Journal of Helminthology

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Population dynamics of a South African isolate of *Steinernema yirgalemense in vitro* liquid culture, using egg yolk as protein source

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Research Paper

Cite this article: Dunn MD and Malan AP (2024). Population dynamics of a South African isolate of *Steinernema yirgalemense in vitro* liquid culture, using egg yolk as protein source. *Journal of Helminthology*, **98**, e64, 1–9
https://doi.org/10.1017/S0022149X24000452.

Received: 23 March 2024
Revised: 05 August 2024
Accepted: 06 August 2024

Keywords:
Egg yolk; population dynamics; EPN; liquid mass culture; Erlenmeyer flask production

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Abstract

The entomopathogenic nematode (EPN), *Steinernema yirgalemense*, is an effective biological control agent against a variety of important insect pests in South Africa. To develop a South African EPN product feasibly in South Africa, EPNs need to be mass-produced. This study aimed to record the population dynamics of *S. yirgalemense* with *in vitro* liquid production in shake flasks, with a protein source of powdered egg yolk. The Erlenmeyer flask results indicated variation between flasks, albeit still achieving high yields. The reasons for attaining such variability in the recovery, yield, and growth stages are unclear, hence requiring further studies seeking to increase consistency. The results obtained indicate that, when IJ recovery is low, yields are also low due to relatively few reproductive adults being present in solution, which, in turn, produces more offspring, which later converts to the desired infective juvenile used for product formulation development. For commercial viability, a consistent production system is required that produces predictable yields. This study showed comparable high yields achieved with the flasks and in an early-stage bioreactor setup, being a positive development for *S. yirgalemense* mass production. Prior to the bioreactor scale-up process, protocol of mass production, the population and growth dynamics of the nematodes in the flask environment requires understanding. This is a positive step, leading to the future commercialisation of a local EPN product.

Introduction

The global agriculture pesticide market is rapidly changing (Tilman *et al.* 2011), with a shift towards the use of eco-friendly pesticide products that reduce environmental and human health toxicity and, more importantly, that assist in rebuilding ancient natural pest defence mechanisms (Warrior 2000). The use of micro-organisms and macro-organisms that are natural enemies of many pests has displayed the potential to disrupt the current pesticide market (Van Lenteren *et al.* 2012; Mishra *et al.* 2014) and to shift the agricultural industry towards more sustainable practices that employ biopesticides, within an integrated pest management (IPM) programme, as the primary form of pest control (Lacey *et al.* 2015). Multiple species of entomopathogenic bacteria, fungi, viruses, and nematodes have been commercialised into marketable formulated biopesticide products (Gil *et al.* 2002; Lacey *et al.* 2015; Hatting *et al.* 2019).

Entomopathogenic nematodes (EPNs) that have displayed great potential as biopesticide products are unique in that they can rapidly disperse and actively seek out host insect larvae in cryptic locations in the soil environment. Furthermore, EPNs have a wide host range, can effectively control economically important insect pests in their different life stages, can be used with existing spray equipment, can recycle in the insect host of a potential long-term sustainable pest control, and, in some cases, are more effective than synthetic products (Peters 1996; Ehlers 2001; Lacey *et al.* 2015). EPNs have an additional advantage over other biopesticides in that they can be mass-produced using existing bioreactor technology, and they can be produced on a large scale in bioprocess plants (Ehlers 2001; Dunn *et al.* 2021a). Multiple species of EPNs of the genera *Steinernema* and *Heterorhabditis* have been mass-produced, using *in vitro* liquid culture technology at high yields (Dunn *et al.* 2021a), and have resulted in the establishment of many commercial enterprises that successfully mass-produce EPNs in bioreactors (Ehlers 1996; Ravensberg 2011; Abate *et al.* 2017).

However, developing a standard protocol and method to mass-produce a locally isolated species requires years of research and development. Furthermore, due to the novelty and commercial sensitivity of such methods, the literature concerning mass production, to scale, in bioreactors is scarce, with the literature involved being dominated by small-scale flask production (Leite *et al.* 2016, 2017; Dunn *et al.* 2021a). The shortcomings of such limited research are exacerbated by the strict environmental regulations governing the use of biopesticides in many countries. The regulations concerned prevent the use of imported biopesticide products

containing exotic species for pest control, and so, native species, with different mass production parameter requirements, must be developed into biopesticide products for use in local markets (Hirao and Ehlers 2010; Abate *et al.* 2017). The research requires considerable capital investment, training, and, more importantly, both time and collaborative research.

EPN research in South Africa began in 2010, with the need to develop a biopesticide product using a local South African species (Ferreira *et al.* 2014a, 2016). Multiple surveys and pathogenic screening tests were conducted to find a species of EPN that was both highly pathogenic to key insect pests and that could be mass-produced (Malan and Hatting 2015; Hatting and Malan 2017; Malan and Ferreira 2017; Dunn 2019; Hatting *et al.* 2019; Dunn *et al.* 2021a, b). In South Africa, *Steinernema yirgalemense* Nguyen *et al.* was shown to be the most promising candidate for commercialisation due to its small size, its high pathogenicity, and its mass production potential (Ferreira *et al.* 2016). Ferreira *et al.* (2016) and Addis *et al.* (2016) successfully mass-produced *S. yirgalemense* in shake flasks, achieving maximum yields of 75,000 infective juveniles (IJs)/mL and 284 113 IJs/mL, respectively. The total yields of the two studies were vastly different, as is often the case with EPN *in vitro* liquid culturing (Ehlers 2001), with them indicating the need to develop a standard protocol of production that could achieve consistent high yields. Crucial to the success of EPN liquid culture technology is the inducement of the synchronised and the rapid recovery of the inoculated IJs, in as short a time as possible.

Nematode recovery is defined as the exit from the developmentally arrested IJ stage, at which time the mouth opens to start feeding and the nematodes concerned start developing into adults (Golden and Riddle 1984; Strauch and Ehlers 1998). Another important consideration is the optimisation of the liquid media (Yoo *et al.* 2001; Gil *et al.* 2002) and the assessing of different ingredients to increase recovery, nematode growth, and yields. Dunn *et al.* (2022) successfully optimised the production method of *S. yirgalemense* by means of increasing the yields through media optimisation, and thereby significantly reducing the costs of production in flasks.

Only with the development of a standard protocol that induces rapid and synchronised recovery, and which produces consistently high yields, can the scaling-up phase to small desktop bioreactors begin. The scale-up from flask to bioreactor requires the consideration of multiple new variables, including the bioreactor design, shape, and type (Ehlers 2001; Dunn *et al.* 2021a). Multiple new variables that need not be considered for the flask production, like aeration, agitation, operating conditions, and the growth dynamics of both nematode and symbiotic bacteria, require evaluation, requiring a new standard protocol, specifically geared towards bioreactor production, to be developed.

The current study aimed to follow the life cycle of *S. yirgalemense* in shake flasks and the use of egg yolk as a protein source in the diet. The goal of the study was to understand better the nematode's development under artificial conditions and to explore the possibility of mass cultivation in a custom-made 10-liter bioreactor with a stirring motor.

Materials and methods

Origin of nematodes, bacteria and insect host

Wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae), were maintained and cultured in ventilated 5-L glass jars, according to the technique of Van Zyl and Malan (2015). Mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), was obtained from a pet store and maintained using a bran diet with carrots for moisture, in well-ventilated plastic containers, also following the technique of Van Zyl and Malan (2015).

Steinernema yirgalemense isolate 157-C (GenBank accession number: EU625295), obtained from Stellenbosch University's nematode culture collection, has been maintained by means of recycling through mealworm or wax moth larvae. IJs harvested from insect cadavers in White traps (Woodring and Kaya 1988) were collected and stored horizontally, in vented culture flasks, at 14°C.

The nematode-associated symbiotic bacteria, *Xenorhabdus indica* Somvanshi *et al.* (GenBank accession numbers: KC479153–KC479158) (Ferreira *et al.* 2014b), was isolated from the haemolymph of the last instar of live inoculated *G. mellonella* (Ferreira *et al.* 2014a). *Galleria mellonella* larvae were inoculated with *S. yirgalemense*, and 18 h after inoculation, the larvae were dipped in a 75% (v/v) ethanol solution, whereupon infected haemolymph was obtained by means of puncturing a proleg of the larvae and streaking a drop of the 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 triphenyltetrazolium chloride), at 28°C for 48 h. A single blue bacterial colony was isolated with a sterile looped streaking rod and added to 30 mL of tryptic soy broth (TSB) in 250-mL Erlenmeyer flasks at 140 rpm on a Junior Orbital Shaker at 28°C for 48 h in a growth chamber. Sterile glycerol (4.5 mL) was added to the flasks (15% glycerol v/v), shaken vigorously and pipetted into 1.5 mL Eppendorf tubes, with their contents being frozen at -80°C (Kaya and Stock 1997). This bacteria stock culture was used throughout the study.

Steinernema yirgalemense and *X. indica* were cultured, using *in vitro* liquid culturing, according to the technique of Dunn *et al.* (2020; 2021b); however, the protein source of soy was replaced with egg yolk. In short, *X. indica* from cryopreserved stock cultures (Kaya and Stock 1997) was inoculated into sterile 250-mL Erlenmeyer flasks, with 30 mL of TSB, for 40–44 h at 28°C on a Junior Orbital Shaker. A 2% (v/v) (600 µL) sample of the TSB and *X. indica* solution was then added to 30 mL of nutrient medium [15.0 g yeast extract (Sigma Life Science), 20.0 g egg yolk, 4.0 g NaCl (AnalaR, BDH Ltd), 0.35 g KCl (AnalaR, Hopkin and William Ltd, England), 0.15g $CaCl_2$ (Merck), 0.1g $MgSO_4$ (PAL Chemicals), and 36 mL canola oil (Canola Oil, SPAR South Africa (Pty) Ltd)], per L of water, in 250-mL Erlenmeyer flasks at 28°C, and agitated at 140 rpm on an OrbiShaker™ (Labotec). After 44 h, 6×10^4 IJs in 1 mL of monoxenically produced IJs (Lunau *et al.* 1993) were inoculated into the nutrient medium with *X. indica* at 25°C. A population ranged between 2.5×10^5, and 3×10^5 IJs/mL was produced after 14 days, whereupon it was stored at 14°C on an open OrbiShaker™ (Labotec), agitated at 100 to 120 rpm. The above-mentioned process provided a stock culture of IJs that was used as inoculum for all subsequent experiments.

Nematode growth assessment in shake flasks

Three Erlenmeyer flasks of 30 mL of nutrient media were inoculated with a 2% (v/v) (600 µL) sample of the TSB and *X. indica* culture. After 44 h, 6×10^4 IJs were inoculated into each flask (2000 IJs/mL) at 25°C, at 140 rpm in the IncoShake incubated chamber. A 200-µl sample was taken from each Erlenmeyer flask, 24 h after IJ inoculation, over a period of 13 days. The samples, accordingly, were diluted, depending on the stage of the life cycle concerned. For the first 3 days, the 200-µl sample was diluted in 20 mL of distilled

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water, with the life stages involved being determined. Recovery or activation of the nematodes was evaluated by means of dividing the number of activated nematodes that had reinitiated their life cycle by the total number of nematodes present.

The presence and density of each life stage, being those of the IJ, the J3/J4, the males, the females, the J1/J2, and the J2D, was also recorded each day by means of enumerating the nematodes in dilution. Two counts were done per flask. On day 14, a 1-mL sample, taken from each flask, was diluted in 100 mL of distilled water. After agitation, five drops of 10 µL were counted, with the number of IJs/mL present then being determined. Two counts per flask were measured for the yields, with the trial being repeated twice on different test dates with a total of nine flasks.

Bioreactor construction and nematode growth assessment

The desktop bioreactor used in the current study was custom-designed by means of trial and error performed in relation to establishing its current form (Dunn 2023). The bioreactor consists of a 10-L cylindrical glass vessel, with a sampling port positioned near the bottom of the vessel. The lid was provided with two ports for the exiting of air, with one being used as an inoculation port. The lid was also supplied with a main opening for the motorised stirrer, which provided the bioreactor with air via two external motor pumps, through sterile silicone tubes and a 0.2- µL filter (Midisart®2000, Sartorius Stedim), at 25 L/min, through a porous sparger. The bioreactor was agitated via an external motor, fitted with two marine impellers. An internal stainless steel baffle was used to disrupt the flow of the liquid. The lid of the vessel was clamped and sealed with autoclavable grease (Figure 1). Thorough sterility trials were performed on the bioreactor using TSB, to ensure a sterile growing environment for the symbiotic bacteria and nematode.

Four 250-mL TSB Erlenmeyer flasks were inoculated with 200 µL of *X. indica* from frozen stock cultures and incubated at 28°C at 140 rpm on a Junior Orbital Shaker for 44 h. Four litres of nutrient medium were added to the bioreactor vessel and autoclaved at 120°C for 20 min. After being allowed to cool down for 24 h, the contents of the bioreactor were agitated during the cooling period, to homogenise the media. After 24 h, 80 mL, or 2% (v/v), of the bacterial TSB culture (51×10^7) (Ferreira *et al.* 2016) was inoculated into the bioreactor under a laminar flow cabinet, to ensure sterility, with the bioreactor then being rotated at 140 rpm, at approximately 25°C to 28°C, for 44 h, on a bench top. After 44 h, 5 000 IJs/mL IJs were inoculated into the bioreactor. For the first 10 days in the bioreactor, the motorised marine impeller was rotated at 60 rpm to reduce the amount of shear force exerted on the female nematodes. The rotation speed was then increased to 120 rpm on day 11, until the termination of the experiment.

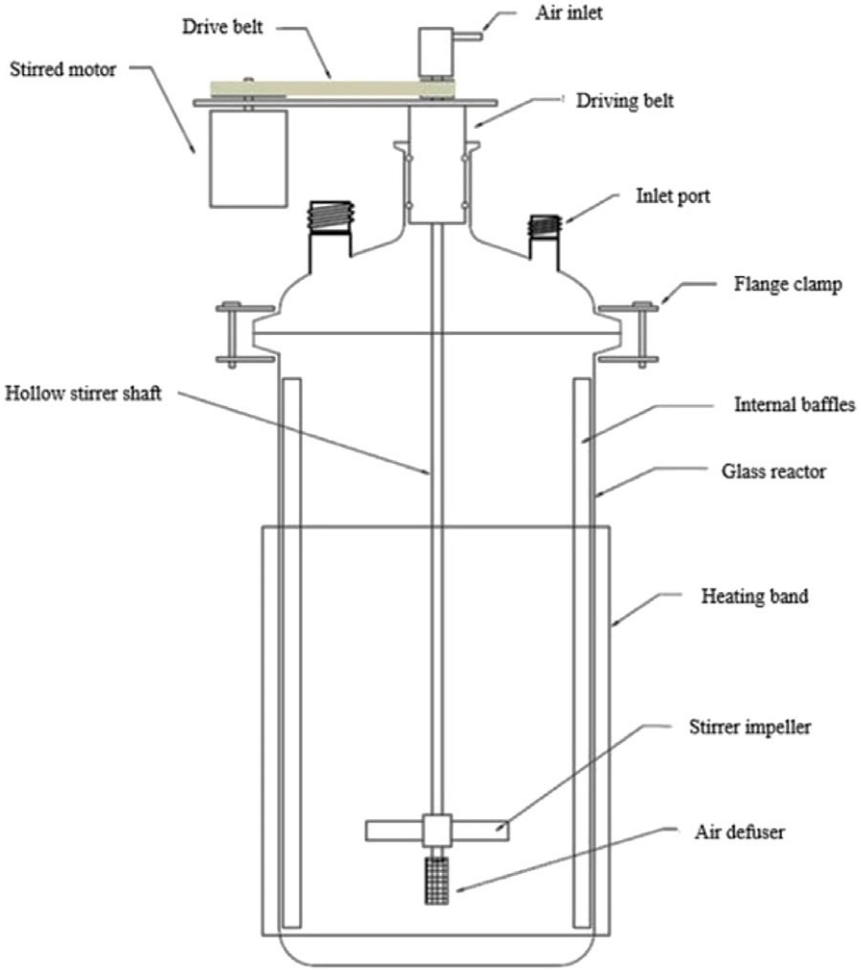

Figure 1. Diagram of a 10-L (glass vessel) custom-designed bioreactor for the mass production of *Steinernema yirgalemense*.

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Samples were taken daily, with 1 mL being diluted accordingly, and with the extent of recovery being measured, as described above. The presence and density of each life stage (IJ, J3/J4, males, females, J1/J2, and J2D) was also recorded each day using dilutions. Two counts were done for each sample taken each day. The total amount of nematode growth of the number of females, males, J1/J2, and IJ/J2D nematodes was presented as life stage/mL. The recovery data were presented as a mean percentage.

Data analyses

The data generated were analysed using the software program STATISTICA, version 14 (StatSoft Inc. 2016). Data from the life stages of the flask trials were assessed by means of a mixed-model analysis of variance (ANOVA) and Fischer's least significant difference (LSD). In each experimental trial, the main effects of the treatment and the test date were analysed separately. If a significant difference was detected between the trials concerned, the data involved could not be pooled, leading to it being analysed separately. The bioreactor growth curve was plotted using a 2D scatterplot and distance-weighted least squares.

Results

Population dynamics of S. yirgalemense *in shake flasks*

Total number of nematodes

Analysis of the data relating to the main effects of the total number of nematodes and days of development involved showed a significant difference ($F_{14, 42} = 6.82$; $\rho < 0.001$) between the three trials. No increase in the nematode numbers was observed on the first three days during recovery and development to J4, males, and females. A significant increase in the total number of nematodes was observed on day 5, however, with little increase being found to occur in the nematode numbers concerned, until day 13. The analysis of the data obtained in relation to the main effects of trials and the final number of IJs showed a significant difference ($F_{2, 7} = 30.64$; $\rho < 0.001$), leading to the data being analysed separately (Figure 2). Regarding the final number of IJs obtained, Trial 1 was significantly different from both Trial 2 ($\rho < 0.001$) and Trial 3 ($\rho < 0.001$), although Trials 2 and 3 were not significantly different ($\rho = 0.959$) from each other. The mean yield on termination of the trials (day 13) for Trial 1 was 423,333 IJs/mL, for Trial 2 was 254,333 IJs/mL, and for Trial 3 was 253,333 IJs/mL (Figure 2). The maximum yield of 456,000 IJs/mL was achieved in Trial 1, with the minimum being achieved in Trial 2, with 231,000 IJs/mL. The population of nematodes began to multiply by day 5 as the adult nematodes involved developed offspring.

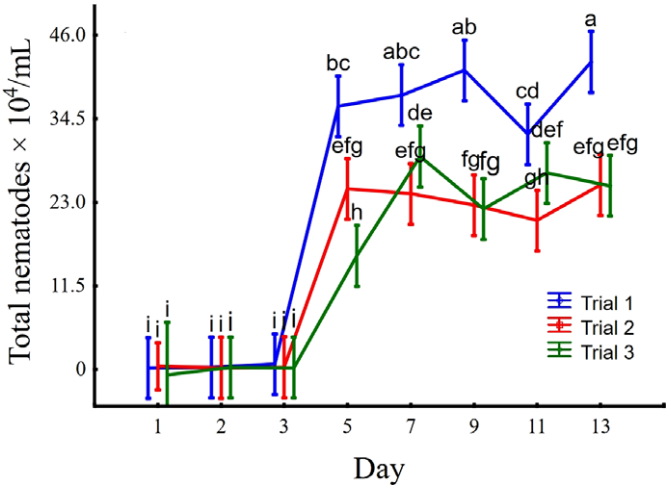

Figure 2. Population growth of all nematode life stages (95% confidence interval) of *Steinernema yirgalemense*/mL for three different trials, using 250-mL Erlenmeyer flasks (mixed-model ANOVA: $F_{14, 42} = 6.82$; $\rho < 0.001$). Letters that are the same indicate no significant difference ($\rho > 0.05$) between the days, the trials, and the total number of nematodes.

Number of IJs recovered

When the recovery of the inoculated IJs was analysed, the main effects of treatment and mean recovery, after three days, showed a significant difference, precluding pooling of the data obtained ($F_{2,6} = 56.18$; $\rho < 0.001$) (Figure 3a). Trial 1 indicated a significant difference in the degree of recovery experienced to that which was obtained for Trial 2 ($\rho = 0.008$) and Trial 3 ($\rho = 0.001$), with both trials showing a significant difference from each other ($\rho = 0.001$). When analysing the data relating to the main effects of treatment and the test date of the three trials in terms of the recovery of the

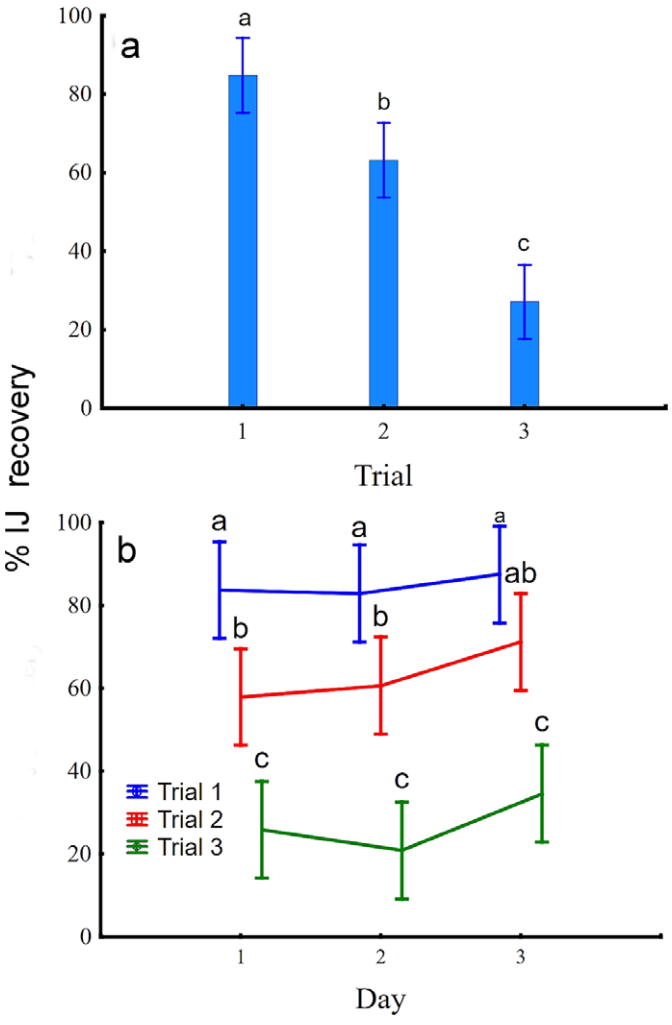

Figure 3. (a) Mean percentage (95% confidence interval) of infective juvenile (IJ) recovery for *Steinernema yirgalemense* in Erlenmeyer flasks (mixed-model ANOVA: $F_{2, 6} = 56.18$; $\rho < 0.001$). (b) Extent of IJ recovery for three separate trials, conducted over a period of 3 days in 250-mL Erlenmeyer flasks (mixed-model ANOVA: $F_{4, 12} = 0.43$; $\rho = 0.784$). Letters that are the same indicate no significant difference ($\rho > 0.05$) between the days, the trials, and the extent of IJ recovery.

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IJs concerned, no significance was found ($F_{4, 12} = 0.43$; $\rho = 0.784$). However, regarding the extent of recovery achieved on day 1, a significant difference was indicated between Trials 1 and 2 ($\rho = 0.004$), Trials 1 and 3 ($\rho = 0.001$), and Trials 2 and 3 ($\rho = 0.001$). For day 2, a significant difference was found between Trials 1 and 2 ($\rho = 0.011$), and between Trials 1 and 3 ($\rho < 0.001$) and Trials 2 and 3 ($\rho < 0.001$). For day 3, no significant difference was found between Trials 1 and 2 ($\rho = 0.052$). However, a significant difference was detected between Trials 1 and 3 ($\rho < 0.001$), and between Trials 2 and 3 ($\rho < 0.001$) (Figure 3). The mean recovery for the three Erlenmeyer flasks for Trial 1 by day 3 was 84.72%, for Trial 2 it was 63.27% and for Trial 3 it was 27.07%. The highest recovery was achieved by Trial 1, with a recovery of 94%, 24 h post IJ inoculation. The lowest recovery achieved was in the Trial 3 flask, with a recovery of 16%.

Number of females

When analysing the data for the three trials, in terms of the main effect of treatment and the total number of females/mL, a significant difference was found between the trials, preventing pooling of the data ($F_{2, 8} = 5.56$; $\rho = 0.033$) (Figure 4a). An analysis of the number of females in the three trials, with the main effects of treatment and test dates, showed a significant difference ($F_{14, 42} = 3.58$; $\rho < 0.001$). On day 1, no significant difference was found between the three trials ($\rho > 0.05$). On day 2, Trial 1 was found to be significantly different from Trial 2 ($\rho = 0.004$) and from Trial 3 ($\rho = 0.001$), with Trial 2 also being found to be significantly different from Trial 3 ($\rho = 0.020$). On day 3, Trial 1 was not found to differ significantly from Trial 2 ($\rho = 0.054$). However, Trial 1 was significantly different from Trial 3 ($\rho = 0.000$) and, similarly, Trial 2 was significantly different from Trial 3 ($\rho = 0.003$) (Figure 4b). The maximum number of reproducing females for the three flasks for each trial was observed on day 3 for all the trials concerned, as the recovery involved was staggered over the first three days post IJ inoculation. By day 3, Trial 1 contained a maximum of 2,250 females/mL, Trial 2 contained a maximum of 2,200 females/mL, and Trial 3 contained a maximum of 800 females/mL. In terms of the mean female concentration for each treatment, by day 3, Trial 1 contained 1917 females/mL, Trial 2 contained 1467 females/mL, and Trial 3 contained 617 females/mL.

Number of males

When analysing the data for the three trials, in terms of the main-effect treatment and the total number of males/mL, no significant difference was found between the trials ($F_{2,7} = 1.88$; $\rho = 0.218$) (Figure 5a). Analysis of the data, relating to the main effects of the treatment and to the test dates for the total number of males present for the three trials, showed a significant difference between the three trials ($F_{14, 42} = 2.21$; $\rho = 0.024$) (Figure 5b). On day 1, no significant

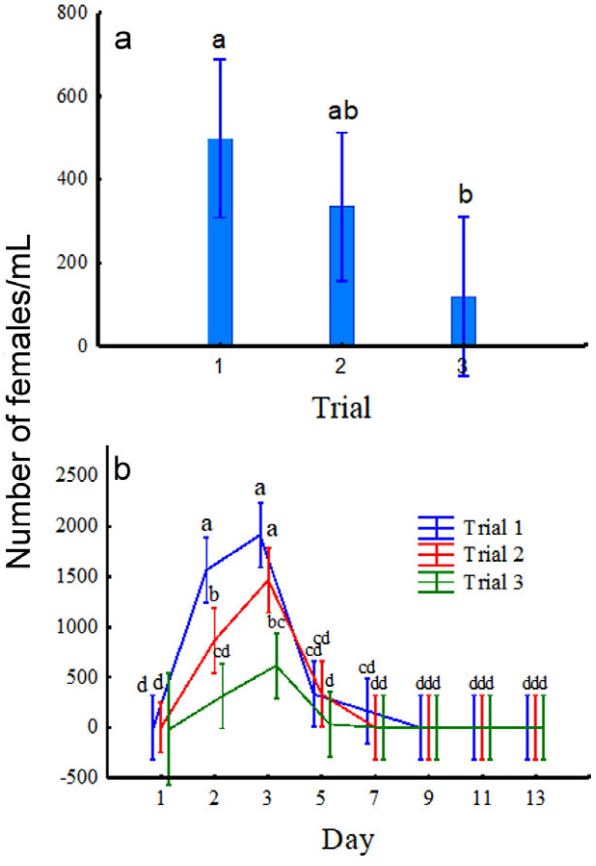

Figure 4. (a) Mean total number (95% confidence interval) of *Steinernema yirgalemense* females/mL held in 250-mL Erlenmeyer flasks, across three trials (mixed-model ANOVA: $F_{2, 8} = 5.56$; $\rho = 0.033$). (b) *Steinernema yirgalemense* females/mL for three trials, over 13 days (mixed-model ANOVA: $F_{14, 42} = 3.58$; $\rho < 0.001$). Letters that are the same indicate no significant difference ($\rho > 0.05$) between the trials, the days, and the number of females/mL concerned.

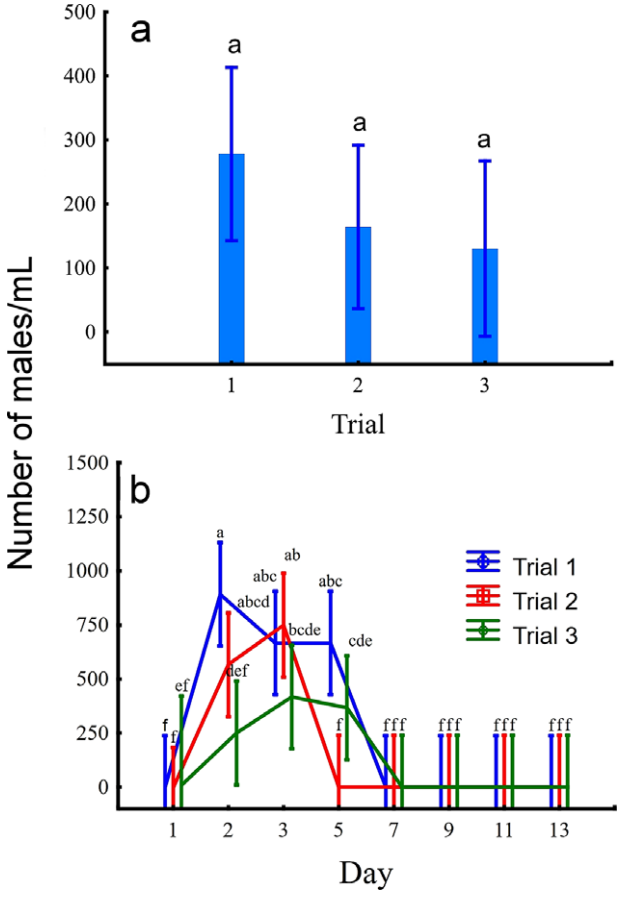

Figure 5. (a) Mean total number (95% confidence interval) of *Steinernema yirgalemense* males/mL across three trials, held in 250-mL Erlenmeyer flasks, on an orbital shaker (mixed-model ANOVA: $F_{2,7} = 1.88$; $\rho = 0.218$). (b) Difference between number of males/mL for the three trials, over 13 days (mixed-model ANOVA: $F_{14, 42} = 2.21$; $\rho = 0.024$). Letters that are the same indicate no significant difference ($\rho < 0.05$) between the trial and the number of males/mL.

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difference was found between the three trials (ρ = < 0.05). On day 2, Trial 1 was found not to be significantly different from Trial 2 (ρ = 0.059). However, although Trial 1 was significantly different from Trial 3 (ρ = 0.001), Trial 2 was not significantly different from Trial 3 (ρ = 0.068). On day 3, Trial 1 was not found to be significantly different from Trial 2 (ρ = 0.623), but Trial 1 was found to be significantly different from Trial 3 (ρ = 0.144); however, Trial 2 was not found to be significantly different from Trial 3 (ρ = 0.055).

Numbers of J1 and J2

When analysing the data for the three trials, in terms of the main effect of treatment and the total number of J1s and J2s/mL present, when disregarding the test date, no significant difference was found between the three trials ($F_{2, 7} = 1.10$; ρ = 0.382) (Figure 6a). When analysing the data for the main effects of treatment and the test dates for the total number of J1s and J2s, however, significant difference was found between the trials ($F_{14, 42} = 15.58$; ρ < 0.001). The presence of J1s and J2s was only recorded on days 5, 7, and 9. On day 5, Trial 1 was found to be significantly different from Trial 2 (ρ < 0.001), with it also being significantly different from Trial 3 (ρ = 0.000). In addition, Trial 2 was also significantly different from Trial 3 (ρ < 0.001). On day 7, Trial 1 was significantly different from both Trial 2 (ρ = 0.000) and Trial 3 (ρ < 0.001). However, Trial 2 was not significantly different from Trial 3 (ρ = 0.624). On day 9, no significant difference was found between the three trials involved (ρ > 0.05) (Figure 6b).

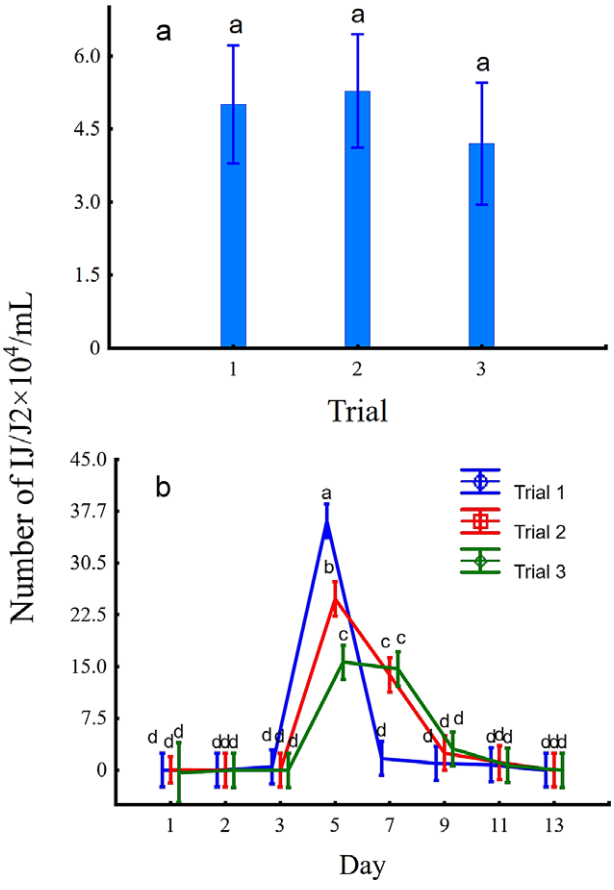

Figure 6. (a) Mean total number (95% confidence interval) of *Steinernema yirgalemense* (J1s/J2s/mL) between three trials in 250-mL Erlenmeyer flasks, on an orbital shaker (mixed-model ANOVA: $F_{2, 7} = 1.10$; ρ = 0.382). (b) The difference between the number of J1s/J2s/mL for the three trials over 13 days (mixed-model ANOVA: $F_{14, 42} = 15.58$; ρ < 0.001). Letters that are the same indicate no significant difference (ρ < 0.05) between the days, the trials, and the J1/J2/mL.

Numbers of J1 and J2D

When analysing the data for the three trials, the main effect of treatment and the IJs/J2D, significant difference was found between the three trials ($F_{2, 7} = 57.11$; ρ < 0.001) (Figure 7a). When analysing the data, the main effects of treatment, and the test dates for the total number of IJs and J2Ds, significant difference could be discerned between the trials involved ($F_{14, 42} = 17.05$; ρ < 0.001) (Figure 7a). For days 1 to 5, no significant difference appeared to be present between the trials (ρ > 0.05). However, significant difference was found between the trials from days 7 to 13. On day 7, Trial 1 showed significant difference from Trial 2 (ρ = 0.001) and from Trial 3 (ρ = 0.001). However, Trial 2 and Trial 3 were not significantly different (ρ = 0.056). On day 9, Trial 1 was significantly different from Trial 2 (ρ = 0.001) and from Trial 3 (ρ = 0.001). However, Trial 2 was not significantly different from Trial 3 (ρ = 0.618). On day 11, Trial 1 showed significant difference to both Trial 2 (ρ = 0.001) and Trial 3 (ρ = 0.021), while Trial 2 showed significant difference to Trial 3 (ρ = 0.003). On day 13, Trial 1 showed significant difference to Trial 2 (ρ = 0.001), and to Trial

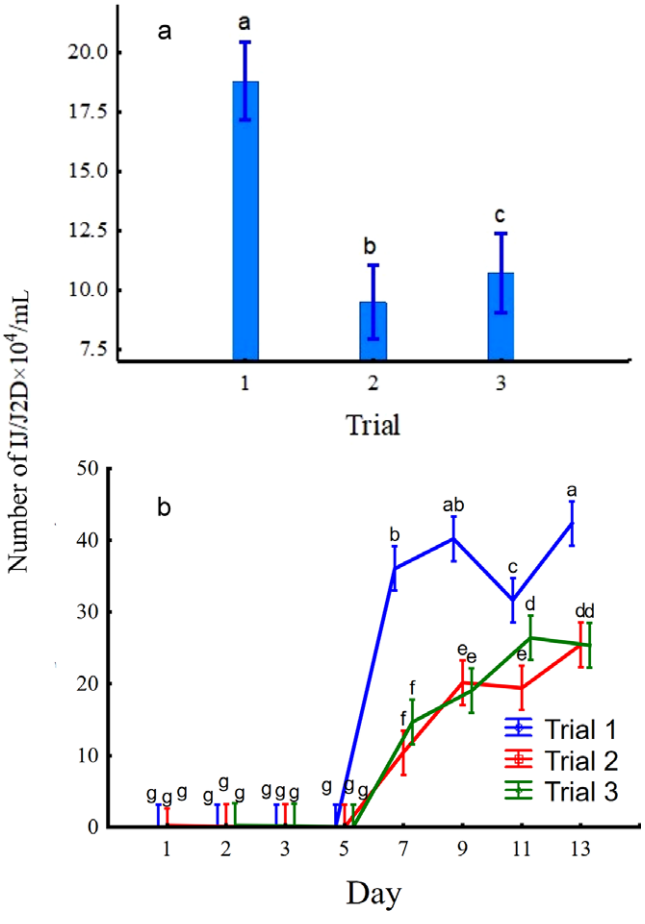

Figure 7. (a) Mean total number (95% confidence interval) of IJs/J2Ds of *Steinernema yirgalemense* (IJs/J2Ds/mL between three trials, in 250-mL Erlenmeyer flasks (mixed-model ANOVA: $F_{2, 7} = 57.11$; ρ < 0.001). Letters that are the same indicate no significant difference (ρ < 0.05) between trial and number of males/mL. (b) Mean total number of IJs/J2Ds/mL) for three trials, over 13 days (mixed-model ANOVA: $F_{14, 42} = 17.05$; ρ < 0.001). Letters that are the same indicate no significant difference (ρ < 0.05) between day, trial, and the number of IJs/J2Ds/mL.

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3 (ρ = 0.001). However, Trial 2 and Trial 3 showed no significant difference to each other (ρ = 0.959) (Figure 7b).

Culture in custom design desktop bioreactors

Out of a total of 11 bioreactor trials, two delivered final high yields of 2.28×10^5 and 2.94×10^5 IJs/mL. The trials were used to depict the growth of the nematodes in the bioreactor vessel (Fig. 8).

Discussion

The current study shows that recovery, population growth, and yield can differ greatly between different trials, presenting an issue of unpredictability when mass-producing *S. yirgalemense*. For the successful *in vitro* liquid culture of EPNs, securing rapid, synchronised, and high recovery results is crucial to the production of many reproducing adult nematodes, with it ultimately leading to high yields of pure IJs (Ehlers 2001; Hirao & Ehlers 2010; Ferreira *et al.* 2016) Asynchronous recovery results in a prolonged process time and in a mixed population of all of the different stages involved, which limits the final IJ yield (Yoo *et al.* 2001; Addis *et al.* 2016). For cost-effective commercial EPN production, even more important than the crucial obtaining of high IJ yields, is ensuring the consistency of yield and the stability of the production process (Ehlers 2001).

However, securing consistency in EPN liquids is difficult, as the recovery involved can be unpredictable, varying between different flasks and different trials, which tends to affect the ability to mass-produce EPNs to scale (Strauch and Ehlers 1998; Ferreira *et al.* 2016). An important aspect of EPN mass production research is providing consistency in the recovery and yield at flask level, prior to the bioreactor scale-up process (Ehlers 2001; Yoo *et al.* 2001; Strauch and Ehlers 1998).

For *in vivo* EPN production, almost 100% of the IJs involved tend to recover within 24 h (Ehlers 2001); however, such is not the case with *in vitro* liquid production due to the absence of the insect haemocoel food signal. However, prior inoculation of the symbiotic bacteria, 24 to 48 h before IJ inoculation, produces an unknown food signal that encourages and initiates recovery of the IJs (Strauch and Ehlers 1998; Aumann and Ehlers 2001; Hirao and Ehlers 2009), although such a food signal results in varied recovery over a few days (Strauch and Ehlers 1998). A similar finding was made for *S. yirgalemense* in the current study, with the results obtained confirming the high recovery rate, and the development of large numbers of reproducing adults, which is critical to the production of high yields, as described by Ehlers (2001), Strauch and Ehlers (1998), and Yoo *et al.* (2001). The recovery was, however, asynchronous, with the recovery ranging from 20% to 90%, illustrating the large variation between the trials involved. Ferreira *et al.* (2016) achieved recovery of 67% after 3 days, with, similarly, Addis *et al.* (2016) observing recovery of 63% to 75% after 72 h for *S. yirgalemense*. Thus, the recovery, in the present instance, was highly variable, which greatly affected the population development of *S. yirgalemense*. Notably, Addis *et al.* (2016) and Ferreira *et al.* (2016), both groups of researchers, used soy as the protein for the nutrient medium in which the nematodes were grown, whereas the present study used egg yolk (Dunn *et al.* 2022).

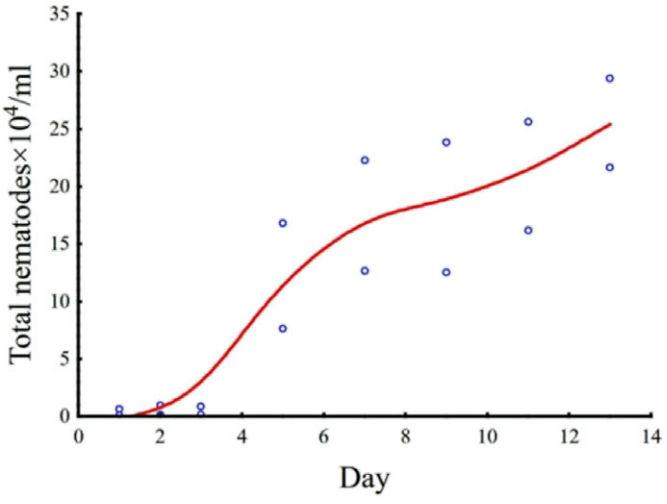

Figure 8. Scatter plot showing the growth of *Steinernema yirgalemense* in 10-L custom-designed bioreactor with 4 L of media, over a period of 14 days.

In the shake flasks, low recovery means the development of comparatively few reproductive females and males, resulting in lower yields than could otherwise be achieved. The results in the current study indicate that lower recovery results in low yield. However, such is not always the case, with the extent of yield attained seeming to be genera- or species-specific. Ehlers *et al.* (2000) found that recovery had no impact on yields for *Heterorhabditis indica* Poinar, indicating that adult nematodes can compensate for low recovery by producing comparatively more offspring per adult. The difference obtained may, then, lie in the mode of reproduction followed, with *Steinernema* spp. requiring sexual reproduction and the copulation of males and females, whereas *Heterorhabditis* spp. reproduce asexually in the first generation. The finding made in the above respect contradicts the work of Johnigk and Ehlers (1999) and Ferreira *et al.* (2014a), in which it was determined that the number of hermaphroditic adults affects the size of the F1 generation, as well as the final yield. The high variation and inconsistency found to be present are concerning from a commercial perspective, as consistency is key to the maintenance of a cost-effective and reliable EPN production process. High recovery tends to lead to a larger F1 population than would otherwise tend to be the case, with the same nutrient provision. Thus, increased recovery usually results in an increased number of reproductive females contributing offspring to the new generation, resulting in increased yields. Furthermore, low recovery can result in the development of an unwanted second generation, when there is a surplus of food or a high bacterial cell density, with a low bacterial cell density inducing the formation of IJs (Strauch *et al.* 1994; Hirao and Ehlers 2010), Fortunately, even with reduced recovery, a second generation was found to be absent from any of the flasks employed for the *S. yirgalemense* trials, with yields exceeding 250,000 IJs/mL being achieved in the case of almost every trial conducted.

The nematode population started multiplying between days 3 and 5, on the sterilisation of the females by the males, with the former beginning to produce F1 progeny of J1 and J2 nematodes, either through egg-laying into the liquid medium or via *endotokia matricida*. By day 5, the number of reproducing males and females had decreased, with the population present consisting of developing J1 and J2 nematodes, as well as of pre-infective J2Ds and new IJs. A new generation of IJs was first observed on day 7, with the population coming to consist only of IJs by day 13. Ferreira *et al.* (2016), similarly, observed population expansion on days 4 and 5, and a new generation of IJs by days 6 and 7. However, in the present study, the population size was substantially larger than it had been for Ferreira *et al.* (2016), leading to the available nutrients being

https://doi.org/10.1017/S0022149X24000452 Published online by Cambridge University Press

consumed more rapidly, and the processing time being decreased from 15 days (Ferreira *et al.* 2016) to 13.

Furthermore, Ferreira *et al.* (2016) found that the number of adult males and females had an initial spike of males and females after recovery, followed by a reduction in the number of females by day 4, which then, subsequently, once more increased between days 7 and 8 due to the development of an unwanted second and third generation. A similar trend was not seen in the current study, in which only one generation of males and females was observed. The second generation witnessed by Ferreira *et al.* (2016) explains the low yield obtained despite the decent recovery rate achieved. In the current study, a second generation did not develop for, possibly, one of two reasons. First, the protein source used in the present research was egg yolk, whereas, in the Ferreira *et al.* (2016) study, soy powder served as such a source. Dunn *et al.* (2022) showed that egg yolk was, indeed, a superior protein source for *S. yirgalemense* production. Second, the method of egg sterilisation employed in the current study and in that of Ferreira *et al.* (2016) differed slightly, in that, in the latter, sterilised eggs were added to agar plates with the symbiotic bacteria, whereas, in the present instance, the eggs were inoculated into flasks. The slight change, coupled with the inferior protein source used, might have influenced the number of nematodes produced in the study conducted by Ferreira *et al.* (2016), which, possibly explains the presence of the second generation.

Developing a standard protocol in flasks has been followed by the development of a standard protocol for bioreactor production, as well as of the optimal design of the bioreactor vessel (Neves *et al.* 2001). Many existing commercial companies (Ehlers 2001; Ravensberg 2011; Devi 2018; Dunn *et al.* 2021a) conduct EPN bioreactor mass production. However, due to the commercial sensitivity of such mass production, the available literature on the subject is scarce (Dunn *et al.* 2021a), and new nematode mass-producing teams tend to have scant access to the appropriate information to guide them in developing new products.

In the current study, a custom-made 10-L bioreactor was designed, with it forming the first step in the scale-up process from flask to bioreactor. In total, 24 bioreactor trials were conducted, of which 11 were successful, in that they achieved a yield of some sort, with a maximum yield of 294,000 IJs/mL being obtained. The population dynamics were only assessed for two runs, as, previously, a sampling port had not been added due to sterility concerns. With the addition of a sampling port, it was possible to obtain the required population growth data.

Conclusion

The mass production of a South African EPN isolate is a positive development for the biopesticide and biocontrol industries of the country, as well as for the sustainable agricultural movement across the entire African continent. Commercialising EPNs as a biopesticide product is a difficult task, with it requiring collaboration with a team of scientists to spur on rapid product development. The results and proof of concept achieved here hold promise for the establishment of a local EPN mass production facility in South Africa. However, the recovery of the IJs and the final IJ yield requires fine-tuning to be able to establish consistently high yield, with low variation between replicates. More in-depth studies of the population dynamics, the nutritional requirements, the oxygen provision, and the nematode behaviour, as well as state-of-the-art commercially available bioreactors for data capture, are required to reduce the variation seen here. However, this study encourages the further development of a local EPN isolate.

Acknowledgements. DG Nel, from the Centre of Statistical Consultation, Stellenbosch, South Africa, for assistance with statistical analysis.

Financial support. This study was funded by Hortgro Science South Africa and by the South African Table Grape Institute (SATI).

Competing interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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