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In vitro liquid culture of the mollusc-parasitic nematode *Phasmarhabditis* (Rhabditida: Rhabditidae)

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Abstract

The success of the mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider) Andrássy (Rhabditida: Rhabditidae), as a biological control agent in Europe has led to worldwide interest in phasmarhabditids as biocontrol agents. In this study, the mass culture potential of three phasmarhabditids, namely *Phasmarhabditis papillosa*, *Phasmarhabditis kenyaensis* and *Phasmarhabditis bohemica*, was assessed. In addition, ten bacterial candidates, consisting of seven associated with slugs and three associated with entomopathogenic nematodes, were investigated. The bacteria were tested for their ability to cause mortality to *Deroceras invadens*, as well as to support nematode growth. Initial mortality studies demonstrated that *Kluyvera*, *Aeromonas* and *Pseudomonas* spp. (AP3) caused 100% mortality when they were injected into the haemocoel of *D. invadens*. However, in growth studies, *Pseudomonas* sp. (AP4) was found to be the most successful bacterium, leading to recovery and reproduction in almost all nematode species, except for *P. kenyaensis*. In flask studies, *P. bohemica*, which showed exceptional growth with *Pseudomonas* sp. (AP1), was chosen for further investigation. The effect of inoculating flasks with different concentrations of *Pseudomonas* sp. (AP1), as well as with different concentrations of *P. bohemica*, was evaluated by assessing the nematode populations for 14 days. The results indicated that the lowest, 1% (v/v), bacteria inoculation led to higher total nematode and to infective juvenile (IJ) yield, with flasks with the highest IJ inoculum (3000 IJs/ml) having a positive effect on the total number of nematodes and IJs in cultures of *P. bohemica*. This study presents improvements for the mass-culturing of nematodes associated with molluscs.

Introduction

Terrestrial molluscs (slugs and snails) (Mollusca: Gastropoda), of which several species are a major pest of many crops, are mostly controlled using chemical molluscicides. One possible alternative for the control of molluscs is biocontrol using mollusc-parasitic nematodes (MPNs), with *Phasmarhabditis hermaphrodita* (Schneider) Andrássy (Rhabditida: Rhabditidae) being one of two MPN species to have so far been commercially developed (Sheehy *et al.*, 2022). The nematode is currently mass-produced and sold by BASF (formally Becker Underwood) and Dudutech, under the tradenames Nemaslug® and SlugTech®, respectively (Rae *et al.*, 2007; Ross, 2019). The nematode is capable of parasitizing various mollusc species from the families Agriolimacidae, Limacidae, Arionidae, Vagnulidae and Milacidae (Wilson *et al.*, 1993; Iglesias & Speiser, 2001; Speiser *et al.*, 2001; Grewal *et al.*, 2003). The nematode is sold in a formulation, with the active ingredient being the infective juvenile (IJ) stage, which, when suspended in water and applied, searches the soil for slug hosts, causing death 4–21 days after infection (Wilson *et al.*, 1993; Tan & Grewal, 2001).

Phasmarhabditis hermaphrodita has been mass-produced using both *in vivo* and *in vitro* methods, although the former production method involves the field collection or rearing of mollusc hosts in the laboratory, which is not economically viable. Therefore, the majority of research so far has focused on the *in vitro* production of the nematode, either in xenic cultures, with a mix of unknown bacteria, or in monoxenic cultures, using only one pure bacterial species (Wilson *et al.*, 1995a). The use of monoxenic cultures, however, is known to offer more predictable results, with it being more effective in producing a high number of IJs, with consistent pathogenicity (Wilson *et al.*, 1995a; Ehlers & Shapiro-Ilan, 2005).

In the search for a bacterial species for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) tested the pathogenicity of nine bacterial isolates, which were isolated from within dauer larvae of *P. hermaphrodita*, by means of injecting 10 µl of each into the haemocoel of *Deroceras reticulatum* Müller (Agriolimacidae). Of the bacterial isolates tested,

only *Aeromonas hydrophila* (Chester) Stanier (Proteobacteria: Aeromonadacaea) and *Pseudomonas fluorescens* (Flügge) Migula (Proteobacteria: Pseudomonadacea) caused significant mortality in the slugs. However, as *A. hydrophila* has not been proven to support the growth of *P. hermaphrodita*, it was not considered as a monoxenic bacterial candidate. Bacterial isolates that supported the growth of *P. hermaphrodita* were then used in monoxenic foam chip cultures and/or liquid cultures, in combination with the nematode concerned, after which the pathogenicity of the nematode was tested on *D. reticulatum* in a soil-based bioassay system. The results indicated that *P. hermaphrodita* caused significant mortality in slugs, producing the highest yields of IJs when grown together with the bacterial isolate *Moraxella osloensis* (Bøvre and Henriksen) Bøvre (Proteobacteria: Moraxellaceae), even though it did not cause significant mortality when injected into the haemocoel of the slug. The above-mentioned bacterial species was, therefore, chosen for the commercial production of *P. hermaphrodita* (Wilson *et al.*, 1995b).

The BASF *P. hermaphrodita* product is produced in large-scale fermenters using *in vitro* liquid culture, like that which is used for the commercial production of entomopathogenic nematodes (EPNs), and in a monotonic association with the bacterium species *M. osloensis* (Morand *et al.*, 2004; Rae *et al.*, 2007). It has however recently been discovered that the bacterial isolate used for the commercial production of *P. hermaphrodita* was possibly misidentified and is *Psychrobacter faecalis* Kämpfer, Albrecht, Buczolits and Busse (Proteobacteria: Moraxellaceae) (Sheehy *et al.*, 2022). Establishing a monotonic nematode–bacteria combination that can be grown in a liquid medium is essential for the consistent production of high IJ yields, and to be able to eliminate the risk posed by the presence of the other pathogenic bacteria in the medium (Poinar & Hansen, 1986; Wilson *et al.*, 1995a). The IJ life stage is produced when stressful environmental factors occur, or when the food sources become depleted (Ross, 2010). Depending on the growth medium concerned, and on the prevailing conditions in the bioreactors, different levels of IJ concentrations are produced, with yields of over 100,000 IJs/ml having been achieved (Glen *et al.*, 1994).

The discovery of the potential of nematodes as the biocontrol agents of insect and mollusc pests has led to widescale interest in the use of various methods for their mass production. In addition, a vast body of research exists on the mass production techniques used for EPNs, which can be translated into the mass-culturing of MPNs. Depending on the costs, time, resources, expertise and amount of product required, EPNs can be mass-produced, using *in vivo* or *in vitro* solid/liquid culturing methods (Ehlers & Shapiro-Ilan, 2005; El-Sadawy, 2011; Abd-Elgawad *et al.*, 2017). Although *in vivo* and *in vitro* solid culturing methods are labour-intensive, they require relatively little capital investment, whereas the *in vitro* liquid culture is the method of choice for large companies in developed countries, with the method requiring sophisticated engineering, a high level of capital investment and running costs, and close monitoring (Ehlers & Shapiro-Ilan, 2005; Lacey & Georgis, 2012; Dunn *et al.*, 2021).

The aim of the current study was to isolate, identify, culture and test the pathogenicity of bacterial isolates capable of supporting the *in vitro* culture of three MPN species, namely *P. papillosa*, *P. kenyaensis* and *P. bohemica*. In addition, the study also focused on optimizing the *in vitro* liquid culture method of successful nematode–bacteria candidates, by means of testing the effect of bacteria inoculum and IJ inoculum density on the total nematode/IJ yield and on the proportion of IJs found to be present after 14 days.

Materials and methods

Source of nematodes

The nematode species used for this study include *P. bohemica* Nermuť, Půža, Mekete & Mráček (Nematoda: Rhabditidae), *P. papillosa* (Schneider) Andrássy (Nematoda: Rhabditidae) and *P. kenyaensis* Pieterse, Rowson, Tiedt, Malan, Haukeland and Ross (Nematoda: Rhabditidae). The *P. papillosa*, which were obtained from a collection held by the Department of Conservation Ecology and Entomology at Stellenbosch University, were initially collected during a survey by Pieterse *et al.* (2017a, b). *Phasmarhabditis papillosa* is a gonochoristic nematode that has been proven to cause mortality in slugs. The *P. bohemica* isolate used in the study, which was obtained from the Biology Centre CAS, Institute of Entomology ASCR, Laboratory of Entomopathogenic Nematodes, Czech Republic, was isolated during a study conducted by Nermuť *et al.* (2017) and is a gonochoristic, facultative MPN. The *P. kenyaensis*, which was obtained from the International Centre of Insect Physiology and Ecology (icipe), Kenya, is a gonochoristic nematode which was obtained during a study conducted by Pieterse *et al.* (2020). Its pathogenicity to molluscs has not yet been tested.

Isolation and source of bacterial isolates

A total of ten bacterial isolates were used in the current study. Five bacterial candidates were isolated by means of dissecting *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Mollusca: Agriolimacidae) which were found dead in the field, by swabbing the intestine and body cavity, and streaking bacteria onto nutrient agar plates (3 g beef extract, 5 g Tryptone, 8 g sodium chloride (NaCl), 15 g agar in 1 l of water, autoclaved for 20 min at 121°C), which were incubated at 25°C for 48 h. The plates were then visually inspected, with different bacterial colonies being sub-cultured onto fresh plates, until only a single species remained on each plate. Five of these isolates were chosen at random for this study. Two additional bacterial isolates, which were obtained from a collection held by the Department of Conservation Ecology and Entomology at Stellenbosch University, were collected during a similar survey conducted by Pieterse (2016). These isolates were initially obtained by swabbing the intestines of dissected *Deroceras reticulatum* (table 1). Three EPN-associated bacterial species were included in the study and were also obtained from the collection held by the same department at Stellenbosch University and were isolated from EPNs in previous studies. *Photorhabdus heterorhabditis* Ferreira, Van Reenen, Endo, Tailliez, Pagès, Spröer, Malan & Dicks was isolated from the EPN species *Heterorhabditis zealandica* Poinar (Ferreira *et al.*, 2014), *Photorhabdus luminescens* subsp. *noenieputensis* Ferreira, Van Reenen, Pagès, Tailliez, Malan & Dicks from *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt (Ferreira *et al.*, 2013a) and *Xenorhabdus khoisanae* Ferreira, Van Reenen, Endo, Spröer, Malan & Dicks from *Steinernema khoisanae* Nguyen, Malan & Gozel (Ferreira *et al.*, 2013b) (table 1).

Identification of bacteria

Bacterial isolates isolated from dissected *D. invadens* in the present study were identified by means of extracting the total genomic DNA, using a Zymo Research fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). The DNA of the 16S rRNA gene was amplified, using the primer

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Table 1. The partial 16S rRNA gene accession numbers of seven bacterial isolates from *Deroceras invadens* collected from sample sites in George, Western Cape, South Africa and three bacterial isolates collected from entomopathogenic nematodes obtained from the collection housed at the Department of Conservation Ecology and Entomology at Stellenbosch University, with United States National Center for Biotechnology Information (NCBI) matches and with identity and coverage of between 98% and 100%.

					NCBI match			
Bacterial isolate	GenBank accession number	Origin	Isolated from	Strain/ isolate	Species/strain	GenBank accession number	Query coverage (%)	Percentage identity (%)
Kluyvera sp.	KX531097	George	*Deroceras reticulatum*	SABACT6	*Kluyvera intermedia* TCM1238	NR112007	–	–
Pseudomonas sp. (AP1)	KX531096	George	*D. reticulatum*	SABACT5	*Pseudomonas fragi* M0421	KF924232	–	–
Pseudomonas sp. (AP2)[a]	MN611311	George	*D. reticulatum*	SA1	*Pseudomonas* sp. H3-5	MN197816	100	99.00
Pseudomonas sp. (AP3)[a]	MN611355	George	*D. invadens*	SA1D1	*Pseudomonas* sp. 36 DCP	MK072848	100	99.78
Pseudomonas sp. (AP4)[a]	MN611356	George	*D. invadens*	SA1Z1	*Pseudomonas* sp. S3Bt34y	MH463748	100	99.28
Aeromonas sp.[a]	MN611353	George	*D. invadens*	SA1	*Aeromonas salmonicida* SHY16-3432	CP038102	100	99.29
Buttiauxella sp.[a]	MN611312	George	Snails		*Buttiauxella* sp. P5	DQ223872	98	99.15
Photorhabdus heterorhabditis	HQ142626	Patensie	*Heterorhabditis zealandica*	SF41	SF41^T	HQ142626	–	–
P. luminescens subsp. *noenieputensis*	JQ424880	Noenieput	*Heterorhabditis noenieputensis*	SF669	AM7^T	JQ424880	–	–
Xenorhabdus khoisanae	HQ142625	Grabouw	*Steinernema khoisanae*	SF87	SF87^T	HQ142625	–	–

[a]Identified during this study.

pair 8F and 1512R (Felske *et al.*, 1997). The amplified products were then sequenced by the Central Analytical Facilities at Stellenbosch University. Sequence traces were inspected and assembled, using the software CLC Main Workbench 7.6.4 (CLC Bio, Aarhus, Denmark, http://www.clcbio/products/clc-main-workbench/) and analysed using the Basic Local Alignment Search Tool of the United States National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (table 1). Sequences were then uploaded to the GenBank database (http://www.ncbi.nlm.nih.gov/) at NCBI.

Bacteria pathogenicity

Bacterial isolates were streaked onto nutrient agar plates (3 g beef extract, 5 g Tryptone, 8 g NaCl, 15 g agar in 1 l water), autoclaved at 121°C for 20 min and grown at 25°C for 24 h. The plates were then washed with 5 ml autoclaved 0.9% saline solution under sterile conditions, with 10 µl of each bacterial isolate being injected directly into the haemocoel of ten *D. invadens* specimens. The control slugs were injected with 10 µl sterile 0.9% saline solution. After having been treated in terms of each bacterial isolate, ten slugs were placed in plastic containers (145 mm × 205 mm × 80 mm) lined with moist tissue paper, each of which was then covered with a perforated lid. The boxes were kept at 18°C for five days, with the slugs being provided with carrot discs as their food source, which was replaced daily. After five days, the number of dead slugs was counted. The experiment was repeated with a fresh batch of slugs.

Bacterial feeding preference

Each of the ten bacterial isolates detailed above was streaked onto five Wout's agar plates (9.5-cm-diam.) in turn and left at 25°C for 24 h (Wouts, 1981). Wout's agar plates were used to support bacterial and nematode growth. A hundred IJs of the three nematode species (*P. bohemica*, *P. papillosa* and *P. kenyaensis*), contained in approximately200 µl distilled water, were added to the plates. Nematodes were washed with distilled water before being added to the plates. After covering the plates with parafilm and leaving them at 18°C for five days, the plates were visually inspected with a light microscope (Leica MZ7s), to determine whether the nematodes had survived, recovered and reproduced in association with the different bacterial isolates. A recovered IJ refers to an IJ that reacts on an environmental stimulus to open its mouth and start feeding. The experiment was repeated three times, on different test dates. The plates were scored based on four categories: dead (0); alive (1); recovered (2); and reproduced (3). Plates categorized as 'dead' contained only dead IJs (100%), whereas those categorized as 'Alive' showed that, despite the IJs involved having survived their treatment, the individuals concerned neither recovered nor developed. Those plates that were categorized as 'recovered' showed that the IJs present had developed into adults, but

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had not reproduced, while the contents of those that were categorized as 'reproduced' showed the presence of adult nematodes, along with next-generation juveniles (J1–J3).

Axenization of nematodes

As *P. bohemica* showed good survival, recovery and reproduction rates among a range of bacterial isolates (see the previous subsection), it was used in the monoxenic cultures. To establish such cultures, the eggs were harvested from adult female nematodes. IJs were added to 9.5-cm- diam. Petri dishes, lined with a piece of moist filter paper, containing freeze-killed *D. invadens*. The Petri dishes were sealed with parafilm and left at 18°C. The IJs of *P. bohemica* were then left for 96 h to develop into adult males and females, and to enable the females to be able to form fertilized eggs. The plates were then washed with saline solution and passed through a 212-µm aperture sieve to remove any life stages other than that of the adult females. The adult females were added to Eppendorf tubes, in which they were lightly crushed for 30 sec with an autoclaved plastic grinding rod. A mixture of bleach and sodium hydroxide (NaOH) (2.25 ml bleach, 0.2 g NaOH in 10 ml distilled autoclaved water) was added to the tubes, with it being left to work on the nematode mix for 8 min. The mixture in the tubes was then centrifuged and washed three times with sterile water, to remove all bleach and NaOH solution, until only a pellet remained with eggs in the water. After pipetting the mixture into 200 µl sterile tryptone soy broth in 24-well plates, they were sealed with parafilm and left for two days at 25°C, so as to allow for testing for bacterial contamination. If, after two days, no bacterial growth had occurred, and the nematodes concerned had hatched out, the J1 and J2 nematodes were used as the inoculation for liquid media culturing.

Establishing a liquid culture

For the liquid culture experiments, the bacterial isolates *Pseudomonas* sp. (AP1) (GenBank accession number: KX531096) and *P. bohemica* were used, as they had demonstrated a good response in the experiment so far (see the bacterial feeding preference subsection above). Previous research on *Steinernema jeffreyense* Malan, Knoetze & Tiedt proved that the use of 30 ml media as opposed to 50 ml media in 250-ml flasks resulted in increased IJ recovery and yield ascribed to efficient oxygen exchange (Dunn *et al.*, 2020). Thirty ml of Luria broth (LB) (10 g NaCl, 10 g Tryptone, 5 g yeast extract/L water, autoclaved at 121°C for 20 min) in a 250-ml Erlenmeyer flask was thus inoculated with *Pseudomonas* sp. (AP1) and incubated on an orbital shaker (140 rpm), at 28°C for 48 h. A 250-ml Erlenmeyer flask containing 30 ml liquid culture medium (LCM) (9 g pig kidney, 17.4 g yeast extract, 8.6 g egg yolk powder, 52.6 g sunflower oil/L water, autoclaved for 20 min at 121°C) was then inoculated with 1.2 ml (4%) of *Pseudomonas* sp. (AP1) in LB. The flask was again incubated on an orbital shaker (140 rpm) at 28°C for 48 h. Once the bacteria had grown for 48 h inside the LCM, the sterile eggs and juvenile stages obtained after the axenization of the nematodes were added to the flasks. The flasks were then incubated at 18°C on orbital shakers at 140 rpm for 14 days, at which point most of the population in the flask were at the IJ stage. The flask involved was used as inoculum for the following experiments, with it coming, hereinafter, to be referred to as the 'inoculum flask'.

Bacterial inoculum concentration

To test the impact of bacteria inoculum density, nine 250-ml Erlenmeyer flasks containing 30 ml LCM were inoculated with three different *Pseudomonas* sp. (AP1) inoculum concentrations, consisting of 1%, 3% or 5%. After three replicate flasks were set up per treatment, *P. bohemica* IJs from the inoculum flask were added to them all, to achieve an initial nematode density of 2000 IJs/ml. All nine flasks were incubated at 18°C on an orbital shaker (140 rpm) that was kept in the dark for 14 days. Every two days, the total number of nematodes and of IJs was recorded by taking a 200 µl aliquot from each flask. Aliquots were then observed under a light microscope (Leica MZ7s) to examine and count the nematodes present in each flask. The experiment was conducted twice.

Nematode inoculum concentration

To test the impact of nematode inoculum density, nine 250-ml Erlenmeyer flasks containing 30 ml LCM were inoculated with 1.2 ml (4%) *Pseudomonas* sp. (AP1) in LB, after which they were incubated on an orbital shaker (140 rpm) at 28°C for 48 h. Three treatments were established using the *P. bohemica* inoculum flask: 1000 IJs/ml, 2000 IJs/ml, and 3000 IJs/ml, with three replicate flasks per treatment. The flasks were incubated at 18°C on an orbital shaker (140 rpm) that was kept in the dark for 14 days. Every two days, the total number of nematodes and of IJs was recorded by taking a 200 µl aliquot from each flask. Aliquots were then observed under a light microscope (Leica MZ7s) to examine and enumerate the nematodes present in each flask. The experiment was conducted twice.

Statistical analysis

Obtaining the bacterial pathogenicity data involved using a generalized linear model with a Poisson distribution and a log link function. The findings made were confirmed by means of conducting a log-likelihood ratio test, giving a Chi-squared value of 2.7844 and $P < 0.001$. The liquid culture data were then analysed, using a repeated-measures analysis of variance, with the compound symmetry assumption being performed on the correlations over the days concerned.

Results

Identification of bacteria

The five bacterial isolates, consisting of three *Pseudomonas* spp. (AP2, AP3 and AP4), an *Aeromonas* sp. and a *Buttiauxella* sp., as used in the current study, were identified from the intestine and body cavity of *D. invadens*, with the 16S rRNA sequences generated being submitted to GenBank (table 1). GenBank details of the additional slug-associated bacterial species and of the EPN-associated bacterial species used in the study are indicated in table 1.

Bacteria pathogenicity

Bacterial isolates were tested on 20 slugs each. All the bacterial isolates tested, apart from for the *Buttiauxella* sp., caused significant mortality ($F_{10,11} = 35.800$; $P < 0.001$) in the slug host *D. invadens* in the current study. *Pseudomonas* sp. (AP3), *Kluyvera* sp. and *Aeromonas* sp. all caused 100% mortality five days after

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they were injected into the slugs. *Photorhabdus heterorhabditis* caused mortality in 16 slugs and *Pseudomonas* sp. (AP4), *Xenorhabdus khoisanae* and *Pseudomonas* sp. (AP1) caused mortality in eight of the 20 slugs studied after five days. *Pseudomonas* sp. (AP2), *Photorhabdus luminescens* and *Buttiauxella* sp. were found to be the least pathogenic, with *Pseudomonas* sp. (AP2) and *P. luminescens* only managing to kill four slugs, and *Buttiauxella* sp. managing to kill none (fig. 1).

Bacterial feeding preference

Phasmarhabditis bohemica was able to reproduce when feeding on the *Pseudomonas* sp. (AP1), (AP2), (AP3) and (AP4) and on the *Buttiauxella* sp., as well as on the *P. luminescens* and *X. khoisanae* from the EPNs. Although the IJs recovered and developed into adults, they did not reproduce when feeding on *Kluyvera* sp. When tested with *Aeromonas* sp. and *P. heterorhabditis*, the nematodes survived, although they remained in their IJ phase (table 2).

Phasmarhabditis papillosa only reproduced when they were tested with *Pseudomonas* sp. (AP4). When tested with *Pseudomonas* species (AP1), (AP2) and (AP3), as well as with *Kluyvera* sp., *Aeromonas* sp. and *X. khoisanae*, the IJs, despite developing into adult males and females, did not reproduce. When tested with *Buttiauxella* sp. and *P. luminescens*, the *P. papillosa* IJs survived, but did not recover. In the case of *P. heterorhabditis*, all the IJs involved died (table 2).

Phasmarhabditis kenyaensis reproduced when tested with *Pseudomonas* sp. (AP1), *Kluyvera* sp. and *Buttiauxella* sp., but died when it was tested with any of the other bacterial candidates (table 2).

Bacterial inoculum concentration

The analysis of the results using different bacterial inoculum concentrations showed a significant difference between the total numbers of nematodes of all developmental stages yielded ($F_{30,180} = 3023$; $P < 0.001$) after 14 days. All flasks displayed exponential growth in terms of the combination of *P. bohemica* and *Pseudomonas* sp. (AP1), regarding the total number of nematodes between days 2 and 6. The rate of growth slowed down between days 6 and 10, and even more between days 10 and 14. The flasks that were inoculated with 3% and 5% bacteria seemingly neared a stationary phase in total nematode numbers by day 14. The flasks containing 1% bacterial inoculum exhibited a significantly higher ($P < 0.05$) total number of nematodes throughout the experimental period than did the other flasks, reaching an average count of 63,126 ± 165 nematodes/ml on day 14. The flasks inoculated with 5% bacteria contained the second highest total amount of nematodes throughout the experiment, reaching an average count of 42,340 ± 167 nematodes/ml on day 14. In contrast, the flasks inoculated with 3% bacteria had the lowest total nematode yield throughout the experiment, reaching only 34,965 ± 146 nematodes/ml by day 14 (fig. 2A).

Analysis of the results using different bacterial inoculum concentrations showed a significant difference between the numbers of IJs ($F_{30,180} = 336.23$; $P < 0.001$) over a period of 14 days. Very low IJ numbers were observed from day 2 to day 6, by which time most of the IJs had recovered and developed into other stages. However, by day 8, the population numbers reached a point where IJ formation was again induced, causing a sharp increase in IJ numbers for all the bacterial concentrations involved. The formation of IJs then slowed down again for all flasks from day 12 onwards, reaching an almost stationary phase by day 14. The flasks inoculated with 1% bacteria had a significantly ($P < 0.001$) higher total number of IJs (61,515 ± 941 IJs/ml) on day 14 than did those that were inoculated with 3% or 5% bacteria (fig. 2B). The flasks inoculated with 5% bacteria had 41,270 ± 610 IJs/ml on day 14, whereas the flasks that were inoculated with 3% had the lowest number of IJs, with a concentration of only 32,909 ± 546 IJs/ml (fig. 2B).

The proportion of IJs analysed showed a significant difference ($F_{30,180} = 38.99$; $P < 0.001$) after the 14-day period. A sharp decrease in the IJ proportion was seen from day 2 to day 4, with the proportion slowing down by day 6, and reaching a low at day 8, as the IJs recovered and the other life stages came to comprise a larger portion of the population than before. However, between days 8 and 12, an exponential increase occurred in the IJ proportion of the population, as overcrowding in the flasks induced the formation of the IJ stage. By day 12, the increase in IJ proportion slowed down, again nearing a stationary phase by day 14. The flasks inoculated with 1% and 5% bacteria had the same proportion of IJs on day 14, both reaching a total of 97 ± 1.5% IJs. The proportion was significantly higher ($P < 0.001$) than it was for the IJ proportion of flasks inoculated with 3% bacteria, which yielded 94 ± 1.5% IJs (fig. 2C).

Nematode inoculum concentration

Analysis of the total number of nematodes of all developmental stages over a 14-day period showed a significant difference ($F_{180,30} = 3023$; $P < 0.001$). The flasks in the IJ inoculum density experiment showed a low total number of nematodes on day 4, which slowly increased between days 4 and 6, and then showed exponential growth between days 6 and 8. However, after day 8, the increase in the total number of nematodes started to slow down, and to near a stationary phase by day 14 in all the flasks. The flasks inoculated with 3000 IJs/ml showed a rise in total nematode numbers after day 6, yielding a significantly higher total number of nematodes (45,156 ± 138 nematodes/ml) by day 14 than did the other inoculum concentrations. Flasks inoculated with 1000 IJs/ml yielded 26,849 ± 154 nematodes/ml, which rate was also significantly higher ($P < 0.05$) than was that of the 24,110 ± 149 nematodes/ml yield of flasks inoculated with 2000 IJs/ml (fig 3A).

The number of IJs present over the 14 days differed significantly ($F_{30,180} = 336.23$; $P < 0.001$), with all the flasks showing a slow decrease in the total number of IJs from day 2 onwards, as the IJs recovered and developed into other life stages. The number of IJs reached a low at day 8, after which a sharp increase in numbers was observed in all the flasks between days 8 and 12, as overcrowding in the flasks induced the formation of IJs. The formation of IJs slowed down from day 12 onwards, nearing a plateau by day 14. The flasks inoculated with 3000 IJs/ml had significantly higher numbers of IJs from day 10 onwards, reaching a high of 37,177 ± 796 IJs/ml by day 14. No significant difference ($P > 0.05$) was found in the total number of IJs in the flasks inoculated with 1000 IJs/ml (22,038 IJs/ml) and in the flasks inoculated with 2000 IJs/ml (21,621 IJs/ml) (fig. 3B).

The analysis of the proportion of IJs showed significant difference over the 14-day period ($F_{30,180} = 38.99$; $P < 0.001$). On days 10 and 12, significant differences ($P < 0.05$) were found in the IJ proportions of the three treatments, with the flasks inoculated with 2000 IJs/ml having the highest proportion of IJs, followed

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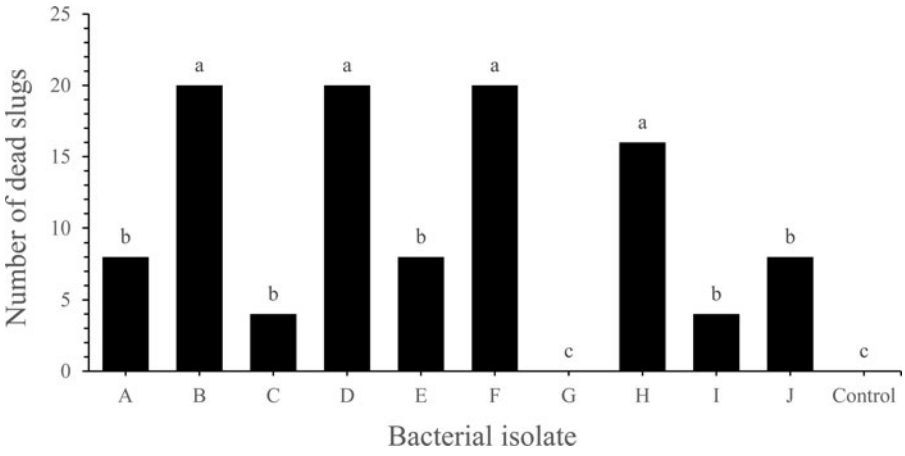

Fig. 1. Mortality of *Deroceras invadens* ($F_{10,11} = 35.800$; $P < 0.01$), five days after being injected with bacterial isolates: (A) *Pseudomonas* sp. (1); (B) *Kluyvera* sp.; (C) *Pseudomonas* sp. (2); (D) *Pseudomonas* sp. (3); (E) *Pseudomonas* sp. (4); (F) *Aeromonas* sp.; (G) *Buttiauxella* sp.; (H) *Photorhabdus heterorhabditis*; (I) *Photorhabdus luminescens*; and (J) *Xenorhabdus khoisanae* and the control (water only). Different letters above bars indicate significant differences ($P < 0.05$) between different bacteria and slug mortality.

by the flasks inoculated with 1000 IJs/ml, with the 3000 IJs/ml flasks having the lowest IJ proportion. However, by day 14, the flasks inoculated with 2000 IJs/ml had a significantly higher ($P < 0.001$) IJ proportion (90 ± 0.7%) than did the other two treatments, which showed no significant difference ($P > 0.05$) in IJ proportion, with both having an average of 82% IJs each (82 ± 1.1% for 1000 IJs/ml and 82 ± 1.9% for 3000 IJS/ml inoculum) (fig. 3C).

Discussion

In the current study *Pseudomonas* sp. (AP1) was the bacterium that was preferred most by all the nematode species, leading to recovery and reproduction in all the species concerned. In their search for a bacterial species suitable for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) tested *A. hydrophila*, finding that it could cause mortality in *D. reticulatum*. Although it was isolated from the intestines of *P. hermaphrodita* IJs, it was not capable of successfully supporting growth in *P. hermaphrodita*. Wilson *et al.* (1995b) also tested two isolates of *P. fluorescens* in foam chip cultures, finding the species concerned capable of supporting the growth of *P. hermaphrodita*. A possible reason for the inability of *P. kenyaensis* to grow on the bacterial isolates from this study is that the bacteria were all isolated locally. Bacterial isolates from Kenya should be tested for their ability to support growth of *P. kenyaensis*.

The pathogenicity tests of the bacterial species isolated from slugs, and of the three bacterial species associated with EPNs, showed that *Pseudomonas* sp. (AP3), *Kluyvera* sp. and *Aeromonas* sp. were most pathogenic of all, causing 100% mortality of the slugs involved. *Buttiauxella* sp. was the least pathogenic of the bacteria tested, as it was found to cause zero mortality. The other bacterial isolates, including the species associated with EPNs, despite causing significant mortality in the slugs, were comparatively less pathogenic. A possible reason for the inability of some bacterial isolates to cause mortality in the slugs is that these bacteria occur naturally in the soil and the slugs have developed immunity to infection.

In EPN studies, the association between the mutualistic bacteria of EPNs is very strong, with it having always been believed that, in the case of *Steinernema*, each nematode species was inevitably associated with its own unique bacterial species (Dreyer *et al.*, 2018). However, *X. khoisanae* has been found to be able to switch between nematode species, and even between distantly related clades (Dreyer *et al.*, 2018). Lee & Stock (2010) show that at least 17 host switches of strains of *Xenorhabdus* species occur between *Steinernema* species, and even between species of different clades. In the case of the slug nematode *P. hermaphrodita*, the association between the nematode and the bacteria concerned is relatively weak, and many bacterial species have been tested to determine the optimum production and viability that could be attained from them being grown together with *P. hermaphrodita* (Wilson *et al.*, 1995b).

While selecting a bacterium for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) found that, when *M. osloensis* was injected into the body cavity of *D. reticulatum*, it was not pathogenic. However, when *P. hermaphrodita* was

Table 2. Ability of different bacterial isolates to support the growth of the nematodes *Phasmarhabditis bohemica*, *Phasmarhabditis papillosa* and *Phasmarhabditis kenyaensis*.

	Growth support score		
Bacterial isolate	*P. bohemica*	*P. papillosa*	*P. kenyaensis*
Aeromonas sp.	1	2	0
Buttiauxella sp.	3	1	3
Kluyvera sp.	2	2	3
Pseudomonas sp. (AP1)	3	2	3
Pseudomonas sp. (AP2)	3	2	0
Pseudomonas sp. (AP3)	3	2	0
Pseudomonas sp. (AP4)	3	3	0
Photorhabdus heterorhabditis	1	0	0
Photorhabdus luminescens	3	1	0
Xenorhabdus khoisanae	3	2	0

Score: 0 = dead; 1 = alive; 2 = recovered; and 3 = reproduced.

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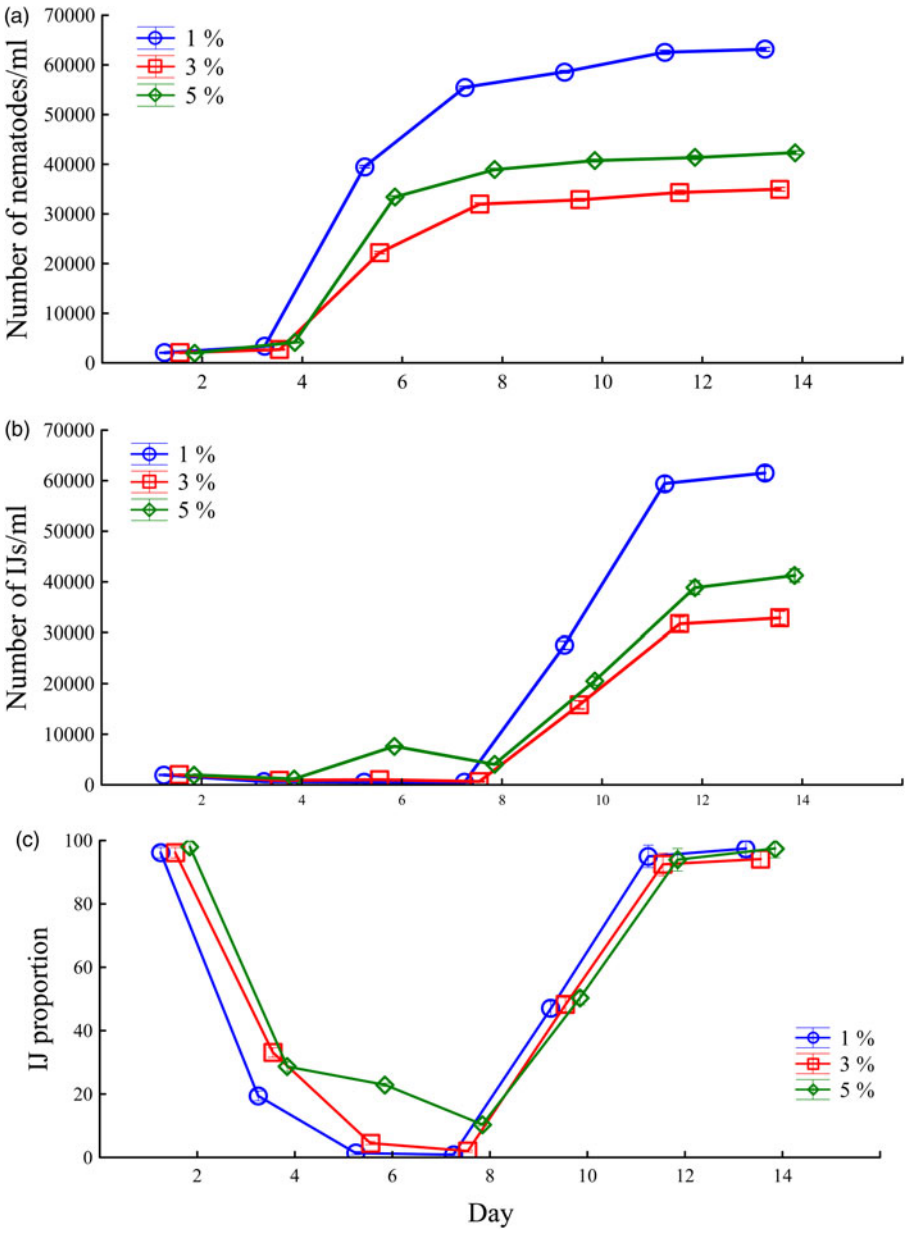

Fig. 2. Effect of different bacterial densities (1%, 3% and 5%) (95% confidence interval) of *Pseudomonas* sp. (AP1) on the development of *Phasmarhabditis bohemica* on: (A) the total number of nematodes/ml (analysis of variance, $F_{30,180} = 3023$; $P < 0.001$); (B) total number of infective juveniles (IJs)/ml ($F_{30,180} = 336.23$; $P < 0.0010$; and (C) IJ proportion in total nematode population ($F_{30,180} = 38.99$; $P < 0.001$) over a period of 14 days.

grown in a monoxenic liquid culture together with *M. osloensis*, and the pathogenicity of the resulting nematodes was retested, the combined force was found to be pathogenic to *D. reticulatum*. Such was especially the case when the pathogenic bacteria species were injected into the body cavities of the slugs concerned. Wilson *et al.* (1995b) concluded that the interaction between the bacterium, the nematode and the immune system of the slug host is important, when testing for the pathogenicity of different nematode–bacteria combinations.

Again, reflecting on previously conducted EPN studies, EPNs have shown not to rely solely on the bacteria concerned to kill the host after infection, but that, after recovery, the IJs tend to release venom proteins that are lethal and that synergistically contribute to the nematode–bacterium complex pathogenicity (Lu *et al.*, 2017). Therefore, the bacteria that were tested for pathogenicity in the current study, by means of being injected into the haemocoel of several slugs, also need to be tested in combination with a slug-parasitic nematode species.

In the present study, *P. bohemica* was established in *in vitro* liquid cultures with the bacterial species, *Pseudomonas* sp. (AP1). The culture methods employed were then further refined by means of testing the effect of bacterial inoculum density and the number of IJs added to flasks on the total nematode yield, and on the IJ yield and proportion. The results obtained showed that the flasks inoculated with 1% bacteria had significantly higher total nematode and IJ yields than did the flasks inoculated with 3% or 5% bacteria. The flasks inoculated with 1% and 5% bacteria had the same IJ proportions by day 14, which were higher than were those proportions that were obtained with those inoculated with 3% bacteria. The higher total nematode and IJ yields obtained with 1% bacteria inoculation confirm the recommendation made by Ehlers (2001) that the inoculum density of the symbiotic bacteria should be between 0.5% and 1% of the culture volume when mass-producing EPNs.

The flasks inoculated with 3000 IJs/ml had the highest yield of nematodes and the highest yield of IJs when compared to the

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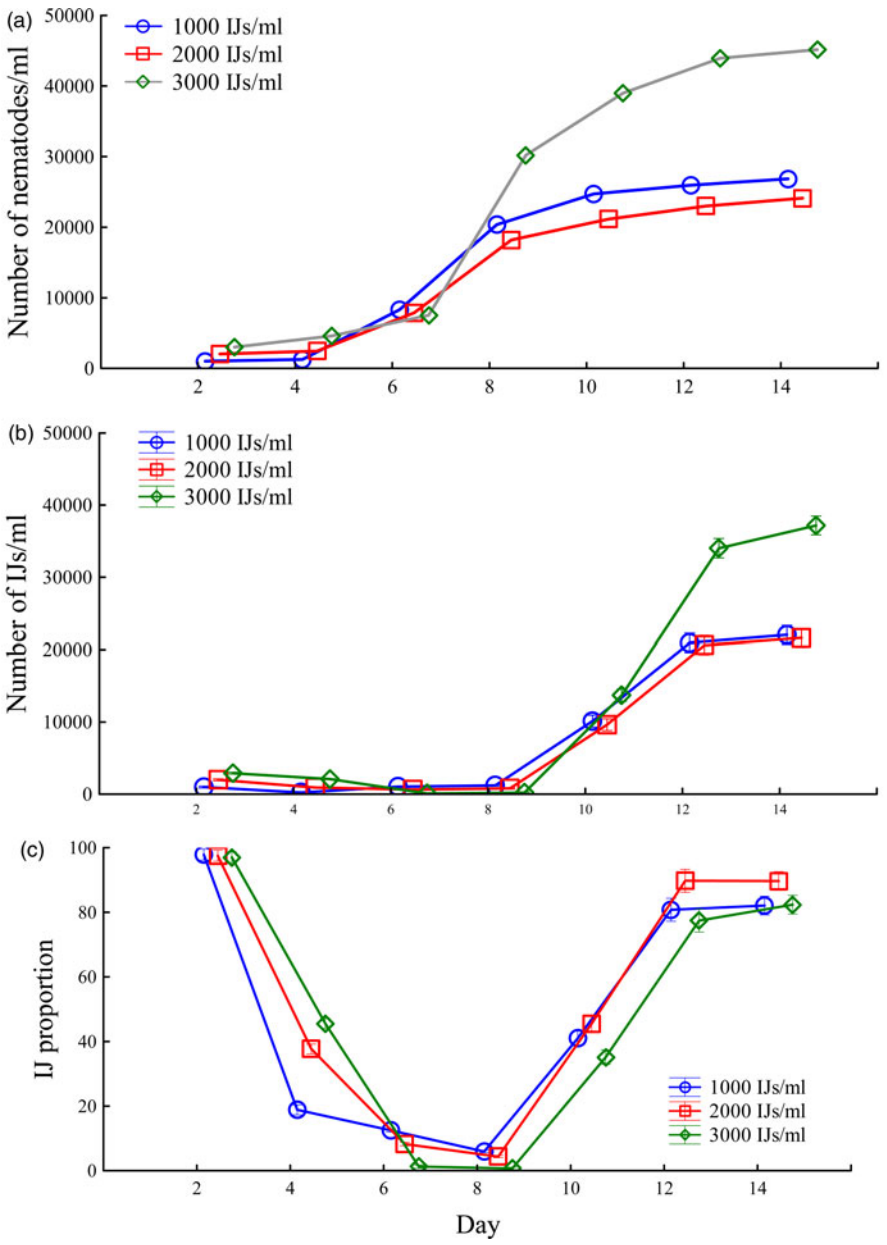

Fig. 3. Influence of different *Phasmarhabditis bohemica* infective juvenile (IJ) inoculum densities (1000, 2000 and 3000 IJs/ml) (95% confidence interval) on: (A) number of nematodes/ml (analysis of variance, $F_{30,\ 180} = 3023$; $P < 0.001$); (B) number of IJs/ml ($F_{30,180} = 336.23$; $P < 0.001$); and (C) IJ proportion in nematode population of monoxenic cultures containing *Pseudomonas* sp. (AP1) ($F_{30,180} = 336.23$; $P < 0.001$), over a period of 14 days.

yield of flasks inoculated with 1000 IJs/ml or 2000 IJs/ml. The flasks inoculated with 2000 IJs/ml, however, had a significantly higher IJ proportion (90%) than did the flasks inoculated with 1000 or 3000 IJs/ml. The results differ from those made in the EPN study conducted by Dunn *et al.* (2020), who found that an inoculum concentration of 1000 IJs/ml gave a higher IJ yield in LCM flasks of *Steinernema jeffreyense* than did a concentration of either 2000 or 3000 IJs/ml. However, the effect of inoculum concentration seems to vary between nematode species, as *Heterorhabditis bacteriophora* Poinar tends to produce optimal yields with intermediate inoculum density, whereas *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding tends to produce higher yields, in higher inoculum concentrations, and *Heterorhabditis indica* Poinar, Karunakar & David appears to be unaffected by inoculum concentration (Han, 1996; Ehlers *et al.*, 2000; Shapiro-Ilan & Gaugler, 2002).

The small volume of media (30 ml) used in the current study is a possible reason for the high IJ yields obtained from all the flasks by day 14. The finding is like the results that were obtained in the EPN study conducted by Dunn *et al.* (2020), who found that 30 ml of LCM produced higher yields of *S. jeffreyense* IJs than did flasks with 50 ml of LCM. Dunn *et al.* (2020) postulate that the higher nematode yield in lower media volumes can be accredited to the greater surface-area-to-volume ratio in the flasks, which leads to the enhanced transfer, or distribution, of oxygen. A possible reason for the large proportion of the IJs present in the cultures is the subsequent overcrowding in the media used, which leads to the accumulation of waste products and ammonia (Shapiro-Ilan *et al.*, 2000; San-Blas *et al.*, 2008). High concentrations of ammonia are produced when nematode populations are overcrowded, which has been proven to induce the emergence of IJs in the EPN *Steinernema feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding (Wright, 2004; San-Blas *et al.*, 2008). Ross

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(2010) also demonstrates that *P. hermaphrodita* tends to secrete a series of small-molecule pheromones when they encounter starvation or overcrowding, which facilitates communication between the nematodes, and which causes the formation of IJs.

In future research, MPNs and bacterial candidates should undergo similar testing, if they are to be developed for commercial production. Such testing is required to determine the ability of the bacteria to support the growth of the nematodes concerned, and to cause mortality in various slug hosts that are known to be pestiferous. The best nematode–bacterium combination should then be grown in *in vitro* liquid cultures, with the pathogenicity of the nematode–bacterium combination being retested for pathogenicity on the relevant slug hosts. If a nematode–bacterium combination is found that grows well and that causes significant mortality in its slug hosts, its production should be optimized by means of testing the effect of different growth conditions on the IJ yield, as was done in the current study.

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Conflicts of interest

None.

Ethical standards

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.

Author contributions

AP, JR and AM conceived and designed the research, while AP conducted the experiments concerned. All the authors contributed nematode isolates. While AP, JR and AM analysed the data obtained, AP wrote the manuscript, which all the authors involved read and approved.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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