved over time. In vitro liquid culture starts with shake flask cultures, which are scaled up to various bioreactor sizes. The population growth of EPNs in liquid culture resembles that of the in vivo life cycle, apart from the all-important step of pre-inoculating the media with the symbiotic bacteria prior to the inoculation of the nematodes, which then replaces the insect as the food source (Ehlers 2001; Shapiro Ilan et al. 2014). Doing the above induces the bacterial food signal that is crucial for EPN development and it replaces the more effective, yet still unknown, haemolymph food signal. Optimising the liquid culture process has been intensively studied (Strauch and Ehlers 2000; Neves et al. 2001; Hirao and Ehlers 2009, 2010; Leite et al. 2016, 2017). The focus of such studies has been directed to improving the essential nutrients, like lipids, proteins and various growth factors, and the culture conditions, such as temperature, inoculum size and the aeration rate for improved yields, quality and costs (Shapiro-Ilan and Gaugler 2002; Surrey and Davies 1996).

Steinernema jeffreyense Malan, Knoetze and Tiedt 2016 (GenBank KC897093), was originally isolated from sandy soil beneath a guava tree, close to the coast in Jeffrey's Bay, in the Eastern Cape province of South Africa (Malan et al. 2016). The nematode belongs to the *Khoisanae* clade, which currently consist of four South African species of which the IJs have a large body length, as in the case of *S. jeffreyense* 926 (784–1043) µm. They also share the same mutualistic bacteria *Xenorhabdus khoisanae* Ferreira, van Reenen, Endo, Sproër, Malan and Dicks 2013, with *Steinernema khoisanae* Nguyen, Malan and Gozel 2006 of the clade (Ferreira et al. 2013).

De Waal et al. (2011) investigated the biocontrol potential of six different locally isolated South African species of EPN against *Cydia pomonella* L. (Lepidoptera: Tortricidae) (codling moth) in the laboratory, using 50 IJ per insect larva and obtained 100% mortality. A semi-field trial, with the three most promising species, showed the highest mortality of 70% of codling moth larvae using *S. jeffreyense*, without being significant different from *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler and Adams 2004. Steyn et al. (2019) assessed the laboratory and field efficacy of *S. jeffreyense* against the *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) (false codling moth) and obtained $> 80\%$ mortality in the laboratory trial with up to 77% mortality in the field. Measured against the success with codling moth and false codling moth for the deciduous fruit industry, the choice of nematode for mass culture is justified.

The main aim of the current study was to successfully mass-culture the local endemic South African EPN species *S. jeffreyense* in liquid culture, with the use of shake flasks on orbital shakers to obtain a pure culture of IJ, $> 1.0 \times 10^5$ IJ ml^{-1}, within a short time (14 days). The influence of the volume of the medium used in the flasks, the initial IJ inoculum density and using different types of shakers as a means of improvement of IJ yield were investigated.

Materials and methods

Origin of organisms used

Tenebrio molitor L. (Coleoptera: Tenebrionidae) (mealworm) was originally obtained from a pet shop. Larvae were cultured according to the technique of van Zyl and Malan (2016). The mealworm larvae were maintained in a well-ventilated plastic container, filled halfway with bran to which a carrot was occasionally added for maintenance of moisture. *Galleria mellonella* L. (Lepidoptera: Pyralidae) were cultured in ventilated glass flasks, starting with eggs according to the technique of van Zyl and Malan (2016).

Steinernema jeffreyense was obtained from the collection of Stellenbosch University, South Africa. The nematode cultures have been maintained by recycling though mealworm or wax moth larvae for the past 12 years, IJ's harvested and then stored in horizontally placed culture flasks at 14 °C.

The nematode associated symbiotic bacteria, *Xenorhabdus khoisanae* Ferreira et al. (2013) (GenBank KX925559), was isolated from the haemolymph of last-instar *G. mellonella*, which were inoculated with *S. jeffreyense* (Akhurst 1980; Dreyer et al. 2017). The infected haemolymph was obtained by puncturing the proleg of a wax moth larva, 18 h after inoculation and by streaking a drop of haemolymph on nutrient agar plates supplemented with bromothymol blue and triphenyltetrazolium chloride (NBTA) (8 g nutrient broth, 15 g agar, 0.25 g bromothymol blue, 1 l distilled H_2O and 0.04 g triphenyltretrazolium chloride). A single bacterial colony was added to 30 ml of TSB (tryptic soy broth) in a 250 ml Erlenmeyer flask on a 140 rpm (0.547 g) Junior Orbital Shaker at 28 °C for 48 h in a growth chamber, after which 4.5 ml sterile glycerol was added to give a 15% glycerol (v/v) stock culture. The bacteria cell concentration of 1×10^7 cells ml^{-1} were determined by counting the cells using a haemocytometer. One ml of the glycerol stock culture were pipetted into 1.5 ml Eppendorf tubes and frozen at − 80 °C (Kaya and Stock 1997) and were used throughout the study.

Monoxenic *S. jeffreyense* and *X. khoisanae* cultures were established using the modified method described by Lunau et al. (1993) and by Ferreira and Malan (2014). In short, a 250 ml Erlenmeyer flask containing 30 ml TSB was inoculated with 200 µl (1×10^7 cells ml^{-1}) of the frozen stock cultures. The Erlenmeyer flasks were incubated for 48 h on a Junior Orbital Shaker at 140 rpm, at 28 °C in a growth chamber, in the dark. A volume of 2 ml (4% v/v) of the bacterial culture was added to 250-ml Erlenmeyer flasks containing 50 ml of liquid media (complex media) and left to grow for 48 h. The complex medium consisted of: 15.0 g yeast extract (Sigma Life Science), 20.0 g soy powder (Health Connection Whole Foods, Diep River, South Africa), 4 g NaCl (AnalaR, BDH Ltd), 0.35 g KCl (AnalaR, Hopkin and William Ltd, England), 0.15 g $CaCl_2$ (Merck), 0.1 g $MgSo_4$ (PAL Chemicals) and 36 ml olive oil (Mild Olive Oil, SPAR South Africa (Pty) Ltd) per litre of water (Ferreira et al. 2014). A sterile three-day-old mixture of sterile eggs and hatched J1 and J2 nematodes were placed in tryptic soy broth (TSB) in 24-well plates according to the technique of Lunau et al. (1993). The Erlenmeyer flasks with bacteria were inoculated with the sterile nematode mixture, incubated in the dark at 25 °C, left in an enclosed, temperature controlled IncoShake for approximately 20 days, until the population consisted solely of IJs. The IJs in the diet were stored in Erlenmeyer flasks, on an open OrbiShaker^{TM} (Labotec) at 120 rpm at 14 °C and were used as the monoxenic pre-culture for all subsequent experiments.

Evaluation protocol

For each treatment, three 250 ml Erlenmeyer flasks were used and IJ recovery was recorded at days 2, 4 and 6, following the IJs being inoculated into the bacterial complex medium. To evaluate recovery, 200 µl of sample was diluted 25 times in distilled water, after which the nematode numbers in 1 ml were counted, using a counting slide and dissection microscope (Leica). Percentage recovery was calculated by dividing the number of recovered nematodes by the total number of nematodes present in 1 ml (Strauch and Ehlers 1998). The IJ of *S. jeffreyense* used in this study belong to the *glaseri* group with a long body length of > 1000 µm enabling J3 and J4 to be visually distinguished during the six-day evaluation period for recovery. Yields were measured after 14 days. The yield in the 1 ml sample taken from each flask was diluted in 100 ml of distilled water. After agitation, five drops of 10 µl were counted and the number of IJ per ml calculated. This procedure was repeated twice.

The nematode reproduction factor (*r*) was calculated according to Maistrello et al. (2010). It was calculated as the ratio between the final yield (IJ ml^{-1}) or final population and the initial inoculum density (IJ ml^{-1}) or the initial population (*Pf/Pi*) of *S. jeffreyense*. This was calculated for every individual flask and for each experiment.

Liquid medium volume

Two volumes of the complex liquid media of 30 ml and 50 ml were used to compare the recovery and yield of IJs of *S. jeffreyense* according to the described protocol, using an IncoShake (Labotec), temperature-controlled, orbital shaker. The liquid culture medium was inoculated with 4% (v/v) of the symbiotic bacteria *X. khoisanae* pre-cultured in TSB medium, equating to 2 ml/50 ml medium and 1.2 ml/30 ml medium, respectively. To obtain an approximate initial inoculum of 2000 IJs ml^{-1} in each flask, 1×10^5 and 6×10^4 IJs were added to 50 and 30 ml media, respectively, which is half the concentration used by Ferreira et al. (2016), working with a smaller *S. yirgalemense*. The nematode-bacteria cultures were left in a temperature-controlled incubating shaker (IncoShake, Labotec) for 14 days at 140 rpm at 25 °C. The experiment was repeated on a different test date.

Type of orbital shaker

Two different types of orbital shakers were used, consisting of an enclosed system IncoShake temperature controlled and an open system OrbiShaker at room temperature. The liquid culture medium of 30 ml was inoculated with 4% (v/v) of the symbiotic bacteria *X. khoisanae*, pre-cultured in TSB medium, as was previously described in the evaluation protocol. In total, 6×10^4 IJs were inoculated into each flask, equating to 2000 IJs ml^{-1}. The nematode-bacterium culture was grown on one of the two shaker setups for 14 days at 140 rpm at 25 °C. The experiment was repeated on three different test dates with a different batch of IJ inoculum.

Inoculum concentration

Three treatments of different IJ inoculum concentrations of 1000 (low), 2000 (medium) and 3000 IJs ml^{-1} (high) were used. The complex medium of 30 ml was inoculated with 4% (v/v) of *X. khoisanae* being pre-cultured in TSB, as was previously described in the evaluation protocol. The three IJ concentrations were added to three 250 Erlenmeyer flasks, with 30 ml of complex medium for each treatment using the IncoShake temperature controlled orbital shaker. The nematode-bacterium cultures were grown for 14 days at 140 rpm at 25 °C. The experiment was repeated on a different test date, with a different batch of IJ inoculum.

Pathogenicity of in vitro cultured IJ

The in vitro cultured IJ of *S. jeffreyense* were tested for pathogenicity, after storage in the diet in Erlenmeyer flasks on an orbital shaker (120 rpm) for a period of ten days and 20 days at 14 °C. Five 24-well bioassay plates were used for each treatment, with 12 alternative wells in each plate lined with filter paper, to which a *T. molitor* larva was added. A concentration of 100 IJ per 50 µl water was added to each well, transferred to a plastic container with a moist filter paper and closed with the lid. To compare, an in vivo cultured *S. jeffreyense,* cultured using *Tenebrio* larvae as host, harvested for IJ after seven days and placed in culture flasks in distilled water and stored at 14 °C were used as inoculum within seven days. For the control treatments, distilled water only was used. The container with the bioassay plates were plates were left in a growth chamber at 25 °C and the percentage mortality determined after 48 h. This experiment was repeated once in time.

Statistical analysis

The IJ recovery was presented as a mean percentage, and the yields as IJs ml^{-1}. Data were assessed by means of ANOVA and Fisher's least significant difference (LSD). For each experiment, the main effects of the treatments and test date were analysed separately. For all experiments, if no significant difference between the test dates were found, the data from the different test dates were pooled and analysed using a one-way ANOVA, except with regards to the media volume experiment, where the main effects could not be interpreted separately. The reproduction factor was assessed by ANOVA and LSD. Pathogenicity of IJ's was assessed by means of ANOVA and Bonferroni's correction.

Results

Liquid medium volume

The test for interactions between the day and treatment indicated a significant difference ($F_{2,\ 26} = 3.765$; $p = 0.03$) and the main effects could be interpreted separately. Thus, a LSD multiple comparisons was done on the interactions to compare the two treatments at each day. At day 2, the treatments do not differ significantly ($p = 0.8769$) with a mean percentage recovery of < 10%. (Figure 1). At day 4, the treatments differed significantly ($p < 0.001$), with a mean recovery of 36.89 ± 1.93% for 30 ml and of 23.17 ± 2.08% for 50 ml. On day 6, the recovery of the IJs was 85.18 ± 2.93% for 30 ml and 75.24 ± 3.08% for 50 ml, which differed significantly ($p = 0.015$) from each other. With regard to the yield of IJ after 14 days, a significant difference ($p = 0.049$) between the two media volumes used, with a mean of 121,833 IJs ml^{-1} (98,000–161,000 IJs ml^{-1}) for 30 ml of complex medium and of 99,876 IJs ml^{-1} (78,000–117,000 IJs ml^{-1}) for the 50 ml complex medium and the interactions could be interpreted. Moreover, when comparing the *r* values, there was a significant difference ($F_{1,\ 13} = 4.706$; $p = 0.049$) between the two treatments (Table 1) with a value of 61 for the 30 ml treatment and 50 for the 50 ml treatment.

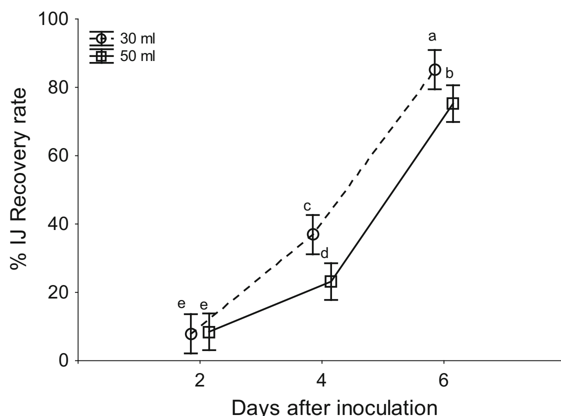

Fig. 1 Mean percentage infective juvenile (IJ) recovery (95% confidence interval) of *Steinernema jeffreyense* over a period of six days, using 30 ml and 50 ml complex medium in 250 ml Erlenmeyer flasks, on an orbital shaker. Letters that are the same indicate no significant difference ($p > 0.05$) in the recovery of IJ over a period of six days

Type of orbital shaker

A test for interactions between day and treatment indicated no significant difference ($F_{2,\ 20} = 1.813$; $p > 0.05$) and the main effect of recovery can be interpreted as changing over time. A LSD multiple comparisons test was conducted to interpret the main effects of treatment (shaker type) and the time (date) of IJ recovery. However no interactions between treatments could be interpreted. On day 2, no significant difference ($p = 0.253$) was found between the two shaker types involved, with a recovery of > 30%, as well as on day 6 ($p = 0.135$), with a recovery between 76 and 86%. However, on day 4, a significant difference ($p = 0.003$) of 54.95% ± 1.93% was found for the OrbiShaker and 76.10% ± 2.08% for the IncoShake (Fig. 2). The percentage recovery after six days for the OrbiShaker was 76.38 ± 2.93%, whereas, for the IncoShake, the percentage recovery was 86.27 ± 3.08%. However, no significant difference ($F_{1,\ 10} = 0.944$; $p = 0.35$) was detected in IJ yield after 14 days for the two shaker setups, which range between 88 and 95 thousand IJs ml^{-1} after 14 days, and there was also no significant difference ($F_{1,\ 10} = 0.944$; $p = 0.354$) between the *r* values for each treatment (Table 1).

Inoculum concentration

A test for interactions between day and treatment indicated no significant difference ($F_{4,\ 30} = 2.091$; $p = 0.107$) and the main effect of recovery could be interpreted as changing over time. A LSD multiple comparisons test was conducted to interpret the main effects of treatment (concentration) and time (date) on the IJ recovery. However no interaction could be interpreted. (Figure 3). On day 2, no significant ($p > 0.05$) difference was found in the recovery > 38% between the three IJ concentrations. However, on day 4, a significant difference ($p = 0.004$) was found between the treatments of medium and high, with the percentage IJ recovery on day 4 for the medium concentration being the highest (83.14 ± 2.0%). On day six, a significant difference ($p = 0.032$) was detected between the treatments of low and high concentrations, with no significant difference ($p > 0.05$) between the other concentrations. The percentage recovery on day 6 was the highest (91.72 ± 2.85%) for the low nematode

Table 1 Effect of different treatments on the mean, minimum and maximum yields (IJ ml^{-1}) for *Steinernema jeffreyense* and the reproduction factor (*r*), based on the final mean yield (*Pf*) and the initial inoculum density (*Pi*)

Experiment	Treatment	Initial inoculum density (IJ ml^{-1})	Mean yield ± SE (IJ ml^{-1})	Min yield (IJ ml^{-1})	Max yield (IJ ml^{-1})	Reproductive factor ± SE (*r*)
1	30 ml	2000	121,833 ± 7400	99,000	154,000	61 ± 3.70[a]
	50 ml	2000	99,875 ± 6922	81,000	112,000	50 ± 4.70[b]
2	Icono shake	2000	95,222 ± 5255	86,333	108,999	47 ± 2.63[a]
	Orbi shake	2000	87,999 ± 5255	74,333	103,666	44 ± 2.63[a]
3	Low	1000	95,222 ± 6526	84,666	104,333	95 ± 3.43[a]
	Medium	2000	94,444 ± 6526	83,999	105,999	47 ± 3.43[b]
	High	3000	90,333 ± 6526	76,666	109,333	30 ± 3.43[c]

Letters that are the same indicate no significant difference ($p > 0.05$) between the *r* values within experiments

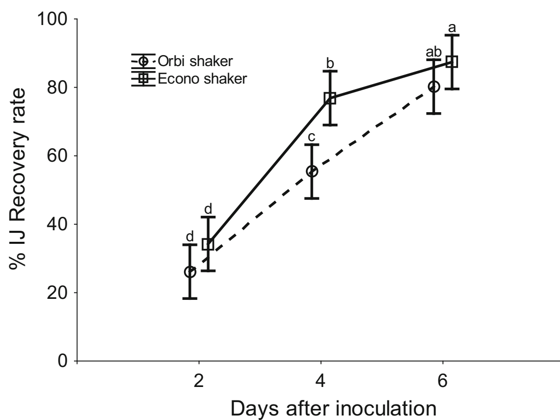

Fig. 2 Mean percentage infective juvenile (IJ) recovery (95% confidence interval) of *Steinernema jeffreyense* over a period of six days, in 30 ml complex medium, in 250 ml Erlenmeyer flasks on two different orbital shakers. Letters that are the same indicate no significant difference ($p > 0.05$) in the recovery of IJ over a period of six days

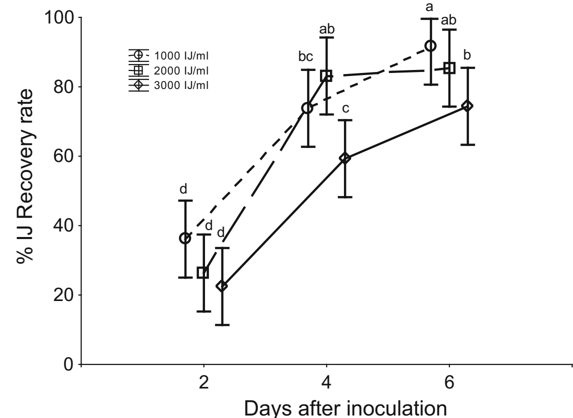

Fig. 3 Mean percentage infective juvenile (IJ) recovery (95% confidence interval) of *Steinernema jeffreyense* over a period of 6 days, inoculated with different concentrations of 1000, 2000 and 3000 IJs ml^{-1}. Letters that are the same indicate no significant difference ($p > 0.05$) in the recovery of IJ over a period of six days

concentration inoculated. However, no significant difference ($F_{2,\ 15} = 0.162$; $p > 0.85$) was found between the three IJ inoculum concentrations regarding the final IJ yield, after 14 days, which range between a mean of 90 and 95 thousand IJ ml^{-1}. However, a significant difference was found between the *r* values. The low inoculum density was significantly different to both the medium and the high ($F_{2, 15} = 97.004$; $p < 0.05$) inoculum densities. Moreover, a significant difference was found between the medium and high inoculum densities ($F_{2, 15} = 97.004$; $p = 0.003$).

Visual observations

For the in vitro liquid culture of *S. jeffreyense*, it was observed that there was a noticeable colour differentiation between successful and unsuccessful cultures. For the initial inoculation of bacteria into the liquid culture media, after two days the media changed from a light beige colour to a brighter yellow colour. This indicated that the bacteria had successfully grown and was confirmed by streaking onto NBTA plates, to ensure that the bacteria were in primary phase. On the other hand, unsuccessful cultures did not change

colour indicating contamination. Moreover, a successful culture would have no visible oil layer at the surface of the liquid media indicating that the bacteria have successfully metabolized the media ingredients and indicated a healthy culture. A colour change was also seen at the end of production after 14 days post-IJs inoculation. A successful culture would change to a dark brown colour and been less viscous whereas an unsuccessful culture would change to a pale white colour and would retain the initial viscosity. When this white colour appeared, the culture flask was discarded. This brown colour of a successful culture indicated that the nematodes had successfully metabolized the media. Moreover, a successful culture would have a pleasant savoury aroma, whereas an unsuccessful culture had a sour milk smell often indicating contamination. These qualitative factors are good indicators of a successful culture run and were used throughout this study as an indicator of quality.

Pathogenicity of in vitro cultured IJ

A total of 94.99 ± 1.84% infectivity was found in *T. molitor* inoculated with ten-day old in vitro cultured nematodes, compared with 86.66 ± 2.22% infectivity for 20 days old in vitro cultures, yet no significant difference was found between these two treatments ($F_{3,\ 16} = 163.80$; $p = 0.061$) (Fig. 4). For the in vivo cultured EPN's, the mortality was 81.66 ± 2.72%, which differed significantly from the in vitro 10-day old culture ($p = 0.0007$), but did not differ significantly from the in vitro 20-days old culture ($p = 0.68$). Natural mortality of 5.83 ± 1.78% was found for the control (Fig. 4).

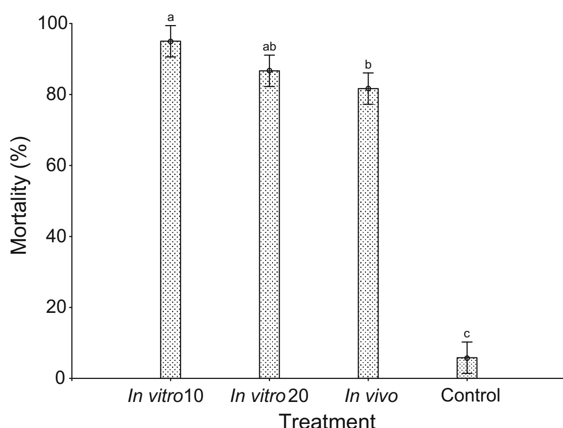

Fig. 4 Mean percentage mortality (95% confidence interval) for *Tenebrio molitor* larvae after exposure to *Steinernema jeffreyense* at a concentration of 100 IJs per larvae in 50 µl. Letters that are the same indicate no significant difference ($p > 0.05$) in the mortality of *T. molitor*

Discussion

The results from the study showed that 30 ml of liquid culture were enough to produce higher *S. jeffreyense* IJ yields compared to 50 ml, in shake flasks. For both volumes, the recovery of IJs was < 10% for day 2, with a substantial increase by day 4, which showed significant difference between the two volumes used. The recovery of the IJs continued to increase, reaching 85% for the 30 ml and 75% for the 50 ml, complex medium on day 6. The recovery period for *S. jeffreyense* was found to be substantially longer than for other steinernematids, being more akin to the recovery period for the *Heterorhabditis* species (Strauch and Ehlers 1998). In this study final yields, after 14 days for each treatment, also significantly differed, with the 30 ml treatment achieving an 18% higher recovery compared to 50 ml. The reproduction factor ratio also provides evidence that suggests that 30 ml is optimal to 50 ml of media volume with the two treatments being significantly different from each other. Moreover, a maximum of 160 thousand nematodes per ml with the 30 ml treatment was achieved in one flask, indicating the potential yield that can be regarded as an achievable benchmark for further improvement.

In previous studies with other EPN species, either 30 ml or 50 ml of liquid culture (Ferreira et al. 2014; Leite et al. 2016) was used. For *S. jeffreyense,* the higher IJ recovery and yield with a lower volume could be ascribed to a greater surface-area-to-volume ratio that improved distribution, or transfer, of oxygen. Oxygen has been shown to play an extremely important role in the success of a culture (Strauch and Ehlers 2000; Neves et al. 2001; Leite et al. 2016), and the supply of oxygen and adequate gas exchange for the developing nematodes is of critical importance. Leite et al. (2016), showed that, when adding thickeners to the medium, the surface area of the medium increases by means of adhering to the flask wall, which, subsequently, improves the culture conditions by increasing the amount of oxygen transfer. These results are supported by Giese et al. (2014) and

Mascarin et al. (2015). Moreover, in the first reports adopting the three-dimensional solid media method of production, in preference to the solid two-dimensional method, polyurethane sponges with a poultry homogenate were used to create a greater surface-area-to-volume ratio, which resulted in an improved gas exchange (Bedding 1981, 1984).

Different laboratories may use different experimental setups for in vitro culture using shake flasks depending on the available apparatus and environmental conditions that it offers. The results indicated that using a closed-system IncoShake did not differ in terms of IJ recovery and yield, compared to using an open system OrbiShaker. A significant difference was anticipated between the two shakers, due to the uniformity of the IncoShake environment, compared to that of the OrbiShaker. The latter was placed on a bench, in an air-conditioned room with a temperature of 25 ± 2 °C. Moreover, the IncoShake was in constant darkness, whereas the OrbiShaker was exposed to artificial light during most of the day. IJs have been shown, through in vivo and in vitro cultures, to require darkness to grow successfully (Stoll 1953; Ehlers 2001). The reasons for the comparable results obtained could be ascribed to the robustness of *S. jeffreyense*. EPNs are known to be adaptable, indicating that slight modifications of their environment have a negligible impact on their growth and development. They might even benefit from an enhanced replica of the natural environment, in cases where there might otherwise be continuous fluctuation in the abiotic factors.

The results of the impact of inoculum density were contrary to what was expected, with a higher yield using a lower inoculum of IJ concentration. This could be ascribed to the decreased availability food for the larger population of nematodes, thus leading to a decreased food signal and to a lower recovery, prior to feeding. For the low IJs ml^{-1} treatment, the smaller initial population density concerned would mean that there would be more food available to the developing nematodes, leading to a stronger food signal and to the recovery of an increased number of IJs (Han 1996; Hirao and Ehlers 2010). This is supported by the *r*-value which is significantly higher for the low inoculum, compared the two other treatments of medium and high. This is an important consideration for mass production in terms of economic factors. As production size moves from laboratory level to industry level, a low inoculum density into large fermenters will be far cheaper than the alternative medium and high inoculum densities.

A higher recovery results in the development of more females and males in the population, finally resulting in the production of more offspring than there might otherwise have been. In situations of over-inoculation, the media might become crowded, which might explain the decreased of recovery. A previous study, Hirao and Ehlers (2010), analysing the effects of inoculum density on the recovery and yield for *Steinernema feltiae* (Filipjev 1934) Wouts, Mráček, Gerdin and Beddin, 1982 and *Steinernema carpocapsae* (Weiser 1955) Wouts, Mráček, Gerdin and Bedding 1982, showed that inoculum density had no impact on the recovery.

The *S. jeffreyense* used in this study showed minimal second-generation development in all of the experiments undertaken. Thus, we were able to use inoculum density as an optimising factor to better synchronise the recovery involved. It is concluded that a higher inoculum density in the case of *S. jeffreyense* negatively affects the recovery, development and overall success of the liquid culture, with a lower inoculum density being optimal. In terms of commercialisation, this is of extreme importance. However, an important factor, when assessing the impact of inoculum density that needs to be taken into consideration, is bacteria concentration. When inoculating at higher densities, one should also increase the bacterial concentration (Han 1996). We used an initial bacterial density of 4% v/v for all treatments and batches, without adjusting for inoculum density. It has been observed that the bacteria in the flask turned from a viscose-yellow to a thinner, brownish colour indicating the nematode consumption of the bacteria supplied, which lead to easy separation of the IJ from the bacteria.

For the liquid culture mass production of EPNs, the first and most important stage of the life cycle is the recovery of the IJ, as this determines the success or failure of the production process concerned (Ehlers 2001). A low recovery of IJs corresponds with a low final yield, as few IJs will then develop into adults, which, in turn, will result in fewer offspring being produced, thus decreasing the number of nematodes contributing to the development of IJs, when the food supply is depleted (Ehlers 2001). In many heterorhabditids, low recovery often results in a second

generation developing into adults, rather than to IJs, thus creating a two-generations process with an extended production time (Ehlers 2001). However, the above was not observed with *S. jeffreyense*, as is so often the case in terms of steinernematids.

Strauch and Ehlers (1998) report recovery of 23 and 45% for two strains of *Heterorhabditis megidis* Poinar, Jackson and Klein 1987, and a mean of 38% for *Heterorhabditis bacteriophora* Poinar, 1976, ranging from 0 to 81%. They also used two *Steinernema* species with a recovery of between 60 and 90%, which did not display the same variability (Strauch and Ehlers 1998). As the variability that was observed in the *Heterorhabditis* species is not reciprocated in the *Steinernema* species, the latter is easier to optimise, as the species concerned possess greater uniformity in their population dynamics than do the former. Strauch and Ehlers (1998) showed that heterorhabditids recover in liquid culture over a period of several days. However, under natural conditions steinernematids can recover within a few hours after reaching the haemocoel of the insect. Thus, it is of crucial importance, when optimising the liquid culture process, to focus on those factors that induce a synchronous recovery process soon after inoculation. The recovery could have an important effect on commercial application, as it would decrease the overall production time. Some of the factors that have been reported to have a significant effect on recovery are oxygen concentration and distribution, inoculum density, increases in symbiotic bacterial cell density, and the addition of glucose or thickeners (Tachibana et al. 1995; Strauch and Ehlers 1998, 2000; Neves et al. 2001; Gil et al. 2002; Leite et al. 2016).

Most of the current study was based on the techniques used for *S. yirgalemense* (Ferreira et al. 2016). However, when comparing the size of the different IJs, the mean body length of *S. jeffreyense* is much longer than that of *S. yirgalemense*, resulting in different demands for the culture concerned. *Steinernema jeffreyense* belongs to the *Khoisanae* group, with its large IJ being $\geq$ 1000 µl in length (Hunt and Khuong 2016), whereas, in the case of *S. yirgalemense*, the mean body length of the IJ is 635 µl (Nguyen et al. 2004). We have successfully shown that, for *S. jeffreyense*, only 1000 IJs ml^{-1} is required to achieve a high recovery rate and yield, as well as to optimise the in vitro liquid culture for the species. The aim with the in vitro culture of *S. jeffreyense* should be to obtain $>$ 160,000 IJs ml^{-1}, whereas, in the case of, for example, *S. yirgalemense*, with its shorter body length, the yield should be approximately double. Ramakuwela et al. (2016) successfully cultured a local species, *Steinernema innovationi* Çimen, Lee, Hatting and Stock 2014, using a solid culture, with a yield of 156,336 IJs ml^{-1}. This nematode species also belong to the *Khoisanae* group, with a large body length, indicating the potential to produce larger yields with *S. jeffreyense*.

The pathogenicity results indicate that IJ of in vitro ten days old cultures differed significantly from those cultured in vivo, with the 20 days old in vitro culture showed no significant difference from the ten days old in vitro or the in vivo cultured IJs. However, contrary to this, some EPN species showed a higher percentage mortality for the in vivo cultured IJ (Converse and Miller 1999). Grewal et al. (1999) found that there was no difference in the infectivity of *S. carpocapsae* produced either in vitro or in vivo. Moreover, the same test was conducted using *S. scapterisci,* which displayed a higher mortality for in vitro produced IJs compared to in vivo. In this study, *S. jeffreyense*, produced in vitro outperformed in vivo produced nematodes with regard to pathogenicity. In a laboratory and field trial, Steyn et al. (2019) compared in vivo and in vitro produced IJ of *S. jeffreyense* to control false codling moth, with in vitro produced IJ providing better pathogenicity in both cases. A higher mortality percentage for in vitro produced IJs also provide promising prospects for upscaling to fermenters and for formulation where IJs often lose their pathogenicity due to sub-culturing and selection of genetic traits that improve mass production but decrease pathogenicity and in formulation where the IJs are subjected to a variety of stressful factors that can decrease their pathogenicity.

This is the first report of the South African isolated *S. jeffreyense* being successfully mass-cultured using in vitro liquid techniques. The culture technique was improved by means of investigating and analysing three factors that might have an influence on the recovery, yield and overall success of a culture. It is also important to note that *S. jeffreyense* showed no adult development after 14 days, which is an important factor for successful formulation. The future optimisation of the culture conditions of *S. jeffreyense* opens further aspects to be investigated, such as the shortening of the IJ recovery time, the effect of the

bacterial concentration and the effect of the media contents of the liquid culture on the yield of IJ.

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Compliance with ethical standards

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

Research involving human and or/animal participants The research does not involve human participant or animals.

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