Selection of a South African *Heterorhabditis bacteriophora* Isolate for *in vitro* Liquid Mass Production for the Control of *Thaumatotibia leucotreta* in Grapevine

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The South African table grape industry requires biological control options for pest insects to maintain export relationships. Entomopathogenic nematodes (EPNs) have shown effective biological control of the economically important table grape pest, *Thaumatotibia leucotreta*, the false codling moth (FCM). Critical to the success of EPNs is their ability to be mass produced using *in vitro* liquid mass production methods. The selection of suitable isolates for mass production is the most important step in the process of developing an EPN biocontrol product for commercial purposes. *Heterorhabditis bacteriophora* is the most common EPN species found in South African soils, with previous research having shown its high pathogenicity against a variety of grapevine pest insects. In this study, four local *H. bacteriophora* isolates were laboratory-screened to select the best candidate in terms of virulence against FCM, and differences in the symbiotic bacteria isolates were also observed. Two of the *H. bacteriophora* isolates, CRI_LC and LLM's symbiotic *Photorhabdus* bacteria, showed slight bioluminescence, while SGI_170 and Px_SPH showed strong luminescence, indicating the biological difference between the symbiotic bacterial species that are associated with the same nematode species. Molecular analysis of the 16S gene indicated three different bacterial species, of which two were the same and two are possibly new. The *H. bacteriophora* isolate, SGI_170, which showed the highest virulence against late-instar FCM larvae, was chosen for the further development of *in vitro* liquid culture.

INTRODUCTION

Thaumatotibia leucotreta (Meyrick) (Lepidoptera: Tortricidae), the false codling moth (FCM), is a pest of economic importance in table grapes, especially in citrus orchards and stone fruit crops (Bloem *et al*., 2007). With regard to the table grape industry, South Africa was the fourth highest exporter of table grapes in the 2022 season, exporting over 400 000 tons (South African Table Grape Industry [SATI], 2023). Thus, the table grape industry influences aspects of South Africa's agricultural economy through international investments and import–export trade (World Citrus Organisation, 2022; SATI, 2023), making the control of FCM an important economic consideration. Because of environmental and human health-related issues with chemical pesticides used to control insect pests, the European Union, one of South Africa's biggest table grape and citrus export partners, has created laws that prohibit the use of certain chemical products. This has affected how South African farmers control FCM, as they can no longer use the same pesticide products they were using previously, and there are few products available in South Africa that meet the organic and environmentally friendly requirements for fruit produce export.

A specific life cycle stage of FCM leaves them vulnerable to parasitism from soil-dwelling biocontrol agents, such as entomopathogenic nematodes (EPNs), fungi and bacteria. FCM lay their eggs on the fruit and, once the eggs hatch, the larvae penetrate the fruit. The fifth-instar (last-instar) larvae emerge from the fruit and burrow into the soil, where they cocoon themselves with their silk mixed with soil debris, whereupon they are also exposed to soil entomopathogens (Daiber, 1979a, 1989). After two to three days, the prepupa in the cocoon turns into a pupa, with a further 12 to 14 days (depending on the prevailing temperature) in the soil before adults emerge from the cocoon and soil, continuing their life cycle above ground (Daiber, 1979b).

EPNs are natural enemies of insects. They occur naturally in the soil environment and are parasites of pest insects. They also pose no threat to humans and the environment, thus making them ideal to use as a biocontrol agent in an integrated pest management strategy (Surrey & Davies, 1996). EPNs are classified in the order Rhabditida and the families Steinernematidae, associated with the mutualistic Xenorhabdus bacteria, and Heterorhabditidae, associated

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with *Photorhabdus* bacteria. EPNs form complex bacto-helminthic relationships with their associated bacteria, which are key to their survival (Ehlers, 2001; Han & Ehlers, 2001). The free-living nonfeeding stage, the infective juvenile (IJ) stage, is an arrested third-stage juvenile and is adapted to adverse conditions in the soil while seeking for an insect host. Once the target host is found, the IJ enters the insect through such natural openings as the mouth or anus. *Heterorhabditis* has a dorsal tooth that allows it to penetrate the soft cuticle by means of abrasion (Surrey & Davies, 1996; Shapiro-Ilan & Gaugler, 2002). Once the EPN is inside the insect, the nematode releases its associated bacteria into the haemocoel of the insect, where the bacteria start to proliferate, killing the insect through induced septicaemia (Surrey & Davies, 1996; Shapiro-Ilan & Gaugler, 2002). A food signal is then released that induces the recovery of the IJs, whereupon they start to feed and emerge from their arrested nonfeeding (dauer) stage (Golden & Riddle, 1984; Ehlers *et al*., 2000). The bacteria also release a cocktail of metabolites that protect the insect cadaver in the soil environment and the internally developing nematodes. The IJs develop into third- and fourth-stage juveniles, and then to males and females in the case of *Steinernema*. In *Heterorhabditis*, the first generation is a self-fertilising hermaphroditic female, while the subsequent second generation produces amphimictic adults (females and males) (Hirao & Ehlers, 2010). The population then continues to grow until the food source is depleted, after which the second-stage juveniles stop feeding and go into an arrested IJ state (Hirao & Ehlers, 2010). The eggs that are retained inside the females develop into juveniles that feed on the mother, which is a process that is known as endotokia matricida, with the juveniles involved emerging as IJs (Hirao & Ehlers, 2010). The IJs then leave the host insect in search of another host, where the cycle continues.

Heterorhabditis bacteriophora are very effective against FCM at low concentrations of IJs (50 IJ/insect), as demonstrated in laboratory screening (Malan *et al*., 2011) and in semi-field trials (Malan & Moore, 2016; Steyn *et al*., 2019). Large-scale field trails with imported *H. bacteriophora* confirm their suitability for application in a systems approach to control FCM in South Africa (Moore, 2021; Moore *et al*., 2024). However, no local *H. bacteriophora* product is currently available, although an exotic registered product can be purchased (Hatting *et al*., 2019).

Even though EPNs can kill the host insect by releasing venomous proteins (Lu *et al*., 2017) and suppressing the host immune system, it is especially highly pathogenic in a synergistic association with the bacteria. As the bacteria also provide the nematodes with nutrition, the relationship between the EPN and its symbiotic bacteria is mutualistic (Ehlers, 2001).

Malan *et al*. (2011) conducted a survey to establish whether any local EPN species would be viable for use against FCM in citrus orchards. Six EPN species, namely *Steinernema khoisanae* Malan *et al*., *Steinernema* yirgalemense Nguyen *et al*., *Steinernema citrae* Stokwe *et al*., *Heterorhabditis bacteriophora* Poinar, *Heterorhabditis zealandica* Poinar and *Heterorhabditis noenieputensis* Malan *et al*., were identified as potential biocontrol agents against FCM in laboratory (Malan *et al*., 2011) and field conditions (Malan & Moore, 2016).

Even though many different indigenous EPN species effectively control the last-instar larvae of FCM in South Africa, *H. bacteriophora* is the most frequently isolated species in vineyards and citrus orchards. *Heterorhabditis bacteriophora* is currently registered as a biopesticide by River Bioscience in South Africa, making it a good candidate for the control of FCM, as well as for mass-culture production, with an abundance of literature available on the mass culture (Surrey & Davies, 1996; Yoo *et al*., 2000, 2001; Malan *et al*., 2006; Hatting *et al*., 2009; Cho *et al*., 2011; Upadhyay, 2015; Malan & Moore, 2016). However, a local isolate of *H. bacteriophora* has not yet been mass-produced in South Africa.

The aim of this study was to select a local South African isolate of *H. bacteriophora* from different localities based on their pathogenicity. Since virulence against FCM is the key criterium for selecting an EPN isolate, the research focused on identifying the *H. bacteriophora* isolate with the highest virulence against FCM. Because the symbiotic bacteria associated with *H. bacteriophora* may not be the same for each isolate, they were separately characterised, as they play a crucial role in pathogenicity.

MATERIALS AND METHODS

Source of insects and nematodes

Thaumatotibia leucotreta final-instar larvae were obtained from XSIT, Citrusdal, Western Cape. Larvae of *Galleria mellonella* L. (Lepidoptera: Phylaridae), the greater wax moth, were obtained from the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Four isolates of *H. bacteriophora* were sourced from the Eastern Cape, Free State and Western Cape provinces of South Africa (Table 1).

Heterorhabditis bacteriophora isolate verification

A single IJ of *H. bacteriophora* was placed in a 10 µl drop of DNA extraction master mix and then cut into pieces using a sterile insulin needle. The DNA extraction master mix consisted of 79 µl nuclease-free water, 20 µl lysis buffer (16 mM $[NH_4]_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween-20) and 1 µl proteinase K. The tubes concerned were transferred to a thermocycler for DNA extraction (Malan *et al*., 2011). The Eppendorf tubes were placed in a thermocycler at 65°C for 1 h, which was followed by 95°C for 10 min, whereupon they were used for PCR amplification. Primers TW81 (1.25 µl) forward 5'-GTTTCCGTAGGTGAACCTGC-3' and AB28 (1.25 µl) reverse 5-5'-ATATGCTTAAGTTCAGCGGGT-3' were used for PCR amplification of the ITS region. To this, 5 µl nuclease-free water, 5 µl DNA and 12.5 µl KAPA master mix were added. The thermocycling conditions for the PCR protocol were an initial temperature of 95°C for 3 min, with the annealing conditions being 35 cycles at 95°C for 20 sec, 48°C for 20 sec and 72°C for 30 sec. The final extension was at 72°C for 5 min, after which the mix was placed on hold at 4°C. The DNA product was visualised using an ethidium bromide agar gel. PCR products were sent to the Central Analytical Service of Stellenbosch University for sequencing. Afterwards, the forward and reverse sequences generated were edited using CLC Main Workbench, version

TABLE 1
Origin of soil samples used to obtain isolates of *Heterorhabditis bacteriophora*.

Isolate	Location	Province	Orchard type	Farm	Collected by	Collected from
SGI_170	Fouriesburg	Free State	Apple	Lone Tree Farm	Justin Hatting	Soil
CRI_LC	Sunday's River Valley	Eastern Cape	Citrus	Olifantskop	Luke Cousins	Soil
Px_SPH	Witzenberg Valley	Western Cape	Apple	Die Eike	Steffan P. Hansen	*Phlyctinus xerophilus*
LLM	Grabouw	Western Cape	Apple	Montieth Trust farm	Letodi L. Mathulwe	Soil

21.0.5, together with the consensus sequence nBLAST function on NCBA, to verify the nematodes' identity.

Isolation of associated *Photorhabdus* symbiotic bacteria
The associated *Photorhabdus* symbiotic bacteria of each *H. bacteriophora* isolate were isolated using *G. mellonella*. The larvae were infected with the different EPN isolates, sealed in a Petri dish to avoid contamination, and stored at 28°C for exactly 18 h. After 18 h, the haemolymph was extracted from the larvae (while still alive) under sterile conditions and streaked onto NBTA [Nutrient Agar (Difco Laboratories), 0.0025% bromothymol blue, 0.04% 2,3,5-triphenyltetrazolium chloride] plates (Akhurst, 1980; Dunn *et al*., 2021). The plates were then sealed and stored at 28°C for 48 h to allow the bacteria to proliferate and form colonies. After 48 h, a single colony of the bacteria was added to 30 ml tryptic soy broth (TSB) flasks and placed on Junior orbital shakers at 140 rpm at 28°C for 48 h. The bacteria were then cryogenically preserved, using glycerol, which was added to the shake flasks (15% v/v), equating to 4.5 ml of glycerol per 30 ml flask. One ml of the glycerol–bacteria mixture was then transferred to the 1.5 ml Eppendorf tubes and stored at -80°C (Kaya & Stock, 1997; Dunn *et al*., 2021). To confirm that the correct bacteria were harvested, bacterial lawns were made using the harvested bacteria of each isolate's associated symbiotic *Photorhabdus* bacteria. The Wouts plates were then stored in a growth chamber at 28°C for two days. In vivo-produced IJs from each isolate (without any sterilisation) were added to their respective Wouts plates, with the presence of the bacteria being confirmed when the nematodes recovered (i.e., opened their mouths and started feeding) and were found to grow on the Wouts plates.

Identification of *Photorhabdus*
The symbiotic bacteria stored at -80°C were thawed, with 200 µl of the bacteria being placed into 30 ml TSB flasks on an orbital shaker at 140 rpm for 48 h at 28°C. After 48 h, the bacteria were ready for use for molecular identification. The bacterial DNA was extracted from the TSB solution using the Xymo Research Quick-DNA™ Fungal/Bacterial Miniprep kit. To ensure that pure DNA was collected, 1 µl of the DNA was placed on a NanoDrop® ND-1000 UV-Vis spectrophotometer, which analyses the purity of the DNA concentrations in a visible UV wavelength range. The purity of the DNA was measured in the 260/280 column, with good purity ranges (A260/A280) of between 1.80 and 2.00 being achieved, with the procedure being repeated for each DNA sample. A polymerase chain reaction (PCR) analysis was then conducted on the symbiotic bacteria, with the 16S genes being targeted for amplification. The reaction components of the PCR analysis included a 5 µl Q5® reaction buffer, 0.5 µl dNTPs, 1.25 µl F8 (forward buffer), 1.25 µl R1512 (reverse buffer), 0.25 µl Q5® high-fidelity DNA polymerase, a 5 µl Q5® high QC enhancer, 11.5 µl nuclease-free water and 0.25 µl template (bacterial) DNA. The summation produced a 25 µl reaction, which could be scaled up, depending on the number of DNA samples involved. The thermocycling conditions for the PCR protocol were kept at an initial temperature of 98°C for 10 min, with the annealing conditions being 30 cycles of 98°C for 10 sec, 57°C for 30 sec and 72°C for 1 min. The final extension was conducted at 72°C for 7 min, after which it was placed on hold at 4°C. A post-PCR check was conducted for both the bacterial DNA and the nematode DNA. Once the presence of DNA bands was confirmed, the PCR product was sent to the Central Analytical Facilities (CAF) DNA Sequencing Unit at Stellenbosch University for sequencing.

Bioluminescence *Photorhabdus* isolates
The associated *Photorhabdus* symbiotic bacteria of each *H. bacteriophora* isolate were streaked onto NBTA plates and stored at 28°C for 48 h to allow colonies to form on the plates. After 48 h, a single colony was placed in TSB and incubated for 48 h at 28°C on an orbital shaker at 140 rpm. Once the bacteria had proliferated, 1 ml of the TSB–bacteria solution was placed into 24-well plates and bioluminescence was calibrated for each sample, using IVIS® Spectrum in vivo imaging.

Pathogenicity bioassay
Late-instar larvae of FCM were used to test the virulence of the different *H. bacteriophora* isolates. The five treatments used in the bioassay consisted of the four *H. bacteriophora* isolates that were selected from the surveys done, with a fifth control treatment consisting of distilled water. For each treatment, five 24-well bioassay plates were used. Whatman 12.7 mm disc filter papers were placed in each alternative well, with the FCM larvae then being placed in each filter paper-containing well. There were 12 FCM larvae per well plate, hence 60 FCM larvae per treatment in total. For the EPN treatments, each well was inoculated with a nematode inoculum of 50 IJs/50 µl, whereas the control bioassay plates were inoculated with only 50 µl distilled water per

well. The concentration formula was used to calculate the nematode inoculum density (Glazer & Lewis, 2000). The five 24-well bioassay plates of each treatment were placed in 2 L ice-cream containers, lined with moistened paper towels (moisture chamber) to ensure high humidity. The containers were then stored in a growth chamber at a controlled temperature of 25°C. The 24-well plates were checked for FCM mortality after 24 and 48 h. The FCM larvae were considered dead if they underwent a colour change and if they no longer moved when stimulated by gentle poking with forceps. FCM larvae in the EPN treatments that were dead were checked for infection by means of dissecting the larvae with the aid of a stereomicroscope. The dead FCM larvae with no EPNs present were considered to have died from natural causes. The bioassays with last-instar FCM larvae were repeated on a different test date with a fresh batch of each *H. bacteriophora* isolate.

Statistical analysis

No mortality occurred in the control group. The data pooled from the four isolates were analysed using R (R Core Team, 2023) and Statistica (TIBCO Software Inc., 2020). A normality test was performed prior to any analysis. The data were normally distributed once the control data had been removed from the pooled data. To determine significant difference among the mortality of the four isolates (treatments), a one-way analysis of variance (ANOVA) was done, with their interactions being significant at $\alpha \leq 0.05$. A Fisher's least significant difference (LSD) test was used as a post-hoc analysis to determine which treatment groups had significant differences from one another.

RESULTS

Verification of *Heterorhabditis* isolates

The IJs of all four isolates were verified to be *H. bacteriophora* species, using the ITS region and the nBLAST function in GenBank. The sequences were submitted on GenBank *H. bacteriophora* isolate Px_SPH, with accession number OP856853, SGI_170, with accession number OR347849, CRI_LC, with accession number OR347816, and LLM, with accession number OR350483. The identity of all isolates submitted on GenBank was a 100% match to *H. bacteriophora*.

Verification of *Photorhabdus*

The associated symbiotic *Photorhabdus* bacteria of each isolate streaked onto the NBTA plates displayed dark red to orange colonies (Fig. 1). The IJs of each isolate were successfully fed on bacterial lawns prepared on Wouts plates using their respective symbiotic *Photorhabdus* bacteria (Table 2). The bacteria for isolate LLM were identified as being *Photorhabdus laumondii* subsp. *laumondii* (Fischer-Le Saux *et al*.) Machado *et al*., whereas the CRI_LC isolate was identified, using next-generation sequencing, as a new species named *Photorhabdus safricana* Machado *et al*. (Machado *et al*., 2018, 2024). The bacteria of isolates Px_SPH and SGI_170 were identified as likely being the same new species, using only the 16S gene (Table 2) and next-generation sequencing (R.R.A. Machado, personal communication).

Bioluminescence of *Photorhabdus* isolates

The bacteria of isolates Px_SPH (Western Cape) and SGI_170 (Free State) were shown to be bioluminescent, whereas the isolates of CRI_LC (Eastern Cape) and LLM (Western Cape) showed low to no bioluminescence (Fig. 2).

Pathogenicity bioassay

Data from the two different test dates were analysed separately and, as no significant ($p > 0.05$) difference was found between the main effects of date and isolate, the data were pooled and analysed. A one-way ANOVA showed a significant difference ($F_{3, 36} = 11.254$; $p < 0.001$) between the means of infection of FCM caused by the different isolates (Fig. 3). After 48 h, SGI_170 had a mean infection rate of 75% ± 18.00%, CRI_LC had 55% ± 23.22%, LLM 44.15% ± 20.43% and Px_SPH 24.17% ± 14.93%. The infection of SGI_170 was significantly higher ($p < 0.001$) than that of the other three isolates. No significant difference ($p = 0.2338$)

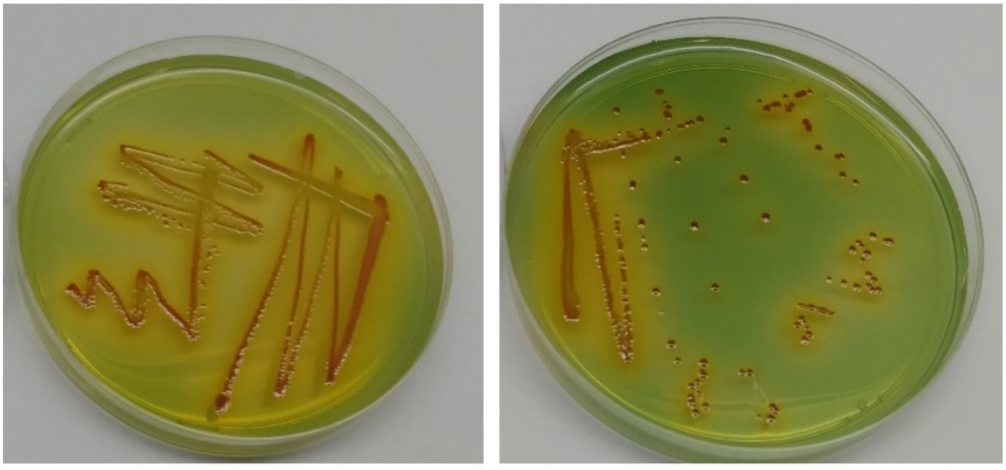
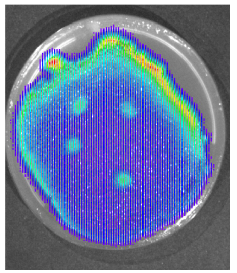
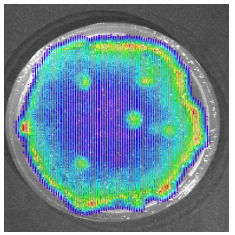
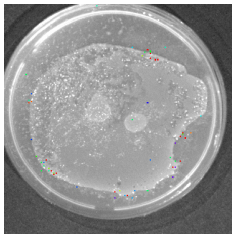
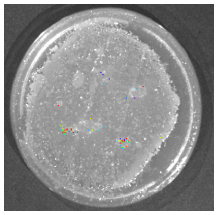


FIGURE 1
Photorhabdus bacteria streaked onto NBTA plates after 24 to 48 h.

TABLE 2
Identification of *Photorhabdus* species isolated from South African *Heterorhabditis bacteriophora* isolates.

Origin	16S	Associated host	Bioluminescence	Wax moth colour	IVIS® image	Growth Wouts plates	GenBank accession number
Witzenberg Western Cape	*Photorhabdus* sp. (Px_SPH)	Apple	Yes	Red		Yes	OP856853
Fouriesburg Free State	*Photorhabdus* sp. (SGI_170)	Apple	Yes	Red		Yes	OR347849
Sunday's River Valley Eastern Cape	*Photorhabdus safricana* (CRI_LC)	Citrus	No	Red		Yes	OR347816
Grabouw Western Cape	*P. laumondii* subsp. *laumondii* (LLM)	Apple	No	Red		Yes	OR350483

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was found between the means of the isolates CRI_LC and LLM. The isolate Px_SPH, which had the lowest infection percentage, was significantly lower ($p < 0.05$) than all the other isolates (Fig. 3). Colonies of the four *Photorhabdus* symbiotic bacteria showed red on NBTA, while the *H. bacteriophora*-infected wax moth larvae all had a similar dark red or brick-red colouration (Fig. 4).

DISCUSSION

Before selecting an EPN isolate for mass culture, it was regarded as being important to conduct a laboratory screening for pathogenicity, comparing not only EPN species, but also different isolates of the same species, to assess pathogenicity against the target pest insect. As *H. bacteriophora* is one of the most common EPN species found in South Africa (Malan

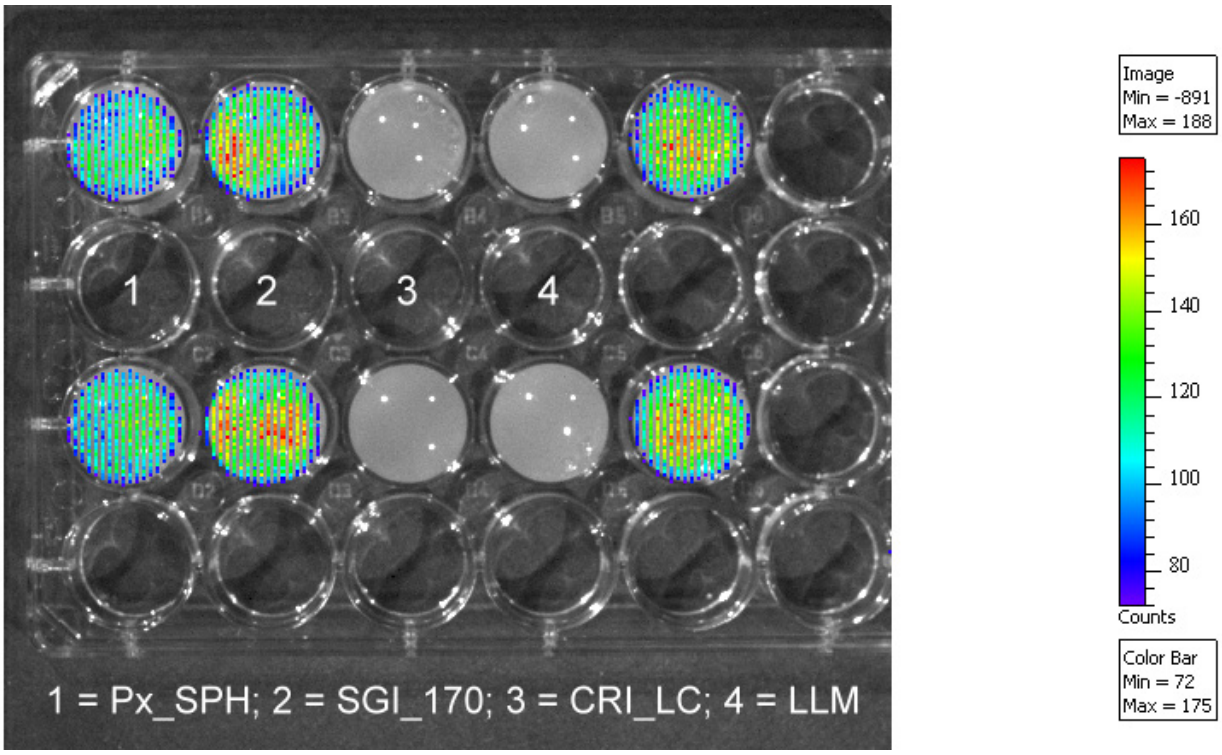

FIGURE 2
Bioluminescence of *Photorhabdus* species from the four selected *Heterorhabditis bacteriophora* isolates.

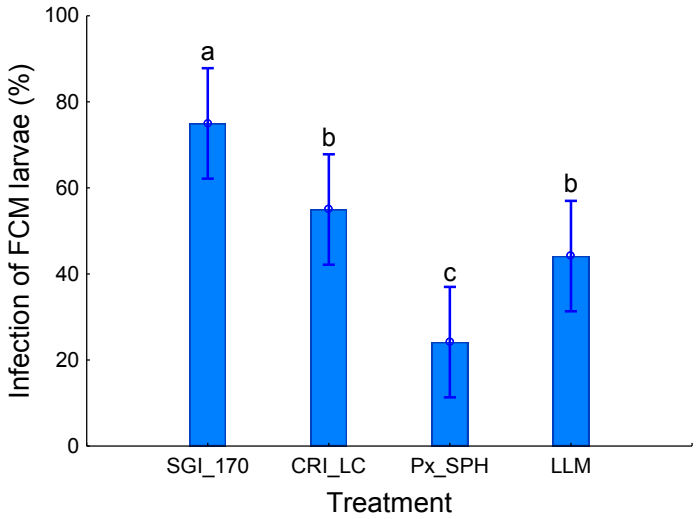

FIGURE 3
Percentage mean infection rate (95% confidence interval) of South African *Heterorhabditis bacteriophora* isolates, at a concentration of 50 IJs/insect against the last-instar larva of *Thaumatotibia leucotreta* (FCM) (one-way ANOVA: $F_{3, 36} = 11.254$; $p < 0.001$). The letters denote significant differences, while bars with the same letters indicate no significant difference ($p > 0.05$) between the treatment and the FCM infection.

FIGURE 4
The dark red colour of the fifth instar, indicating that the wax moth larvae were infected with *Heterorhabditis bacteriophora.*

et al., 2011) and one of the most successful commercially available nematode species (Heve *et al*., 2018; Modic *et al*., 2020), it has been selected as a potential species for mass-culture in South Africa.

The bacterial colonies of the four *Photorhabdus* symbiotic bacteria on the NBTA plates were all red, while the wax moth larvae, infected with the four different *H. bacteriophora* isolates, all had a similar dark red or brick-red colouration. Although the colour displayed by the EPN-infected wax moth larvae has been used to distinguish between different *Photorhabdus* subspecies for *Heterorhabditis zealandica* Poinar, the same cannot be done for the bacteria associated with *H. bacteriophora* (Booysen *et al*., 2022).

IVIS imaging of the bacteria in the TSB and on the agar plates showed differences between the *Photorhabdus* bacteria associated with each nematode isolate in terms of bioluminescence. Bioluminescence was found in two of the *Photorhabdus* symbionts (SGI_170 and Px_SPH), while two only showed slight traces of bioluminescence (CRI-LC and LLM). Generally, all *Photorhabdus* species are assumed to show bioluminescence. However, another non-bioluminescent *Photorhabdus* was described as P. heterorhabditis subsp. *aluminescens* by Machado *et al*. (2021), named by the fact that it does not show any bioluminescence. A recent study by Cassells *et al*. (2024) demonstrates the potential for bioluminescence aposematism, combined with chemical defences produced by *Photorhabdus*, to deter slug scavengers from feeding on insect cadavers infected by bioluminescent bacteria.

Molecular analysis of the 16S gene further confirmed that the two low-luminescent bacteria were two different species, with one identified as *P. safricana* and the other as *P. laumondii* subsp. *laumondii* (Machado *et al*., 2024). The other two bioluminescent isolates are an unknown, new *Photorhabdus* species. Recently, housekeeping genes and 16S RNA sequences were found not to be robust enough to identify *Photorhabdus* to species and subspecies level. Booysen *et al*. (2022) indicated the association of *H. bacteriophora* with seven different *Photorhabdus* species, including three subspecies of *P. laumondii.* All species of the *Photorhabdus* and *H. bacteriophora* combinations were associated with different countries. The results found by Booysen *et al*. (2022) concur with the results found in the present study, but with the same two unknown *Photorhabdus* occurring in different provinces (Western Cape and the Free State), and the two with low bioluminescence, *P. laumondii* subsp. *laumondii* (LLM) in the Western Cape and *P. safricana* in the Eastern Cape, occurring in citrus. In a previous study in South Africa, the symbiotic bacteria of *H. bacteriophora* isolate SF351 were identified as *P. luminescens* subsp. *laumondii* (Geldenhuys *et al*., 2016) from a vineyard in Wellington in the Western Cape, with the name being elevated by Machado *et al*. (2018) to *P. laumondii* subsp. *laumondii*. The result obtained agrees with the occurrence of the associated bacteria LLM found in apple orchards in the Western Cape.

The isolates SGI_170 and Px_SPH of *H. bacteriophora*, from apple orchards in the Free State and the Western Cape, respectively, showed the highest infection (75%), whereas Px_SPH showed the lowest (24%). Although the two isolates concerned originated from different provinces in South Africa, they share the same unknown *Photorhabdus* isolate. A possible reason for the difference in pathogenicity may be ascribed to the fact that Px_SPH was recently isolated directly from a *Phlyctinus xerophilus* weevil, whereas SGI_170 was isolated directly from the soil and then recycled many times

through wax moth larvae (Hatting *et al*., 2009; Hansen *et al*., 2023). The isolates CRI-LC and LLM share the same *Photorhabdus* species, *P. laumondii*, although with different subspecies. However, no significant difference was found in the pathogenicity between the two subspecies.

A low concentration (50 IJs/insect) of different *H. bacteriophora* isolates was used to obtain a good indication of the difference in pathogenicity between the four isolates with FCM larvae as the host. The infection rate of SGI_170 was found to be in line with other studies with similar bioassay protocols (Geisert *et al*., 2023). Host insect susceptibility to EPN infection might also affect infectivity. FCM are susceptible to several EPNs, including S. yirgalemense, *H. zealandica*, *Steinernema* litchi Steyn *et al*. and *Steinernema* jeffreyense Malan *et al*. (Steyn *et al*., 2017, 2019). Laboratory bioassays conducted with such EPN species resulted in high mortality of >80% (Steyn *et al*., 2017, 2019), with such mortality rates all being higher than what was achieved in this study with *H. bacteriophora*. One explanation for such mortality rates could be that *H. bacteriophora* might have a lower infectivity rate compared to the other EPN species. However, due to the ubiquitous spread of *H. bacteriophora* across South Africa and its registration as a biopesticide, as well as due to the body of research that is currently available, *H. bacteriophora* is taken to be the species of choice for mass in vitro culture.

As with the study conducted by Steyn *et al*. (2017), 50 IJs/50 µl were inoculated per well. Geisert *et al*. (2023) obtained a high mortality against corn rootworm larvae when inoculated with 60 IJs and 120 IJs per well, with between 80% and 100% mortality being obtained. Wells inoculated with 30 or fewer IJs had a mortality of < 50% (Geisert *et al*., 2023). From this, the inference can be made that inoculating a high number of IJs per well per larva would likely result in an increased mortality rate. The reason for isolates having reduced mortality could be that the FCM were less susceptible to infectivity compared to the other isolates concerned. The isolate with the second highest mortality was CRI_LC, at 50%.

The results of the present study indicate the importance of the different associated symbiotic bacteria associated with the same nematode species. Future studies should identify the associated bacteria of *H. bacteriophora* at both species and subspecies level, before comparing different isolates of the same species of *H. bacteriophora* with each other. Significantly, imported *H. bacteriophora* could be associated with different species of *Photorhabdus*, which could not be regarded as the same biological system, despite being identified as the same nematode species. The isolate SGI_170, with its associated unknown *Photorhabdus* species of symbiotic bacteria, was selected for in vitro liquid mass-culture of *H. bacteriophora* in terms of virulence against FCM larvae during laboratory screenings. As the ease of in vitro mass culture is the second most important characteristic for in vitro liquid culture, the next step in this study should be to select between the two best-performing *H. bacteriophora* isolates.

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