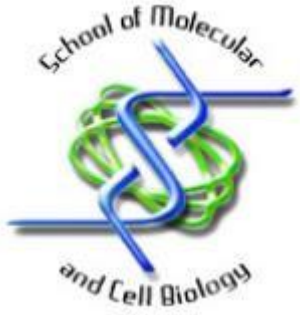


Isolation, identification and characterisation of entomopathogenic nematodes; with a potential to be used as biological control agents of problematic insects in agricultural industries.

Dissertation by: Miss Tiisetso Elizabeth Lephoto (0713627e)

Supervisor: Prof VM Gray

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the degree of Master of Science.

Contents Page

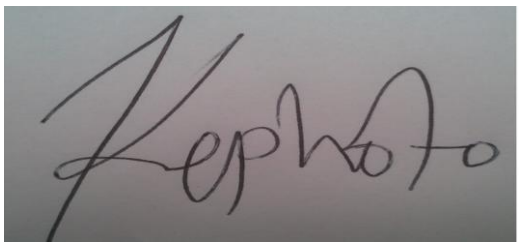
Declaration

I Tisetso Elizabeth Lephoto, (student number: 0713627e)

I hereby declare the following:

- I am aware that plagiarism is wrong and declare that this dissertation is written in my own words and have referenced and cited all sources used.
- This research and all experiments were done by me, with a complete thesis written by me.
- I submitted this dissertation for Master of Science degree at the University of the Witwatersrand, Johannesburg
- The dissertation has not been submitted before for any degree in any other University

Tiisetso Elizabeth Lephoto

Signature: 

Date: 10 May 2013

Dedication

I dedicate this work to my loving parents Mr David Lephoto and Mrs Monica Lephoto who always put my education first and continue to support me, my fiancé Mr Lebang Nong for his outmost support and motivation which kept me going through out my research days, I thank you.

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Most of all I thank my parents Mrs Monica and Mr David Lephoto and my fiancé Mr Lebang Nong for the love, care and support throughout my MSc.

~~~~~~~~I thank God for the strength,wisdom and his love ~~~~~~~~
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Research outputs:

Authors: Miss TE Lephoto and Prof VM Gray

Conference 1: GDARD Research Annual 2012, 6 June

Venue: St Georges Conference Hall, Midrand

Poster presentation:

Isolation, identification and characterisation of entomopathogenic nematodes and desiccation tolerance of cultured EPNs with a potential to be used as biological control agents in agricultural industries

Conference 2: 4th Cross Annual Symposium 2012, 19-22 October

Venue: Wits professional hub

Poster presentation:

Isolation, identification and characterisation of entomopathogenic nematodes, behaviour studies and desiccation tolerance of cultured EPNs with a potential to be used as biological control agents in agricultural industries

Conference 3: MRBT Research conference 2012

Venue: Wits professional hub

Poster presentation:

Isolation, identification and characterisation of entomopathogenic nematodes, behaviour studies and desiccation tolerance of cultured EPNs with a potential to be used as biological control agents in agricultural industries

Papers 1:

Desiccation tolerance of *H. bacteriophora* and *S. australe*: In preparation for publication

Paper 2:

Behaviour studies of *H. bacteriophora* and *S. australe*: In preparation for publication

Abstract:

The purpose of the study was to isolate and identify indigenous nematode species and use them as model organisms for studying the responses of indigenous entomopathogenic nematodes (EPNs) to soil desiccation, soil rehydration, and also to study their behaviour with regards to the infection and location of insect larvae, *Galleria mellonella* in an 18cm column filled with sterilised sandy loamy soil. Two unknown nematodes were isolated from soil samples collected in Walkerville, South of Johannesburg and their sequences were found to have high affinity to *Steinernema australe* (accession number FJ235125) and *Heterorhabditis bacteriophora* isolate 56-C (FJ217351) when aligned with existing sequences in the NCBI database. Furthermore, a polymerase chain reaction was used to amplify the 16S rDNA region in order to identify bacterial symbiots of these EPNs. Symbiotic bacteria isolated from the *Heterorhabditis spp* had high affinity to *Photorhabdus sp carborca* (JF12345). Desiccation tolerance studies revealed that EPN5T and EPN3T were able to withstand desiccated conditions or continuous dehydration for 20 days. EPN5T caused 80% larval mortality after day 20 when the last row of Petri dishes was rehydrated. EPN3T caused only 60% larval mortality. All of the sandy controls gave 0% larval mortality by day 20. Comparative dose-response assays involved exposing *G. mellonella* insect larvae to different IJs concentrations (0, 5, 25, 100, 300 and 500 IJs/ml) were carried for both EPN5T and EPN3T. Larval mortality was recorded daily over a week. Insect mortality was high for both *S. australe* and *H. bacteriphora*, at 100IJs\ml, 300 IJs\ml and 500 IJs\ml. Mortality was observed within 48 to 96 hours and insects larvae showed signs of infection after 48hours. Significant differences were observed at EPN concentrations containing 5 IJs/ml and 25 IJs/ml. At these low IJ concentrations *H. bacteriphora* was able to kill 20% of the larvae by day 3 while *S. australe* displayed mortality only after day 4 and 5 for the two respective IJ concentrations. The significance of this observation was supported by the two-way Post-hoc analysis. Further studies were conducted to investigate the effect of soil humidity on EPNs behaviour in a column of soil. Results supported that humidity was crucial for EPNs locomotion or mobility and infection efficiency, as 100% mortality was observed in all columns by day 4. The study also investigated different IJ application concentrations in order

to determine suitable field application doses. Even at the lowest IJ concentration of both EPNs 33.3% mortality was observed in each column. *H. bacteriophora* displayed increased mobility because high mortality percentage was obtained within 48hours in almost all three arenas of the column, proving that this species is an effective cruiser foraging deeper into the soil. *S. australe* was capable of cruising further down to 18cm searching for host as high mortality was observed even at the deeper or the bottom arena near the bottom of the soil column. Lastly field capacity was determined where the sandy loamy soil was saturated with water and allowed to drain so as to remove excess water from the 8 vertical columns for 48hours, resulting in a soil moisture content associated with the soil's field capacity for water. Overall results of this study gave ideas for IJ formulation IJ storage and IJ application strategies of the identified EPNs which proved to have promising potential as biological control agents.

Chapter 1: Literature review

1. Introduction

1.1 What is biological control?

Biological control (BC) is an activity that involves the use of natural enemies of insect pests to reduce bad effects of insect pest (Flint et al, 1998). These effects include destroying important crops and plantation, plant growth destruction or development infections caused by pests (Flint et al, 1998). BC can really have a positive impact in agriculture or any other industry if interactions between the natural enemies and pest populations are well established. Environmental factors, nutrition and migration have major implications on the interaction of natural enemies with the pests. BC processes can be properly understood if chemical, physical and biological interactions are understood in soil, in order to directly understand how entomopathogenic nematodes react when applied on plants with the aim to attack and kill insect pests (Georgis et al, 2006). The higher the pest population the greater the damage of crops, while when pest population is low then tolerance may be obtained. The main objective of pest management projects is to ensure that pest populations are reduced and crop damage is also directly reduced so that people can purchase good quality fruits, vegetables and other plant products (Flint et al, 1998).

Because of health-related reasons, the use of chemical pesticides which include insecticides, herbicides, fungicides and acaricides will eventually diminish as more natural biological agents are identified and applied. In addition to natural agents, there is a concept of biological plant protection which is the use of biological processes to improve plant growth and modify plants to be resistant against disease and also develop effective ways to suppress pests (Georgis et al, 2006).

The main aim of BC is to use natural enemies to destroy or reduce pests by disrupting their reproduction and then ultimately decrease pest population (Flint et al, 1998). Natural enemies play an important role in pest management and can be used to specifically target unwanted host in an ecosystem hopefully without having adverse outcomes such as

destroying crops themselves. These enemies can be parasites or predators both with the mission to attack and kill pests. Bacteria, fungi and nematodes can be used as natural enemies. Several nematodes species have been identified to have the potential to successfully kill specific insect host in a few days after invasion, and examples include *Heterorhibditis* and *Steinernema* species (Jagdale, 2009).

Very often natural enemies are not noticed by pest managers and are left unidentified even though it is a fact that every pest has its own natural enemy that can decrease its population when conditions are favourable. Usually natural enemies are destroyed by the application of synthetic chemical pesticides and also unfavourable weather conditions which also contribute to them not being noticed by pest managers. A recent example includes cockroaches and woodlice which are controlled by a specific species of nematodes and the research behind this was strongly supported by the Association for Industrial Research (Flint et al, 1998).

The problem with synthetic chemical pesticides has been reported based on animal and human health implications, as well as the ecological problems caused by application of these synthetic chemicals. The accumulation of these chemicals in humans can interfere with most of the biological processes and pathways; affecting several proteins involved in cell growth and repair because they can induce DNA mutations in human cells, (Segal et al, 2000). These pesticides further affect the ecosystem in nature whereby they kill the beneficial insects as most of them are not host specific. Another disadvantage is associated with the resistance of the insects towards the synthetic chemical pesticides whereby even at the highest concentration of the chemical, the insects rapidly grow and continue damaging crops (Georgis et al, 2006). A better way of controlling and reducing pests had to be found and thus the use of biological agents has so far not shown any adverse consequences towards human health and they are therefore considered safe for human health as well as the environment. The identification of the naturally occurring enemies is important in taking optimum advantage of the use of biological control (Flint et al, 1998) and this is why this research aims to identify entomopathogenic nematodes which will be tested for their ability to be used for biological control in agricultural industries.

1.2 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are non-segmented roundworms also known as threadworms or eelworms. They have an excretory, nervous, digestive and reproductive

system but lack the circulatory and respiratory system. The alimentary tract consists of the mouth, buccal cavity or stoma, oesophagus, intestines, rectum and the anus respectively. They range in size from 0.3mm to 10mm long and they can be more or less cylindrical (Flint et al, 1998).

EPNs are found in various locations and can survive externally on insect exoskeleton or internally in the reproductive, respiratory, digestive or excretory system. Generally some nematodes reside in very dry areas including deserts and most have the ability to tolerate environmental stresses which include anoxybiosis, thermobiosis and desiccation (Grewal, 2000).

It is evident that the free-living stage of the EPNs during the life cycle is the most infective and effective stage where the free-living infective juveniles can search and move into the soil in search of insects to infect (Spence et al, 2008). EPNs utilise the insect cadaver as their nutrient source and also feed on the microflora and microfauna associated with the cadaver (Flint et al, 1998). The use of molecular biology techniques and genomics has improved the efficiency and ability to identify EPNs which can be used as biological control agents and understand their ecology, behaviour and how they respond to climate change through screening of related genes (Segal et al, 2000).

1.3 Why EPNs can be considered effective biological control agents?

Any natural enemy is considered effective if it can successfully reduce pest populations in a specific ecosystem without harming beneficial organisms. Below is a general life cycle of EPNs showing what happens upon invasion of host by infective juveniles, and what they benefit from invading the insects (Georgis et al, 2006).

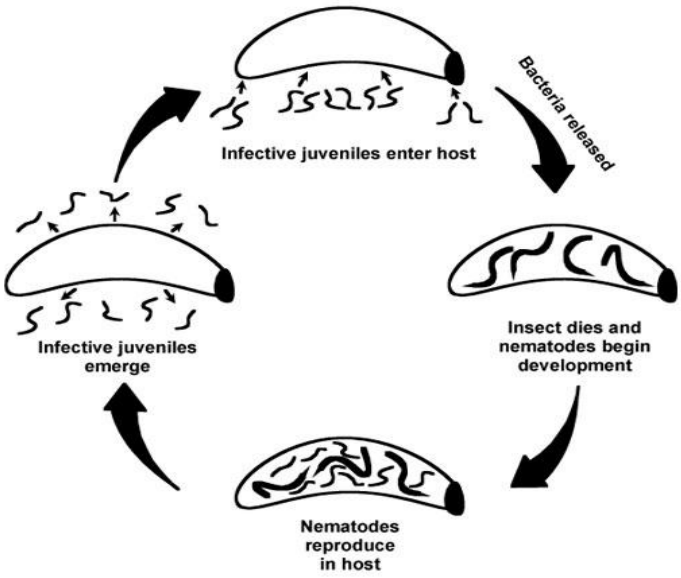

Figure 1.1.3 EPNs life cycle: Infective juveniles (IJs) enter the insect and release enterobacteria into the hemocoel of the host which then kill the insect. New generation of EPNs feed on the insect cadaver and proliferate together with the symbiotic bacteria. IJs are then released into the soil and they migrate in search for new live host (Adams et al, 2009).

Entomophathogenic nematodes genera *Steinernema* and *Heterorhabditis* have a symbiotic relationship with enterobacteria *Xenorhabdus* and *Photorhabdus* respectively, (Stuart et al, 2006). These bacteria are capable of killing host within 24-48 hours after they have been released into the insect's gut. Through this life cycle, EPNs have thus far being recognised as the best candidates for biological control of problematic insects (Adams et al, 2009).

Most nematodes are amphimictic (requiring both sexes to reproduce) such as the *Steinernema* genera while *Heterorhabditis* nematodes are hermaphrodites or parthenogenetic (requiring females only) (Griffin et al, 1994). Most of these identified EPNs live for several weeks to months in the infective stage so they can persist for a long time in the soil. Their life cycle also contribute to why they are cheap and easy to culture, sub-culture and maintain hence they are considered the best biological control organisms which can be used to replace or substitute for harmful synthetic chemical pesticides (O'Leary et al, 2001).

1.4 Environmental factors affecting nematode-insect interactions

Several species have been successfully identified and they are currently commercially used in many agricultural industries, forestry and in medical entomology (Adams, 2009). EPNs interact with environments which include the insect hemocoel, soil pore space or river bottom and these environments are referred to as microenvironments (Webster, 1973). In natural habitats, EPNs interact with various and numerous hosts as they have a have a broad host range and, thus this makes them highly suitable as biological control agents. The interaction of EPNs with insects can be limited mainly by climate and soil type. Moisture is important and soil texture is important. Ecological barriers can also interfere with the infection process of insect host by EPNs (Susurluk, 2003). In the absence of hosts or under unfavourable seasonal conditions such as draughts, the free living infective juveniles need to survive in the soil until the conditions became favourable again. Free living infective juveniles therefore need to be able to survive desiccation as soil moisture levels decline (Stuart et al, 2006).

Even when EPNs are parasitic, that does not necessarily imply that they will be effective as biological control agents. Factors such as nematode motility can contribute to the decreased infection rates for example; a parasitic group of nematodes was unable to kill black flies because of their inability to swim (Flint et al, 1998). These factors can be taken into consideration in order to understand how nematodes interact with insects in the natural environment. Such knowledge also contributes to the development of effective EPN products and also identifying suitable season and weather for EPNs application.

1.5 Application Technology and environmental considerations

One strain of a specific nematode species can be used to control various insect pests in agricultural industries, forestry and other fields where needed. The efficacy of pest management and control depends vastly on the methods of application of EPNs and these methods include the use of spray equipments and several irrigation and pumping systems (Shapiro-Ilan et al, 2006). Equipments and techniques used to apply specific biological control agents influence and also increase the chances of proper contact between the nematode and the insect (Jagdale et al, 2009). If one applies biological control of insects into the correct system in the correct way then they can expect it to be an economically and environmentally successful process.

It is of critical importance to analyse the environment in which the EPNs will be applied and take into consideration factors which may drastically affect their infectivity. Soil moisture

and water content, temperature and ultraviolet radiation are some of the environmental factors which may impact on the efficacy of EPNs.

1.6 Storage and formulation of EPNs and development of industrial product

When developing an industrial product, one should take into consideration that EPNs are highly sensitive to ultra violet radiation, high humidity and desiccation. In order to optimize the strategies which are necessary for increasing the effectiveness of these nematodes one must critically focus on the timing of application and type of formulations (Shapiro-Ilan, 2000). Several formulations which have been used before include clay, polyetherpolyurethane sponge, anhydrobiotic nematodes, bait and activated charcoal (Grewal, 2000).

In order for nematodes to be considered efficient and successful pest control agents they should reach the end user in good condition since there will be no point in commercializing them if they die before use. Good storage and formulation strategies can be developed in order to ensure nematode survival and maintenance of increased infectivity (Grewal, 2000). This is why it is crucial to have an understanding of nematode ecology and also analyze which environmental factors affect their activity and infectivity (Lewis et al, 2006).

Nematodes survival must be evaluated at varying temperatures and then ultimately identify the maximum survival temperature and also the mortality temperature. Once that information is obtained, researchers and pest managers will have the approximate idea of which climatic conditions are suitable for the application of nematodes in the field (Shapiro-Ilan et al, 2006). Other factors which must be taken into consideration include pH and salt concentration and these factors will obviously differ depending on the sensitivity of each EPNs species. pH can affect bacterial growth associated with the nematodes and their survival in storage facilities (Grewal, 2000) .

Furthermore, a good strategy for formulation development would involve establishing in what medium infective juveniles would survive desiccation. Experiments can be conducted to test which type of formulation material will result in increased or decreased infectivity thus desiccation studies are relevant as they provide a lot of insights in this research. Formulation material and content is also vital for the prolonged survival of nematodes (Shapiro-Ilan, 2000). Addition of preservatives can affect the survival of nematodes and also aerated water

which plays a role in the reproduction, growth and survival of nematodes (Shapiro-Ilan, 2000).

1.7 The contribution of genetic engineering in biological control and nematodes behaviour

In order to make EPNs better and effective biological control agents, behaviour studies are crucial in order to understand what stimulates the nematodes to move towards the host and ultimately infect it (O'Leary et al, 2001). Previous studies have focused carefully on the interaction of the EPNs with host where experiments were done to observe and record the movement of EPNs in soil and how deep they forage into the soil (El-Borai et al, 2011). Once the data had been interpreted and analysed, specific genes can then be isolated and analyzed in order to further understand the molecular reasoning behind EPNs behaviour. Once the genes have been isolated, genetic engineering can be employed to improve the function and expression of those isolated genes and therefore directly improving the effectiveness of EPNs, (Segal et al, 2000).

Genetic engineering is further used to modify and improve beneficial characteristics of EPNs and thus increase their resistance against harsh environmental conditions and nematicides. These genetic modifications require genetic screening and isolation of the EPNs populations with beneficial characteristics. EPNs can be modified for heat tolerance and an example includes the EPN *Heterorhabditis bacteriophora* (Segal et al, 2000) which was manipulated to withstand extremely hot conditions and still exhibited a good infection potential.

Ongoing research has revealed that the biggest challenge hindering EPNs reaching their complete biological control potential is their sensitivity to extreme environmental conditions. In this case, genetic engineering can be applied as an improvement tool of already existing beneficial traits or the introduction of new useful genes in the genome (Segal et al, 2000). IJs which cannot tolerate extremely hot temperatures result in failure of their application on exposed surfaces including soil surfaces which can have very high temperatures. Temperature on its own can affect nematodes movement, infectivity, life cycle, reproduction, development and most of all their survival (Susurluk, 2003). Most EPNs studies are done on nematodes which originate from the northern hemisphere and can practically tolerate temperatures between 18^{o}C to 28^{o}C. This information is of great importance in warehouses used to store nematodes in agricultural industries (Segal et al, 2000).

In genetic improvement protocols different mutant strains may act as genetic markers. In order to obtain genetic variation the amphimictic nematodes individuals can be used for cross fertilisation and transfer of genetic markers and swapping genetic material. On the other hand hermaphroditic nematodes are important in the maintenance of homozygous line (Segal et al, 2000). Genetic engineering has already been tried on some nematode species including *Heterorhabditis bacteriophora* which was considered to be the perfect candidate because of the existence of special traits which included the ability to withstand desiccation (Grewal, 2000).

1.8 Summary

Overall, insect pests have caused major damage to commercial crops and this has affected the economy of most countries since the agricultural sectors generate a lot of income. A pest can be beneficial in its own environment but as soon as it has harmful implications to people and the ecosystem then it is considered problematic. In order to protect crops from pathogens, synthetic chemical pesticides were developed (Flint et al, 1998). Unfortunately the use of these chemicals has raised major concerns in human and livestock health as well as the ecosystem. Another problem which was reported early by most farmers was the development of pest resistance to pesticides. These problems have led to the use natural enemies to control and reduce pest populations (Flint et al, 1998) thus this project focuses on the use of identified EPNs in the study as biological control agents. This project further aims to evaluate the identified EPNs potential to replace synthetic chemical pesticides in the fields. Several techniques including soil baiting, the white trap method, DNA isolation, Polymerase chain reactions and DNA sequencing were employed in the identification process of the unknown nematodes. Once specific EPNs were identified a series of experiments including the comparative dose assay, desiccation tolerance and behaviour studies were conducted in order to understand the effectiveness of the identified species. Results of the study and observations gave ideas for formulation and storage strategies.

1.9 Research motivation

1.9.1 The utilization of indigenous EPNs as biocontrol agents of insect crop pest requires the development of rapid identification procedures such as the PCR

amplification of the 18S rDNA. In this investigation this procedure was used to identify EPNs isolated from local soils collected in the Johannesburg region.

1.9.2 The effective utilization of indigenous EPNs requires a good using of their behaviour, tolerance to desiccation and their effectiveness in relation to the application dose. Understanding the induction of desiccation tolerance and recovery from dehydration will be important for the development of formulations and storage strategies.

1.10 Objectives

- ✓ 1.9.1Isolate, identify and characterise EPNs from collected soil samples.
- ✓ 1.9.2Investigate desiccation tolerance of the identified EPNs
- ✓ 1.9.3Investigate EPNs behaviour and dose response and foraging strategies

1.10Aims

- Isolation of infective juveniles (IJs) from infected dead larvae using the White trap method
- Molecular identification of nematodes using PCR amplification of the 18S rDNA and DNA sequencing
- Identification with the aid of morphological analysis by light and scanning electron microscopy
- Comparative dose assay, effective concentration of the EPNs
- Perform dehydration survival studies
- Behaviour studies (Investigate whether the identified nematodes can bait on target pest host, what stimulates them to move towards the host and how far deep down the soil can they forage bearing in mind factors such as soil moisture which may affect EPNs movement)

1.11 Research impact and feasibility and economic benefits

- This research has major positive environmental and health impacts as it can contribute to the introduction of an alternative way for the controlling and reducing of insect pests without leading to any adverse consequences towards human or domestic animal health.
- It has social development impacts, whereby farmers will be trained and educated about the application and use of these new and improved biological control methods. Jobs will be created since there will be a need for facilitators.
- Impacts on maintaining a healthy environment free from synthetic chemicals which can harm and destroy ecosystems and biodiversity.
- EPNs are relatively cheap to isolate from the natural environment and also to maintain at low cost, therefore EPNs as biological control agents can be cost effective.

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Chapter 2

Identification and characterisation of entomopathogenic nematodes

2.1 Introduction

2.1.1 Entomopathogenic nematodes and pest control

Several entomopathogenic nematode (EPNs) species have been successfully identified and used commercially in agricultural industries, forestry and medical entomology (Grewal et al, (2001). EPNs of the genera *Steinernema* and *Heterorhabditis* which have a symbiotic relationship with the enterobacteria, *Xenorhabdus* and *Photorhabdus* species *r*espectively, have already been used in other countries to control insect pests (Rosa et al, 2004). Further studies are currently being conducted to increase the possibility of identifying more species with improved and higher potential to control and diminish harmful pests which cause economic, health and environmental conflict (Gaugler et al, 2002).

Classification of nematodes also depends on the type of relationship they have with their host so if an insect is found dead upon invasion by the nematodes then they can be considered parasitic. To prove or disprove parasitism, Koch's postulates can be used as they are the main prerequisites for the confirmation of pathogenicity. Consequences of parasitism include delayed development and sterility, reduced fight activity and other morphological and physiological alterations in insects (Kaya et al, 1997).

For the past 11years EPNs have been used for controlling soil and cryptic pests in Australia, Asia and Europe. Synthetic chemical pesticides not only raised concerns about the negative impacts they have on human health and biodiversity but also have implications on soil and water pollution as these chemicals accumulate in soil and get washed into nearby rivers and dams (Grewal et al, 2001). This has led researchers to identify alternative, healthy and safe ways of controlling pests without harming the environment.

Several insects can be controlled by EPNs; however for the past years the use of EPNs has been limited due to lack of feasible formulation and storage methods which are important to understand prior implementation (Grewal et al, 2001). However, before one can consider strategies on formulations and other methods necessary for the application of EPNs, the life cycle of these organisms must be well understood.

2.1.2 Typical EPNs life cycle

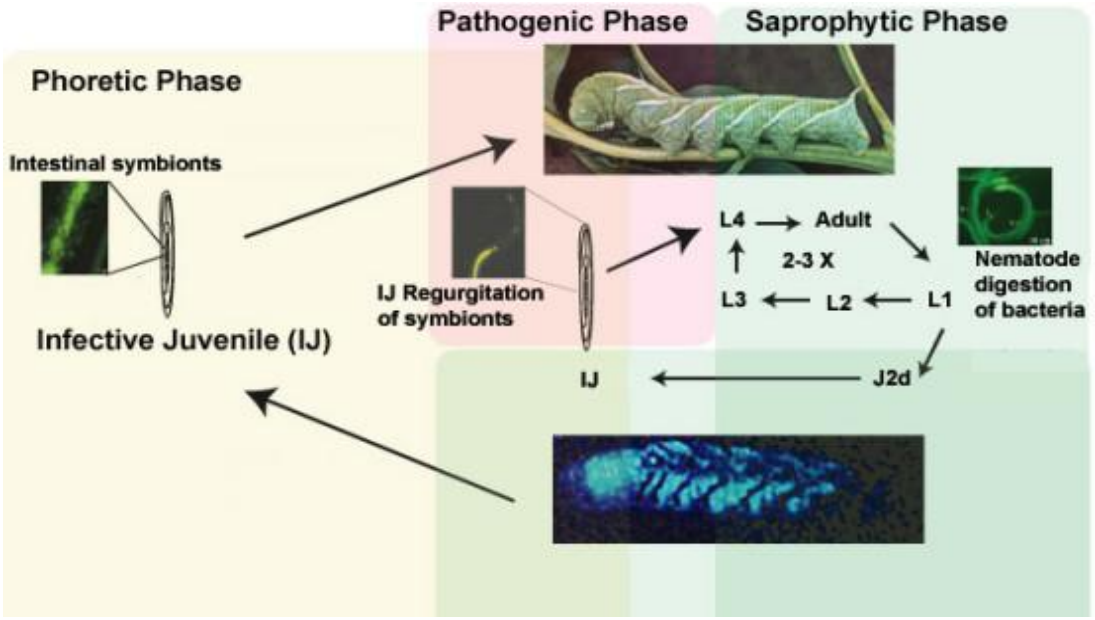


Figure 2.1 EPNs typical life cycle is divided into the egg, 4 juvenile stages and the adult stage (Gaugler, 2002).

The non-feeding IJs start the life cycle of EPNs, as they can survive for longer in the soil; searching for insect hosts to infect. The 3rd stage IJ of *Heterorhabditis* enters the insect by penetrating the cuticle. Generally EPNs can enter their host through all openings such as the mouth, spiracles and anus. Once the IJs are inside the host, they mainly attack the hemocoel where they expel the infectious symbiotic bacteria which carry powerful toxins capable of weakening the insect and swiftly killing it within 24-48 hours. Favourable conditions are created once the insect host dies, as IJs start feeding on the bacteria and cadaver; then proceeding to reproduce, giving rise to 2-3 generations of EPNs inside the insect cadaver. The 3rd stage IJs released can survive for longer in the soil environment while other EPNs in other stages of the life cycle may die when nutrients are depleted (Grewal et al, 2001).

2.1.3 Host range of EPNs

Each EPNs species has a relationship with a one precise bacterial species resulting in a symbiotic relationship (Hazir et al, 2003). However, the same cannot be said about bacteria as one bacterial species can create unions with more than one nematode species.

Due to the fact that nematodes kill their host so quickly, leaving no chance for the development of any relationship between the host and parasite; this fact allows EPNs to kill a lot of different hosts from various species and thus they are considered to have a broad host range (Andalo et al, 2006). For example, experiments have revealed that *S.carpocapsae* is capable of killing over 250 different insect species belonging to more than 75 families in 11 orders. However; in field application studies, different results were observed due to limited nematode-host contact and interaction and also due to UV-radiation, desiccation stress and temperature fluctuations which contribute to a decrease in infectivity of EPNs (Stuart et al, 2006).

2.1.4 Molecular identification and phylogenetic analysis.

A polymerase chain reaction is done to amplify the internal transcribed spacer (ITS) region; the non-functional DNA between the ribosomal RNAs (rRNAs) on transcripts. This region is highly conserved in most nematode species and thus it can be used to identify newly isolated nematodes. The *Steinernematidae* family was reported to consist of the genera, *Steinernema* with more than 40 recognized species. The family *Heterorhabditidae* consists of 1 genus, *Heterorhabditis*, which consists of 11 described species (Glazer et al, 2000). This is why this project also aims at identifying more EPNs species and using phylogenetic analysis to understand the relationships between the identified EPNs species with other species which have already been identified and saved in the NCBI database.

2.2 Materials and methods

2.2.1 Breeding and maintaining insect larva

2.2.1.1 *Galleria mellonella*

Order: Lepidoptera

Family: Pyralidae

Common name: Greater wax moth

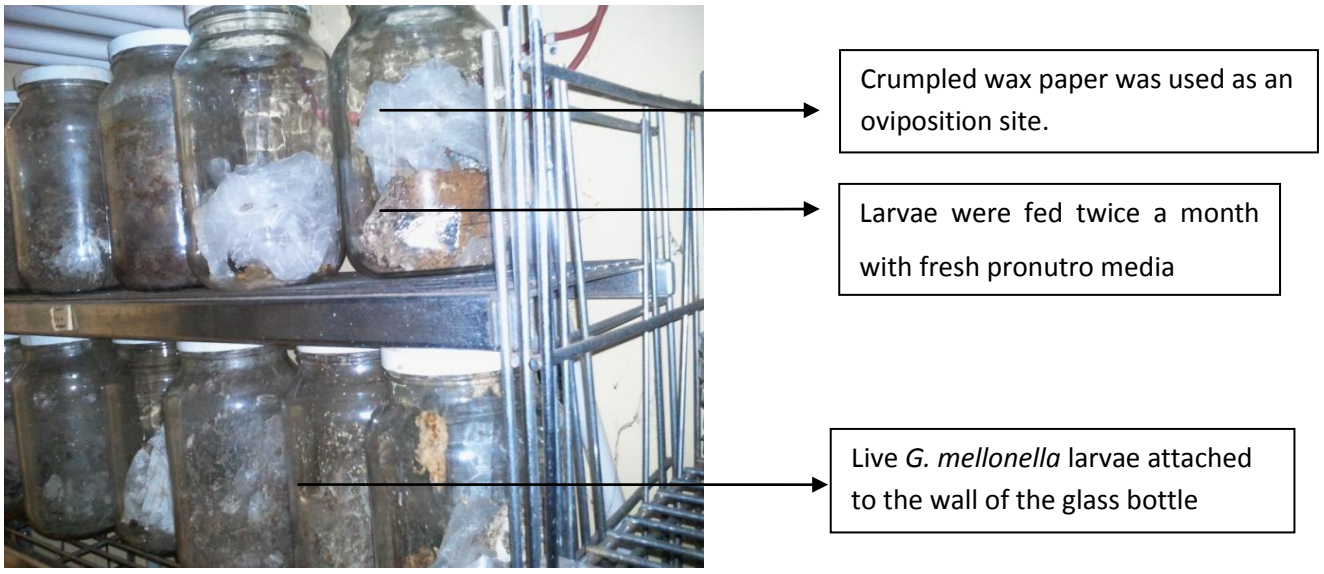


Figure 2.2.1.1 Adult wax moths (male and female) and larva kept in 3L glass bottles stored at room temperature in an incubator.

G. mellonella was reared in the laboratory as shown in the above image. Adult female wax moths mated with males and then laid eggs to give rise to *G. mellonella* larvae. Adult wax moths (male and female) and larva kept in 3L glass bottles, stored at room temperature in an incubator (25°C). The cultures were maintained by continuous supply of nutrient media made from pronutro and honey (see appendix B) and sub-cultures as soon as the bottles were overcrowded, (Kaya et al, 1997).

2.2.1.2 *Tenebrio. Molitor* (*T. molitor*)

Order: Coleoptera

Family: Tenebrionidae

Common name: Yellow wheat mealworm

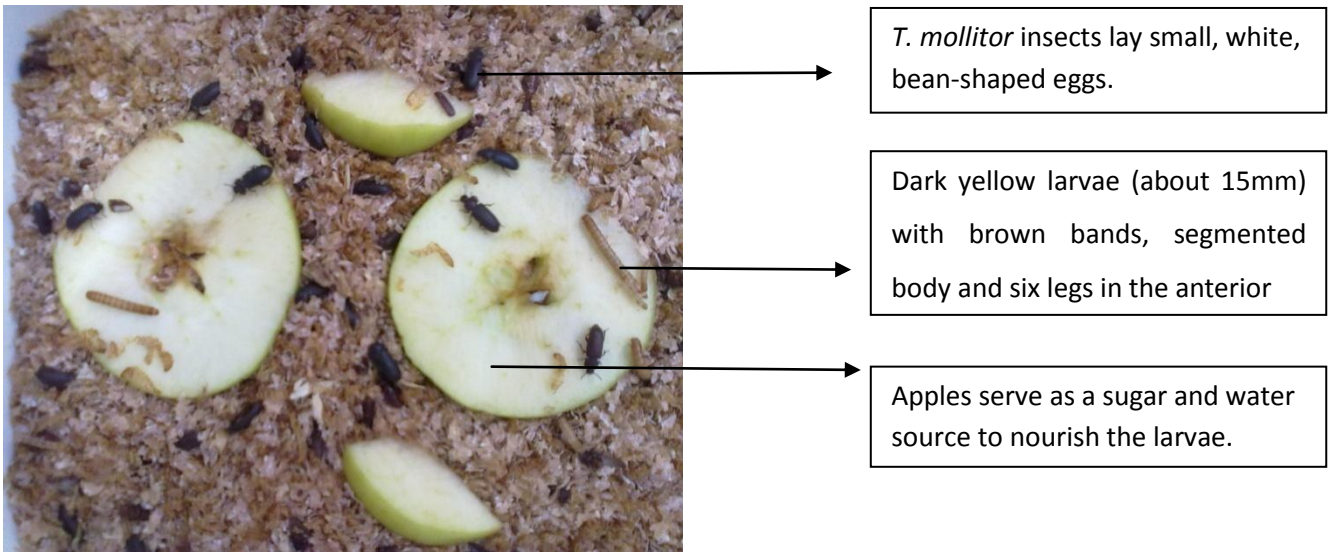


Figure 2.2.1.2 the mealworm larvae were reared in the laboratory and incubated at room temperature in an incubator.

T. molitor was also used as host insect for propagating EPNs. They were reared in the lab in 2 litre plastic containers and fed wheat bran.

2.2.2 Collection of soil samples

Figure 2.2.2 collected soil samples stored at room temperature. They were obtained from road number 6, 7, Wadie Street, Boundary road and Grasmere road in Walkerville, south of Johannesburg and used for isolation of nematodes.

A hand shovel was used to collect the soil and with reference to (Kaya et al, 1997) at each area, soil was collected to a depth of 15 cm (Stuart *et al*, 2006). The soil samples were kept in separate plastic containers and stored at 22-25°C (optimum temperature for

nematodes) during transport to the laboratory. Prior baiting, water was added to give a moisture content not greater than 8% moisture and the samples were then stored at room temperature overnight (Kaya et al, 1997).

2.2.2.1 Baiting Technique: Extraction of the nematodes from the soil samples adapted from (Kaya et al, 1997).

Soil samples were baited with *Galleria mellonella*, the greater wax moth and *T*. molitor the yellow meal worm. About 20 insect larvae of each species were placed on top of each soil sample and the tub turned over then stored in an incubator set at 25^{o}C. Observations were done daily to monitor the infected and dead larvae. This method is also called the soil baiting technique and it was used for isolating EPNs from soil. The symptoms of cadavers after infection were also recorded and used for diagnosis of EPNs induced infection. All the dead larvae were collected after 48-72 hours then placed at room temperature to allow them to dry for at most 48 hours at room temperature. These dried infected larvae were then placed on white traps to allow the emergence of nematodes.

2.2.2.2 Isolation of infective juveniles (IJs) from dead larvae using the White trap method

The modified traps as described in (Kaya et al, 1997) were used for isolation of IJs from dead larvae suspected to be infected with EPNs. The modified White trap consists of two Petri dishes of different sizes. A small (60 mm in diameter) plastic watch dish was inverted and covered with a moist filter paper and then placed within a Petri dish of 90 mm diameter. The outer Petri dish was filled with sterile distilled water up to 2 mm and each dead insect larva was placed on top of the moist filter paper as demonstrated in the figure 2.2.2.2a. White traps were then placed on a bench at room temperature during warm summer days and during cold winter days a heater was used to keep the room temperature at 25 ^{o}C. The emergence of the IJs from the infected cadaver into the water was greatly favoured by temperature between 25°C-30°C. Precautions were taken to make sure that the filter was not too moist as that can introduce excess water into the insect cadaver while on the trap, leading to death of EPNs before they emerge.

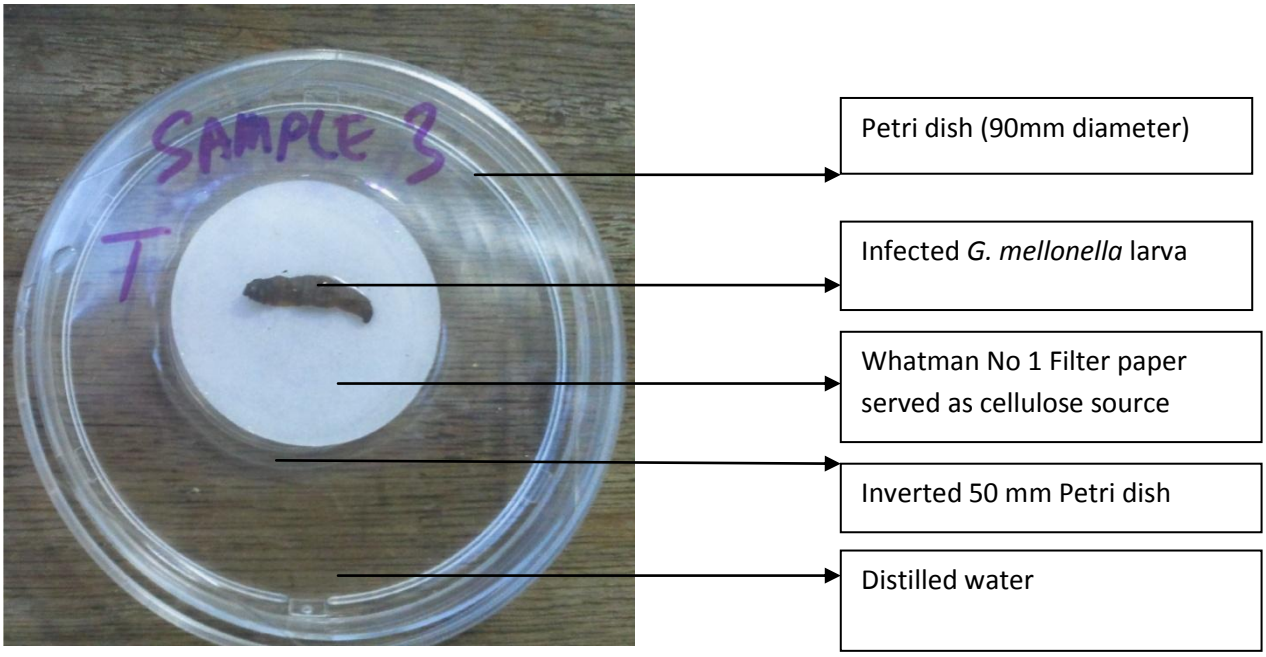


Figure 2.2.2.2a Representative image of a White trap used to collect IJs from insect cadavers.

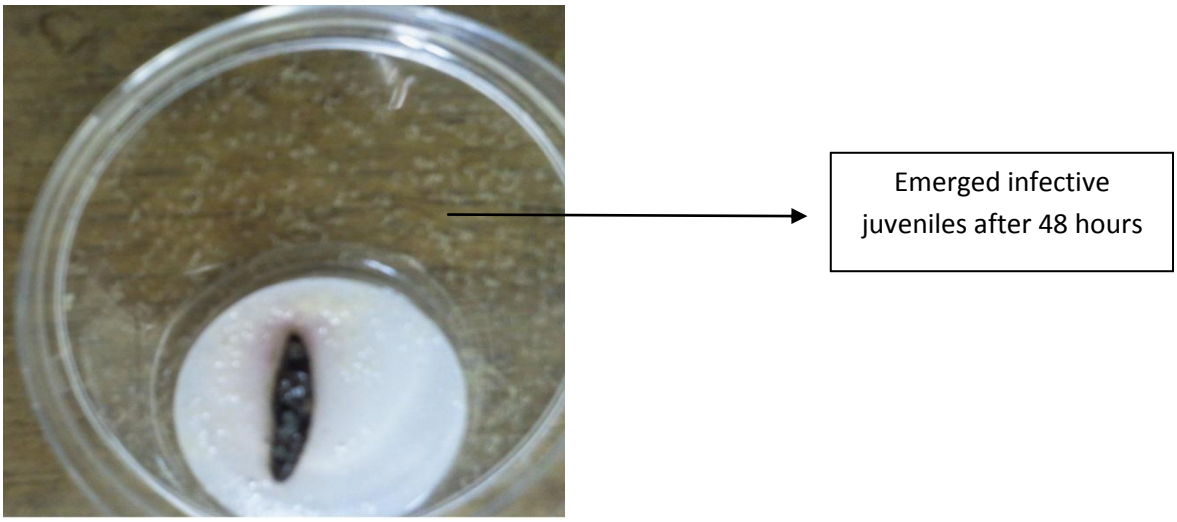


Figure 2.2.2.2b Representative image of IJs emerging from the insect cadaver migrating into the water in the 90 mm Petri dish.

2.2.3 Molecular identification of nematodes

2.2.3.1 Genomic DNA extraction from nematodes

Genomic DNA was extracted from nematodes using a protocol from Puregene® DNA Purification Kit,

Gentra systems 2003. Nematodes were collected from White traps under sterile conditions under a laminar flow hood, and placed in sterile 1.5 ml Eppendorf tubes. The nematodes were rinsed three times using approximately 4ml distilled water per wash. Nematodes were centrifuged at 14000rpm for 10 minutes and a pellet was formed. The tubes were then placed on ice for 30 seconds to allow cooling and reduction of genomic material degradation. And excess water was removed. Nematode pellet was re-suspended in 1 ml distilled water and transferred into a sterile 1.5 ml Eppendorf on ice. Samples were centrifuged at 13000-16000 rpm for 3 minutes then placed on ice for at least 30 seconds and the supernatant was discarded. 600µl cell lysis solution was added and tubes were inverted several times. Into each tube 3µl of proteinase K solution (from kit) was added and the tubes were inverted gently 25 times. The samples were incubated at 55˚C for 3 hours, until the tissue particulates have dissolved were inverted periodically. 3µl of RNase A solution was added into the cell lysate, and inverted 25 times then incubated at 37˚C for 15-30 minutes. Samples were cooled to room temperature and 200µl of protein precipitation solution was added to the RNase A treated cell lysate and vortexed at high speed for 20 seconds. The tubes were centrifuged at 13000-16000 rpm for 3 minutes. A tight protein pellet was formed. The supernatant containing the DNA was poured into a sterile 1.5ml centrifuge tube containing 600µl 100% isopropanol and then inverted gently 50 times. Tubes were centrifuged at 13000-16000 rpm for 1 minute; the DNA was visible as a white pellet. The supernatant was poured off and drained the tube on clean absorbent paper. Into the tube 600µl of 70% Ethanol was added and inverted the tube to wash the pellet gently. The tubes were centrifuged at 13000-16000 rpm for 1 minute and carefully poured off the ethanol. The tubes were inverted and drained on an absorbent paper again and allowed to air dry for 10-15 minutes. 100µl of DNA hydration solution was added and the DNA was rehydrated by incubating the sample 1 hour at 65˚C. The tubes were tapped to aid dispersion of the DNA which was then store at 4˚C.

0.5% agarose gel was prepared in order to confirm the presence and quality of the extracted DNA. 0.25g of agarose power was dissolved in 50ml of 1XTBE buffer and 1µ of ethidium bromide was added and mixed gently. The gel was left to solidify with the well comb inserted. For each 10µl sample, only 3µl of loading dry was added and then samples were run on the gel for 30minutes at 90 volts immersed in 1XTBE, with constant current.

2.2.3.2 PCR amplification of nematode ITS rDNA region using universal primers and gene sequencing.

Primer information:

TW81 Forward Primer

5’-GCGGATCCGTTTCCGTAGGTGAACCTGC -3’

Tm (°C)=71.94

AB28 Reverse Primer

5’-GCGGATCCATATGCTTAAGTTCAGCGGGT -3’

Tm (°C)=68.87

Table 2.2.3.2a PCR recipe showing the quantity of each reagent required for a reaction to amplify the ITS rDNA region.

PCR contents in a microtube	Quantity (µl)
PCR Master mix	25
Nuclease free water	22
Forward primer TW81	1
Reverse primer AB28	1
DNA (ng)	1

Total reaction volume= 50µl

Samples were mixed gently and put under the following conditions to amplify the ITS region found between the 18S and 28S rDNA:

Initial denaturation before cycling: 94°C for 5 minutes

25 cycle amplification series:

Denaturation at 95°C for 60 seconds

Annealing at 64°C for 60 seconds

Extension at 72°C for 120 seconds

Final extension after cycling: 72°C for 10 minutes

1% agarose gel was prepared in order to confirm the presence of the desired amplified region. 0.5g of agarose power was dissolved in 50ml of 1XTBE buffer and 1µ of ethidium bromide was added and mixed gently. The gel was left to solidify with the well comb inserted. For each 10µl sample, only 2µl of loading dry was added and then samples were run on the gel for 45minutes at 90volts immersed in 1XTBE, with constant current.

Post successful polymerase chain reactions, PCR products where then sent to Inqaba Biotechnical Industries (Pty) Ltd; South Africa for sequencing. The nucleotide sequence alignment website of the National Centre for Biotechnology Information (NCBI-BLAST) was used to identify the unknown species of nematodes.

2.2.3.3 Identification by morphological analysis:

2.2.3.3.1 Light microscopy and specimen preparation

Killing and fixing nematodes modified from (Kaya et al, 1997)

Nematodes were placed in a Petri dish suspended in 1ml distilled water. 3-4ml of 100ºC TAF was added and left for 24 hours. TAF was replaced with double-strength TAF and stored at 4ºC to relax nematodes for up to one hour. 65ºC TAF was added and then the fixative was allowed to infiltrate for at least 24 hours. Most of the fixative was then removed.

Processing nematodes to pure glycerine:

Fixed nematodes were transferred to a Petri dish containing 0.5ml of solution I. 5ml of 95% ethanol was placed the watch glass containing the nematodes in the desiccator. The desiccator was placed in an oven preheated to 35ºC for 12 hours. The watch glass with nematodes was then removed from the desiccator. The watch glass was filled with solution II and placed in a glass Petri dish. The Petri dish was left partially open to allow for slow ethanol evaporation. The Petri dish containing the watch glass was placed in an oven preheated to 40ºC for 3 hours.

Water preparation alternative method

Fresh IJs were collected directly from the white traps and placed on a clean slide then covered with a cover gently. The IJs were then viewed under a light microscope connected to a digital camera and images were captured.

2.2.3.3.2 Scanning electron microscopy:

1ml of EPNs was collected directly from white traps and transferred into 1.5mL Eppendorf tubes and heated to kill them at 80°C for 5 minutes. IJs were rinsed with Ringers solution (pH 7.3) three times with 5 minutes between each rinse. IJs were then fixed in 8% glutaraldehyde for overnight (glutaraldehyde 25% EM grade, diluted in Ringers solution). IJs were further rinsed with distilled water three times and dehydrated with 30, 50, 70, 90, 95, and 100% ethanol at 10 minutes interval sequentially. Each sample was critically point dried with CO2 for 2 hours and mounted on SEM stubs, coated with palladium, and scanned using FEI QUANTA scanning electron microscope fitted with a digital camera.

Once the microscopy preparations were done, EPN3T and EPN5T images were captured EPN3 and analysed to assist in the identification process of the unknown nematodes. The table below summarises some of the general morphological features which can aid in identification of EPNs. Morphological identification in this study is useful to confirm EPNs identity after molecular methods have been employed and crucial for characterising the species.

Table 2.2.3.3 General morphology of entomopathogenic nematodes, *Steinernema species* (Edgington et al, 2009) and *Heterorhabditis bacteriophora* (Nguyen and Smart, 1996).

Steinernema australe		*Heterorhabditis bacteriophora*	
Adult Females	Adult Males	Adult Female and hermaphrodites	Adult Males
Anterior region (Head)		Anterior region (Head)	
6 Labial papilla, 4 cephalic papillae, 2 amphids and a mouth.	6 Labial papilla, 4 cephalic papillae and raising perioral disc	oesophagus without metacorpus, and enlarged basal bulb	Single testis; Bursa peloderan usually has 9 pairs of genital papillae
Posterior region (Tail)		Posterior region (Tail)	
Pointed tail and phasmid present before the end of the tail. Vulval flap	Blunt tail Spicule present	Pointed tail, females have ovaries, hermaphrodites have ovotestis and amphidelphic have a vulva median	Spicules present sometimes curved, paired or separated. Blunt tail in males
Additional characteristics Cheilorhabdions present Epiptygma		Additional characteristics Gubernaculum present Fat bodies	

Fat bodies	
Infective Juvenile (3rd stage)	Infective juvenile (3rd stage)
Head has a mouth, amphid, 4 celaphalic papillae and one line of lateral field.	Pointed tail, 2 cuticular sheaths covering the IJ, mouth and anus closed, immature oesophogus. Symbiotic bacteria in intestine present

2.2.3.4 Phylogenetic analysis

- Sequences obtained from Inqaba Biotech were edited using Chromas sequence analysis program. The following taxa were used for the construction of the phylogenetic tree for *Heterorhabditis*: *H. bacteriophora isolate* (Genbank accession number FJ217351), *isolate 117-C* (EU860183), *strain 51-C* (EU796074), *isolate arak1* (JF920961), *strain N-Arg* (HQ225906), strain N-NC1 (EF043445), *strain N-GPS23* (HQ225847), *isolate SF291* (FJ346825), strain J172 (EU716335), *strain 65-C* (EF530041), *strain IRAZ4* (FJ860043), *strain IRAZ8* (FJ860046), *isolate 175-C* (FJ360727), *H. georgina* (HQ225885), *H. species QZL-2011* and *Caenorhabditis elegans* (EU196001) was used as the outgroup taxa.

- The following taxa were used for the construction of the phylogenetic tree for *Steinernema*: *S. australe isolate* (Genbank accession number FJ235125), *S. diaprepsi* (GU173995), *S. brazilense* (FJ410325), *S. kushidai* (GQ497741), *S. karri* (GQ497742), *S. khoisanae* (DQ314287), *S. akhursti* (DQ375757), *sp.3SS-2003* (AY171286), *S. glaseri* (GU173998), *S. unicornum isolate* (GU191461), *S. apuliae* (HQ416968), *S. diaprespesi strain polk* (AF440764), *S. longicaudum* (AY170337), *S. guangodonyense* (AY170341), *S. khoisane* (EU727169) and *Caenorhabditis elegans* (EU196001) was used as the outgroup taxa.

The above EPNs sequences were derived from the National Center for Biotechnology (NCBI), using a tool called BLAST (Basic Local Alignment Tool), web address (blast.ncbi.nlm.nih.gov), edited using Chromas programme and DNAMAN v4.0 programme was used for phylogenetic tree construction. The Genbank sequences were used as references to establish the consensus sequences for the isolated EPNs.

2.3 Results

2.3.1 Symptoms, infection analysis and emergence of IJs from infected larvae.

Infection symptoms vary depending on different genera and species which infected the insect larvae. Yellowing and development of dark black spots is highly associated with the *Steinernema* genera. Maroon colour development observed after 24-48 hours of infection is associated with infections caused by *Heterorhabditis*. Figure 2.3.1a clearly shows the observed symptoms after 48 hours of infection.

Figure 2.3.1a Different infection symptoms observed when *G. mellonella* larvae were infected with pure cultures of isolated EPNs. A:maroon wax moth larva assumingly infected with *Heterorhabditis* genera. B: yellow and with black spots wax moth larva assumingly infected with *Steinernema* genera after 48 hours.

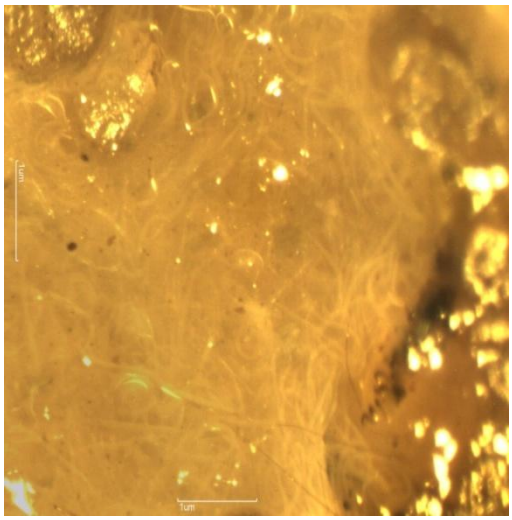


Figure 2.3.1b Image showing IJs at their early development stage emerging from a larvae infected with EPN 3T taken under a light microscope with a compatible digital camera. Nematodes emerge from the infected host within 24-48hours when placed on a white trap.

Once the physiological and morphological information was collected, molecular techniques were employed to confirm the placement of EPNs into genera and to identify the unknow species isolated form soil samples.

2.3.2 DNA extraction, PCR and Sequencing results of samples EPN3T and EPN5T

The quality of the extracted DNA was confirmed by agarose gel electrophoresis (figure 2.3.2a). The DNA is not degraded and thus has a high molecular weight as seen in the below image. This DNA was then used for further analysis.

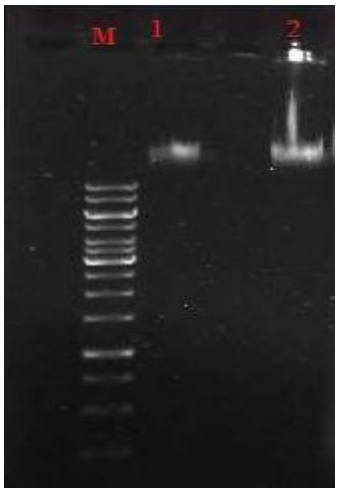


Figure 2.3.2a 1% Agarose gel electrophoresis of DNA extracted from unknown nematode species on

lane1 EPN3T, lane 2 EPN5T and M 100bp plus molecular weight marker.

Extracted DNA from EPN3T and EPN5T was amplified using universal primers targeting the 18S rDNA ITS region crucial for identification of nematodes.

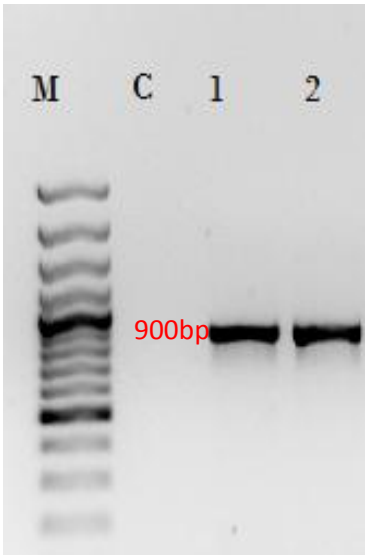


Figure 2.3.2b The 18S rDNA ITS region PCR products resolved on a 2% agarose gel. M: 100bp plus molecular weight marker, C: control lane (no DNA), 1: EPN3T and 2: EPN5T.

2.3.3 Sequencing of the 18S rDNA ITS region

Sequences obtained from Inqaba were used for sequence alignment against the Genebank sequence data base and thereby establish the taxonomic affinities of the isolated unknown species.

ACTTTGTGATTTTTAATGATTGTGCGAATGAGCGGAACGGCACAGGTTGCGT
TTCTAAGTGTCGATTCCGTTCGCTGAACGGCTTTGAATGGTTTCTATAGGTGT
CTGGAGCAGCTGTATGAGCGTGGTGTGGTGAAGGACATTTGACATCTTATGC
CAGTGTGTGTGAAATACGCGTCTCTTGTATTTTGTTGCTCGCTGGTGATGAGA
ATCAAAGAGGTCAGTCGGAGACCCGCCATTCACAAACCTACCATTATTTTAC
TTGATCATGCTCCATGCGCTTGTATGGTTGCAAACAAAGTTATCAAGTCTTAT
CGGTGGTCACTCGGTTCGTAGTTCGATGAAAAACGGGGCAAAAACCGTTAT
TTGGCGTGAATTGCAGACTATTGAACGCTAAAATTTTGAACGCAAATGGCAC
TATCAGGTTTATATCTGATAGTATGTTTGGTTGAGGGTCGATTAATTCGTTACT
TGCAGTCGGCTTCGACTGTTTCTTCGATTAGCTACTCTTGCGTGGAGTACCTT
TTCGGCGTGTGTCGCTTCATTGCGATAGGCTAATGGAGAGTTCCGGAATGCG
AGCGTTTCTTTCGCTTTCGGAATTTTCTGCTATCATATCGGT

Figure 2.3.3a The sequence of the unknown isolate; EPN3T had high affinity to *Steinernema autrale* with the accession number FJ235125. *Steinernema australe* isolate is suspected to be a new species identified in South Africa. It was first identified in 2009 in Chile were the soil sample was collected close to Isla Magdalena Island in the Pacific Ocean. Sequences of the ITS region have confirmed that this is a valid species.

GGGTATGCTTTGATCACGAGAGATCGGTACCAATGGAATCAGGCTTGTTC
TTGAWTTCAATCGGTTTCTCACCCCATCTAAGCTCATGGAGAGGTGTCTA
GTCCTAATTGGAGTCGCTTTGAGTGACGGCTATGAAAATTGGGTATGTTC
CCCGTGAGGGTCGAGCATAGACTTTATGAACAGTGCTGGAGCTGTCGCC
TCACCAAAAAATCATCGATAACTGGTGGCTATGTGTGACATTAGTCACATA
GGTATCTGCTGATGCAGAGAGCCTTAATGAGTTGTTCGTGTCATCTGACC
TACAACCGCCACTATCGGTAAATCAACCCAATTAACTTGTTTCTTGTGTCG
TGTTAATACATACTGGCAAAGTGTATTAGCTTTAGCGATGGATCGGTTGAT
GTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTTCGCGC
AGACGCTTAGAGTGGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTC
CCCTTCGGCACGTCTGGCTCAGGGTTGTTTAATAAGCGAAAGTGTTGAA
AGTTCATTAAACGAGAGTTCGGTGATACTGACAACACTGCGTCGATCGG
GTACTGTTGAAAGTACCCCGTTCAAGTATCTTTATGGGGCAACATGTCTT
GTATACGGAGACATGAAAGATATTAAGAGTATATACCTGTGGATGCCCAC
GTATGAAATATGACGTGTCGTATACACGGCTAGGAGGTATGTCTCAGATG
AATTTGTTTATGCAACCTGAGCTCAGTCGTGATTACCCGCTGAACTTAAG
CATT

Figure 2.3.3b The sequence of the unknown isolate; EPN5T sequence had high taxonomic affinity to *Heterorhabditis bacteriophora* isolate 56-C with the accession number FJ217351.

Both the identified species belong to different nematode families and genera. The results were confirmed by re-collecting the soil samples from the same regions as before and then re-isolation the species. Several other species were isolated but difficult to maintain and harvest in vitro thus isolates EPN3T and EPN5T were selected to be further used in the study and tested for their ability to be used as reliable biological control agents.

Table 2.3.3 A summary of EPNs identified through DNA sequencing, including the location and area co-ordinates of where they were collected

Sample label	Identified species	Region collected
EPN3T	*Steinernema australe isolate*	Road number 6
EPN5T	*Heterorhabditis bacteriophora isolate*	Wadie road

2.3.4 Phylogenetic analysis

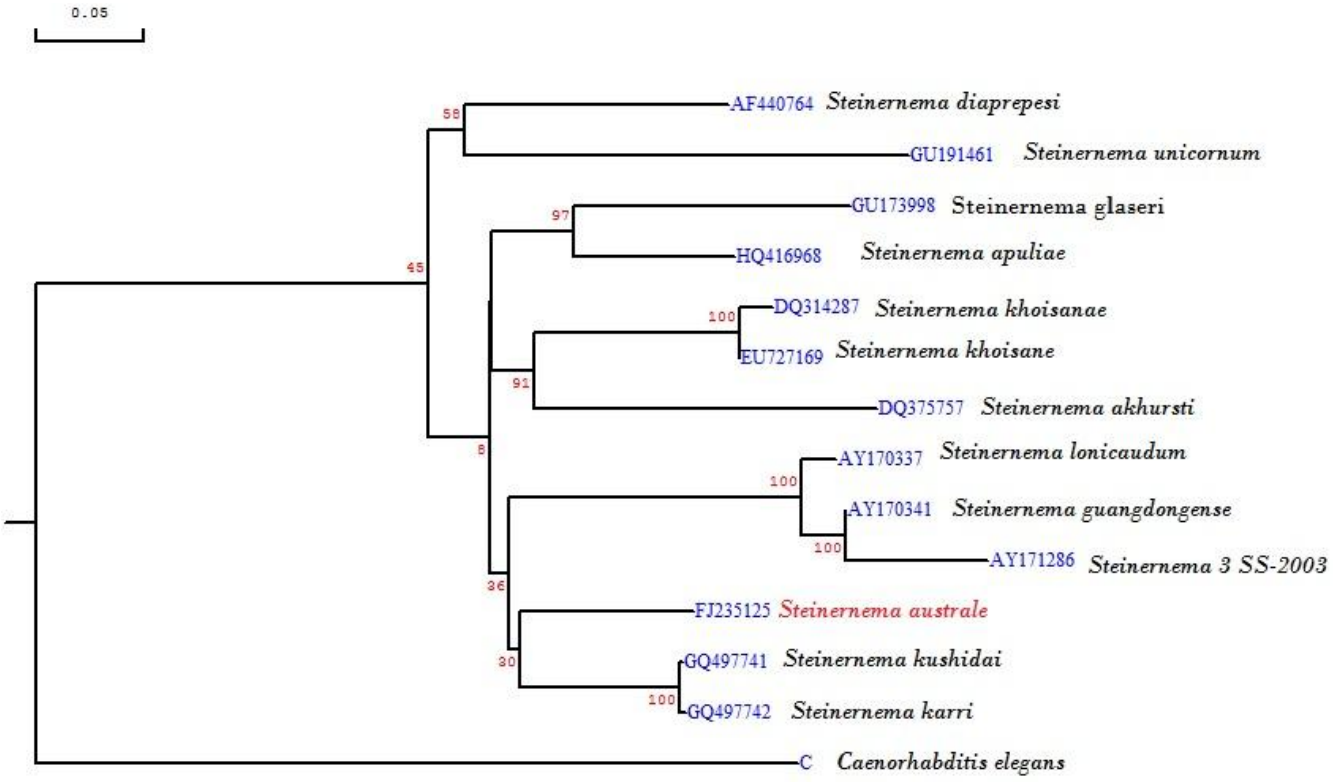


Figure 2.3.4a Phylogenetic tree showing the relationship between the initially unknown species highlighted in red (*Steinernema australe*) and 13 other species with accession numbers indicated in blue, where the analysis was based on the 18S rDNA ITS region of the EPNs. *C. elegans* were used to root the tree and the bootstrap values are based on 1000 replications on the nodes. Bar scale 0.05 substitutes per nucleotide position. The phylogenetic tree was constructed using the DNAMAN programme (v4.0).

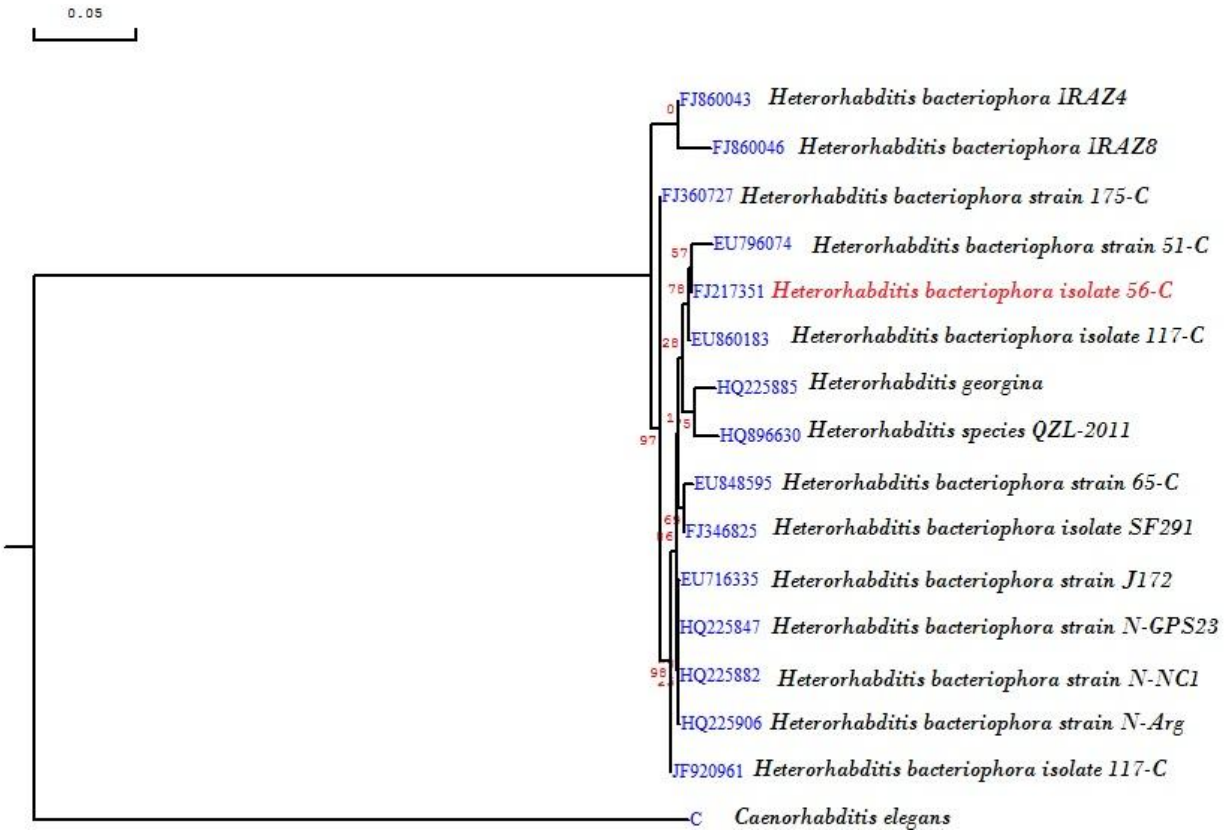
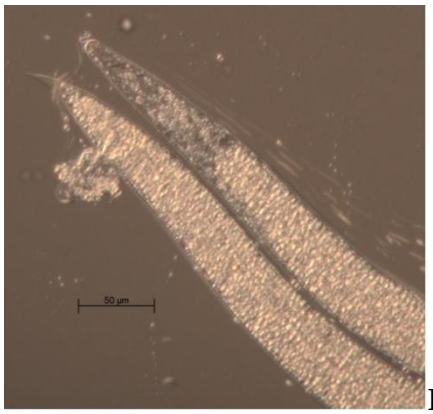
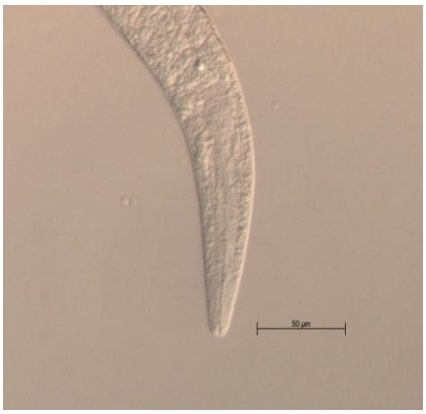
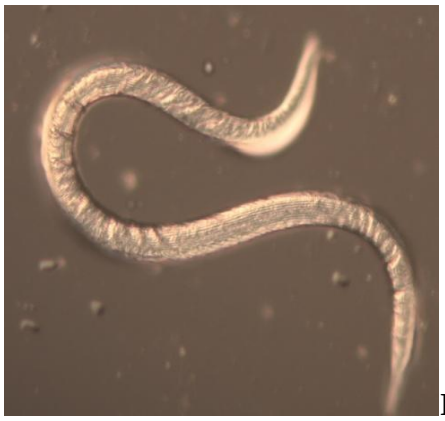
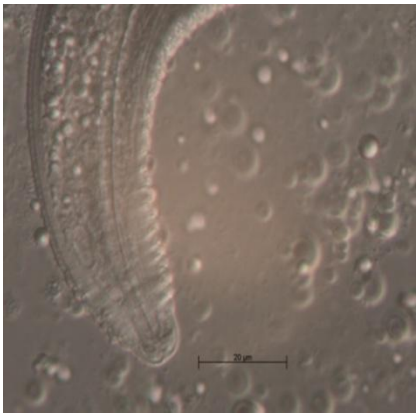


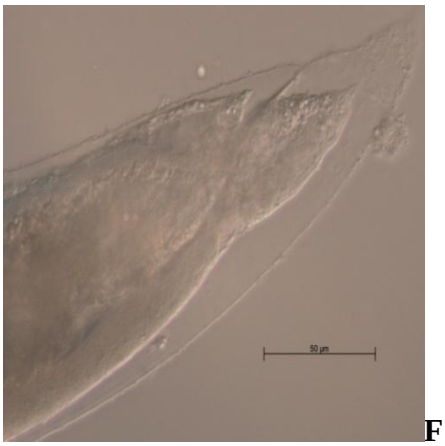
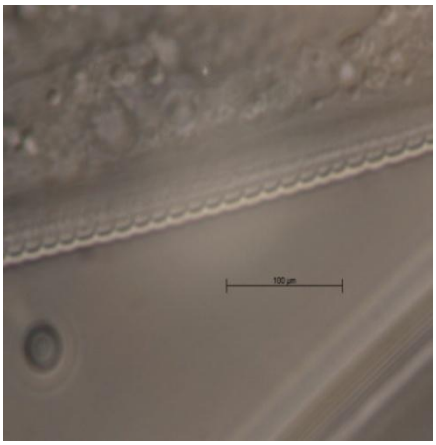
Figure 2.3.4b Phylogenetic tree showing the relationship between the initially unknown species highlighted in red (*Heterorhabditis bacteriophora*) and 14 other species with accession numbers indicated in blue, where the analysis was based on the 18S rDNA ITS region of the EPNs. *C. elegans* were used to root the tree and the bootstrap values are based on 1000 replications on the nodes. Bar scale 0.05 substitutes per nucleotide position. The phylogenetic tree was constructed using the DNAMAN programme (v4.0).

2.3.5 Morphological identification by light microscopy:

Light microscopy is an important tool in the identification of nematode species as it provides an idea of structure and existing organs within the nematode. Through this analysis, once can also characterise and understand different potentials displayed by the nematodes linked with structure, e.g. ability to withstand dry conditions can be linked with a thick cuticle.

A  **B** 

C  **D** 

E  **F** 

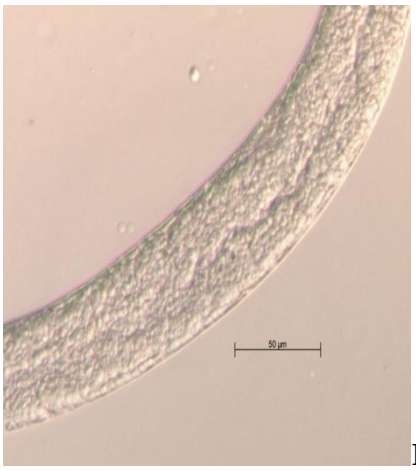
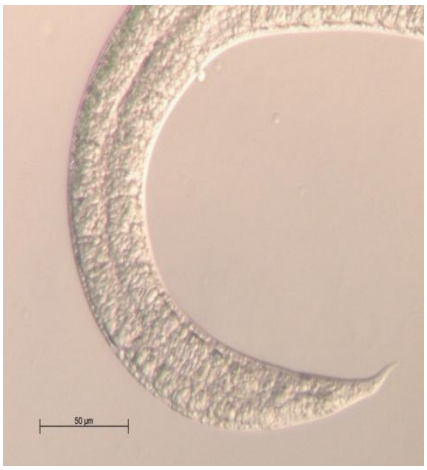
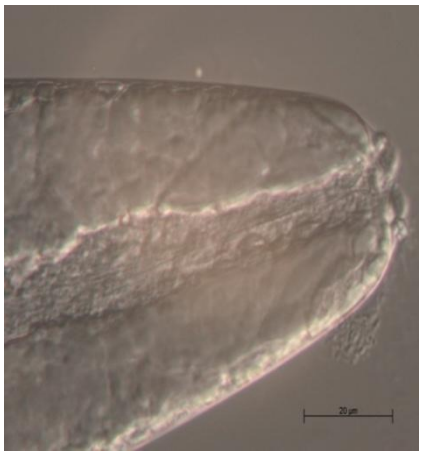
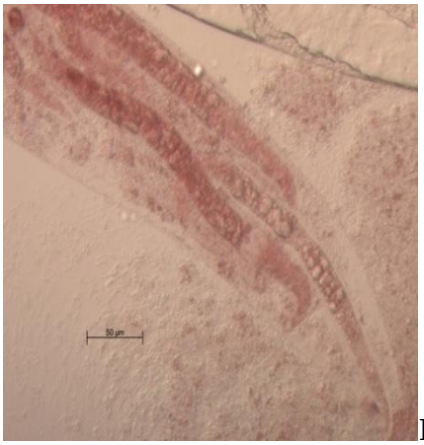
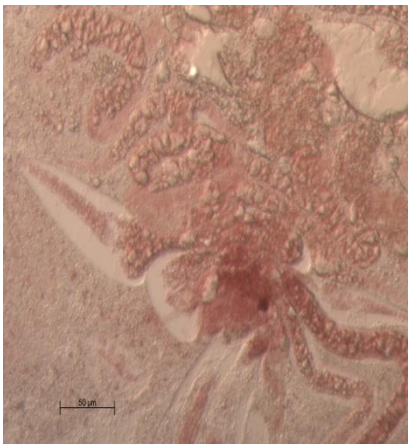
G  H 

Figure2.3.5a: Steinernema australe with A: showing the male and female mating, with fat bodies expelled from the vulva, B: the head highlighting the mouth, stylet, esophagus and metacorpus, C: Infective juvenile, D: mouth area of an adult with fat bodies, E: tail of a female adult, F: thick cuticle of an adult body, G: intestines and H: pointed female tail

A  B 

C  **D** 

E  **F** 

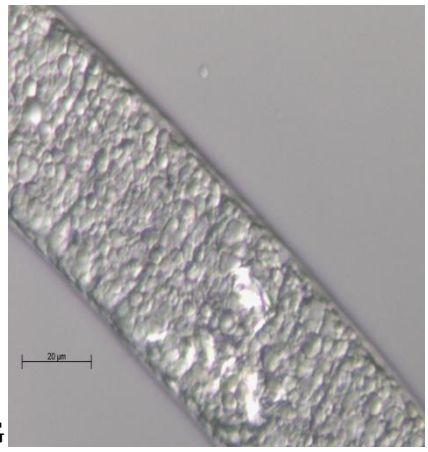
G 

Figure 2.3.5b: *Heterorhabditis bacteriophora* isolate. A: Infective juvenile with a fluorecent symbiotic bacteria inside the body hypothesized to be a *Photorhabdus spp* , B: pointed female tail, C: head showing the esophagus, metacorpus and parts of the intestines, D: head area exaggerating the mouth and distinct lips of an adult, E: female adult with IJs, F: rupturing female adult releasing IJs and its symbiotic bacteria and G: body of an adult highlighting the fat bodies and the fluorescent bacteria.

2.3.6 Morphological identification by scanning electron microscopy (SEM)

Analysis by the use of SEM provides an understanding of the surface structure of the nematodes.

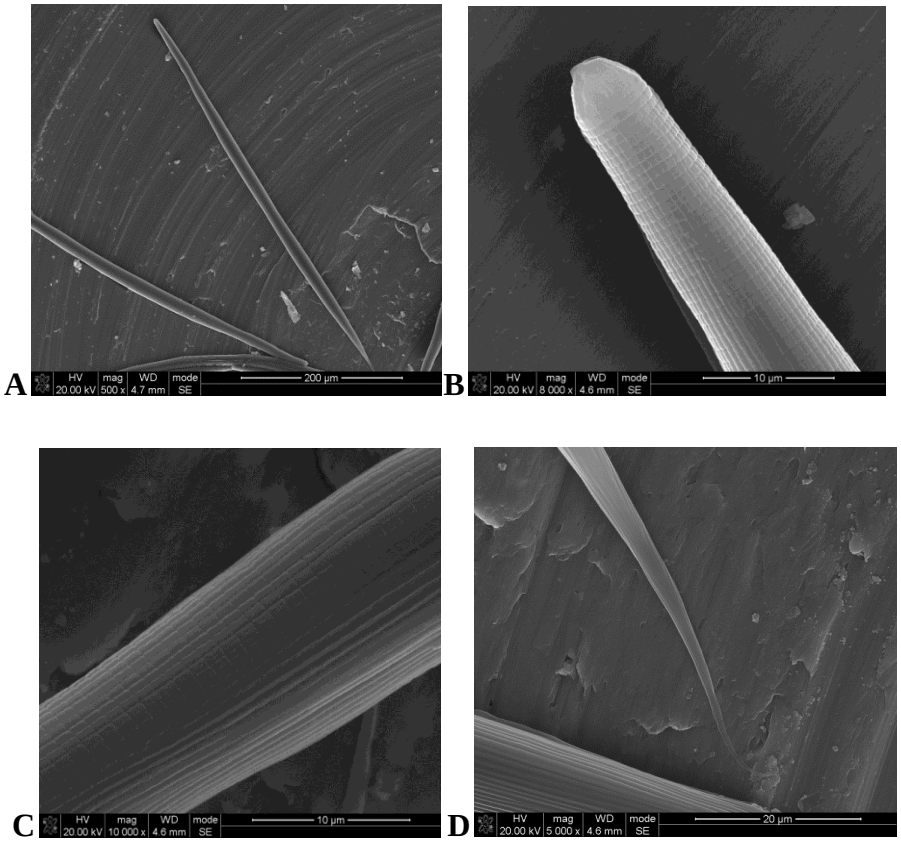
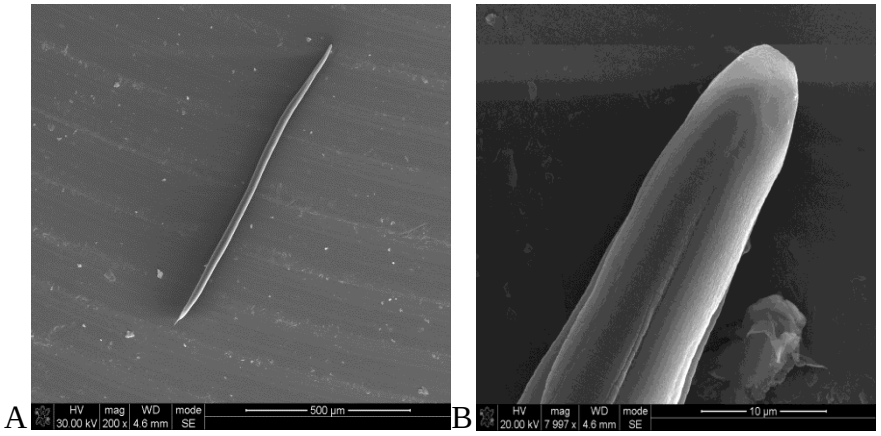


Figure 2.3.6a *Steinernema australe*: A: infective juveniles, B: tessellate anterior region with the mouth and papillae, C: radial symmetry and tessellate body and D: pointed tail of a female.



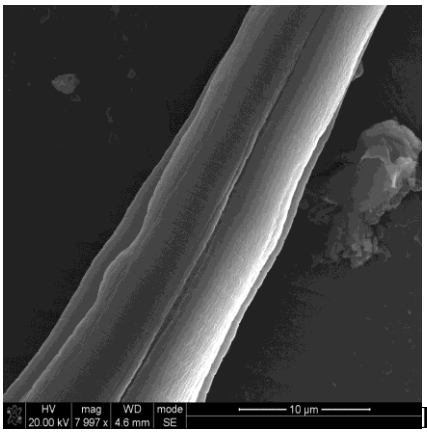
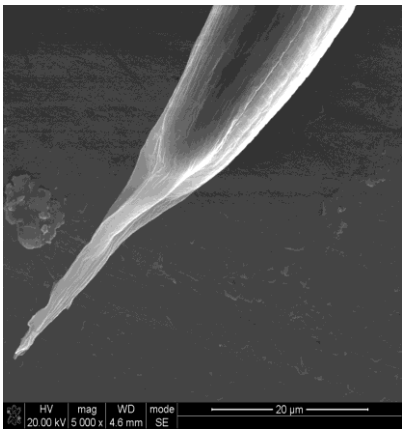
C  D 

Figure 2.3.6b *Heterorhabditis bacteriophora* isolate surface photos. A: Infective juvenile, B: tessellate anterior region with the mouth and papillae, C: longitudinal ridges in the dorsal and D: pointed tail.

2.4 Discussion

The unknown isolates EPN3T and EPN5T were successfully identified by employment of reliable molecular techniques and certified by morphological and phylogenetic analysis. EPN3T was identified to be *Steinernema australe* with the accession number FJ235125. According to the phylogenetic tree seem in figure 2.3.4a, *S. australe* is closely related to *S. kushidae* (GQ497741) and *S. karri* (GQ497742) and *C. elegans* were used as an out group. EPN5T was identified to be *Heterorhabditis bacteriophora isolate 56-C* with the accession number (FJ217351) which as seen in figure 2.3.4b is closely related to *H. bacteriophora strain 51 C* (EU796074) and *H. bacteriophora isolate 117-C* (EU860183).

The molecular and phylogenetic analysis confirmed that the isolate EPN3T is a *Steinernema* as assumed earlier due to yellow and black spots development on the infected dead larvae, similarly EPN5T isolate is a *Heterorhabditis* species, which was assumed earlier due to the maroon colour development on the infected host. Furthermore, microscopy results support the identity of the unknown nematodes and according to table 2.2.3.3 EPN3T is a *Steinernema* and EPN5T is a *Heterorhabditis*. Fat bodies were seen under the light microscope in both isolates which may have implications on their metabolism and nutritional requirement. Figure 2.3.5a image **F** highlights the thick layer of cuticle which may put the species into an advantage to survive harsh environmental conditions and protect the species from being harmed by other microorganisms. These EPNs are symbiotically associated with bacteria which live in their gut as seen in figure 2.3.5b image **E**, with the bacteria naturally in red (no staining was done), which may be supporting the reason why insect larvae infected with *H. bacteriophora* turn break red or maroon after infection. Females of both species had pointed tails and males had blunt tails.

Soil dwelling stages of insects can be controlled by EPNs which specifically target insects and use the host increase IJs populations. Most insects at early developmental stages are susceptible to infection by EPNs and thus EPNs were the considered to be the best candidates to solve pest problems (Dillion et al, 2006).

The morphology is characterised by morphometrics of the IJ with a thick or double epiptygmata which is also evident in figure 2.3.5a F in first generation females, which may support that the species isolated may be indeed similar to *S. australe* isolated from sandy loamy soil collected from Chile, Island Magdalena (Edgington et al, 2009). The IJs are also characterised by a very long body ranging between 1116 to 1484 µm and thus classified under the *glaseri*-group. *S. australe* IJ exhibits a long tail and posses an excretory pore towards the anterior far from the posterior region. First generation males have long spicules between (55-78) µm and long gubernaculums (36-51) µm.

H. bacteriophora IJs have a sheath protecting the entire body of the nematode, a tessellate on the anterior region and is characterised by longitudinal ridges throughout almost the entire body length as seen in figure 2.3.6 b C. The IJ, and the third stage which develops from it, have a dorsal tooth on the labial region. The fourth stage has low lips and labial papillae, and a rounded oral aperture. It may be suspected that the IJ in figure 2.3.5b D is a hermaphrodite as it has huge lips with prominent labial papillae at the apex of the lips. The described features confirm that the isolated species is *H. bacteriophora* (Malan et al, 2011).

2.5 Conclusion

Steinernema australe was first isolated in 2009 form soil samples collected from Chilean Island, Isla Magdalena located in the Pacific ocean. This species is symbiotically associated with *Xenorhabdus magdalenensis* (Edgington et al, 2009). This species is able to survive in its environment because the first generation of females have a double epiptygmata (Edgington et al, 2009) which is also seen in figure Figure 2.3.5a image **F**. Soil in Isla Magdalena is soft or loamy and the island is lightly covered by vegetation (Edgington et al, 2009). Similarly, soil collected from Walkerville in the south of Johannesburg, South Africa was sandy loamy, fertile and suitable for vegetation.

Heterorhabditis bacteriophora isolate 56-C was isolated from the Eastern cape in Knysna with Genebank accession number FJ217351 (Malan et al, 2011) and its morphometrics and similarities in the 18S rDNA may certify that unknown EPN5T is indeed a *Heterorhabditis bacteriophora* isolate 56-C.

The 18S rDNA ITS region sequencing confirmed the identification of *Steinernema australe* and *Heterorhabditis bacteriophora* isolate 56-C. These families of EPNs are found in soils all over the world (Malan et al, 2011). Both the identified EPNs were isolated from sandy loamy soil, also supporting the fact that IJs can survive, persist and scatter successfully in the natural environment and this why they can be trusted as potential biological control agents in agricultural industries.

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Chapter 3

Identification of bacteria symbiotically related to *Heterorhabditis bacteriophora* and *Steinernema australe* based on 16S rDNA Sequence.

3.1 Introduction

Photorhabdus and *Xenorhabdus* species are symbiotically associated with *Heterorhabditis* and *Steinernema* EPNs species respectively (Adams et al, 2006). These species of bacteria are both involved in insect pathogenicity and thus enabling nematodes they live in to be potential candidates for biological control of insects (Ciche et al, 2006). Bacterial symbiots live inside the intestine of IJs which provide them with shelter and transport.

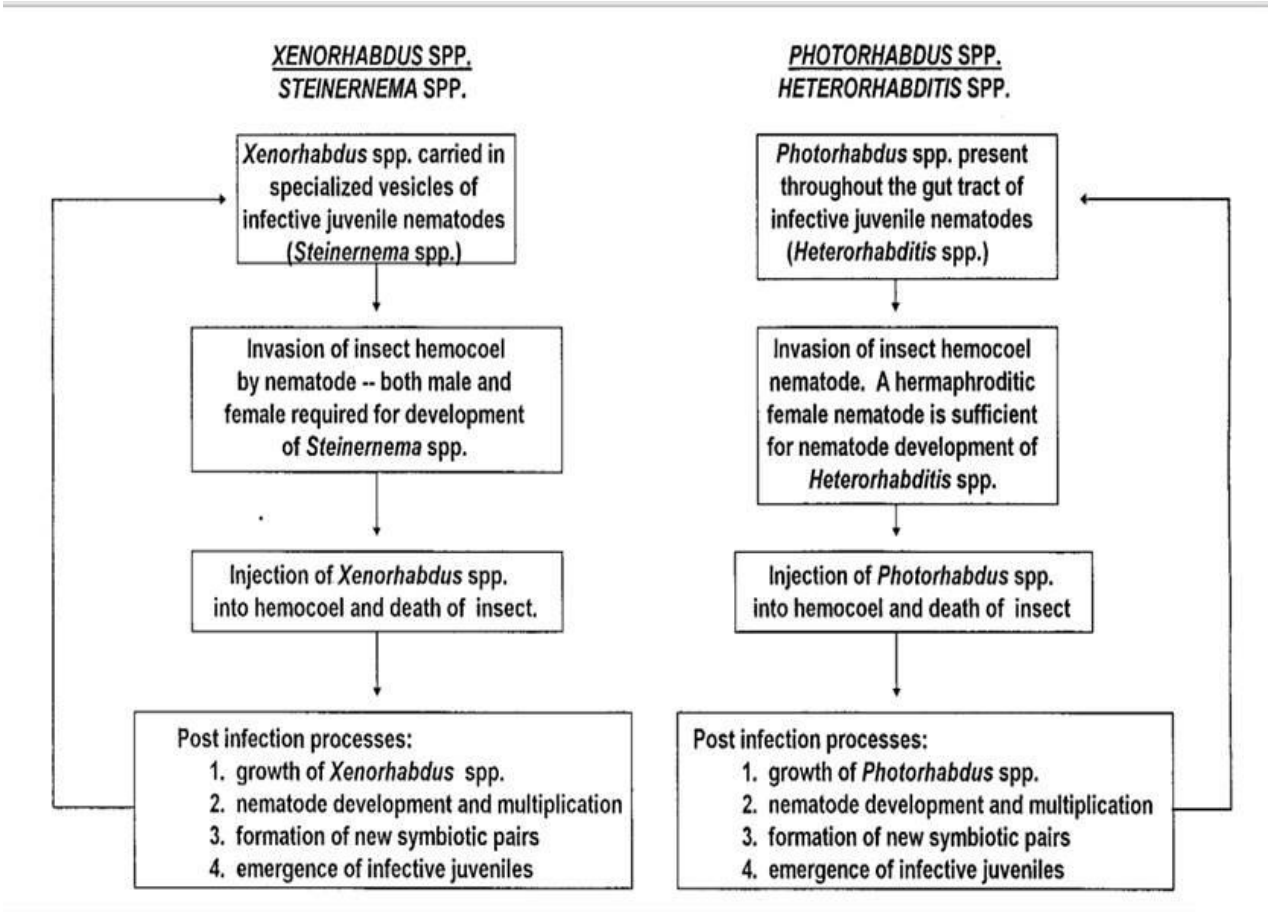


Figure 3.1 This diagram shows the life cycles of bacterium-nematode symbioses of the Xenorhabdus-Steinernema and Photorhabdus-Heterorhabditis and highlights all the distinct differences and similarities between the two genera (Forst and Nealson, 1996).

Photorhabdus and Xenorhabdus kill most insects when they are released in the hemolymph (Fischer-Le Saux et al, 1999). Once the nematodes have invaded the insect's hemocoel, they release symbiotic bacteria which carry immune depressors which are involved in blocking the activity of phospholipase A2 of the insect and thus protecting the nematodes and the bacteria themselves from attack and degradation (Ji et al, 2004). The infected insect becomes more susceptible to saphrophytic pathogens and becomes weaker. Previous studies have shown that gram negative *Xenorhabdus nematophila* is symbiotically associated with *Steinernema carpocapsae*. These enterobacteria are capable of protecting themselves and the IJs which have invaded the insect, by inducing immunodepression of the invaded insect host. *X. nematophila* secretes antibiotics and hydrolytic enzymes into the hemolymph of the insect, killing the host within 16hours after invasion. *X. nematophila* also secretes active immunodepressive agents which protect the association of the nematodes-bacteria from insect's immune response upon invasion (Park et al, 2004).

Symbiotic bacteria produces antibiotics (antibacterial compounds) such as xenocoumacins, indoles and dithiolopyrrolones which assist to retain the monoxenic conditions (Ji et al, 2004). The mechanism of antibacterial compounds was hypothesized to block protein and RNA synthesis by increased production of guanosine-30, 50-bispyrophosphatase (ppGpp), a regulatory protein. Metabolic products of these symbiotic bacteria are of major importance in agriculture and also have a medical potential (Ji et al, 2004).

Photorhabdus species have catalase activity and produce hydroxystilbenes, an example includes P. luminescens which also has anthraquinones pigments compounds. *Xenorhabdus* species also produce hydroxystilbenes and have catalase activity but exhibit no pigmentation compounds. Both these generas may have urease activity, indole production and aesculin hydrolysis depending on the species (Gaugler, 2002).

Example of exoenzymes include lecithinase secreted by *X. nematophila* which was observed to be involved in degrading the insect's phospholipids and therefore proving lipids to the *Steinernema* which are necessary for growth and reproduction. This enzyme could not be detected in several strains of *Photorhabdus*. The enzyme protease secreted by both *Photorhabdus and Xenorhabdus* was believed to be involved in the breakdown of the insect's proteins. This result is advantageous for nematodes since the breakdown provides nutrition for the nematodes and thus favouring their growth before the exit the

insect cadaver (Gaugler, 2002).

Bacteria alone cannot enter the insect's hemocoel and fail to penetrate the alimentary canal without the nematodes (Grewal et al, 2001). This implies that EPNs serve as vectors and mode of transport for the bacteria to enter the insects, where upon entry; they infect the host and start dividing, at the same time preparing favourable conditions for the nematodes to also start reproducing in the cadaver (Fischer-Le Saux et al, 1999). Specific insects have been recorded to be susceptible to several *Xenorhabdus* species and their immune system is not strong enough to resist infection, resulting in death of the host. Existing bacteria in the insect cadaver then takes the opportunity to feed on nutrients found in the hemocoel and start dividing, resulting in numerous colonies which then interact and associate with the new generations of IJs (3^{rd} stage) which later emerge as populations increase in the hemocoel and survive in the soil until new hosts are invaded. Almost the similar interactions appear in *Photorhabdus* bacteria which are classified under gammaproteobacteria and have shown agricultural importance when associated with Heterorhabditis. An example of genes involved includes the Lux genes secreted by several Phorhabdus species which have been reported to be expressed during degradation of the insect hemocoel.

In this study, a polymerase chain reaction (PCR) was employed to amplify the 16S rDNa region (a small part of the 30S subunit) using universal primers. The PCR results were sequenced and used to identify bacteria symbiotically associated with the EPNs species of interest. Except for the spacer sequence between the 16S and 23 S regions, the 16S and 23 rDNA regions are highly conserved across most bacterial species. 16S rDNA is used in taxonomy and molecular phylogeny, (Adams et al, 2006).

3.2 Materials and methods

3.2.1 Surface sterilisation of nematodes (Kaya et al, 1997).

Fresh infective juveniles were collected from white-traps and transferred into clean falcon tubes and allowed to sediment. The excess water was removed and the EPNs sediment was immersed in 10ml of 0.1% sodium hypochlorite and left for 1 hour. The IJs were transferred into 10ml fresh 0.1% sodium hypochlorite solution and left for 3 hours. IJs were then rinsed twice with Ringer's solution under a laminar flow hood. Sterile IJs were carefully suspended in 1ml of sterile nutrient broth in a micro tube and crushed with a sterile motor. The homogenate was aseptically transferred into a sterile Mc Cartney bottles with about 10ml of nutrient broth. Bacteria were grown in the dark on a shaker for 24-48 hours at 25-28°C. In order to allow colony growth the bacteria from the nutrient broth was streaked on Mc Conkey and NBTA streak plates and incubated at 25°C for 24hours. Pink or red colonies were isolated and plated on clean Mc Conkey and NBTA streak plates and the pure cultures were used for further

analysis

3.2.2 Genomic extraction from bacteria

The bacterial genomic DNA was extracted using ZR Bacterial DNA Kit, # D6005. Bacteria were grown on NBTA and Mc Conkey plates and an isolated bacterial colony was picked and suspend in a ZR BashingBeadTM Lysis Tube and vortex at maximum speed for 5minutes, this aided in gently breaking down the bacterial wall in order to release nucleic material. The ZR BashingBeadTM Lysis tube was centrifuged at 10 000 rpm for 1 minute. Up to 400µl of the supernatant was transferred into a Zymo-Spin TM IV Spin Filter in a Collection Tube and centrifuged at 7000 rpm for 1 minute. 1200µl of Fungal/ Bacterial DNA binding buffer was added to the filtrate in the Collection Tube and transferred 800µl of the mixture to a Zymo-SpinTM II Column in a Collection Tube and centrifuged at 10000rpm for 1 minute. The flow was discarded from the Collection Tube and 200µl DNA Pre-Wash Buffer was added to the Zymo-SpinTM II Column in a new Collection Tube and centrifuged at 10000rpm for 1 minute. 500µl of Bacterial DNA Wash Buffer was added to the Zymo-SpinTM II Column and centrifuged at 10000rpm for 1 minute. The Zymo-SpinTM II Column was transferred to a clean 1.5 ml microcentrifuge tube and added 100µl DNA Elution Buffer directly to the column matrix. The tube was centrifuged at 100000rpm for 30 seconds to release the DNA from the matrix. The DNA was stored at 4°C to be used for further analysis.

3.2.3 PCR amplification of the 16S rDNA region using universal primers:

Table 3.2.3 PCR recipe showing the quantity of each reagent required for a reaction to amplify the 16S rDNA region.

PCR contents in a microtube	Quantity µl
PCR Master mix	25
Nuclease free water	22
Forward primer EUB968	1
Reverse primer UNIV1382	1
DNA/1 colony	1

Total volume= 50µl

Primer information:

EUB968 Forward primer

5’-ACGGGCGGTGTGTRC-3’

Tm (°C)=62

UNIV1382 Reverse primer

5’-AACGCGAAGAACCTTAC-3’

Tm (°C)=66

Samples were mixed gently, making sure that no air bubbles were formed as they can inactivate the enzymes (DNA polymerase) in the master mix. The following conditions were used to amplify the 16S rDNA region of the bacterial isolate:

Initial denaturation before cycling: 95 ºC for 3 minutes

35 cycle amplification series:

Denaturation at 94ºC for 30 seconds

Annealing at 60ºC for 45 seconds

Extension at 72ºC for 90 seconds

Final extension after cycling: 72ºC for 7 minutes

3.2.4 Agarose gel electrophoresis of PCR products

2% agarose gel was prepared in order to confirm the presence of the desired amplified region. 1g of agarose power was dissolved in 50ml of 1XTBE buffer and 1µ of ethidium bromide was added and mixed gently. The gel left to solidify with the well comb inserted. For each 10µl sample, only 2µl of loading dry was added and then samples were run on the gel for 45minutes at 90volts immersed in 1XTBE, with constant current.

Post successful Polymerase chain reactions, PCR products where then sent to Inqaba Biotechnical Industries (Pty) Ltd; South Africa for sequencing. The National Centre for Biotechnology Information (NCBI-BLAST) was used to identify the unknown species.

3.2.5 Phylogenetic analysis

- Sequences obtained from Inqaba Biotech were edited using Chromas sequence analysis program. The following taxa were used for the construction of the phylogenetic tree for *Photorhabdus*: *P. sp. caborca* isolate (Genbank accession number JQ912644), *P. asymbiotica* (AY278672), *P. sp SF41* (HQ142323), *P. luminescence* (FJ87473), *P. temperate strain* P7 (AY278666), *P. temperate strain* Wx12 (AY278665), *P. temperate strain* Wx11(AY278664), *P. temperate strain* Wx9 Hyper (AY278662), *P. luminescence subsp. luminescence* (AY278641), *P. luminescence subsp. luminescence* (AY278640), *Uncultured P. sp.clone9* (DQ314767), *P. temperate strain* (AY278658) and *Caenorhabditis elegans* (EU196001) was used as the outgroup taxa.

The above stated bacteria sequences were derived from the National Center for Biotechnology (NCBI), using a tool called BLAST (Basic Local Alignment Tool), web address (blast.ncbi.nlm.nih.gov), edited using Chromas programme and DNAMAN v4.0 programme was used for phylogenetic tree construction. The NCBI sequences were used as references to establish the consensus sequences for the isolated bacteria.

3.2.6 Light microscopy and specimen preparation (Kaya et al, 1997).

Killing and fixing nematodes

Nematodes were placed in a Petri dish suspended in 1ml distilled water. 3-4ml of 100ºC TAF was added and left for 24 hours. TAF was replaced with double-strength TAF and stored at 4ºC to relax nematodes for up to one hour. 65ºC TAF was added and then the fixative was allowed to infiltrate for at least 24 hours. Most of the fixative was then removed.

Processing nematodes to pure glycerine (Kaya et al, 1997)

Fixed nematodes were transferred to a Petri dish containing 0.5ml of solution I. 5ml of 95% ethanol was placed the watch glass containing the nematodes in the desiccator. The desiccator was placed in an oven preheated to 35ºC for 12 hours. The watch glass with nematodes was then removed from the desiccator. The watch glass was filled with solution II and placed in a glass Petri dish. The Petri dish was left partially open to allow for slow ethanol evaporation. The Petri dish containing the watch glass was placed in an oven preheated to 40ºC for 3 hours.

Water preparation alternative method

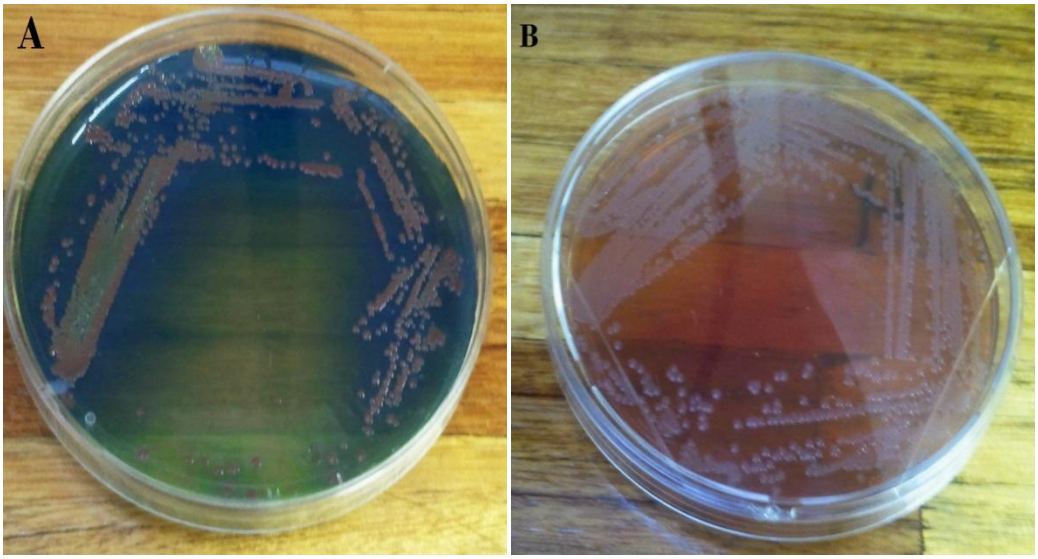
Fresh IJs were collected directly from the white traps and placed on a clean slide then covered with a cover gently. The IJs were then viewed under a light microscope connected to a digital camera and images were captured.

3.3 Results:

3.3.1 Symbiotic bacteria grown on selective media

Mc Conkey agar was used to isolate gram negative bacteria with a potential to ferment lactose, thus dropping the pH of the media resulting in the growth of red/pink colonies which may be suspected to be associated with the EPNs (Kaya et al, 1997).

Bromothymol Blue agar was used to also isolate gram negative bacteria and have a potential to absorb bromothymol blue thus also resulting in blue- green colonies with a red centre.



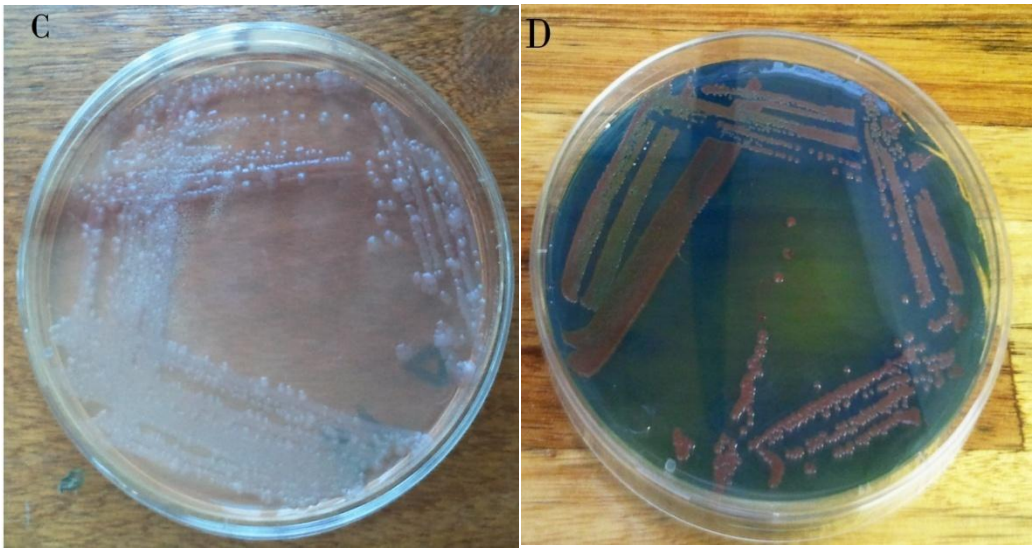


Fig 3.3.1 EPNs symbiotic bacteria cultured in Mc Conkey and NBTA plates. A and B: Symbiotic bacteria isolated from *H. bacteriophora* A: with reddish-green colonies with a red centre on NBTA and B: deep pinkish-red colonies on Mc Conkey agar. C and D Symbiotic bacteria isolated from *S. australe* C: Ma Conkey agar with pink colonies and D: greenish-red with a red centre on NBTA.

Bacteria can occur in two phases, phase I and phase II which may enable one to identify the desired bacterial species and characterise them on by simple streaking on to MacConkey and NBTA agar plates which are greatly used in selecting bacteria according to lactose fermentation and ability to absorb bromothymol blue and reduction of triphenyltetrazolium chloride (TTC) which results in a red colour of colony surrounded with clear zones seen mostly in *Xenorhabdus* species (Kaya et al, 1997).

Table 3.3.1 Summary of the isolated bacteria from EPNs characterised according to phase variants described by (Kaya et al, 1997).

	H. bacteriophora symbiotic bacteria	*S. australe* symbiotic bacteria
McConkey colony phase, colour and shape	Phase I, pink and red, circular colonies	Phase I, pink and irregular and some circular colonies
NBTA colony phase, colour and shape	Phase I, green/red colonies surrounded by a clear zone	Phase I, green/red colonies surrounded by a clear zone

3.3.2 Amplification of the 16S rDNA region

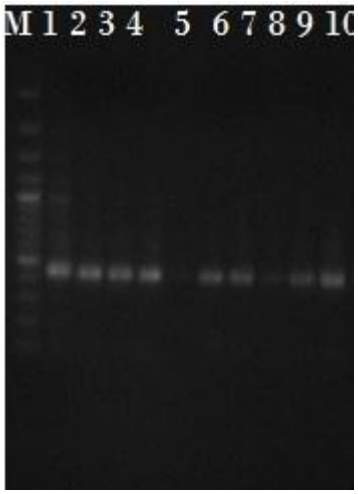


Figure3.3.2 2% Agarose gel electrophoresis showing the PCR products of unknown bacteria isolated from EPN3T and EPN5T. Noting that all PCR products are between 400bp to 500bp, approximately the size of the 16S rDNA conserved in most bacterial species. M: 100bp DNA ruler, 1-4: PCR products from bacteria associated with *H. bacteriophora*, 5: control lane and 6-10 PCR products from bacteria associated with *S. australe.*

PCR products from bacteria associated with *S. australe* gave unexpected results. This is due to fungal contamination which was difficult to hinder as spores can still survive even after treatment with ethanol or detergents. The isolation step was repeated several times however, EPNs could no longer be re-covered since Steinernema is very sensitive and therefore difficult to harvest and maintain. White traps were contaminated with fungi and nematodes stopped emerging even after troubleshoot methods have been tried to resuscitate the IJs.

3.3.3 Sequencing of the 16S rDNA of bacteria

Sequences obtained from Inqaba Biotech were used to search the National Centre for Biotechnology (NCBI), using a tool called BLAST (Basic Local Alignment Tool), web address (blast.ncbi.nlm.nih.gov), edited using Chromas programme and thus establish the taxa of the isolated unknown species.

CATGCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTC
CAATCCGGACTACGACAGACTTTGTGTGTTCCGCTTGCTCTCGCGAGGTCGCTTC
ACTTTGTATCCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGA
CTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCC
ACCTTCACGTGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAAC
CCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCASAGCTC
CCGAAGGCACAACCTCATCTCTGAGGTCTTCTCTGGATGTCAAGAGTAGGTAAGG
CCTTTYCCCGTTA

Figure 3.3.3 The above sequence of symbiotic bacteria associated *Heterorhabditis bacteriophora* had high affinity to *Photorhabdus sp Carborca* with the accession number JF12345.

Table 3.3.2 A summary of bacterial species symbiotically associated with EPN3T and EPN5T which were identified through DNA sequencing.

Sample label	Identified bacterial species	Region collected
EPN3T	*Xenorhabdus* unidentified isolate	Road number 6
EPN5T	*Photorhabdus sp. Caborca*	Wadie road

3.3.4 Phylogenetic analysis

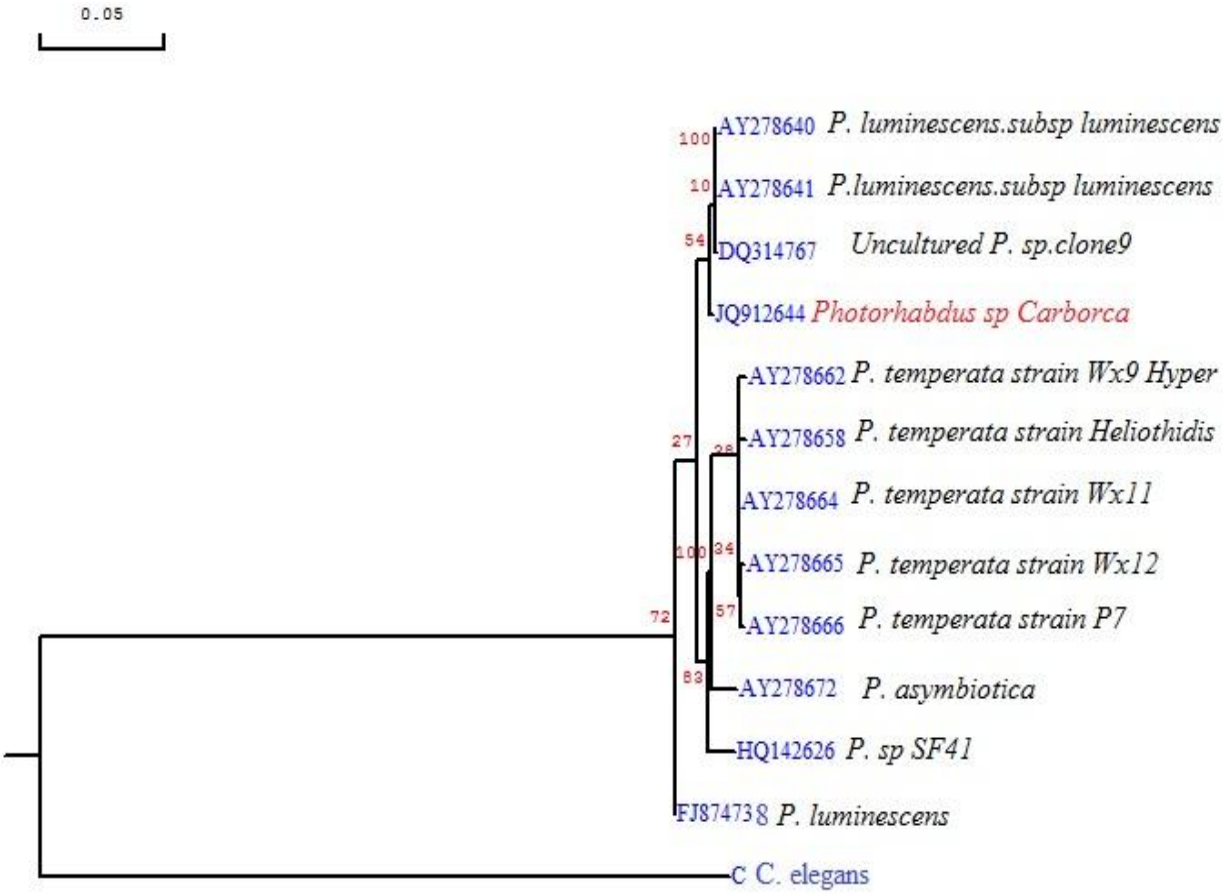


Figure 2.3.4a Phylogenetic tree showing the relationship between the initially unknown species highlighted in red (*Photorhabdus sp Carborca*) and 12 other species with accession numbers indicated in blue, where the analysis was based on the 16S rDNA ITS region of the EPNs. *C. elegans* were used to root the tree and the bootstrap values are based on 1000 replications on the nodes. Bar scale 0.05 substitutes per nucleotide position. The phylogenetic tree was constructed using the DNAMAN programme (v4.0).

3.3.5 Light microscopy

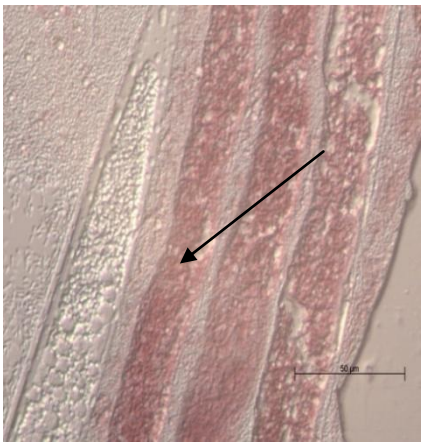


Figure 3.3.5 *Heterorhabditis bacteriophora* isolate 56-C symbiotically associated with *Photorhabdus sp. Carborca.* The arrow is pointing at *Photorhabdus* bacteria with red pigments inside IJs which have not emerged from the adult female.

3.4 Discussion

The relationship between *Photorhabdus and Xenorhabdus*; *Heterorhabditis* and *Steinernema* respectively, is not obligate because of their ability to be grown without the nematodes in the laboratory (Gaugler, 2002). Nematodes and bacteria symbiotic relationships are highly specific however; several different *Steinernema* species can for association with the same *Xenorhabdus* species. This is different in *Heterorhabditis* as one species is restricted to only associate with one species of *Photorhabdus* (Gaugler, 2002).

Phylogenetic results showed that the unknown species suspected to be *Photorhabdus* aligned closely with *Photorhabdus sp carborca* (JQ912644). *P. sp carborca* belongs in the kingdom of bacteria, Proteobacteria division, Gammaproteobacteria class, Enterobacteriales order, belongs to Enterobacteriaceae, genus Photorhabdus and *P. sp carborca* species. These bacteria reside in the gut of *Heterhabditidae*. This group of bacteria is capable to secreting toxins such as proteic which degrades the hemocoel and body of the insect within 48 hours of the bacteria being released into the bloodstream of the insect host.

P. sp carborca isolated from *H. bacteriophora* was able to ferment lactose, thus dropping the pH of the media resulting in the growth of red/pink colonies on the McConkey agar plate. This bacterial species can be classified under the phase I colony morphology and characteristics as it was able to absorb

bromothymol blue thus also resulting in blue- green colonies with a red centre as see in figure 3.3.1 and table 3.3.1. According to the phylogenetic tree seem in figure 3.3.4b *P. sp carborca* is closely related to the *Uncultured Photorhabdus sp clone9* (DQ314767), *Photorhabdus luminescence subsp luminiscence* (AY278640) and *Photorhabdus luminescence subsp luminiscence* (AY278641) and since the identified species has a close relation with *Photorhabdus luminescence subsp luminescence* that may account for the fluorescent characteristic as seen in light microscope images seen in figure 3.3.5. This species also produces red pigmentations visible in figure 3.3.5 which account for the brick red infected insect cadaver.

The unknown *Xenorhabdus* did not have any close or high affinity with the expected species, further studies will attempt again at isolating the unknown species.

3.5 References

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Chapter 4

Desiccation stress tolerance of entomopathogenic nematodes: *Steinernema australe and Heterorhabditis bacteriophora*

4.1 Introduction

4.1.1 Anhydrobiosis of EPNs

EPNs can survive harsh environmental conditions such as desiccation; this is because they are able to enter a dormant state which allows them to withstand most stresses exerted by climatic or environmental changes (Chen et al, 2003). Dormancy is divided into diapauses and quiescence. When IJs enter diapauses; an arrested development state which they will not be resuscitated even when the environmental conditions are brought to normal but will only exit this arrested state when specific requirements are made available (Grewal, 2000). This dormancy state allows the nematodes to survive but it is not favourable as it has critical limitations and no guarantee of IJs resuscitation. Quiescence dormancy is more promising and is a state whereby an IJ has modified its metabolism by lowering its rate to unexpected harsh environmental conditions but when the conditions are back to normal the state can easily be reversed. However, if the unfavourable weather conditions persist for longer, some IJs can enter cryptobiosis where there is no quantifiable metabolism (Grewal et al, 2006).

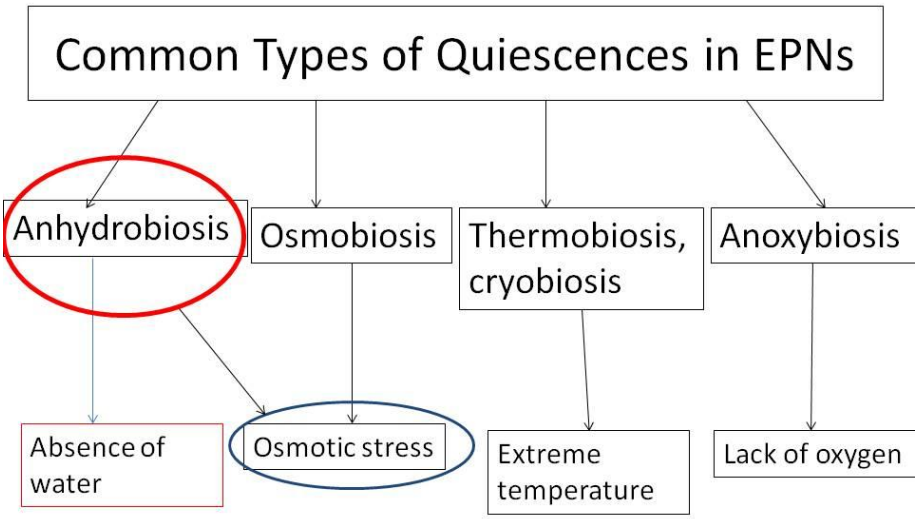


Image developed by T.E Lephoto, information adapted from (Grewal et al, 2006)

Figure 4.1.1 Types of quiescence linked with the environmental conditions which cause them.

In the above figure, unfavourable environmental conditions which cause the linked quiescence types may dependently exist in affecting EPNs survival due to the conditions interacting in the natural environment. An example is observed between desiccation which commonly goes together with pre-exposure to osmotic stress because dry soil has lower thermal conductance and a higher rise in thermal pressure (Grewal et al, 2006).

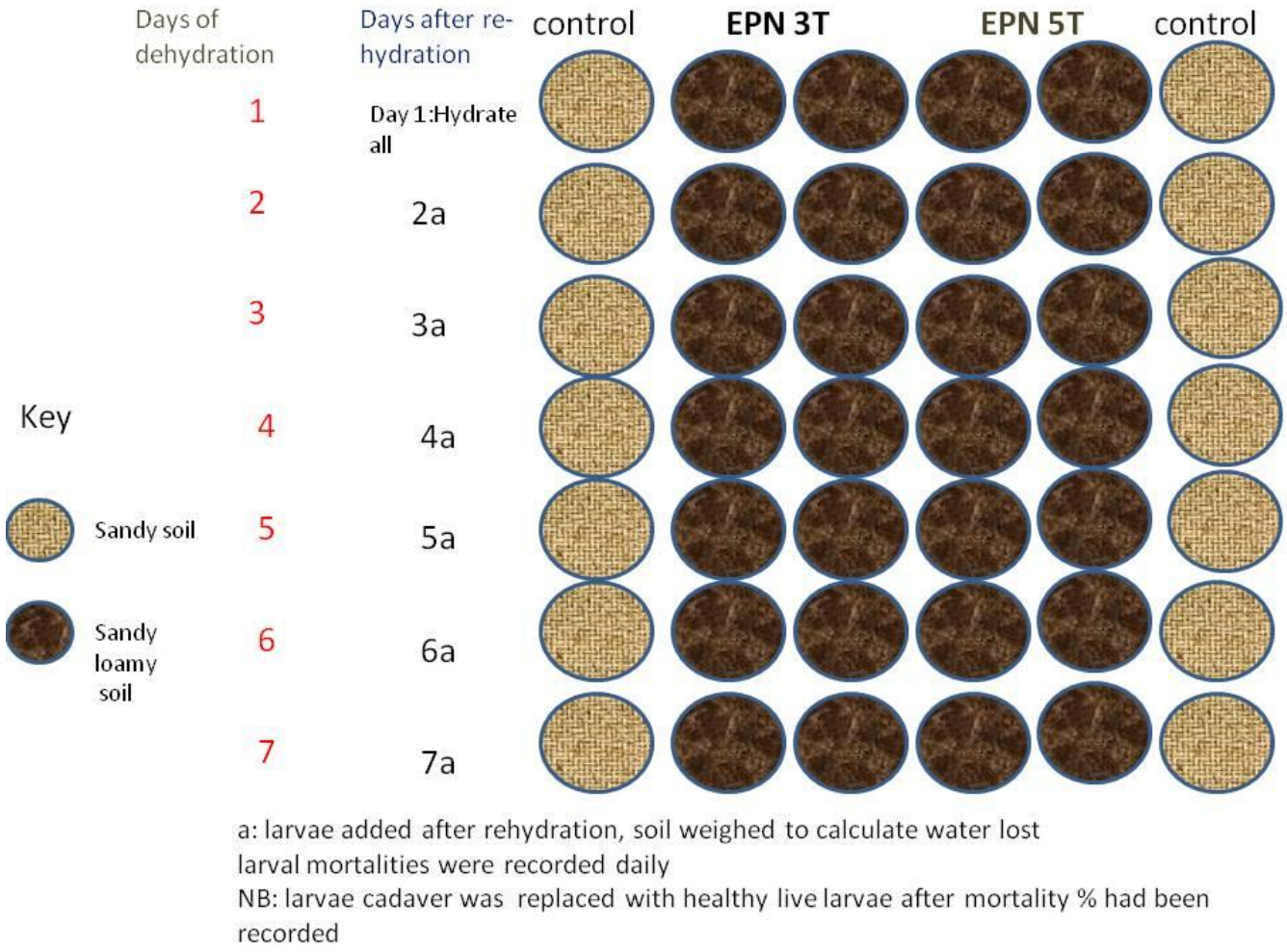
EPNs survive desiccation stress through anhydrobiosis as they slowly lose water stored in their bodies. Only limited stages in the nematode's life cycle tolerate desiccation, such as the free-living IJs. Anhydrobiosis is very crucial in the efficacy of EPNs in dry weather conditions (Grewal et al, 2006). These conditions are mostly experienced in South African provinces which mainly include Limpopo, Mpumalanga and Gauteng. Several other factors affect the efficacy of EPNs including soil type, organisms present in the soil, availability of nutrients and many more (Strauch et al, 2000).

EPNs are grouped under quiescent anhydrobiotes because they need some degree of water to survive. Generally all nematodes are found in aquatic areas and require to be surrounded by water for locomotion and survival (Grewal, 2000). When nematodes undergo anhydrobiosis, some species coil up in order to reduce the amount of water that can be lost, but this does not apply for all species (Grewal et al, 2006).

This study aimed at investigating desiccation stress tolerance of *Steinernema australe and Heterorhabditis bacteriophora* through a series of dehydration and rehydration experiments.

4.2 Materials and methods

4.2.1 Desiccation tolerance of the identified entomopathogenic nematodes



(Graphics developed by TE Lephoto)

Figure 4.2.1 Desiccation-stress tolerance of EPN 3T and EPN 5T representative image displaying days of dehydration and rehydration also recording moisture loss and mortality percentage daily in each Petri dish. The sandy soil used for control purposes was river stand collected from the Spruit in the south of Johannesburg. The sandy loam soil was collected from the site where soil samples were collected mentioned in chapter 2 which was used for the isolation of EPNs. 28 Petri dishes with sterilised sandy loamy soil (experiment) and 14 Petri dishes with sterilised sandy (control) were inoculated with the *Heterorhabditis* species (EPN5T) IJs and another 28 (experiment) and 14 (control) were inoculated with *Steinernema* species (EPN3T) 100IJs/ml in all Petri dishes. On day 1, all dishes were brought to the

same moisture of 10%, with only the first row baited with 10 *G. mellonella* larvae on day 1. On day 2 the moisture loss was calculated by weighing the dishes to determine the amount of water lost in the first and second row; followed by rehydration with the volume that raised the moisture back to 10%. This was followed by baiting row 2 on day 2 and the same process was done with the remaining 14 rows over a period of 2 weeks as seen in figure 4.2.1. By day 8 the dehydration and rehydration was complete and the dishes were dried for 6 days and then the same process of rehydration was resumed to resuscitate the IJs, continuously recording moisture lost daily on every set of dishes per row. Observations were done inspecting susceptibility and mortality of the larvae to EPN 5T and 3T within 7 to 21 days of loamy soil dehydration and rehydration. Only 10 insect larvae were added per dish and this whole experiment was repeated 2 times with 2 replicates per EPNs species to determine the mean percentage for each day. To confirm that the larvae were killed by EPNs infection, white traps were done to recover the IJs.

The sandy soil used for the control was river stand collected from the Spruit in the south of Johannesburg. The sandy loam soil used in the experiment was collected from Wadie road in Walkerville, South of Johannesburg. The soil was rich, fertile and suitable for plantation with the ability to hold water and remain moist.

4.3 Results

Summary of desiccation tolerance of *H. bacteriophora* and *S. australe* experiments results.

4.3.1 Desiccation tolerance of *H. bacteriophora* on sandy loamy soil (experiment)

Table 4.3.1 Desiccation tolerance of *H. bacteriophora* on sandy loamy soil reflected by the mortality percentage of *G. mellonella* larvae recorded after daily dehydration and rehydration over a period of 20 days.

		Mortality percentage(%) of larvae recorded after rehydration daily							6 day dehydra-tion	Mortality percentage(%) of larvae recorded after rehydration daily					
Days of dehydration	Days after re-hydration →	1	2	3	4	5	6	7	8-14	15	16	17	18	19	20
1		0	60	100	0*	50	100	0	Dry	0	20	30	50	40	60

2		0	30	60	100	0*	30	100	Dry	0	20	20	30	40	50
3		0	30	50	70	100	-	-	Dry	0	10	20	30	40	50
4		0	10	30	50	80	100	-	Dry	0	0	20	20	30	40
5		0	10	20	40	60	80	100	Dry	0	10	20	30	30	40
6		0	0	10	30	50	60	80	Dry	0	20	30	30	50	80
7		0	0	10	20	50	50	70	Dry	0	20	30	40	60	80
		A	B	B	B	B	B	B	C **	D	E	E	E	E	E

A: larvae added after rehydration; B: % larval mortalities after rehydration; C: dehydration for 6 day period; D: rehydration and larval addition, and E: % larval mortalities after rehydration

* *fresh larvae added and after collecting dead larvae

4.3.2 Desiccation tolerance of *H. bacteriophora* on sandy soil (control)

Table 4.3.2 Desiccation tolerance of *H. bacteriophora* reflected by the mortality percentage of *G. mellonella* larvae recorded after daily dehydration and rehydration over a period of 20 days.

		Mortality percentage(%) of larvae recorded after rehydration daily							6 day dehydra-tion	Mortality percentage(%) of larvae recorded after rehydration daily					
Days of dehydration	Days after re-hydration →	1	2	3	4	5	6	7	8-14	15	16	17	18	19	20
1		0	80	100	0*	60	100	0	Dry	0	0	0	0	0	0
2		0	60	60	100	0*	60	100	Dry	0	0	0	0	0	0
3		0	60	60	80	100	-	-	Dry	0	0	0	0	0	0
4		0	60	60	80	80	100	-	Dry	0	0	0	0	0	0
5		0	30	40	40	60	80	100	Dry	0	0	0	0	0	0
6		0	0	10	30	30	60	80	Dry	0	0	0	0	0	0
7		0	0	10	20	50	60	60	Dry	0	0	0	0	0	0
		A	B	B	B	B	B	B	C **	D	E	E	E	E	E

A: larvae added after rehydration; B: % larval mortalities after rehydration; C: dehydration for 6 day period; D: rehydration and larval addition, and E: % larval mortalities after rehydration

* *fresh larvae added and after collecting dead larvae

4.3.3 Desiccation tolerance of *S. australe* on sandy loamy soil (experiment)

Table 4.3.3 Desiccation tolerance of *S. australe* on sandy loamy soil reflected by the mortality percentage of *G. mellonella* larvae recorded after daily dehydration and rehydration over a period of 20 days.

		Mortality percentage(%) of larvae recorded after rehydration daily							6 day dehydr -ation	Mortality percentage(%) of larvae recorded after rehydration daily					
Days of dehydration	Days after re-hydration →	1	2	3	4	5	6	7	8-14	15	16	17	18	19	20
1		0	60	80	100	0*	40	60	Dry	0	10	20	30	40	50
2		0	60	60	90	100	-	-	Dry	0	10	20	20	30	40
3		0	30	40	50	70	100	-	Dry	0	0	30	30	40	40
4		0	30	30	60	70	90	100	Dry	0	0	20	30	30	40
5		0	20	30	40	60	60	80	Dry	0	10	20	30	40	50
6		0	0	20	30	40	60	80	Dry	0	20	20	30	50	60
7		0	0	20	30	40	50	70	Dry	0	20	30	40	50	60
		A	B	B	B	B	B	B	C **	D	E	E	E	E	E

A: larvae added after rehydration; B: % larval mortalities after rehydration; C: dehydration for 6 day period; D: rehydration and larval addition, and E: % larval mortalities after rehydration

* *fresh larvae added and after collecting dead larvae

4.3.4 Desiccation tolerance of *S. australe* on sandy soil (control)

Table 4.3.4 Desiccation tolerance of *S. australe* on sandy loamy soil reflected by the mortality percentage of *G. mellonella* larvae recorded after daily dehydration and rehydration over a period of 20 days.

		Mortality percentage(%) of larvae recorded after rehydration daily							6 day dehydration	Mortality percentage(%) of larvae recorded after rehydration daily					
Days of dehydration	Days after re-hydration →	1	2	3	4	5	6	7	8-14	15	16	17	18	19	20
1		0	60	80	100	*	0	40	Dry	0	0	0	0	0	0
2		0	60	80	100	*	0	40	Dry	0	0	0	0	0	0
3		0	60	80	80	100	-	-	Dry	0	0	0	0	0	0
4		0	40	60	60	80	100	-	Dry	0	0	0	0	0	0
5		0	0	30	40	60	80	80	Dry	0	0	0	0	0	0
6		0	0	10	20	30	50	60	Dry	0	0	0	0	0	0
7		0	0	10	30	40	50	50	Dry	0	0	0	0	0	0
		A	B	B	B	B	B	B	C **	D	E	E	E	E	E

A: larvae added after rehydration; B: % larval mortalities after rehydration; C: dehydration for 6 day period; D: rehydration and larval addition, and E: % larval mortalities after rehydration.

4.3.5 Moisture loss during desiccation

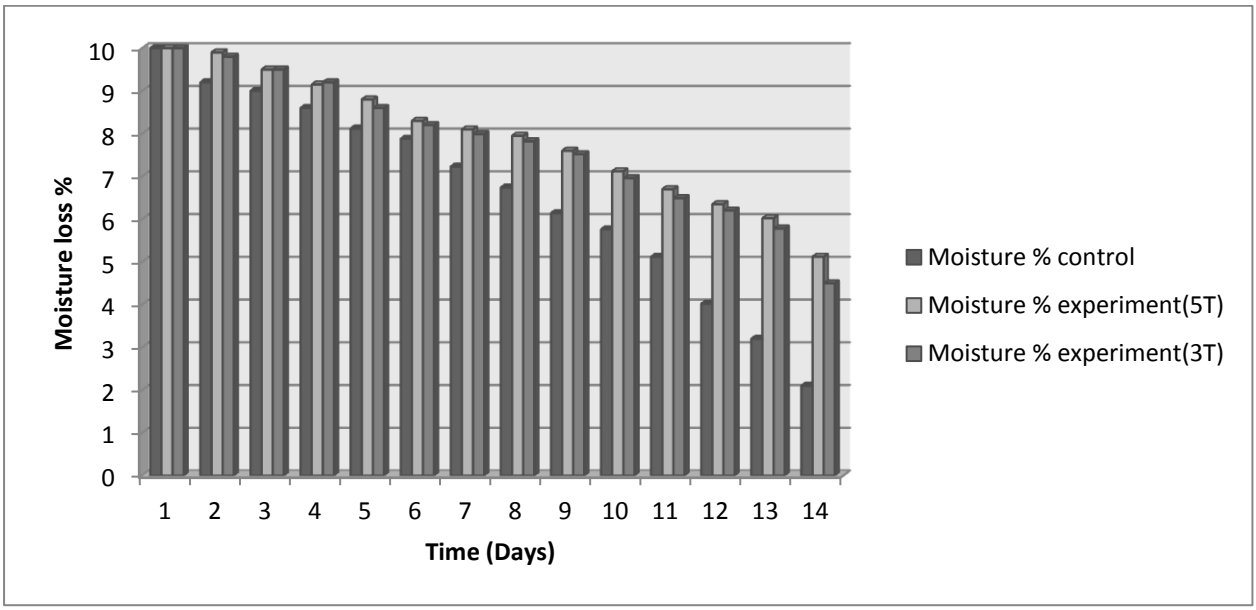


Figure 4.3.5 Graphical representation of moisture lost daily for 14 days measured before rehydration of the sandy loamy soil inoculated with EPN 3T (*S. australe*) and 5T (*H. bacteriophora*) over 14 days and averaged moisture loss for both controls. The initial moisture in all the Petri dishes was of the soil was 10%.

4.4 Discussion

Previous studies have shown that most *Steinernema* and *Heterorhabditis* species can survive for 14 to 21 days on dry soil compared to when they are subjected to exposed surfaces where they can only survive for only a few hours (Grewal et al, 2006). This evidence highly supports results obtained in this study as seen in table 4.3.1 and 4.3.3, under slow drying conditions. *Steinernema australe* and *Heterorhabditis* genera were able to withstand desiccated conditions for 20 days; however *Heterorhabditis species* had showed a slightly higher percentage of mortality than *S. australe*. Furthermore; (Grewal et al, 2006) also highlighted that *H. bacteriophora* IJs have an increased desiccation tolerance than *H. poinar* which also support the observed results in this study where the *bacteriophora* species expressed strong tolerance to desiccation.

The desiccation tolerance depends on the type of species, morphology and genetics. EPNs sheath or cuticle has been recommended to decrease the rate at which water is lost from IJs during dehydration in *H. bacteriophora* but the same cannot be said about *S. carpocapsae* because the sheath was not observed in this species even though the rate of water loss was lower (Grewal et al, 2006). In this current study it was observed that *S. australe* had a slightly higher moisture loss than *H. bacteriophora* as seen in figure 4.3.5. The reason behind this observation is beyond the thickness of the cuticle since in chapter 2 figure

2.3.5a showed a thick cuticle layer of *S. australe* also suggesting that permeability of the cuticle might have been the key feature in slightly increasing the rate of water loss in *S. australe.*

Another explanation for desiccation tolerance lies in genetics and the temporal up-regulation of stress-related genes, implying that gene expression patterns are modified in proteins which aid in enabling the EPNs to survive stress (Gal et al, 2003). Environmental conditions which EPNs are subjected to thus induce the expression of genes which can help them survive or tolerate the pressure. Harsh environmental conditions such as extreme heat and cold, pH increase and decrease; have triggered the development of an infective stage of EPNs called "dauer juvenile". This infective juvenile stage is very unique, allowing the IJs to forage and persist in the soil for longer periods searching for host even without nutrition supply (Gal et al, 2003). Tolerance to desiccation stress is different in varying EPNs species across both commonly known genera. *S. carposapsae* was reported to be one of the species with a high degree of desiccation tolerance and it was then proposed that the species must be genetically modified to further improve its special feature in of tolerating desiccation stress (Grewal et al, 2006). This can be done for most species which display similar traits and promising traits, including *H. bacteriophora* and *S. austral*e.

Studying genes which play a role in modifying EPNs in order to allow them to adapt to harsh environmental conditions is of great importance and will thus create further understanding of biochemical and molecular processes that take place in EPNs cells (Perez et al, 2003). This knowledge can contribute to the improvement of formulation strategies and methods, as well as mass production advancements (Strauch et al, 2003). Understanding desiccation stress tolerance of EPNs can be of promising hope to formulation and application strategies of EPNs to control problematic insects.

Further reasoning to why both identified species in the study were able to withstand desiccation for a considerable length of time can be explained by the presence of Polyols in the EPNs. Polyols (alcohol which has many hydroxyl groups) and sugars have been identified and characterised in nematodes which undergo anhydrobiosis. These molecules such as trehalose and glycerol protect the biological membranes and intracellular proteins during desiccation. Hydroxyl groups of trehalose replace water in the phospholipid bilayer during desiccation resulting in a crystallised bilayer (Grewal et al, 2006). This can be hypothesised to be true with respect to *H. bacteriophora* and *S. australe* isolated in the study.

From the findings in this study it is evident that both EPNs are effective and have a satisfactory potential to tolerate dry conditions for a longer period (Serwe-Rodriguez et al, 2004). This brings both species to an advantage of being used as biological control agents in agriculture supported by suitable formulations

and storage conditions. The study also proves that soil type and suitable moisture are important factors for mobility and infectivity of EPNs (Serwe-Rodriguez et al, 2004).

4.5 Conclusion

H. bacteriophora and *S. australe* are possible candidates for biological control of problematic insects since they can persist in dehydrated soil for at least 20 days. This brings the species to an advantage of being used as a biological control in agriculture supported by suitable formulations and storage conditions.

4.6 References

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Chapter 5:

Behaviour studies of *Heterorhabditis bacteriophora* and *Steinernema australe.*

5.1 Introduction

Increased pest populations in agriculture and forestry can lead to drastic economic loss, especially in countries which depend on agriculture, forestry and tourism. In Europe, pine weevils were chemically controlled by insecticides and estimations showed that if pests were not controlled, over 140 million Euros per year would have been lost due to this problematic pest. EPNs were then recommended by the sustainable forest management under the European Union in order to limit the danger that comes with the use of synthetic chemical pesticides. The sustainable forest management then highlighted the importance of also understanding EPNs behavior and application time in order to efficiently use them as reliable control agents (Dillion et al, 2006).

In order to understand the efficiency and ability of the EPNs to infect their host and how they search for host and food, it is crucial to study the group of activities and types of responses which are decoded by their nervous system in response to internal and external stimuli and also understand nematode-insect interactions. EPNs behaviour studies can assist in understanding their foraging strategies which are crucial for determining infection rate (O'Leary et al, 2001). There are ambush predators which infect more insects on the soil surface and cruising predators infect more insects found deep in the soil (Susurluk, 2003). Most *Heterorhabditis* species are classified under cruise foraging strategy as they go deeper into the soil, searching for host. While most *Steinernema* species are grouped under ambushers as they target insect found on the soil surface, which are very mobile (Dillion et al, 2006).

Types of behaviour play a role in the classification of nematodes since they can be grouped according to how they respond to various stimuli. Alterations in behaviour of specific species can be induced by chemical and physical stimuli, therefore suitable environmental factors must be identified in order to accommodate and favour EPNs successful activity. Like human beings and other vertebrates, nematodes use receptors, the central nervous system and specific transcription factors for signal transduction and cell communication (O'Leary et al, 2001).

Having a better understanding on EPNs behaviour can contribute in improving application of these biological control agents. Field application studies are done to monitor the impact EPNs have on target and non-target pest insects (Shapira-Ilan et al, 2006). An example includes *Steinernema carpopsae* attempt to control adult carabid beetles in field trials. Unfortunately no effect or impact was observed

from this trial. This now raised concerns on host specifity (Grunner et al, 2006).

Important things to do and understand about field application of EPNs on problematic soil organisms.

- One needs to establish which application is better, wet or dry?
- Habitat of host/target and its interactions with EPNs must be well understood
- Factors supporting EPNs population increase in that specific environment must be identified

Integrated pest management has already adopted and employed the use of biological control agents; the aim now is to identify suitable agents which can contribute greatly in reducing pest populations of insects. In the field, factors such as problematic target insects, crop to be protected, type of application method and field size must be well understood for determining the concentration of IJs to be applied (Grunner et al, 2006).

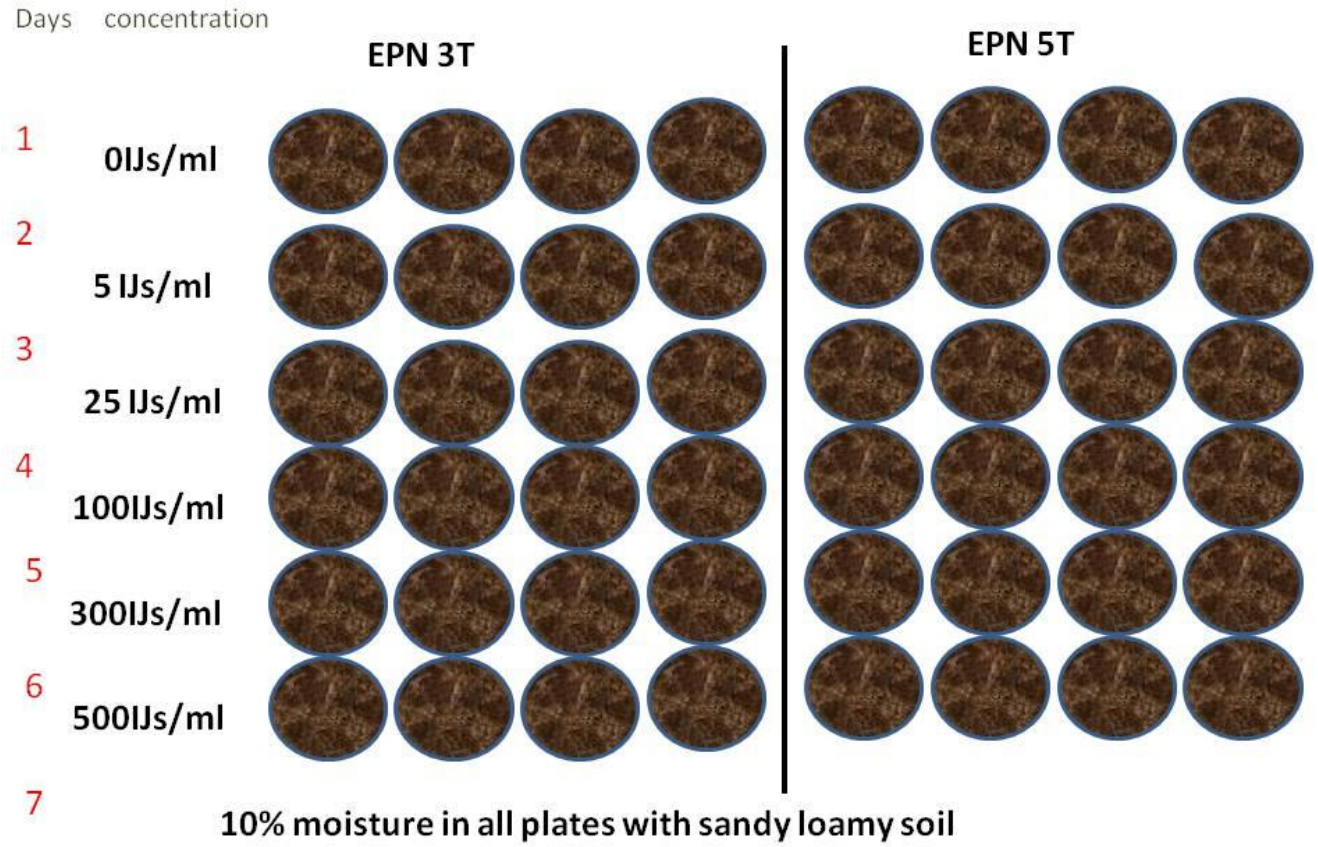
EPNs can be applied in more or less similar technology as synthetic chemical insecticides. These methods include electrostatic sprayers with tip openings of 0.05mm. Previous studies showed that method of application does not always significantly affect viability and concentration of EPNs unless if they are blocked from passing through the opening of the application apparatus (Shapira-Ilan et al, 2006).

In order to have an idea of how EPNs behave several investigations were done using the two identified species in the study, *H. bacteriophora* and *S. australe.*

5.2 Material and Methods

5.2.1 Comparative dose assay

Comparative dose-response assay involved exposing *G. mellonella* insect larvae to different IJs concentrations which were 0, 5, 25, 100, 300 and 500 IJs/ml and larvae mortality was recorded daily over a week as seen in the diagram below.



(Graphics developed by T.E Lephoto)

Figure 5.2.1 Comparative dose response of EPN5T *H. bacteriophora* and EPN3T *S. australe.*

For each species, sterile sandy loamy soil was distributed into 24 90mm Petri dishes, with 10% moisture and inoculated with 5, 10, 25, 50, 100, and 500 IJs immersed in 1ml of sterile distilled water, except for the control with 0IJs/ml. 10 larvae per plate were added and the plates were left on the bench at room temperature (25°C). The experiments were repeated four times.

5.2.2 Investigating the behaviour of EPNs in sandy loamy soil and foraging strategies

Fresh IJs were collected from white traps and rinsed once with Ringer solution. Sandy loamy soil was autoclaved for 30minutes at 121°C and the soil was dried in a 37°C oven for 48hours. In each designed column as seen in 5.2.2 below, 164g of soil was added and adjusted the moisture to 10% with sterile water. The column had 3 sections with varying distances in between and 1 wax moth larvae with the mouth area facing outward away from the soil was placed in each and sealed the ends to prevent them

from moving out. Each section was sprayed with 500µl of water to allow EPNs to swim towards the host, with the control not sprayed with any water. Surface application was mimicked by only adding 100 IJs/ml to the surface of the soil in the vertical column. The top of the vertical column was loosely closed with a compatible lid to allow some degree of aeration but limiting high loss of humidity and also to avoid suffocating the IJs. The experiment was conducted at room temperature 25+/-2°C. Two EPN species were used, EPN3T and EPN5T and 4 vertical columns were used for each species. Mortality rate was recorded over 7 days and dead larvae were collected and placed on white traps to confirm cause of infection.

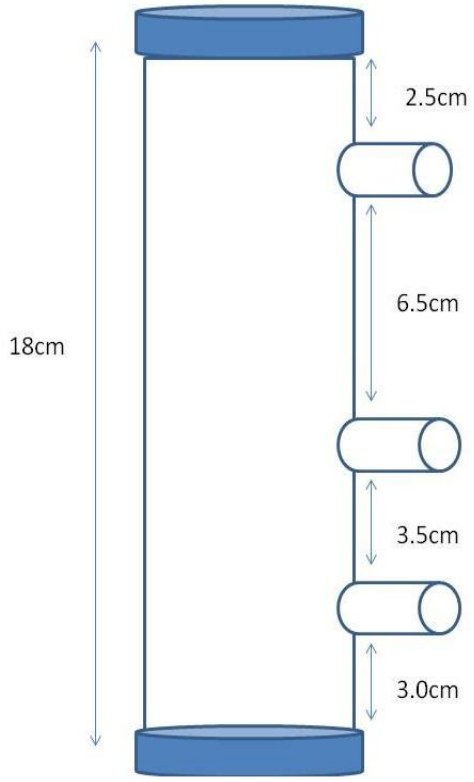


Figure 5.2.2 An 18cm vertical column used to study behaviour of nematodes with 3 arenas at varying distances in which insect larva were placed.

Table 5.2.2 A summary of material measurements used in the investigations.

Material	Weight in grams (g)
Vertical column and lids	120.45
Larvae	-/+ 0.14
Stopper	1.25
Soil to fill column	137.39
Total weight before hydration	327.06

5.2.3 Investigating the effect of soil humidity on EPNs behaviour

Materials and methods same as in 5.2.2 with the following modification: Each vertical column was set up at different moisture which included 4%, 6%, 8% and 10% respectively for each species using the ratio below to calculate the moisture percentage. The mortality ratio observed in 5 days also recorded the moisture lost daily.

Ratio for calculating moisture percentage

1g=1%

5.2.4 Investigating different application concentrations in order to determine suitable field application dose.

Materials and methods same as in 5.2.2 with the following modification: Each vertical column was set up at different IJs concentrations ranging from 5, 10, 25 and 50IJs/ml for both species and then further increasing the concentration to 50, 100, 200 and 400IJs/ml respectively for each species. The mortality percentage was recorded daily.

Application and dose was worked out as follows:

Example:
To calculate IJs/cm2, 250 000 IJs per m 2 was divided by the number of c m 2 in one m 2 which is (100 cm x100 cm = 10 000 c m 2 in 1 m 2)
Therefore: 250 000 IJ/ (100x100) = 25 IJs per cm 2.
The recommended dose form literature is 2.5 x 10 9 IJs per hectare. There is 10 000 m 2 in 1 hectare. So the recommended dose of EPNs per m 2 is 2.5 x 10 9 / 10 000 which gives 250 000 IJs per m 2

5.2.5 Determining field capacity

Soil is said to be at field capacity when all the excess water has been drained after it had been saturated with water. Field capacity was determined after sandy loamy soil saturated with water and excess water was drained from the 8 vertical columns. The average initial weight of soil saturated with water and also weight after draining excess water was calculated.

5.3 Results

5.3.1 Comparative Dose assay

It is of crucial importance that the application dosage is well calculated also depending on the nature of the identified nematodes and size of the field, taking into consideration the target insect host. The results below summarise a series of IJs concentration suitable for effective increased insect host mortality.

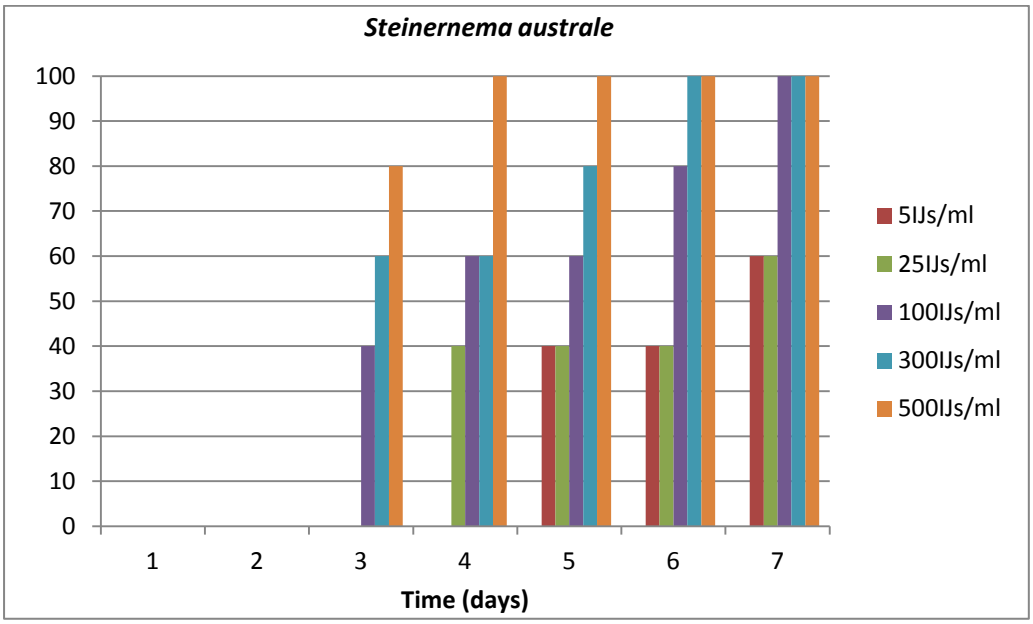


Figure 5.3.1a Cumulative percentage mortality of host (wax moth) in response to *Steinernema australe* IJ dose. Statistical analysis and interpretation of the results above recorded in table 5.3.1a and 5.3.1b in Appendix C (supplementary information).

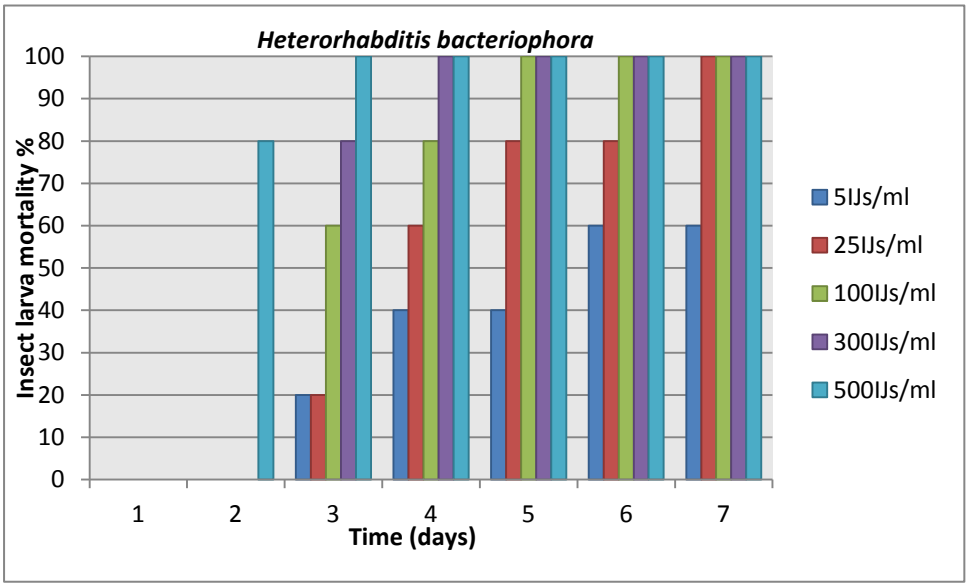


Figure 5.3.1b Cumulative percentage mortality of host in response to *Heterorhabditis bacterophora* IJ dose. Statistical analysis and interpretation of the results above recorded in table 5.3.1c and 5.3.1d in Appendix C (supplementary information).

5.3.1.1 Investigating the behaviour of EPNs in sandy loamy soil and foraging strategies

Table 5.3.3.1 Mortality percentage of *G. mellonella* recorded over 5 days after inoculating the soil in the column with EPN3T and EPN5T on 2conditions: Dry and humid arenas/sections with larvae and column moisture of 10%.

Column	EPN3T Mortality %						EPN5T Mortality %				
[100IJs/ml] in all.	Days						Days				
	1	2	3	4	5		1	2	3	4	5
1a	0	0	66.6 x,y	66.6 x,y	100 x,y,z		0	0	66.6 x,y	100 x,y,z	100 x,y,z
2a	0	0	33.3 x	66.6 x,y	100 x,y,z		0	0	66.6 x,y	100 x,y,z	100 x,y,z
3b	0	0	33.3 x	33.3 x	100 x,y,z		0	0	66.6 x,y	66.6 x,y	100 x,y,z
4b	0	0	33.3 x	33.3 x	100 x,y,z		0	0	33.3 x	66.6 x,y	100 x,y,z

Moisture status: a=humid arena with larvae, b=dry arena with larvae

Observed mortality: x= first top arena, y=second middle arena, z=third bottom arena

5.3.1.2 Investigating the effect of soil humidity on EPNs behaviour

Table 5.3.1.2 The effect of soil humidity on EPNs behaviour were each vertical column was set up at different moisture contents ranging from 4%, 6%, 8% and 10% and the mortality % was recorded daily.

Column moisture %	EPN3T Mortality %						EPN5T Mortality %				
[100IJs/ml]	Time (Days)						Time (Days)				
	1	2	3	4	All infected larvae showed known symptoms by day 3		1	2	3	4	All infected larvae showed known symptoms by day 3
A:10% moisture*	0	0	66.6 x,y	100 x,y,z			0	0	100 x,y,z	100 x,y,z	
B:8% Moisture*	0	0	66.6 x, y	100 x,y,z			0	0	100 x,y,z	100 x,y,z	
C:6% moisture *	0	0	66.6 x,y	100 x,y,z			0	0	66.6 x,y	100 x,y,z	
D:4% moisture*	0	0	33.3 x	100 x,y,z			0	0	66.6 x,y	100 x,y,z	

Observed mortality: x= first top arena, y=second middle arena, z=third bottom arena

(*) The arena was humid to allow efficient EPNs movement

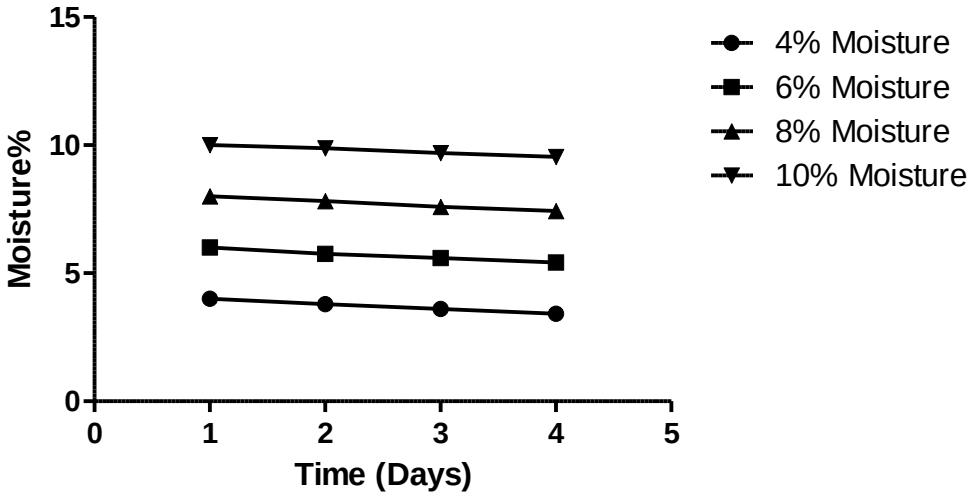


Figure 5.3.1.2 Graphical representation of the total soil moisture lost daily from the vertical columns with inoculated with EPNs

5.3.1.3 Investigating different application concentrations in order to determine suitable field application dose.

Table 5.3.3.3a Summary of results showing results obtained from using different application concentrations 50, 100, 200 and 400 IJs/ml. This is to determine the suitable field application dose of EPNs. Insect larvae mortality % was recorded daily in each arena of the column.

Column IJs concentration	EPN3T Mortality %						EPN5T Mortality %				
10% moisture	Time (Days)						Time (Days)				
	1	2	3	4	All infected larvae showed known symptoms by day 2		1	2	3	4	All infected larvae showed known symptoms by day 2
A=[50IJs/ml]	0	0	33.3 x	100 x,y,z			0	33.3 x	100 x,y,z	100 x,y,z	
B=[100IJs/ml]	0	33.3 X	66.6 x, y	100 x,y,z			0	66.6 x,y	100 x,y,z	100 x,y,z	

C[200IJs/ml]	0	33.3 x,y	66.6 x,y	100 x,y,z			0	66.6 x,y	100 x,y,z	100 x,y,z	
D=[400IJs/ml]	0	66.6 x,y	100 x,y,z	100 x,y,z			0	66.6 x,y	100 x,y,z	100 x,y,z	

Observed mortality: x= first top arena, y=second middle arena, z=third bottom arena

Table 5.3.3.3b Summary of results showing results obtained from using different application concentrations 5, 10, 25 and 50 IJs/ml. This is to determine the suitable field application dose of EPNs. Insect larvae mortality % was recorded daily in each arena of the column.

Column IJs concentration	EPN3T Mortality %						EPN5T Mortality %				
10% moisture in all coulmns	Time (Days)						Time (Days)				
	1	2	3	4	All infected larvae showed known symptoms by day 2		1	2	3	4	All infected larvae showed known symptoms by day 2
A=[5IJs/ml]	0	0	33.3 x	33.3 x			0	33.3 x	33.3 X	33.3 x	
B=[10IJs/ml]	0	33.3 X	33.3 x,	33.3 x			0	33.3 X	33.3 x,y	66.6 x,y	
C[25IJs/ml]	0	33.3 X	33.3 x	100 x,y,z			0	33.3 X	66.6 x,y	100 x,y,z	
D=[50IJs/ml]	0	33.3 x,	66.6 x,y,	100 x,y,z			0	66.6 x,y	66.6 x,y,	100 x,y,z	

Observed mortality: x= first top arena, y=second middle arena, z=third bottom arena

5.3.2 Field capacity evaluated on sandy loamy soil

Days	EPN5T soil weight grams (g) in a column							
Vertical columns	A	B	C	D	E	F	G	H
1	350.01	344.46	358.69	354.36	341.24	366.23	350.12	337.64
2	Excess water drained from the column for 2 days to determine the field capacity							
3								
4	346.19	336.84	355.79	346.99	337.46	359.58	339.68	331.83
Average initial (i) weight of soil saturated with water =350.34grams			Average final (f)weight (g) of soil after draining water =344.30grams			Therefore the weight of soil at field capacity= 344.3grams		

5.4 Discussion and conclusion

The behaviour of EPNs can affect their efficacy since some EPNs look for host on the soil surface and do not apply other foraging strategies to search further. Several *Steinernema* species have been grouped under ambushers since they tend to dwell on the soil surface and not forage deeper into, examples include *S. carpopasae* and S. *scapterisci*. However results obtained in tables 5.3.3.1, 5.3.3.2 and 5.3.3.3a show that *S. australe* is capable cruising further down to 18cm searching for host as mortality was observed in the last bottom arena. Most if not all *Heterorhabditis* species cruise deeper into the soil searching for host, examples include *H. bacteriphora* which is classified as a cruiser, (Grewal et al, 2001). This supports the results obtained in tables 5.3.3.1, 5.3.3.2, 5.3.3.3a and 5.3.3.3b were the isolated *H. bacteriophora* in this study displayed increased mobility, high mortality percentages obtained within 48hours in almost all arenas, proving that this species is an effective cruiser foraging deeper into the soil, (Susurluk, 2003).

Insect mortality was high for both *S. australe* and *H. bacteriphora*, at 100IJs\ml, 300 IJs\ml and 500 IJs\ml observed within 48 to 96 hours and insects larvae showed signs of infection after 48hours. Significant difference was observed at concentrations 5IJs/ml and 25IJs/ml since *H. bacteriphora* was able to kill 20% of the larvae by day 3 while *S. australe* displayed mortality by day 4 and 5 for respective concentrations. This observation is supported by the Two-way Post-hoc analysis in table 5.3.3.3a and 5.3.3.3b. From these results it can be hypothesised that *H. bacteriphora* is more virulent than *S. australe* due to its ability to infect *G. mellonella* in 48-72 hours even at lowest IJs concentration.

Application humidity of EPNs before and after; aids in assisting their locomotion in soil as they search for host. Humidity is also important for infection efficiency and persistence, (Lewis et al, 2006). This is supported by results seen in table 5.3.3.1 where increased mortality is observed in columns with moist arenas.

Soil-type highly influences and affects EPNs infectivity and efficacy. Soil characteristics such as particle size, organic matter in the soil are associated with the texture of the soil and its ability to hold moisture. The smaller the soil pores, the lower the locomotion rate of EPNs, thus sandy loamy soil is considered favourable for most cruiser EPNs such as *H. Bacteriophora* identified in this study. Sandy loamy soil also has a great potential to retaining moisture and thus directly influencing EPNs mobility and survival, (Koppenhofer etal, 2006). The ability to retain moisture also has a close link to understanding field capacity. Soil is said to be at field capacity when all the excess water has been drained after it had been saturated with water as seen in table 5.3.4. The average initial weight of soil saturated with water was 350.34grams, weight after draining excess water was 344.30grams, and therefore weight of the soil in the columns at field capacity was 344.30 grams.

5.5 References

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Research summary

S. australe and *H. bacteriophora* symbiotically associated with *Xenorhabdus spp* and *P. carborca* respectively, which were isolated form sandy loamy soil collected from Walkerville, South of Johannesburg. From desiccation tolerance analysis, comparative dose assay and behaviour studies it can be concluded that both EPNs have a promising potential to control insects. Behaviour and comparative doss response revealed that any concentration between 100-500IJs/ml is effective for application doss of both species with soil moisture content kept between 4-10%. Another aim for behaviour studies was to investigate the effect of different application strategies tested on the concentration of IJs viability and valuate IJs efficiency by observing how far they can travel to find host in soil. At field capacity, soil has water and air which can be considered favourable and ideal for plantation and supporting crop growth.

Findings of this study have implications on field application; highlighting the importance of soil moisture and IJs concentration. Mainly this study gave an idea of on how the isolated EPNs behave, pointing out specific foraging strategies. Further future studies will be done to understand the molecular biology of EPNs behavior.

Application of these EPNs can lead to changes and drastic decrease of insect populations in both favourable and dry environmental conditions as seen in chapter 4 where both identified EPNs were able to withstand desiccation for 20days.

For biological control of insects, the wisest and best strategy to control adult insects of any species is to target the early developmental stages as they are more prone and easy to infect. This is why in this study *G. mellonella* insect larvae is used for all experimental work as they are more susceptible than a fully developed insect.

Overall, both identified species have the outmost potential to be applied as biological control agents in Agriculture.

Appendix A

Functions of reagents

1. Agarose= creates a matrix in which shorter molecules move faster and migrate farther than longer ones depending on the percentage of the gel. Low % gels can be used to separate large DNA fragments while high % gels will used to separate small DNA fragments.

2. Tris/borate/EDTA buffer (TBE) = contains a mixture of Tris base, boric acid and EDTA. TBE has higher buffering capacity and be re-used. EDTA is to protect the nucleic acids against enzymatic degradation.

3. TAE buffer= has the lowest buffering capacity and provides the best resolution for larger DNA (>20kb) i.e. lower voltage applied and more time taken to run the gel but best products are obtained. It contains a mixture of Tris base, acetic acid and EDTA.

4. Tris= tris (hydroxymethyl) aminomethane, maintains the proper pH for the DNA

5. EDTA= ethylenediaminetetraacetic acid acts to chelate (bind up) ions in the solution and disrupt the activity of any DNAse which may cleave the DNA so it chalates divalent cations such as Mg++

6. 6X loading dye= was used for loading DNA samples into wells on agrarose gel electrophoresis and labelling the DNA sample so that it is visible as the gel runs. It contains EDTA, glycerol, bromphenol blue and 10 mM Tris-HCl (pH8.0).

7. 1kb DNA ladder= ruler, is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb.
8. Ethedium bromide (EtBr) = is an intercalating agent i.e. it gets inserted into adjacent bases in DNA sequences. It is added before the gel polymerises. When EtBr bound to DNA is irradiated by UV light, it fluoresces at visible wavelength.

9. Chloroform= separates contaminants from DNA by forming an organic phase and thus leaving DNA in the aqueous phase.
10. RNase A= is an enzyme used which was used in genomic DNA isolation and it digests/degrades RNA which may contaminate the isolated genomic DNA.
11. Absolute ethanol= precipitate and recover DNA.
12. 70% ethanol= was used to wash the DNA pellet
13. Nuclease free water= was used to re-suspend the DNA, and it is safer to use to reduce the risk of DNA cleavage by DNases.
14. Phenol: removes proteins
15. Chloroform-isoamyl alcohol: separates nucleic acids from proteins
16. Proteinase K: degrades proteins
17. 5X TBE= electrophoresis buffer. 1X TBE was used as a running buffer at low voltage of 50 volts to prevent overheating and denaturation of small DNA fragments

Appendix B

Media for feeding the greater wax moth larvae

500g ProNutro (Multivitamin bran substituted with ProNutro (original flavour)
200ml pure natural honey
200ml glycerol
5 teaspoon yeast extract
200ml boiled distilled water
48
1 teaspoon benzoate

Mix honey, glycerol and ProNutro together. Add yeast extract, boiling water and benzoate to ProNutro mixture. Mix contents thoroughly, place mixture in tin foil and seal adequately and autoclave the media at 121˚C and 15 psi for 25 minutes and feed to larvae after cooling.

Selective media EPNs symbiotic bacteria

NBTA selective media

1 litre nutrient agar
0.04g triphenyltetrazolium chloride (TTC)
0.025g bromothymol blue (BTB)
Mix nutrient agar and BTB and autoclave the media at 121˚C and 15 psi for 15 minutes. Add TTC, just before pouring into Petri dishes; ensuring that the autoclaved media is less than 50˚C. TTC will break down if added when medium is too hot. Swirl to mix and dispense into sterile Petri dishes and leave to solidify.

McConkey agar selective media

Composition (g/l)

20.0g Peptone
10.0g Lactose
1.5g Bile Salts
5.0g Sodium Chloride
49
0.03g Neutral Red
0.0001g Crystal Violet
13.5g Agar
Weigh out McConkey agar powder and suspend in 1000ml distilled water. Boil whilst stirring until completely dissolved and autoclave at 121ºC and 15 psi for 15 min. Cool to 45 – 50ºC. Mix properly and dispense into sterile Petri dishes and leave to solidify.

0.1% jik solution for infective juvenile sterilization
34ml distilled water
1ml 3.5% jik
Autoclave distilled water. Mix jik and distilled water in bottles and autoclave at 121˚C and 15 psi for 15 min.

Recipe for *in vitro* culture media

Nutrient Broth
4.0% (W/V) Canola oil
25mg/ml glucose
Weigh out nutrient broth powder and suspend in desired volume of distilled water. Add glucose. Mix well and dispense adequate amounts into volumetric flasks. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth and autoclave at 121˚C and 15 psi for 15 minutes

5X TBE
54g Tris base
27.5g Boric acid
20ml 0.5M EDTA pH 8.0
Mix with 1L distilled water and autoclave at 121˚C at 15psi for 20 minutes

Appendix C

Supplementary information

Table 5.3.1a A two-way ANOVA analysis for *Steinernema australe* comparative dose assay

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Column Factor	86.13	< 0.0001		
Time (Days)	7.14	0.0107		
Source of Variation	P value summary	Significant?		
Column Factor	***	Yes		
Time (Days)	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Column Factor	5	27333	5467	38.44
Time (Days)	3	2267	755.6	5.313
Residual	15	2133	142.2	
Number of missing values	0			

Table 5.3.1b Two-way Bonferro post-hoc test for *Steinernema australe* comparative dose assay showing exactly where the significance between the difference concentrations.

Two-way ANOVA

Source of Variation	% of total variation	P value
Column Factor	86.13	< 0.0001
Time (Days)	7.14	0.0107

Source of Variation	P value summary	Significant?
Column Factor	***	Yes
Time (Days)	*	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Column Factor	5	27333	5467	3
Time (Days)	3	2267	755.6	5
Residual	15	2133	142.2	

Number of missing values 0

Bonferroni posttests

Mortality% at 0IJs/ml (Control vs Mortality% at 5IJs/ml

Time (Days)	Mortality% at 0IJs/ml (Control	Mortality% at 5IJs/ml	Difference	9
4.000	0.0	0.0	0.0	-6
5.000	0.0	40.00	40.00	-2
6.000	0.0	40.00	40.00	-2
7.000	0.0	60.00	60.00	-1

Time (Days)	Difference	T	P value	S
4.000	0.0	0.0	P > 0.05	n
5.000	40.00	2.372	P > 0.05	n

6.000	40.00	2.372	P > 0.05	n
7.000	60.00	3.558	P < 0.05	*
Mortality% at 0IJs/ml (Control vs Mortality % at 25IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 25IJs/ml	Difference	9
4.000	0.0	40.00	40.00	-2
5.000	0.0	40.00	40.00	-2
6.000	0.0	40.00	40.00	-2
7.000	0.0	60.00	60.00	-1
Time (Days)	Difference	T	P value	S
4.000	40.00	2.372	P > 0.05	n
5.000	40.00	2.372	P > 0.05	n
6.000	40.00	2.372	P > 0.05	n
7.000	60.00	3.558	P < 0.05	*
Mortality% at 0IJs/ml (Control vs Mortality % at 100IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 100IJs/ml	Difference	9
4.000	0.0	60.00	60.00	-1
5.000	0.0	60.00	60.00	-1
6.000	0.0	80.00	80.00	1
7.000	0.0	100.0	100.0	3
Time (Days)	Difference	T	P value	S
4.000	60.00	3.558	P < 0.05	*
5.000	60.00	3.558	P < 0.05	*
6.000	80.00	4.743	P<0.01	*
7.000	100.0	5.929	P<0.001	*

Mortality% at 0IJs/ml (Control vs Mortality % at 300IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 300IJs/ml	Difference	9
4.000	0.0	60.00	60.00	-1
5.000	0.0	80.00	80.00	1
6.000	0.0	100.0	100.0	3
7.000	0.0	100.0	100.0	3
Time (Days)	Difference	T	P value	S
4.000	60.00	3.558	P < 0.05	*
5.000	80.00	4.743	P<0.01	*
6.000	100.0	5.929	P<0.001	*
7.000	100.0	5.929	P<0.001	*
Mortality% at 0IJs/ml (Control vs Mortality % at 500IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 500IJs/ml	Difference	9
4.000	0.0	100.0	100.0	3
5.000	0.0	100.0	100.0	3
6.000	0.0	100.0	100.0	3
7.000	0.0	100.0	100.0	3
Time (Days)	Difference	T	P value	S
4.000	100.0	5.929	P<0.001	*
5.000	100.0	5.929	P<0.001	*
6.000	100.0	5.929	P<0.001	*
7.000	100.0	5.929	P<0.001	*

Table 5.3.1c ANOVA analysis for *Heterorhabditis bacterophora* comparative doss response assay

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Column Factor	95.42	< 0.0001		
Time (Days)	1.78	0.0546		
Source of Variation	P value summary	Significant?		
Column Factor	***	Yes		
Time (Days)	ns	No		
Source of Variation	Df	Sum-of-squares	Mean square	F
Column Factor	5	31283	6257	102.4
Time (Days)	3	583.3	194.4	3.182
Residual	15	916.7	61.11	
Number of missing values	0			

Table 5.3.1d Two-way Bonferro post-hoc test for *Heterorhabditis bacterophora* comparative doss assay showing exactly where the significance between the difference concentrations.

Table Analyzed	Data 1	
Two-way ANOVA		
Source of Variation	% of total variation	P value
Column Factor	95.42	< 0.0001
Time (Days)	1.78	0.0546

Source of Variation	P value summary	Significant?		
Column Factor	***	Yes		
Time (Days)	ns	No		
Source of Variation	Df	Sum-of-squares	Mean square	F
Column Factor	5	31283	6257	1
Time (Days)	3	583.3	194.4	3
Residual	15	916.7	61.11	
Number of missing values	0			
Bonferroni posttests				
Mortality% at 0IJs/ml (Control vs Mortality% at 5IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality% at 5IJs/ml	Difference	9
4.000	0.0	40.00	40.00	-0
5.000	0.0	40.00	40.00	-0
6.000	0.0	60.00	60.00	1
7.000	0.0	60.00	60.00	1
Time (Days)	Difference	T	P value	S
4.000	40.00	3.618	P < 0.05	*
5.000	40.00	3.618	P < 0.05	*
6.000	60.00	5.427	P<0.001	*
7.000	60.00	5.427	P<0.001	*
Mortality% at 0IJs/ml (Control vs Mortality % at 25IJs/ml				

Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 25IJs/ml	Difference	9
4.000	0.0	60.00	60.00	1
5.000	0.0	80.00	80.00	3
6.000	0.0	80.00	80.00	3
7.000	0.0	100.0	100.0	5

Time (Days)	Difference	T	P value	S
4.000	60.00	5.427	P<0.001	*
5.000	80.00	7.236	P<0.001	*
6.000	80.00	7.236	P<0.001	*
7.000	100.0	9.045	P<0.001	*

Mortality% at 0IJs/ml (Control vs Mortality % at 100IJs/ml

Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 100IJs/ml	Difference	9
4.000	0.0	80.00	80.00	3
5.000	0.0	100.0	100.0	5
6.000	0.0	100.0	100.0	5
7.000	0.0	100.0	100.0	5

Time (Days)	Difference	T	P value	S
4.000	80.00	7.236	P<0.001	*
5.000	100.0	9.045	P<0.001	*
6.000	100.0	9.045	P<0.001	*
7.000	100.0	9.045	P<0.001	*

Mortality% at 0IJs/ml (Control vs Mortality % at 300IJs/ml

Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 300IJs/ml	Difference	9
4.000	0.0	100.0	100.0	5
5.000	0.0	100.0	100.0	5

6.000	0.0	100.0	100.0	5
7.000	0.0	100.0	100.0	5
Time (Days)	Difference	T	P value	S
4.000	100.0	9.045	P<0.001	*
5.000	100.0	9.045	P<0.001	*
6.000	100.0	9.045	P<0.001	*
7.000	100.0	9.045	P<0.001	*
Mortality% at 0IJs/ml (Control vs Mortality % at 500IJs/ml				
Time (Days)	Mortality% at 0IJs/ml (Control	Mortality % at 500IJs/ml	Difference	9
4.000	0.0	100.0	100.0	5
5.000	0.0	100.0	100.0	5
6.000	0.0	100.0	100.0	5
7.000	0.0	100.0	100.0	5
Time (Days)	Difference	T	P value	S
4.000	100.0	9.045	P<0.001	*
5.000	100.0	9.045	P<0.001	*
6.000	100.0	9.045	P<0.001	*
7.000	100.0	9.045	P<0.001	*