INVESTIGATING FORMULATION STRATEGIES AND DESICCATION TOLERANCE OF A SOUTH AFRICAN ENTOMOPATHOGENIC NEMATODE (RHABDITIDA: HETERORHABDITIDAE)

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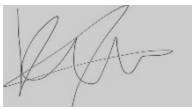
DECLARATION

- I hereby declare that this Dissertation is my own, unaided work and material obtained from other sources have been referenced and citied.
- The experimental protocols, analysis of the results and write up of this dissertation were done by myself
- It is being submitted for the degree of Master of Science with dissertation at the University of the Witwatersrand, Johannesburg
- This work has not been submitted before for any degree or examination at any other university

Keneilwe Ranakabae

Signature: 

Date: 21 May 2018

DEDICATION

This work is dedicated to my late mother, Malisebo Adelina Ranakabae, for all her efforts in enabling me to get an education. Your spirit lives on. To the almighty God, all glory and honour belongs to you.

I thank you

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ABSTRACT

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are excellent biological control agents of lepidopteran and coleopteran soil dwelling insects. Their successful potential as biological control agents is attributed to the bacterial symbiont contained in their guts, together the dual kill insect host in 24 hours. The aims of the dissertation were to investigate the effects of nematode infectivity against susceptible hosts in different formulations as well as its tolerance to desiccation. Soil samples were collected from Brits, North-West province, South Africa. Insect baiting technique and White trap methods were used to recover nematodes from the collected soil samples. Preliminary identification based on symptoms revealed a red/maroon colour on EPN infected cadavers which indicated that the isolated EPN specie belonged to a *Heterorhabditis* genus. For molecular based confirmation of taxonomic affinities, genomic DNA was extracted, followed by PCR and sequencing of the 18S rDNA. BLASTn sequence results revealed that the presumptive *Heterorhabditis* sp. had a 99 % sequence affinity to *Heterorhabditis bacteriophora_ isolate UP2A2* (MF033536.1*)*, the evolutionary distance of the two species was 0.022 revealing evolutionary relatedness. *Heterorhabditis* sp. and *Heterorhabditis _ isolate UP2A2* were clustered together in the same clade confirming the presumed *Heterorhabditis* sp. as a *Heterorhabditis bacteriophora* isolate B1. *Heterorhabditis bacteriophora* isolate B1 infected larvae were dissected to isolate the associated endosymbiotic bacteria and a drop of hemolymph was streaked onto sterile plates of nutrient bromothymol blue-triphenyltetrazolium chloride agar (NBTA) and MacConkey which were then stored in the dark at room temperature. After 4 days of incubation, preliminary identification of the associated bacteria was performed by observing the colony morphology. Single small sized colonies of bacteria were green with red centres on NBTA and red on MacConkey agar plates confirmed that the isolated bacterial colonies displayed phenotypic features expected of bacteria belonging to *Photorhabdus* genus. Molecular identification revealed the presumed *Photorhabdus* spp. to be 99% closely related to *Photorhabdus_ temperate_subsp._khani_NC19* (KF740642) in sequence. *Heterorhabditis bacteriophora* isolate B1 was able to tolerate desiccation up to 15 days and following exposure to desiccation treatment the IJs induced 100% larval mortality within 96 hours post exposure to desiccation following resuscitation by rehydration. Infective juveniles desiccated in host cadavers embedded in loam soil and vermiculite emerged at a faster rate on larval cadavers

placed on saturated White traps at a mean rate of 16.67 IJs/day whereas emergence of injective juveniles from undesiccated host cadavers emerged at a mean rate of 10 IJs/day. The EPN IJ populations formulated in pure loam and sandy soil induced 80% and 50% larval mortality on day 2 of exposure to *Galleria mellonella* larvae. Cumulative mortalities reaching a 100% larval mortality were induced on days 5 and 6. EPN infected cadavers were desiccated for 31 days by incubating them at 16 ºC, 25 ºC, and 37 ºC. Previously desiccated EPN infected cadavers which were stored at temperatures 25 ºC and 37 ºC induced 100% larval mortality following rehydration in water over a period of 6, 8 and 24 hours. Desiccated IJs formulated in host cadavers could serve in formulation and application technology. Applying desiccation tolerant and native strains of EPNs is important for the eradication of susceptible insect pests.

1. CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Insect pests such as Armyworm larva, Japanese beetle and White flies amongst others, attack economical significant crops causing yield losses in agriculture, especially in the absence of insecticide application (Du Plessis and Goldblatt, 2015). The effects and implications of insect pests on crop yield has been for decades a subject of concern which researchers have studied with the aim to formulate environmentally friendly solutions for the eradication and control of these crop insect pests (Sharma *et al*., 2012). In an attempt to control insect pests, following the massive expansion of mono-crop production, the manufacture and application of pesticides which was first introduced in 1947 has since grown into a massive and very profitable international agrochemical industry (Gatehouse *et al*., 2011). Moreover, the management of insect pests has further expanded and augmented through various strategies such as crop rotation, the introduction of pest-resistant crops, timing of field operations, amongst others (Plant Protection Directorate, 2008) thus forming a broad spectrum of pest control methods. Together, the combined application of the latter strategies constitutes what is now known as Integrated Pest Management (IPM), which altogether aims to supress pest populations below the crop economic injury level (Plant Protection Directorate, 2008).

1.1.1. Biological Control

Based on environmental concerns and issues with human health and non-target organisms, biological control has provided an eco-friendly means of eliminating insect pests' populations in agroecosystems (Lacey *et al*., 2001). Using biological organisms as agents against insect pests has to be justified by their efficacy as pest control agents as well as economic viability in terms of lowering costs of crop pest control. For the most part, biocontrol agents which have been in the market have shown to be cost effective as well as more effective in their killing mechanisms in comparison to chemical pesticides (Lacey *et al*., 2001). The current status is that farmers are reluctant on using or even considering using biological means for pest control which include genetically modified crops and biological organisms to overcome pests, it is very important to understand the definition and scope of biological control, its concept as well as the importance of it.

1.1.1.1. Definition of biological control

Biological control is an approach or strategy which uses living biological organisms to eradicate specific pests which are deemed problematic in agricultural settings. This approach is one of the key components in programs that manage the existence and spread of agricultural crop pests and has been in existence and employed worldwide for over centuries (Sweetman, 1958). The use of microorganisms in biological control were first introduced by Agostino Bassi, Louis Pasteur, and Elie Metchnikoff in 1956-1975, and although the widespread use of biological control tactics had been in existence, the discovery and application occurred at different times for different countries. The introduction and use of biological control of pests in Europe, for example started in the 1990s (Lenteran *et al*., 1997) while the first usages of the term , biological control was established at the University of California by Smith in 1999. Today, many countries are using a variety of natural enemies to control diptera, coleopteran and lepidopteran insects (Lacey *et al*., 2001) which are found in various agricultural areas such as orchards, turf and lawn, greenhouses and forestry (Burges, 1981; Tanada and Kaya, 1993; Lacey and Kaya, 2000). According to Greathead, (1995) and Gurr *et al*., (2000) the permanent reduction of pest populations, which includes approximately 165 pest species has been achieved since about a 120 years ago.

'Entomopathogens' describes natural enemies used as biological control agents against problematic insects and some of the common entomopathogens used include fungi, viruses, protozoa, bacteria as well as nematodes (Lacey *et al*., 2001). The natural enemies, with their respective attacking and killing mechanisms and factors, disrupt the reproduction activity, diminish the growth and maturity of crop insect pests (Flint *et al*, 1998).

1.1.1.2. Importance of entomopathogens as biocontrol agents

'Entomopathogenic' has also become a term used in pathology and parasitology. It describes microorganisms which have the ability to infer diseases on insect hosts as well as cause death (Onstad *et al*., 2006). Entomopathogens in comparison to conventional chemical pesticides provide more benefits from an environmental health and risk perspective. These organisms have been considered safe, with no reports of ill effects on non-target organisms and humans (Lacey *et al*., 2001). Pesticide residues on food and soil contamination decrease with the application of entomopathogens. They also provide specificity against susceptible and suitable pests. Most biocontrol agents can be applied on the field using suitable conventional

equipment, they can also be mass produced on artificial media in various bioprocess configurations. They have the advantage of being formulated in various substrates which improves their shelf life, while their infectivity is not lost under long storage periods. Unlike chemical pesticides which offer short term control of pests, biocontrol agents provide long-term and permanent control of specific pests (Lacey *et al.*, 2001).

1.1.2. Evolution of nematodes and their association with insects

Nematodes are moulting animals with sizes ranging from 0.2mm to 6m (Blaxter and Denver, 2012). The general anatomical structure of the organisms consist of an excretory system, digestive system and a nervous system (Barbercheck, 2005). They are a diverse and abundant group of animals found in marine and terrestrial habitats across the globe (Blaxter and Koutsovoulos, 2014). It has previously been reported that most plants and animals are in association with at least one species of a parasitic nematode, the association has also been discovered in human populations and in overall, 25000 nematode species have been estimated to have a parasitic relationship with vertebrates and some of these species, however remain undescribed (Dobson *et al*., 2008). Because of the association that nematodes have with plants and animals, they are often regarded as important regulators of plant and animal within the ecosystem (Blaxter and Koutsovoulos, 2014).

Nematodes can be regarded as either predators or parasites (free-living) of which most species of nematodes are predators of vertebrates and invertebrates, plants and while some obtain their nutritional nourishment from microorganisms such as fungi and bacteria (Dillman *et al*., 2012). Nematode-arthropod associations have been grouped into 4 categories; 1) phoretic (nematodes are transported by an insect), 2) necromenic (nematodes obtain nourishment from insect cadavers), 3) facultative parasitism (nematodes obtain nourishment from living insects) and 4) entomopathogeny (nematode is an obligate parasite of insects and uses bacteria to kill insects) (Dillman *et al*., 2012). The evolution of nematode parasitism has been stipulated in reports to have occurred sequentially as described in the latter and according to figure 1.1 below. Moreover, the interaction of nematodes is not restricted to insects, they also interact with bacteria in one of the three ways described below; 1) parasitism (bacteria causes disease in nematodes), 2) trophism (nematodes feed on bacteria) and 3) mutualism (nematodes and bacteria cooperate in the benefit of both organisms) (Dillman *et al*., 2012).

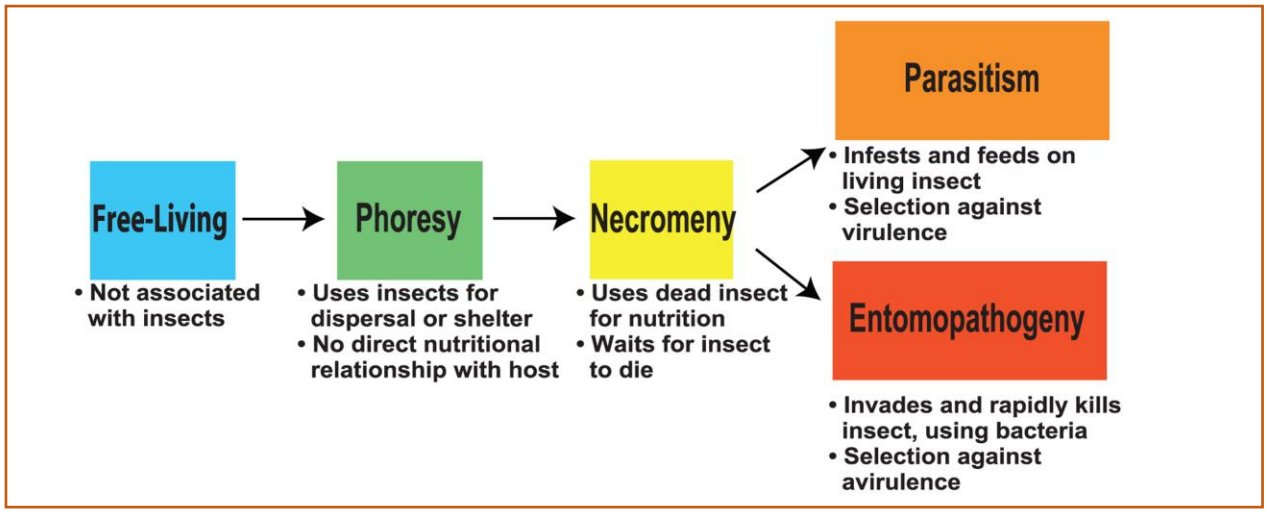

Figure 1.1: Evolution of nematode-insect associations. Dillman et al., 2012 doi:10.1371/journal.ppat.1002527.g001

1.1.3. Entomopathogenic nematodes

1.1.3.1. Taxonomy

Nematodes belong to the phylum Nematoda and families, Steinermatidae, Heterorhabditidae, Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae and Rhabtidae (Kaya *et al.*, 1997). The latter EPN families are often used for various research, however, nematodes in the families Steinermatidae and Heterorhabditidae have received the most interest in biocontrol development (Perez *et al.*, 2003). Since 2012, 78 EPN species have been isolated and identified, 15 belonging to Heterorhabditidae and 63 belong to Steinernematidae (Dillman *et al.*, 2012).

1.1.3.2. Host range and distribution

Entomopathogenic nematodes are soil dwelling insects which are obligate parasites of a broad range of soil borne pests. Insect pests controlled by entomopathogenic nematodes include army worms, filth flies, flea beetles, cat flea, crown borers, german cockroaches and many more (Smart, 1995). These EPNs mainly attack and target the larval stages and in some instances, adult stages of lepidopteran, coleopteran and diptera insect pests (Nickdel and Nicknam, 2015). *Heterorhabditis* and *Steinernema* EPNs are distributed worldwide (Hominick *et al.*, 1996; Hominick, 2002; Adams *et al.*, 2006). In South Africa, EPNs have been isolated in the provinces; Eastern Cape, Western Cape and Mpumalanga from citrus orchards with the aim of controlling a false codling moth (FCM), *Thaumatotibia leucotreta*, a key pest of citrus in South

Africa (Malan *et al*., 2011). *Steinernema sangi*, a new EPN species was also isolated from Walkerville in Gauteng (Serepa and Gray, 2013).

1.1.4. Parasitism by entomopathogenic nematodes and bacterial symbiont

In the bacteria-EPN symbiosis complex, bacterial symbionts, hosted in the gut of EPNs infective juvenile stage, which is the only stage that resides outside of its host and is non-feeding, contribute the EPNs infectivity and pathogenicity by killing the insect host through septicaemia when the IJs are introduced into the insect cavity, in addition, the bacteria also provides nutrients and creates a suitable environment for EPNs reproduction (Poinar and Thomas, 1966, 1967; Akhurst and Boemare, 1990; Boemare *et al.*, 1997). Entomopathogenic nematodes (EPNs) are responsible for transporting the bacterial symbionts from its host and introducing it to a new insect's hemocoel. In addition, EPNs protect the bacterial symbionts from host defence mechanisms which can be induced as a response to fight infection (Poinar and Thomas, 1966, 1967; Milstead, 1979; Dunphy and Thurston, 1990). Together, the dual bacterial-EPNs symbiotic partnership kills a susceptible insect host within 24-48 hours following infection (Hu *et al.*, 1999).

Furthermore, in the bacterial-EPNs symbiotic relationship, bacterial symbionts produce antimicrobial metabolites which attack potential opportunistic microorganisms in the insect cadaver, EPNs produce immune depressive factor, protease, which facilitates the release of bacteria in the insect hemocoel (Götz *et al*., 1981). One of the putative microbiocides that have been identified are derived from precursor metabolites such as indole, which is derived from tryptophan and 3, 5-dihydroxy-4-isoprylstillbere (ST) (Hu *et a*l., 1999). These two metabolites have not only been shown to be toxic to other microorganisms but have been reported to affect the egg hatching process of plant parasitic nematodes, fungal and bacterial feeding nematodes. The metabolites also paralysis movement of other nematodes and affect their viability against and their host (Hu *et al.,* 1999).

A high specificity exists between EPNs and their associated insect pathogenic bacteria. Generally, *Steinernema* spp. and *Heterorhabditis* spp. are exclusively associated with *Xenorhabdus* spp. and *Photorhabdus* spp. respectively (Boemare and Arkhurst, 2006). Their high specificity has been proven from conducted surveys and gnotobiologal experiments. Gnotobiologal studies revealed that *Photorhabdus* spp. do not support cultures of *Steinernema* spp. in-vitro (Arkurst, 1983). The association between EPN and bacteria are mutually

beneficial, however, *Photorhabdus* and *Xenorhabdus* spp. have the advantage to be cultured in-vitro in bacteriological media (Boemare and Arkhurst, 2006).

The entomopathogeny of *Steinernema* and *Heterorhabditis* first starts with 1) the carriage of the pathogenic bacteria by the infective juveniles (IJs) ; 2) active host seeking and penetration by the IJs; 3) release of the bacteria into the insect host haemolymph; 4) death of the insect host; 5) nematode reproduction and bacterial proliferation using cadaver tissues as nutrients; 6) re-association of bacterial symbionts with new generations of IJs; and 7) emergence of IJs from the nutrient depleted cadaver in search of new insect hosts (figure 1.2) (Chaston and Goodrich-Blair, 2010; Kaya and Gaugler, 1993).

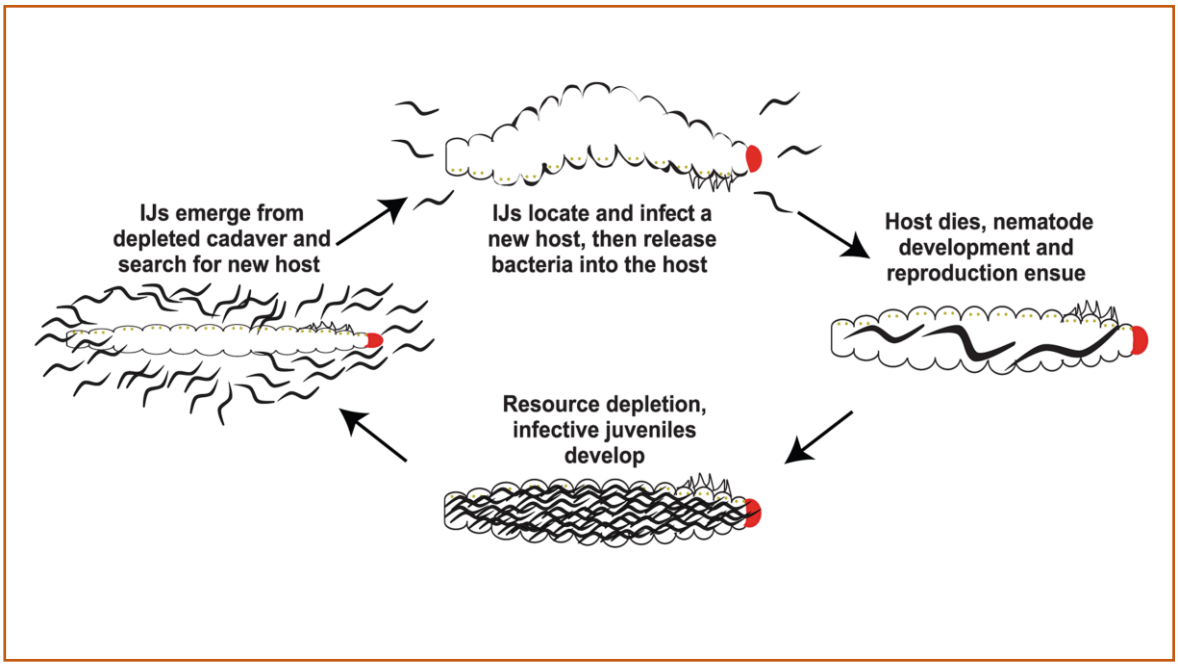

Figure 1.2: The entomopathogeny of *Steinernema spp*. and *Heterorhabditis spp.* Dillman et al., 2012 doi:10.1371/journal.ppat.1002527.g001

1.1.5. Biology of endosymbiotic bacteria; *Xenorhabdus and Photorhabdus*

Xenorhabdus and *Photorhabdus* bacteria are gram negative and are characterized by the presence of non-fermentative rods (Koppenhöfer, 2007). The bacteria belong to the family Enterobacteriacae, Proteobacteria class and γ-subclass (Boemare and Arkhurst, 2006). The bacteria has been reported to have been found in nature but only associated with an EPN partner (Lengyel *et al*., 2005; Tailliez *et al.,* 2006). Three species of *Photorhabdus* genera and approximately 23 species and 15 subspecies of *Xenorhabdus* genera have been identified (Arkhurst *et al*., 2004). In the years 2005 and 2006, 14 new species in the genera *Xenorhabdus* were identified (Lengyel *et al*., 2005; Tailliez *et al*., 2006). Currently, two bacterial symbionts

associated with EPNs native to South Africa (SA) have been described thus far. The respective bacterial symbionts are; *Photorhabdus luminescens* subsp and *Xenorhabdus khoisanae* (Ferreira *et al*., 2013). *Photorhabdus luminescens* subsp was isolated from a previously described South African (SA) native *Heterorhabditis noenieputensis* (Malan *et al.*, 2013), similarly *Xenorhabdus khoisanae* was also isolated from a SA native EPN species *Steinernema khoisanae*, described by Nguyen and colleagues in 2006.

1.1.6. Phase variation

Photorhabdus and Xenorhabdus genera of bacteria have the ability to produce two variants; form I and form II. Form I is regarded as the primary form and Form II a secondary form (Ferreira and Malan, 2014). Form I is associated with EPNs whereas form II only appears when subjected to artificial media (Akhurst 1980). Moreover these two phases are reported to have differences in morphological and physiological traits.

Form I (primary) cells are larger and motile because of the presence of a peritrichous flagella while form II (secondary) cells are small and non-motile (Givaudan *et al*., 1995). Form II colonies of the genera *Xenorhabdus* lack pigmentation on nutrient agar plates, in contrast, *Photorhabdus* form II colonies show pigmentation but this is dependent on the type of species that is cultured in-vitro on nutrient agar (Akhurst and Boemare, 1988; Boemare and Akhurst 1988; Boemare *et al*., 1997). Secondary cells have the ability to adsorb dyes supplemented in nutrient agar, and produce enzymes and antibiotics important in pathogenicity against insect pests, these characteristics are absent in form I cells. Moreover, form II cells, particularly associated with *Photorhabdus* spp. are bioluminescent (Akhurst 1980, 1982; Boemare and Akhurst 1988; Forst *et al*., 1997). *Xenorhabdus* form II cells produce OpnB, a protein which functions to protect the outer membrane of the bacteria during its stationary phase of growth (Vogyi *et al*., 2000).

Respiratory enzyme activity is elevated in form II cells of *Photorhabdus luminescens* and *Xenorhabdus nematophila*, because of this, form II cells are capable of taking up more nutrients as compared to form I cells which lack this ability (Smigielski *et al*., 1994) . Both cell forms (I and II) are pathogenic against the model insect host, *Galleria mellonella* (Akhurst 1980), however under in vitro and in-vivo culturing, form II cells of *Photorhabdus* fail to support the potential of *Heterorhabditis* EPNs to reproduce into other generations as well as to mature and develop (Gerritsen and Smits 1993, 1997). In contrast, *Xenorhabdus* n*ematophila* spp. support

the growth as well as production of an associated EPN, *Steinernema* spp. both under in-vivo (Sicard *et al*., 2005) and in-vitro culturing conditions (Ehlers *et al*., 1990; Volgyi *et al*., 2000).

1.1.7. Entomopathogenic nematodes in biological control

An increasing interest in EPNs for their potential as biocontrol agents is attributed to their ability to forage either passively or actively through soil for suitable insect hosts (Lewis *et al*., 1992; Gaugler, 1990), their broad host range (Smart, 1995) as well as the high virulence trait which is mainly due to the mutualistic relationship they have with bacterial symbionts they carry in their intestines (Poinar, 1979). Entomopathogenic nematodes (EPNs) can be mass produced without major challenges (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993). The biggest hindering factor for EPNs are extreme temperatures, ultraviolet (UV)-radiation and desiccation (Kaya *et al*., 1997). Most EPN species have been reported to be effective in temperatures above 16 ºC (Lacey *et al*., 1998), and all EPN species require a small film of water to search for a suitable insect host and thus infect and kill it (Wright *et al*., 2005).

Soil moisture is a vital factor which adversely affect EPNs activity and infectivity (Kaya, 1990) although EPN species have been reported to survive rapid and gradual decline in moisture (Womersley, 1987). An example of an EPN species which are tolerant to desiccation include *Steinernema carpocapsae* and some *Heterorhabditis* species (Salame and Glazer, 2015). Apart from environmental limitations, the use of exotic EPN species to control native pests have also been reported, one of the limitations are that exotic EPNs affect non-target organisms, thereby negatively affecting these organisms (Ehlers, 2005), bringing in exotic EPNs further displaces the future of identifying native species which can be used to control specific pests (Malan *et al*., 2011). Strict South African regulations (no person shall import an exotic insect or animal into the republic, no person is also allowed to import anything into the republic that is not determined by the minister by notice in the gazette) on importations is another factor limiting the use of exotic EPNs in the local environments (Amendment of Act 18 of 1989 under the agricultural pest act, no. 36 of 1974). In light of this, there is an opportunity to explore the field of nematology in the context of the African continent to isolate more EPN species, identify and further characterize with the possibility of increasing the spectrum of their biocontrol potential.

1.1.8. Induction of cryptobiotic anhydrobiosis in response to desiccation

Cryptobiotic anhydrobiosis is a reversible inactive state which allows EPNs to escape the seasonal effects of extreme soil dehydration which is typically associated with temperate grasslands in summer rainfall regions (Crowe *et al.,* 1992). Following the seasonal rehydration of grassland soils after a winter period of dehydration, EPNs are able to fully recover following prolonged exposure to critically low soil water potentials. Post recovery, by rehydration of soils, EPNs are able to proceed with their normal metabolic processes (Crowe *et al*., 1992). It has been previously reported that cryptobiotic anhydrobiosis is important for EPNs storage stability (Grewal, 2000) and an extended shelf life. This state improves and increases EPNs average survival under desiccated soil, up to approximately 30 years (Wharton, 1986; Womersley *et al*., 1998). During cryptobiotic anhydrobiosis, EPNs not only undergo temporary physiological changes, they also undergo behavioural changes such as coiling, forming large clumps and aggregating together, this assists EPNs to survive and tolerate desiccation (Sean *et al*., 2001). As a biochemical response to dessication, EPNs synthesise high levels of trehalose and glycerol (Sean *et al*., 2001), they induce expression sequence tags (EST), hydrophilic proteins as well as membrane proteins (Tyson *et al.,* 2006), which further assist EPNs with survival under dehydrated environments for prolonged periods (Crowe *et al*., 1992). Trehalose, a non-reducing sugar, functions in stabilizing EPN lipids and proteins during dessication (Womersley and Higa, 1998; Crowe and Crowe, 1984). Coiling reduces EPN surface area and protects their cuticle from exposure to dehydration, this in turn decreases drying of EPNs (Shannon *et al*., 2005).

When recovering from previously desiccated soil, EPNs have the ability to uncoil within a couple of minutes to hours at maximum (Treonis and Wall, 2005). The recovery of EPNs from dehydrated soil was conducted by Treonis and Wall, (2005), using density centrifugation technique with sucrose solution. Experiments conducted proved the theory of EPNs having the ability to uncoil when they are rehydrated and are able to resume with their metabolic activities (Sean *et al*., 2001). All stages of EPNs have been identified to coil during cryptobiotic anhydrobiosis and this represents a behavioural consistency of EPNs in their response to desiccation. The coiling process by EPNs has been observed in both *Steinernema* and *Heterorhabdtidis* genera (Treonis and Wall, 2005). Soil dryness and EPNs coiling are believed to consistently co-occur. Entomopathogenic nematodes (EPNs) readily coil without difficulties when soil humidity is relatively low. In most cases, sand, which dries up quickly compared to

slit and clay have shown to promote stronger and more immediate coiling responses in EPNs (Freckman *et al*., 1987; Demeure *et al.,* 1979; Townshend, 1984).

1.1.9. Formulation and application technology of entomopathogenic nematodes

Entomopathogenic nematodes (EPNs), which have a successful history in controlling insect pest damaging economically important crops, have introduced a new area of speciality; formulation and application technology (Ansari *et al*.,2006). Formulation of EPNs in various substrates has been considered a feasible method because of EPNs ability to be mass cultured and produced in-vivo (Baur *et al*., 1997). Formulation, is the combination of a formulation media in which the IJs are embedded in (Georgis and Kaya, 1998). Formulation media can range from inert carriers as well as organic products. The significance of formulation is the ability of the media to simulate natural processes under which EPNs are conditioned to survive for lengthy periods under extremely stressful environmental conditions, furthermore, formulation improves the infectivity and survival of EPNs (Lacey *et al*., 2010; Baur *et al*., 1997) as well activate defence mechanisms in plants against attacking insect pests (Dembilio *et al.,* 2009).

One of the commercialized EPN formulation which is in the market is Biorend R® Palmeras. This product consists of *S. carpocapsae* EPN and an adjuvant made up of chitosan (Lacey *et al*., 2009). Chitosan, a biodegradable product, in this particular formulation functions to activate defence mechanisms in the plant, foster root development as well as increase lignification in the plant (Hadwiger *et al*., 1981). Entomopathogenic nematodes (EPNs) are reported to have a shelf life of 6 months at least, and it is therefore imperative for researchers to consider properties of formulation media which will increase the shelf life of EPNs to more than 6 months and, furthermore retain its pathogenicity and viability (Georgis, 1990) under transportation and as well under conditions of unstable climate change (Ansari *et al.,* 2006).

Recent investigations have evaluated the effectiveness of EPNs against insect hosts when formulated as liquid suspensions over cadaver formulation with the addition of adjuvants and it was found that formulation, which includes the addition of anti-desiccant agents improves EPNs infectivity and survival, mainly because the adjuvant agents provide adequate moisture for EPNs to survive until they can infect their host larvae during application (Lacey *et al.,* 2010). Desiccation of EPNs, which reduces metabolism and reserves energy has proved to be

one of the most efficient type of formulation which extends shelf-life of EPNs (Georgis, 1990; Lacey *et al*., 2010). Understanding the physiology of EPNs and its tolerance to desiccation enables researchers to invent formulations which will introduce partial anhydrobiosis in EPNs, promoting survival under stressful environments (Grewal, 2000).

Research Motivation

- ✓ Understanding the behaviour, physiology of entomopathogenic nematodes and the survival mechanisms that they utilize in response to desiccation will aid in understanding and establishing suitable EPN formulation media. Suitable formulations which will aid and stimulate maximum survival and infectivity of entomopathogenic nematodes during application and are attributed with long shelf life during transportation and storage are important in biological control
- ✓ Entomopathogenic nematodes which are tolerant to environmental stresses such as desiccation are suitable for effective control of soil dwelling insects found in different geographical locations.
- ✓ Isolation and application of native entomopathogenic nematode strains which have the ability to tolerate and have no challenges to adapt to local environmental changes are more preferable because they show high efficacy to control pests in the local environment as well as targeting specific insect pests.
- ✓ Identification of entomopathogenic nematodes and their bacterial symbionts by establishing taxonomy and phylogenetic affinities using PCR and sequencing of the ribosomal DNA has proven to be an efficient method of identification.

Aims

- Isolate, identify and investigate desiccation tolerance of entomopathogenic nematodes native to South Africa
- Investigate the influence of formulation media on IJ infectivity following recovery through rehydration from the desiccated state.
- Isolate and identify an entomopathogenic nematode associated insect pathogenic bacterial symbiont

Objectives

- Isolation and molecular characterization of a South African entomopathogenic nematode using 18S rDNA molecular marker
- Isolation and molecular characterization of an associated bacterial symbiont using 16S rDNA molecular markers
- Desiccation tolerance of an entomopathogenic nematode
- Evaluation of infectivity of a formulated entomopathogenic nematode

Research experimental design

Chapter 2

- Addresses the collection of soil samples, recovery and isolation of the EPNs and identification using an 18S molecular marker

Chapter 3

- Addresses the isolation of the endosymbiotic bacteria on NBTA and MacConkey agar media and molecular identification using the 16S rDNA molecular marker

Chapter 4

- Investigates the infectivity following the application of aqueous suspensions of IJs to inert carriers and Infectivity of IJs in storage media under selected storage conditions

Chapter 5

- Addresses testing the effect of water loss over time on EPN viability in different substrates and comparing the rate of infective juveniles emergence from infected cadavers exposed to desiccation.

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2. CHAPTER 2: ISOLATION AND MOLECULAR CHARACTERIZATION OF ENTOMOPATHOGENIC NEMATODES

2.1. Introduction

Entomopathogenic nematode (EPN) parasites exposure as biocontrol agents of insects started in the 1930s but were known since the 17th century (Smart, 1995). The first EPN that was described by Steiner was *Steinernema* which was found to be lethal and infected Japanese beetle (Smart, 1995). Entomopathogenic nematodes (EPNs) are non-segmented, microscopic "round worms", composed of an excretory, digestive, reproductive and nervous system (Kaya and Stock, 1997). They inhabit diverse habitats and are found in soil and aquatic habitats in the subtropical, tropical and temperate regions (Adams *et al*., 2006).

Steinernematidae and Heterorhabditidae families of nematodes are often studied due to their success as potential biocontrol agents. Both EPN families of EPNs are incorporated in insect pest management programs (Smart, 1995). Isolation and description of entomopathogenic nematodes has been accomplished within South Africa in provinces; Gauteng, KwaZulu-Natal, Western Cape and Eastern Cape (Malan *et al*., 2006, 2011; Hatting *et al*., 2009). It is of paramount significance to isolate native strains which have the ability to tolerate and have no challenges to adapt to local environmental changes (Torrini *et al*., 2015), this is because native species show high efficacy to control pests in the local environment as well as targeting specific insect pests (Grewal *et al*., 2001).

The success of *Heterorhabditis* and *Steinernema* as biological control agents is attributed to their distinctive relationship with an associated bacterial endosymbiont carried by the infective juvenile stage of EPNs (Kaya and Stock, 1997). Entomopathogenic nematodes (EPNs) of *Steinernema* spp. carry a bacteria of *Xenorhabdus* spp. Similarly, EPNs of *Heterorhabdtidis* are associated with a bacteria of *Photorhabdus* spp., this partnership enables EPNs to rapidly kill a susceptible insect within 24-48 hours post infection (Stefanovska and Lewis, 2012). Recently, a new described EPN species *Oscheius* spp. has been identified and described to be in association with pathogenic bacteria, just like *Steinernematid* and *Heterorhabtid* species, to parasitize insect hosts (Dillman *et al*., 2012).

2.1.1. Preliminary identification of EPNs

In preliminary identification of EPNs, the colour change observed on the larvae is used as a key in identifying specific EPN genera (Torrini *et al*., 2015), this is often conducted using an insect baiting technique. A specific colour change observed on the larvae has been associated with the genera responsible for infection, for example, brown/tan colour on the larvae is indicative of *Steinernema* infection and a maroon/black is indicative of a *Heterorhabditis* infection (Woodring and Kaya, 1988).

2.1.2. Molecular identification of EPNs

Molecular based methods have provided accurate and rapid identification of EPN species (Nguyen and Hunt, 2007). Molecular techniques in the identification of EPN species and strains have proved to be of great use in taxonomy of EPNs (Hafner and Nadler, 1990). Key molecular methods, which have been employed in the identification of EPNs are PCR amplification, sequencing of the ITS region and the 18S rDNA (Torrini *et al*., 2015).

Molecular markers; 18S and 28S are used based on the fact that they flank a highly conserved region; 5,8S rDNA region which holds low levels of variation (Adams *et al.*, 1998; Darissa and Iraki, 2014). Previous and more recent studies have identified new EPN species as well as resolving their variability between species and strains through sequencing of ITS, 18S, 28S rDNA regions. Based on the flanking region contained in these regions, molecular primers are also generated based on the conserved trait of this region, which allows molecular taxonomic studies to be conducted (Reid *et al*, 1997). The latter regions are also useful in phylogenetic analysis not only limited to EPNs but many other eukaryotic organisms (Tubeville *et al*., 1991).

The current study aims to isolate and characterise entomopathogenic nematodes using the ITS and 18S rDNA molecular markers

2.2. Materials and Methods

Insect baiting technique is used to recover infective stages of EPNs from soil using a susceptible insect host as bait (Bedding and Akhurst, 1975) and White traps to recover IJs from infected larvae (White, 1927). The most common and available insect host used in biological control studies is the 4th instar stage *Galleria mellonella* larvae. It is commonly used because it is easy to rear and easily accessible and produces high yield of EPNs in vivo (Ehlers and

Shapiro-Ilan, 2005). The greater wax moth larvae have been used in insect pathogenic studies, including nematode studies (Andrejko and Mizerska-Dudka, 2011).

2.2.1. In-vivo rearing of *Galleria mellonella* larvae

Kingdom: Animalia │Phylum: Arthropoda │Class: Insecta │Order: Lepidoptera │Family: Pyralidae │Subfamily: Galleriinae │Genus: *Galleria* │Species: *G. mellonella*

Common name: Greater wax moth

Figure 2.1: Healthy uninfected *Galleria mellonella* adult larvae

Galleria mellonella was reared in the laboratory by placing adults (male and female) moths in 3L Consol® glass bottles (11cm diameter and 23 cm height) with prepared artificial media adapted from Woodring and Kaya (1988). Wax paper which functions in facilitating the recovery of eggs (oviposition) laid by adult female moths was inserted into the glass bottle. Larval growth and development was maintained by continuous supply of *Galleria mellonella* nutritional media (Appendix I). Larvae were collected from wax paper.

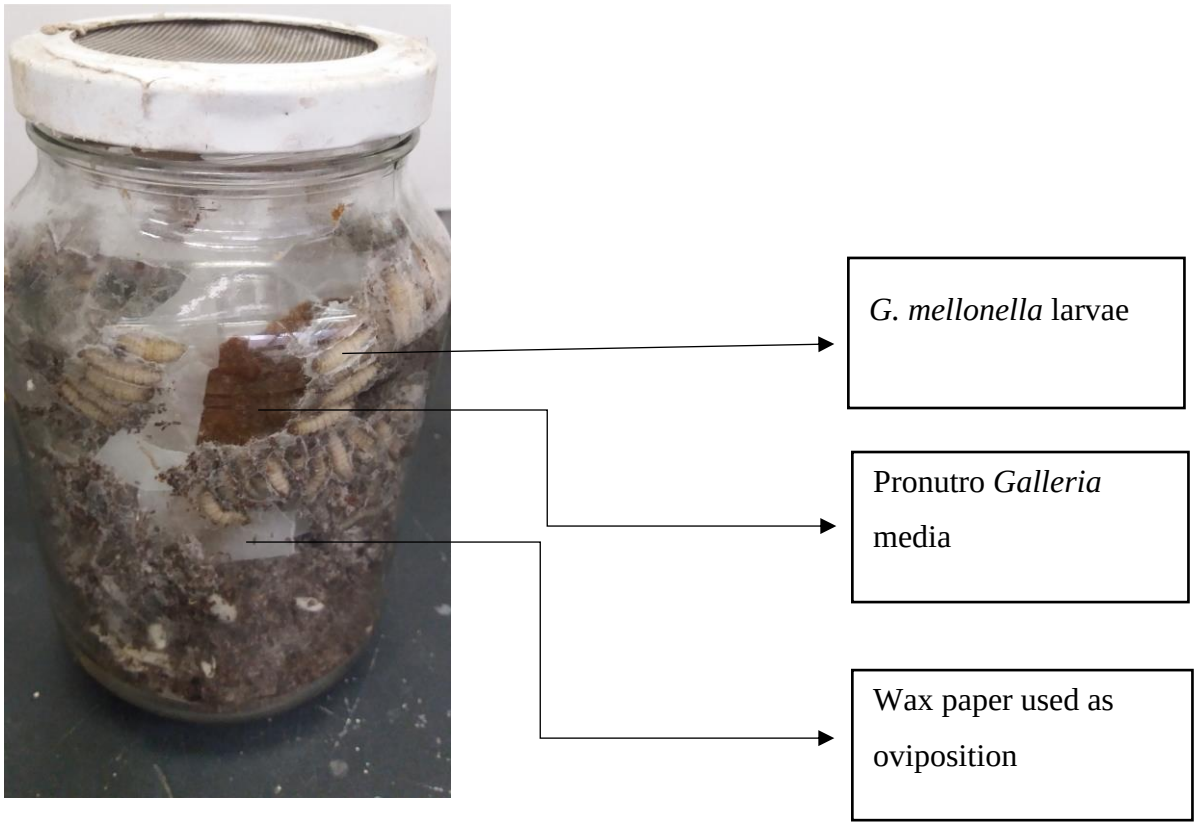

Figure 2.2: In-vivo rearing of *Galleria mellonella* larvae in 3L Consol® glass bottle

2.2.2. Isolation of entomopathogenic nematodes from infected larvae

2.2.2.1. Soil Sampling

An ethanol sprayed spade was used to excavate soil from the ground (10 -20cm deep). The soil samples were collected from Brits area, located in the North West province, South Africa. GPS coordinates (25°36'28.7"S 27°47'45.1"E). A total of 20 soil samples were collected from 8 locations in the same area and transferred into 2L plastic containers which were transported to the laboratory and stored at 22-28ºC, which is the optimum temperature for EPNs (Thanwisai *et al*., 2012). The location that the soil samples were collected from were labelled on the 2L plastic containers and locations in which EPNs had been recovered from was also recorded.

A

B

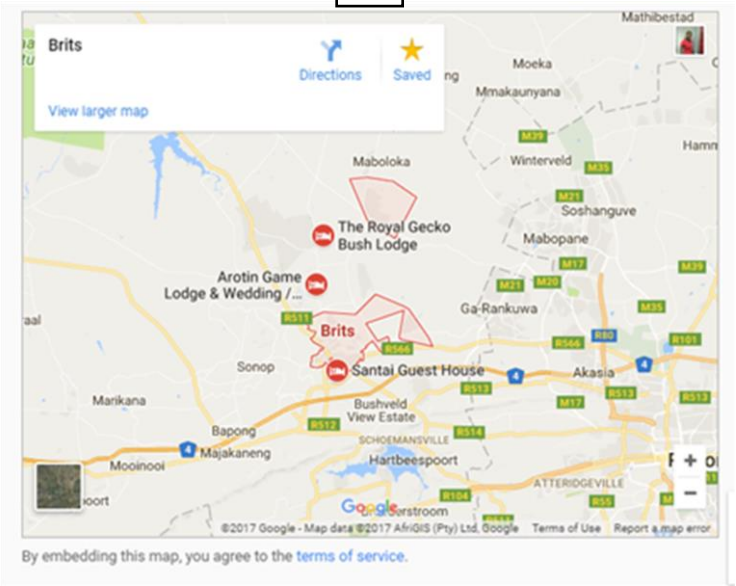

Figure 2.3: A) Soil samples from the collection site, B) Geographic location of Brits where soil samples were collected from (B), source: maps.google.co.za/maps

2.2.2.2. Insect Baiting Technique- Entomopathogenic nematode recovery

Sterile water was added to soil to bring moisture level to 10%. This degree of water potential facilitates EPN movement between the soil particles in searching for insect host to kill and infect (Alekseev *et al*., 2006). Ten *Galleria mellonella* instar larvae were placed on top of the soil sample and 10 embedded in soil. Dead larvae, showing signs of EPN infection were collected from the soil samples and replaced with live larvae to extract more EPNs from soil. Signs of infection were reflected by a change in colour on the larvae, usually red/maroon/black for *Heterorhabditids* and ocher/brown/black for *Steinernematids* (Kaya and Stock, 1997; Woodring and Kaya, 1988). Dead larvae with symptoms of EPN infection were collected from the samples and studied for primary identification.

Figure 2.4: Insect baiting technique used for the recovery of EPNs from soil using *Galleria mellonella larvae*

2.2.2.3. Infective juveniles' recovery from EPN infected host: White trap Method

Infected larvae were sprayed with 70% ethanol and placed on a modified White trap to collect IJs (Kaya and Stock, 1997). The White trap method consists of a 90mm Petri dish, 60mm disk, and Whatman No 1 filter paper (White, 1927). A small disc plate was inverted and placed in a large Petri dish. Autoclaved distilled water was added into the latter dish up to 2mm. Moist Whatman filter paper was placed on an inverted small disc plate to facilitate IJ emergence from the larvae. Dead larvae suspected of EPN infection were place onto the Whatman filter paper. The lid of the large Petri dish was replaced and water levels in the Petri dish monitored daily. The emerging IJs were collected from the surrounding water in the large Petri dish.

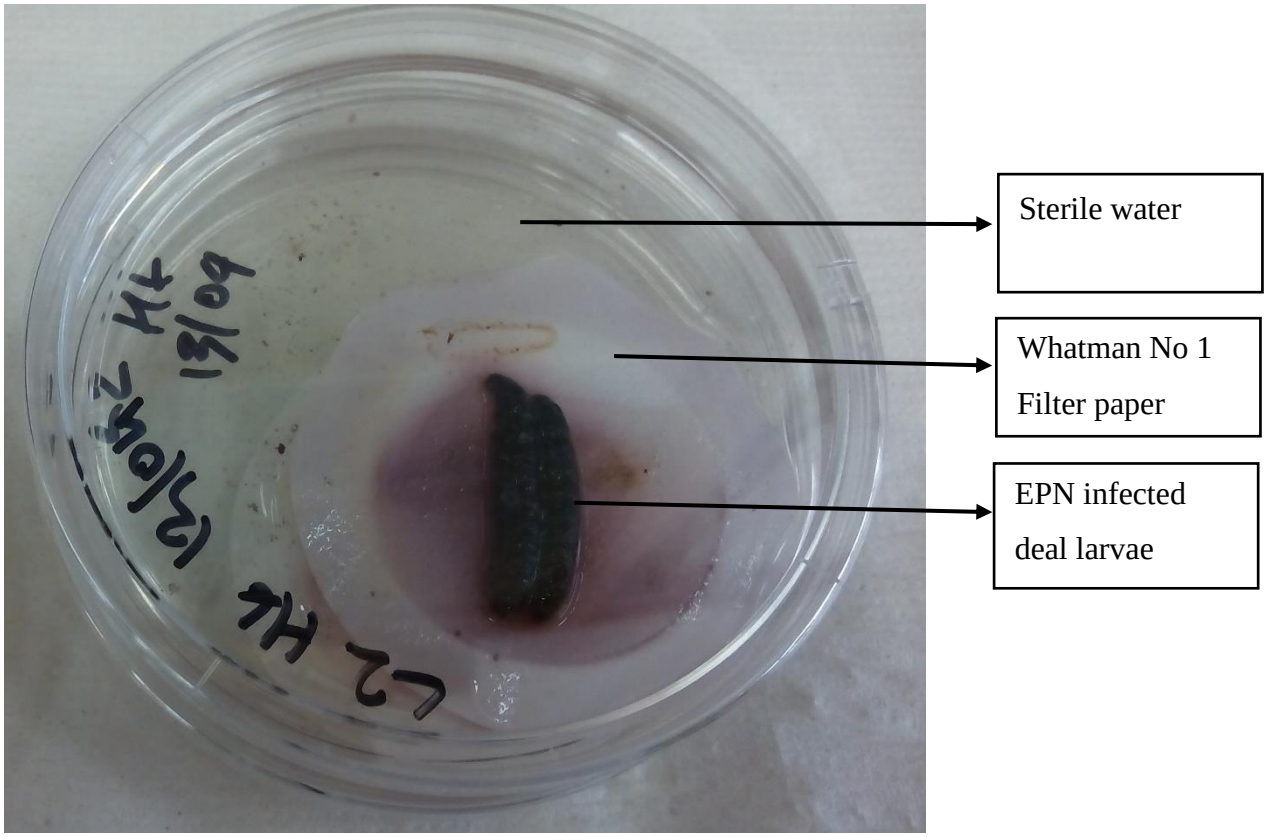

Figure 2.5: White trap method used to recover infective juveniles from EPN infected larvae

2.2.3. Molecular identification of entomopathogenic nematodes

2.2.3.1. Genomic DNA extraction

Genomic DNA of Infective juveniles (IJs) from White traps was extracted using Gentra system protocol (Appendix II) according to the manufacturer's instructions.

2.2.3.2. PCR amplification of the ITS and 18S rDNA

Polymerase chain reaction (PCR) was conducted to amplify the 18S and 28S regions of the isolated EPN species using the oligonucleotide universal forward (TW81: 5'-GTTTCCGTAGGTGAACCTGC-3', Tm: 62ºC and reverse primer (AB28: 5'ATATGCTTAAGTTCAGCGGGT-3', Tm: 60 ºC (Joyce *et al*., 1994). The primers with the latter respective sequences were synthesized at Inqaba Biotechnological Company.

The PCR reaction was prepared by the addition of a master mix reagent, EPN genomic DNA, reverse and forward primers and nuclease free water. The EPN genomic DNA was not added in the control tube (Appendix VI)

2.2.3.3. Sequencing of the ITS region and 18S rDNA

The PCR products of the 18S rDNA amplification were purified and sequenced using Sanger sequencing technology at Inqaba Biotechnical Industries. The rDNA sequences from Inqaba were edited and base calls verified with Bioedit version 7.0.4 (Hall, 1999).

2.2.3.4. Identification of the EPN species: NCBI BLASTn

Final edited EPN sequences were aligned with EPN sequences deposited in NCBI GenBank. The BLAST-based tool, 'highly similar sequences' was used to calculate the percentage similarity of the query sequence (undescribed isolate) to the identified EPN species deposited in the GenBank.

2.2.3.5. Multiple Sequence Alignment of sequences

EPN sequences with a percentage identity of 95% to the query sequences were selected and loaded onto molecular evolutionary genetics analysis, version 6.0 (MEGA 6.0) program. The sequences of identified EPN species as well as the undescribed isolated EPN specie were aligned with Clustal W. *Caenorhabditis elegans* was used as an outgroup taxa. Furthermore, the evolutionary divergence between the species were analysed using pairwise distance matrix in MEGA 6.0 (Tamura *et al*., 2013).

2.2.3.6. Phylogenetic tree analysis

The evolutionary relationships between the aligned isolates were inferred using Kimura-2 parameter (Kimura, 1980) with bootstrap replications of 1000 base substitutions. The phylogenetic trees were based on the Maximum Likelihood method.

2.3. Results

2.3.1. Symptoms of EPN infection

The first route of identification was by monitoring and observing the colour change caused by the EPN genera infecting the host larvae. The colour change observed from the larvae baited on L2 soil samples were red to maroon in colour throughout the sampling process (figure 2.6A),

same symptoms were observed in re-infection experiments. Infective juveniles and adult nematodes emerging from the infected larvae were observed as a confirmation of infectivity caused by a *Heterorhabditis* species (figure 2.6B)

A

B

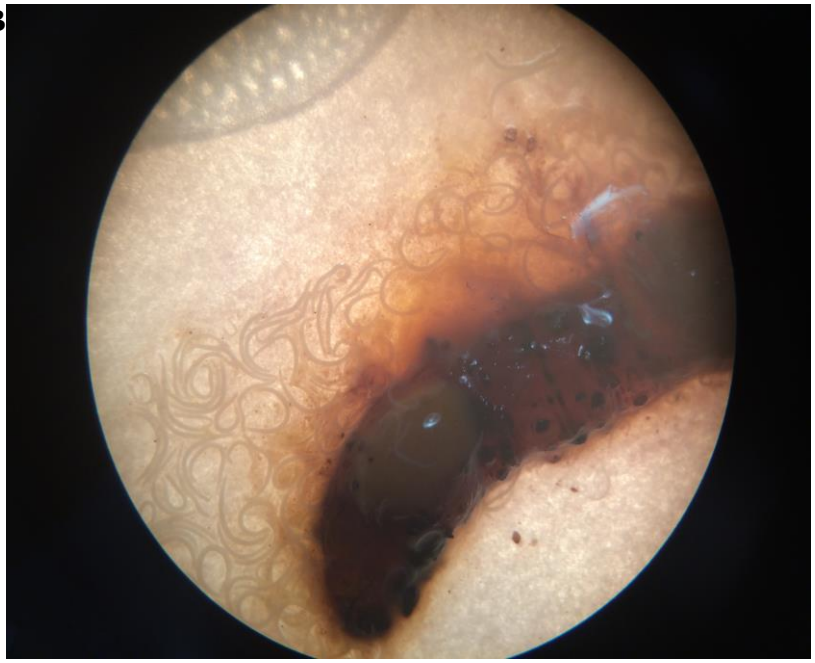

Figure 2.6: A) Colour change (red to maroon) of the larvae indicative of *Heterorhabditis* spp. infection, B) Adults and Infective juveniles emerging from the infected larvae after 24-48 hours, under a dissecting microscope.

Eight soil samples from different locations were positive for the presence of EPNs, in particular EPNs isolated from L2 were persistent throughout sampling. Suspected *Heterorhabditis* spp. (table 2.1) based on the development of a maroon colour on the larvae were recovered from soil samples collected from one collection site.

Table 2.1: Soil samples collected from different locations indicating the occurrence of EPNs

Location	Number of samples	Negative/Positive for EPN	Isolated EPN species
L1	2	Positive	*Heterorhabditis sp.*
L2	4	Negative	-
L3	2	Negative	-
L4	2	Negative	-
L5	2	Negative	-
L6	2	Positive	Unidentified. Full recovery was affected by fungal contamination
L7	2	Negative	-
L8	4	Positive	Unidentified. Full recovery was affected by fungal contamination

2.3.2. Sequencing of the ITS and 18S rDNA of the unknown isolates

Sequence length of isolate B1 was 767bp (Appendix VII) and had a high affinity to *Hetorhabditis* species in NCBI BLASTn search results. A 99% sequence affinity was observed between *Heterorhabditis* isolate B1 and *Heterorhabditis bacteriophora_ isolate UP2A2* with accession number MF033536.1. Nucleotide matches were from position 1-60 in the query sequence to position 46-105 in the subject query and a 1% chance mutation resulted between nucleotide AG

2.3.3. Evolutionary divergence

Evolutionary divergence on the variation of the ITS regions between identified *Heterorhabditis* species and undescribed *Heterorhabditis* isolate B1 were analysed using pairwise distance matrix in MEGA 6. The lowest evolutionary divergence distance was observed between undescribed *Heterorhabditis* isolate B1 and *Heterorhabditis bacteriophora*_ isolate UP2A2, *Heterorhabditis bacteriophora*, and *Heterorhabditis bacteriophora i*solate DO 6e (0.002; 0.002;0.002) suggesting close relation between the three *Heterorhabditis bacteriophora* species.

2.3.4. Phylogenetic analysis

Phylogenetic relationships analysis was conducted by MEGA6 and Maximum Likelihood method was used to identify the isolates to species level.

Heterorhabditis isolate B1 was clustered in the same clade as *Heterorhabditis bacteriophora*_isolate UP2A2, *Heterorhabditis bacteriophora*, *Heterorhabditis bacteriophora* isolate DO 6e. A 70% support amongst the latter species indicated a strong evidence that the sequences of the species clustered together to the exclusion of any other sequence. Moreover, the undescribed *Heterorhabditis* isolate B1 was more closely clustered with *Heterorhabditis bacteriophora*_ isolate UP2A2 in figure 2.7.

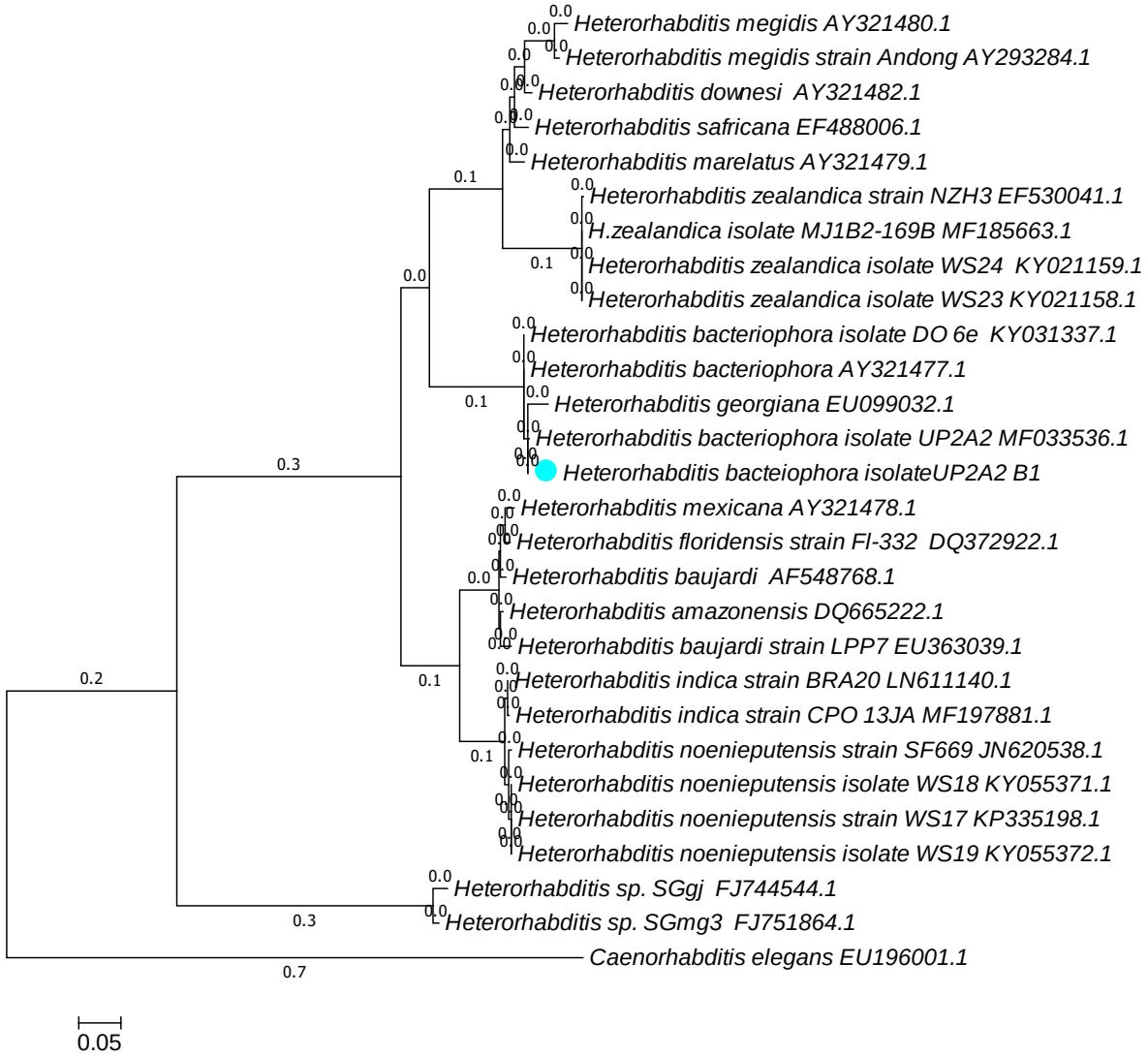

Figure 2.7: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history of Heterorhabditis species was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-3408.9288) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 456 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

2.4. Discussion

The current study isolated and characterized an EPN specie from Brits in the North West Province, South Africa. Entomopathogenic nematodes (EPNs) are widely distributed organisms occupying different soil habitats (Hatting *et al*., 2009). Based on the findings reported by the different researchers, more *Steinernema* species have been recovered more than *Heterorhabditis* spp. (Hominick, 2002), this was proven by Hatting *et al.,* (2009) who recovered 43% of *Heterorhabditis* spp. and 55.7% of *Steinernema* spp. Out of a total of 20 soil samples collected, eight soil samples (40%) were positive for the presence of EPNs, however consistent EPN infection and identification was established in 2 soil samples (10%) from L2 location site. The low percentage from our results was also observed from a study conducted by Hatting *et al.,* (2009) which reported 5.2% of soil samples collected in South Africa to be positive for the presence of EPNs. However, in a study conducted by Rio and Cameron, (2000), EPNs were recovered from 69% of the soil samples collected in Pennsylvania. The differences are indicative of the fact that more provinces and different locations with distinct vegetation need to be explored for isolation and identification of EPNs. *Heterorhabditis* genera were isolated from the EPN positive samples and our results are supported by Kaya, (1990) and Rio and Cameron, (2000) where in both their studies, Heterorhabdtids were isolated from one geographical location.

The manifestation of a red to maroon colour on the infected larvae confirms a *Heterorhabditis* infection in figure 2.6 (Woodring and Kaya, 1988) and the colour change has been reported to be caused by the associated bacterial symbiont which confers the EPN virulent against insects pests (Woodring and Kaya, 1988).Insect baiting technique and preliminary identification of EPNs based on symptoms allowed for the successful identification of an EPN of *Heterorhabditis* genera in the current study.

Molecular identification of EPNs is crucial in studying and understanding their behaviour and habitat preference and geographical location (Alper Susurluk and Toprak, 2006). An entomopathogenic nematode species isolated in the study was described by analysing the ITS region and of 18S rDNA sequences.

In NCBI blast search sequence results between *Heterorhabditis* isolate B1 and *Heterorhabditis bacteriophora*_isolate UP2A2 was 99%. The high sequence similarities were further validated using pairwise distance matrix to assess the evolutionary divergence between the species and according to the pairwise distance matrix, *Heterorhabditis* isolate B1

and *Heterorhabditis bacteriophora* _ isolate UPA2A2 were in close proximity with regards to the evolutionary sequence information, the observed distance (0.002) was the lowest. These results were further confirmed to analyse their phylogenetic relationships by construction of maximum likelihood evolutionary trees, phylogenetic tree construction were conducted because this is a reliable method to assess described and undescribed species, most conclusions are drawn from phylogenetic relationships (Alper Susurluk and Toprak,2006)

*Heterorhabditis bacteriophora*_ isolate UP2A2 clustered in close proximity in the same clade with *Heterorhabditis* isolate B1 with bootstrap percentage support of 70%. The branch length of the above mentioned species was short, indicating that evolutionary change between the species was not abundant.

BLAST search results, pairwise distance matrix and phylogenetic relationship between the undescribed *Heterorhabditis* species and the identified species confirm that *Heterorhabditis* isolate B1 could possibly be a *Heterorhabditis bacteriophora* sp.

The identified EPN isolate in the study, *Heterorhabditis* isolate B1 was isolated from Brits area located in the North West province, South Africa. *Heterorhabditis bacteriophora*_ isolate UP2A2 was isolated from grapevine soil and identified by Malan, (2017) from South Africa, Carpe Diem, Upington. The presence of this EPN species from different habitats and location leads us to deduce and expand on the biodiversity and geographic distribution of EPNs. It's worth noting that *H.bacteriophora* isolates were collected in 4 provinces with distinctness in biogeography and habitat diversity (Hatting *et al*., 2009). Spaull, (1991) recovered *H. bacteriophora* for the first time in Kwazulu-Natal, and Malan *et al*., (2006) recovered it from the Western Cape and Hatting *et al*., (2009) recovered it from Free State and Mpumalanga.

The isolated EPN species was confirmed as Heterorhabditis *bacteriophora* isolate B1 based on its 18S rDNA. More molecular markers are recommended for full validation of identification and further characterization.

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3. CHAPTER 3: ISOLATION AND MOLECULAR CHARACTERIZATION OF AN EPN ASSOCIATED BACTERIAL SYMBIONT

3.1. Introduction

Bacterial species of *Photorhabdus* and *Xenorhabdus* are members of Enterobacteriacae family, the bacteria are gram, negative and non-fermentative rod shaped cells (Koppenhöef, 2006). Entomopathogenic nematodes (EPNs) of *Heterorhabditis* and *Steinernema* are mutually associated with bacteria of the genera *Photorhabdus* (Boemare and Mourant, 1993) and *Xenorhabdus* (Thomas and Poinar, 1979), respectively. The EPN, which hosts an associated bacterial symbiont acts as a vector and transports it into the insect cavity, (Ferreira and Malan, 2014), in turn the bacterial symbiont creates a suitable atmosphere by secretion of endo and exotoxins inside of the insect cadaver to allow the EPN to develop and reproduce into thousands of progeny. Toxins secreted by the endosymbiont main function are to overcome the host immune system (Boemare *et al.,* 1997). Together, virulent factors produced by EPNs and bacterial symbionts induce septicaemia on the insect host usually between 1-2 days post exposure (Forst and Clarke 2002). Free living forms of the bacterial symbionts have not been isolated in either soil or water emphasizing the fact that both EPNs and bacterial symbionts are dependent on each other for survival, persistence and infectivity (Forst *et al,* 1997).

3.1.1. Taxonomy of *Xenorhabdus* and *Photorhabdus*

Xenorhabdus and *Photorhabdus* bacteria are non sporulating bacteria (Koppenhöf, 2006) and are negative for oxidase reaction. They are furthermore regarded as chemoorganotrophic heterotrophs (Grimont *et al*., 1984). These bacterial species are characterized by rod shaped cells. They belong to group 5, subgroup 1 of Enterobacteriaceae (Holt *et al*., 1994).

Photorhabdus and *Xenorhabdus* spp. produce phase variants; phase I and phase I. Phase I form is only specifically associated with EPNs and phase II arises when in artificial culture (Arkhurst, 1980). Determination of the phase variants can be conducted by streaking bacteria onto MacConkey and nutrient bromothymol triphenyltetrazolium agar (NBTA) (Boemare and Arkhurst, 1988). The adsorption of dye; indole in MacConkey agar and Bromothymol blue and triphenyltetrazolium in NBTA is what identifies bacteria into a specific species (Fischer-Le Saux *et al*., 1999; Boemare and Arkhurst, 1988).

Molecular techniques such as DNA: DNA hybridization, Random amplification of polymorphic DNA (RAPD), Enterobacterial repetitive intergenic consensus sequence (ERIC) and 16S rDNA are used in the identification and description of the bacterial symbionts further down to species and strain level (Tailliez *et al*., 2006; Boemare and Arkhurst, 2006: Fischer-Le Saux *et al*., 1999).

The aim of the current study was to isolate a bacterial symbiont associated with an isolated and identified *Heterorhabditis bacteriophora* isolate B1 using NBTA and MacConkey agar medium and to furthermore characterize the symbiont using 16S rDNA.

3.2. Materials and Methods

3.2.1. Symbiotic bacterium isolation from hemolymph of infected larvae

The bacterial symbiont was isolated from EPN infected larvae using haemolymph method Adapted from Arkhurst, (1980)

Galleria mellonella 4th instar larvae used to isolate symbiotic bacteria were recovered from Koch postulates 48hrs post-infection. The recovered cadavers from Koch postulates were dipped into 95% ethanol and rinsed 3 times in sterile distilled water. Cadavers were cut open with a sterile sculpt and needle with careful attention to not rapture the midgut of the larvae. The insect haemolymph was collected using a needle connected to a syringe. The haemolymph was transferred into sterile Eppendorf tubes with ample deionised water. The suspension was mixed with a pippete to achieve a homogeneous haemolymph suspension. A drop of haemolymph was streaked onto MacConkey and NBTA agar plates to obtain pure bacterial colonies. Plates were sealed with parafilm and incubated in the dark at 25ºC for 1-3 days and development of phase variants (I and II) were monitored daily. The morphology of phase I variants from NBTA and MacConkey were examined and scored according to the scoring method used by Boemare and Akhurst, (1988).

3.2.2. DNA isolation

Pure colonies of the bacteria were picked with a sterile loop and suspended in sterile deionised water. The genomic bacterial DNA was extracted using the protocol from ZR Fungal/Bacterial DNA Kit No. D6005 (Appendix IV). All procedures were followed as stipulated by the manufacturer's instructions.

3.2.3. Sequencing of the 16S rDNA

Polymerase chain reaction (PCR) was conducted to amplify the 16S rDNA of the isolated EPN species using the oligonucleotide universal primers (EUB968: 5'-ACGGGCGGTGTGTRC-3', Tm: 62ºC) and reverse primer (UNIV1382: 5'-AACGCGAAGAACCTTAC-3', Tm: 66ºC) (Brunel *et al.*, 1997).

The PCR products of the 16S rDNA amplification were purified and sequenced using Sanger sequencing technology at Inqaba Biotechnical Industries. The rDNA sequences from Inqaba were edited and base calls verified with Bioedit version 7.0.4 (Hall, 1999).

3.2.4. Evolutionary divergence and phylogenetic relationships

Final edited sequences obtained were aligned with bacterial sequences deposited in NCBI GenBank. The BLAST-based tool, 'highly similar sequences' were used to calculate the percentage similarity of the query sequence (undescribed isolate) to the identified bacterial species deposited in the GenBank. Bacterial sequences with percentage identity of 95% to the query sequences were selected and loaded onto molecular evolutionary genetics analysis, version 6.0 (MEGA 6.0) program. The sequences of identified bacterial species as well as the undescribed bacterial symbiont species were aligned with Clustal W. *Escherichia coli* was used as an outgroup taxa. Furthermore, the evolutionary divergence between the species were analysed using pairwise distance matrix in mega 6.0 (Tamura *et a*l., 2013).The evolutionary relationships between the aligned isolates were inferred using Kimura-2 parameter (Kimura, 1980) based on the Maximum Likelihood method with bootstrap replications of 1000 base substitutions.

3.3. Results

3.3.1. Phenotypic characterization

The isolated bacterial symbiont colonies were red in colour, small and circular in shape in MacConkey agar plates (table 3.1, figure 3.1B), in the NBTA agar medium plate, green colonies with red centres were observed, these colonies were circular in morphology and small to medium in sizes (table 3.1, figure 3.1A).

Table 3.1: Characterization of *Photorhabdus spp*. phase variants adapted from Arkhurst (1980, 1986) and the isolated bacterial symbiont in the study

Characteristics	**Phase I**	**Phase II**	**Isolated symbiont from *H.bacteriophora* isolate B1**
Colony morphology in MacConkey	Granular, convex and circular with irregular margins Cells are small to middle in size	Flat, translucent with irregular margins The diameter of the cells is big	Small sized circular cells
Colony morphology in NBTA	Granular, convex and circular with irregular margins Cells are small to middle in size	Flat, translucent with irregular margins The diameter of the cells is big	Small sized and some medium sized cells Cells circular with irregular margins
Pigmentation in MacConkey	Red-brown, red	Off-white, yellow	Red
Pigmentation in NBTA	greenish with reddish-brown centres Colonies surrounded by clear zones in the agar	Colonies shaded from red to rust	Green with red centres Clear zones in the agar plate
Positive(+)/Negative (-)for Adsorption of dyes: Indole(MacConkey),	+ for indole + for Bromothymol blue	-Indole -For Bromothymol blue	+ for indole +Bromothymol blue

Bromothymol blue(NBTA)			

A

B

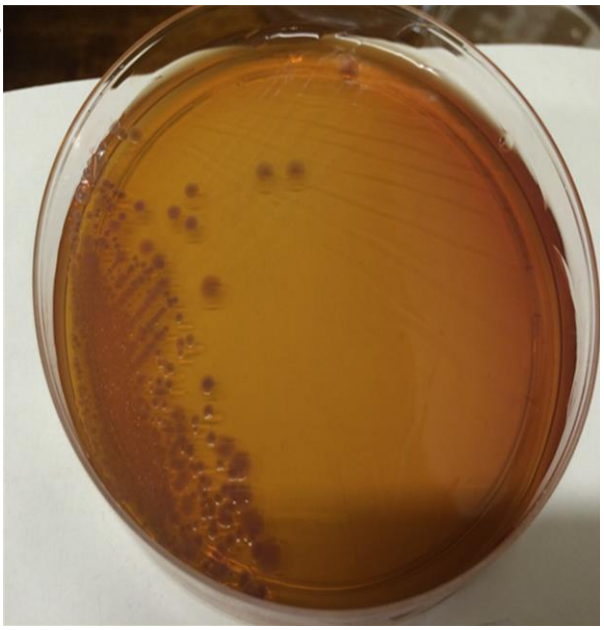

Figure 3.1: A) Phase I variation colonies represented by green circular colonies with red centres with clear zones around colonies in NBTA agar medium, B) Red circular colonies in MacConkey agar medium

3.3.2. Molecular identification of isolated bacterial symbionts

3.3.2.1. Evolutionary divergence and phylogenetic relationships

Undescribed *Photorhabdus* sp. was extremely divergent with the rest of the species from *Photorhabdus* genera with distances between 3 and 4 (table 3.2). Phylogenetic relationships established on the construction of a phylogenetic tree showed that the undescribed *Photorhabdus* sp. clustered together on the same clade with *Photorhabdus temperate subsp khanii NC19* (KF740642) (figure 3.2) however the long branches between the two species indicated evolutionary divergence. The unresolved polytomy with species KF740642 was also as a result of the different 16S rDNA sizes of the two species, the undescribed *Photorhabdus* species nucleotide size was 515bp (Appendix VIII) and the size of KF740642 was 768bp

Table 3.2: Estimates of Evolutionary Divergence between *Photorhabdus* sequences

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Maximum Composite Likelihood model (Kimura, 1980). The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 319 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.*, 2013).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. AY526333.1_Photorhabdus_asymbiotica_strain		1.875	1.871	1.871	1.871	1.871	1.872	1.870	1.816	1.875	1.816	1.875	1.870	1.865	1.865	1.880	1.870	1.921	1.865	2.553	1.689	2.179	1.856	1.865	1.639	1.597
2. Z76744.1_P.luminescens_(strain_Brecon)	3.365		0.005	0.005	0.005	0.004	0.005	0.003	0.013	0.000	0.013	0.003	0.003	0.006	0.006	0.003	0.004	0.005	0.005	2.275	1.385	2.359	2.182	0.006	1.355	1.262
3. KY290645.1_Photorhabdus_luminescens_subsp._kayaii_strain_PL-Hb-G	3.365	0.009		0.000	0.000	0.003	0.004	0.006	0.013	0.005	0.013	0.006	0.006	0.008	0.008	0.006	0.007	0.008	0.008	2.263	1.390	2.360	2.175	0.008	1.359	1.254
4. HM140700.1_Photorhabdus_luminescens_subsp._kayaii_strain_LB04	3.365	0.009	0.000		0.000	0.003	0.004	0.006	0.013	0.005	0.013	0.006	0.006	0.008	0.008	0.006	0.007	0.008	0.008	2.263	1.390	2.360	2.175	0.008	1.359	1.254
5. GU080061.1_Photorhabdus_luminescens_subsp._kayaii_strain_3209	3.365	0.009	0.000	0.000		0.003	0.004	0.006	0.013	0.005	0.013	0.006	0.006	0.008	0.008	0.006	0.007	0.008	0.008	2.263	1.390	2.360	2.175	0.008	1.359	1.254
6. EU930343.1_Photorhabdus_luminescens_strain_C8406	3.372	0.006	0.003	0.003	0.003		0.003	0.005	0.013	0.004	0.013	0.005	0.005	0.007	0.007	0.005	0.006	0.007	0.007	2.260	1.389	2.362	2.177	0.007	1.358	1.255
7. EU930336.1_Photorhabdus_luminescens_strain_KR04	3.379	0.009	0.006	0.006	0.006	0.003		0.006	0.013	0.005	0.013	0.004	0.006	0.006	0.006	0.006	0.007	0.008	0.006	2.260	1.389	2.359	2.176	0.006	1.358	1.255
8. AB355865.1_Photorhabdus_sp._OnKn2	3.364	0.003	0.013	0.013	0.013	0.009	0.013		0.012	0.003	0.012	0.004	0.000	0.005	0.005	0.004	0.003	0.004	0.004	2.263	1.381	2.359	2.192	0.005	1.352	1.262
9. NR_044412.1_Photorhabdus_temperata_subsp._cinerea_strain_3107	3.363	0.039	0.039	0.039	0.039	0.043	0.039	0.036		0.013	0.000	0.012	0.012	0.012	0.012	0.013	0.013	0.013	0.012	2.160	1.296	2.446	2.377	0.012	1.323	1.354
10. AY278647.1_Photorhabdus_luminescens_subsp._laumondii_strain_Brecon	3.365	0.000	0.009	0.009	0.009	0.006	0.009	0.003	0.039		0.013	0.003	0.003	0.006	0.006	0.003	0.004	0.005	0.005	2.275	1.385	2.359	2.182	0.006	1.355	1.262
11. KU240002.1_Photorhabdus_sp._SN259	3.363	0.039	0.039	0.039	0.039	0.043	0.039	0.036	0.000	0.039		0.012	0.012	0.012	0.012	0.013	0.013	0.013	0.012	2.160	1.296	2.446	2.377	0.012	1.323	1.354
12. JX221723.1_Photorhabdus_luminescens_strain_SG-HR4	3.372	0.003	0.013	0.013	0.013	0.009	0.006	0.006	0.036	0.003	0.036		0.004	0.005	0.005	0.004	0.005	0.006	0.004	2.275	1.385	2.356	2.181	0.005	1.355	1.262
13. NR_116512.1_Photorhabdus_luminescens_subsp._hainanensis_strain_C8404	3.364	0.003	0.013	0.013	0.013	0.009	0.013	0.000	0.036	0.003	0.036	0.006		0.005	0.005	0.004	0.003	0.004	0.004	2.263	1.381	2.359	2.192	0.005	1.352	1.262
14. HM140702.1_Photorhabdus_luminescens_subsp._thracensis_strain_LB03	3.371	0.013	0.019	0.019	0.019	0.016	0.013	0.010	0.036	0.013	0.036	0.010	0.010		0.000	0.007	0.006	0.007	0.005	2.270	1.378	2.376	2.192	0.000	1.349	1.259
15. NR_029012.1_Photorhabdus_temperata_subsp._thracensis_strain_39-8	3.371	0.013	0.019	0.019	0.019	0.016	0.013	0.010	0.036	0.013	0.036	0.010	0.010	0.000		0.007	0.006	0.007	0.005	2.270	1.378	2.376	2.192	0.000	1.349	1.259
16. Z76749.1_P.luminescens_(strain_Q614)	3.366	0.003	0.013	0.013	0.013	0.009	0.013	0.006	0.043	0.003	0.043	0.006	0.006	0.016	0.016		0.005	0.006	0.006	2.275	1.381	2.348	2.173	0.007	1.359	1.266
17. JN834009.1_Photorhabdus_luminescens_strain_VITICRI	3.357	0.006	0.016	0.016	0.016	0.013	0.016	0.003	0.039	0.006	0.039	0.009	0.003	0.013	0.013	0.009		0.005	0.005	2.263	1.382	2.359	2.195	0.006	1.353	1.261
18. FJ006727.1_Photorhabdus_luminescens_strain_SRK6	3.492	0.010	0.019	0.019	0.019	0.016	0.019	0.006	0.043	0.010	0.043	0.013	0.006	0.016	0.016	0.013	0.010		0.006	2.253	1.350	2.366	2.281	0.007	1.322	1.262
19. MF114105.1_Photorhabdus_luminescens_strain_namnaonensis_PB45.5	3.370	0.009	0.019	0.019	0.019	0.016	0.013	0.006	0.036	0.009	0.036	0.006	0.006	0.010	0.010	0.013	0.009	0.013		2.252	1.381	2.367	2.192	0.005	1.352	1.262
20. photorhabdus isolate1492	4.542	3.754	3.765	3.765	3.765	3.757	3.757	3.750	3.774	3.754	3.774	3.754	3.750	3.904	3.904	3.754	3.750	3.742	3.746		2.202	2.780	2.708	2.270	2.241	2.073
21. AY278496.1_Photorhabdus_asymbiotica_subsp._australis_strain_9802892	3.103	2.659	2.646	2.646	2.646	2.654	2.654	2.663	2.485	2.659	2.485	2.659	2.663	2.567	2.567	2.663	2.656	2.567	2.663	3.990		2.057	2.282	1.378	0.019	1.422
22. KF218576.1_Photorhabdus_temperata_subsp._thracensis_strain_DSM_15199	3.858	4.138	4.122	4.122	4.122	4.130	4.122	4.138	4.281	4.138	4.281	4.130	4.138	4.289	4.289	4.131	4.138	4.145	4.137	4.865	3.744		3.730	2.376	2.019	2.221
23. KF740642.1_Photorhabdus_temperata_subsp._khanii_NC19	3.548	3.800	3.779	3.779	3.779	3.787	3.779	3.805	4.130	3.800	4.130	3.792	3.805	3.789	3.789	3.795	3.813	3.969	3.797	4.569	4.354	6.251		2.192	2.177	1.774
24. AJ560634.1_Photorhabdus_luminescens_subsp._thracaensis	3.371	0.013	0.019	0.019	0.019	0.016	0.013	0.010	0.036	0.013	0.036	0.010	0.010	0.000	0.000	0.016	0.013	0.016	0.010	3.904	2.567	4.289	3.789		1.349	1.259
25. AY278514.1_Photorhabdus_heterorhabditis_strain_Q614	3.065	2.648	2.635	2.635	2.635	2.643	2.643	2.652	2.558	2.648	2.558	2.648	2.652	2.560	2.560	2.643	2.645	2.560	2.652	4.066	0.073	3.673	4.216	2.560		1.474
26. NZ_CCQX01000094.1_Escherichia_coli_strain	3.001	2.347	2.373	2.373	2.373	2.364	2.373	2.347	2.545	2.347	2.545	2.356	2.347	2.356	2.356	2.339	2.356	2.347	2.356	3.839	2.719	4.046	3.284	2.356	2.766	

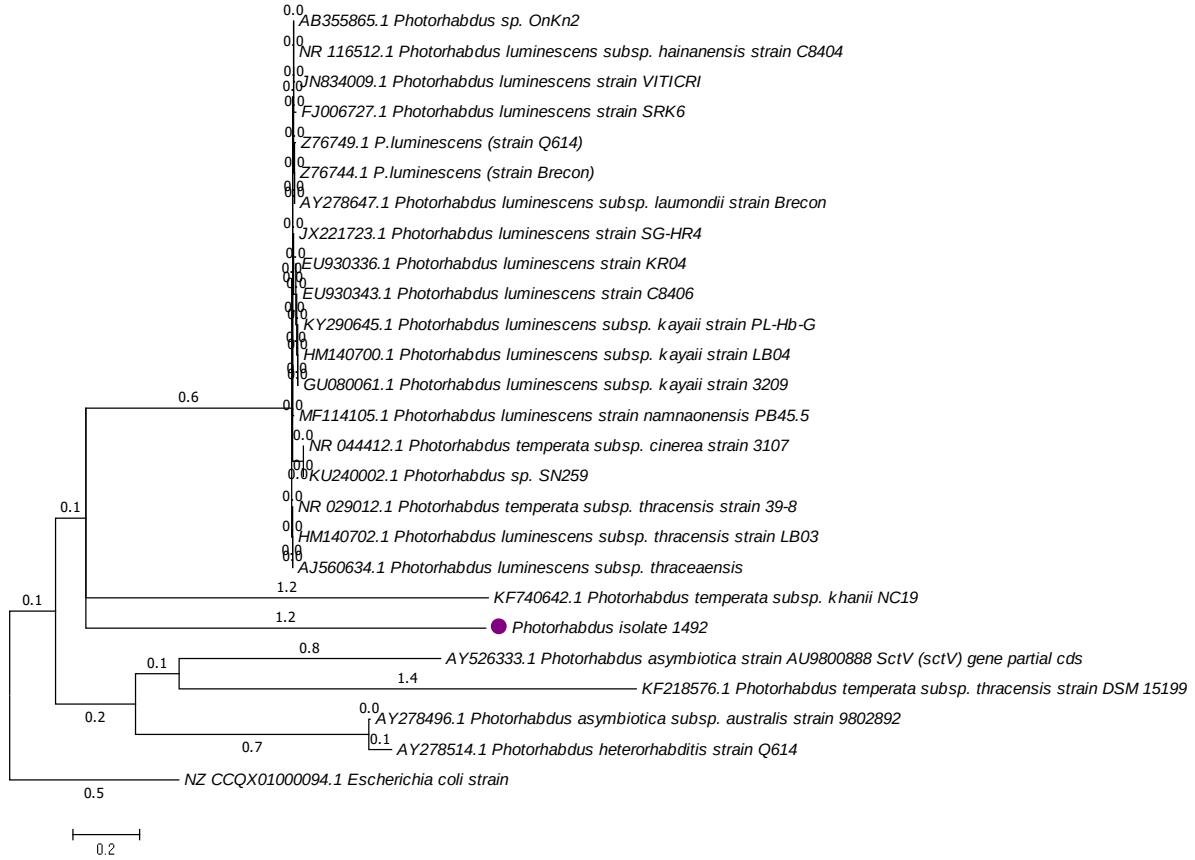

Figure 3.2: Molecular Phylogenetic analysis of *Photorhabdus species* by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1990). The tree with the highest log likelihood (-3215.6601) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 319 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

3.4. Discussion

Phase variation, a genetic instability induced by bacteria in response to environmental changes plays a pivotal role in characterising bacteria (Brunham *et al*., 1993; Dybvig, 1993; Robertson and Meyer, 1994). Results obtained in the study show the absence of phase variation due to the homogeneous colonies in the plate which were greenish with red centres (figure 3.1A), representing the primary phase (I) according to (Arkhurst, 1980; 1986), and hence phase II was not observed. The morphology and pigmentation of colonies in the plates in figure 3.1 are suspected to represent colonies of *P.temperata* variation I. Isolation of phase II variation in *Photorhabdus* spp. has not been reported in many studies (Forst *et al*, 1997).

Results from NCBI blast results showed a 99% high affinity of an undescribed bacterial symbiont to *Photorhabdus temperate subsp khanii* NC19 (KF740642). Furthermore the two species clustered together in the same clade, however because of the low bootstrap value, which was less than 65% and the long branches as well as the high evolutionary distance, there is a possibility of genetic variation amongst the bacterial symbionts. Molecular work on characterising this bacterial symbiont based on its 16S rDNA indicated that the isolated bacterial symbiont might possibly be a new species of bacteria due to the distance in relation to other *Photorhabdus* species. More accurate and reliable molecular analysis such as DNA hybridization and RFLP are recommended for further validation and conclusions in future studies.

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4. CHAPTER 4: THE INFLUENCE OF FORMULATION MEDIA ON EPN INFECTIVITY

4.1. Introduction

Previously, entomopathogenic nematodes (EPNs) were formulated as aqueous suspensions in carriers such as vermiculite, alginate gels, sponges and liquid concentrates (Georgis, 1990; Grewal, 2002). A recent formulation method involved the use of desiccated host cadaver infected with EPNs or host cadavers formulated with materials that protect desiccated host cadavers against rapture and sticking (Ansari *et al*., 2008). The goal of EPN formulation for commercialization purposes is to ensure longevity of the EPNs and its shelf life. It is also imperative that the formulation media produced and commercialised is easy to mix and apply in the field for consumers (Baur *et al.,* 1997). Longevity of EPNs has been attained by storing EPN infected cadavers in optimum temperatures which delay the emergence of IJs until a time of application occurs (Ansari *et al*.,2009), as well as dehydrating EPNs-reducing their metabolic rate (Georgis and Dunlop,1994).

Application of different formulations (liquid suspensions, infected host cadavers, desiccated EPNs) has to be rapid to ensure immediate activation of EPNs post application and the ease of which consumers apply the formulation should require less labour and allow them to work at their own convenience (Georgis and Kaya 1998). A good formulation according to researchers is one which provides EPNs with a longer shelf, one that will provide and maintain adequate moisture for survival and infectivity of the EPNs (Kaya *et al*, 1984; Lacey *et al*., 2006). Adequate temperature for storage of the EPNs in formulations has also been highlighted to be one of the limiting factors in most formulations, and studies have shown that formulated EPNs have to be stored in optimum temperatures specific to the EPN species niche, which allow survival and do not impede the efficacy and infectivity of the EPNs (Lacey and Unrah, 1998).

The type of soil that EPNs are formulated in also affects their persistence and survival and to maximise EPNs infectivity, it is important to formulate EPNs in soils such as loam soil which have the ability to retain water (Portillo-Aguilar *et al*., 1999) unlike sandy soil which has little water retention capacity, resulting in dehydration occurring at a faster rate (Hara *et al*., 1991).

In application and formulation, the substrates used to formulate EPNs should promote longevity and survival of EPNs. Transporting formulated EPNs should also be considered in

that their pathogenicity and viability will still be retained prior to application (Gerogis, 1990). All these factors should primarily be the building blocks to consider in order to promote the success of EPNs as biological control agents.

The aim of experiments undertaken here were to investigate the influence of different formulation material on EPN infectivity and the effect of storage temperature on EPN pathogenicity.

4.2. Materials and methods

4.2.1. Infectivity of an EPN formulated in inert carriers

4.2.1.1. Comparison of vermiculite and loam soil as formulation carriers or substrates for infective juveniles (IJs)

A mass of 55g of vermiculite and loam soil were each moistened with sterile water and mixed in a container. The level of moisture content was 10% for both carriers. Following this, both vermiculite and loam soil were inoculated with 1000 IJs/ml. Each substrate was transferred into the wells and one larvae per well placed on the substrate inoculated with IJs. The plates were covered with parafilm and needle picked to ensure aeration for EPN IJs and its host. Plates were stored at room temperature and the capacity of IJs to cause larval mortality was recorded daily for 6 days. Three replicates were prepared for each of the formulation substrate tested. IJs were not added in the control plates.

A

B

4.2.1.2. Evaluation of polyutherane sponges for the formulation of IJs.

Figure 4.1: A) *Galleria mellonella* larvae infected with IJ aqueous suspension mixed with loam soil and B) vermiculite.

Polyutherane sponges were cut into cubes (1 cm 3 × 9) which could fit in flasks. Distilled water was poured on the little pieces of sponge and inoculated with 1000 IJs/ml and mixed. Following this, sponge cubes were then incubated for 1 hour for complete penetration by EPN IJs. After this period of incubation, sponge cubes of sizes (1 cm 3 × 9) were transferred into flasks and 10 larvae were used as bait. The opening of the flasks were covered with foil, which was needle picked in the centre to ensure oxygen flow. The treatment had 3 replicates and flasks were stored at room temperature for 7 days, with mortality of the host recorded daily. To confirm entomopathogenicity, the cadavers were placed on White traps to harvest IJs. The harvested IJs were used for Koch postulates for further validation. IJs were not added in the control flasks.

In the second trial, Petri dishes were used. A sheet of sponge of the size 800 × 535mm was placed in the Petri dish and inoculated with an aqueous suspension of 1000IJs/ml followed by hydrating the sponge with 5ml of distilled autoclaved water. Five *Galleria mellonella* larvae were placed on the inoculated sponge sheet and the Petri dish closed. Mortality of the host was recorded daily.

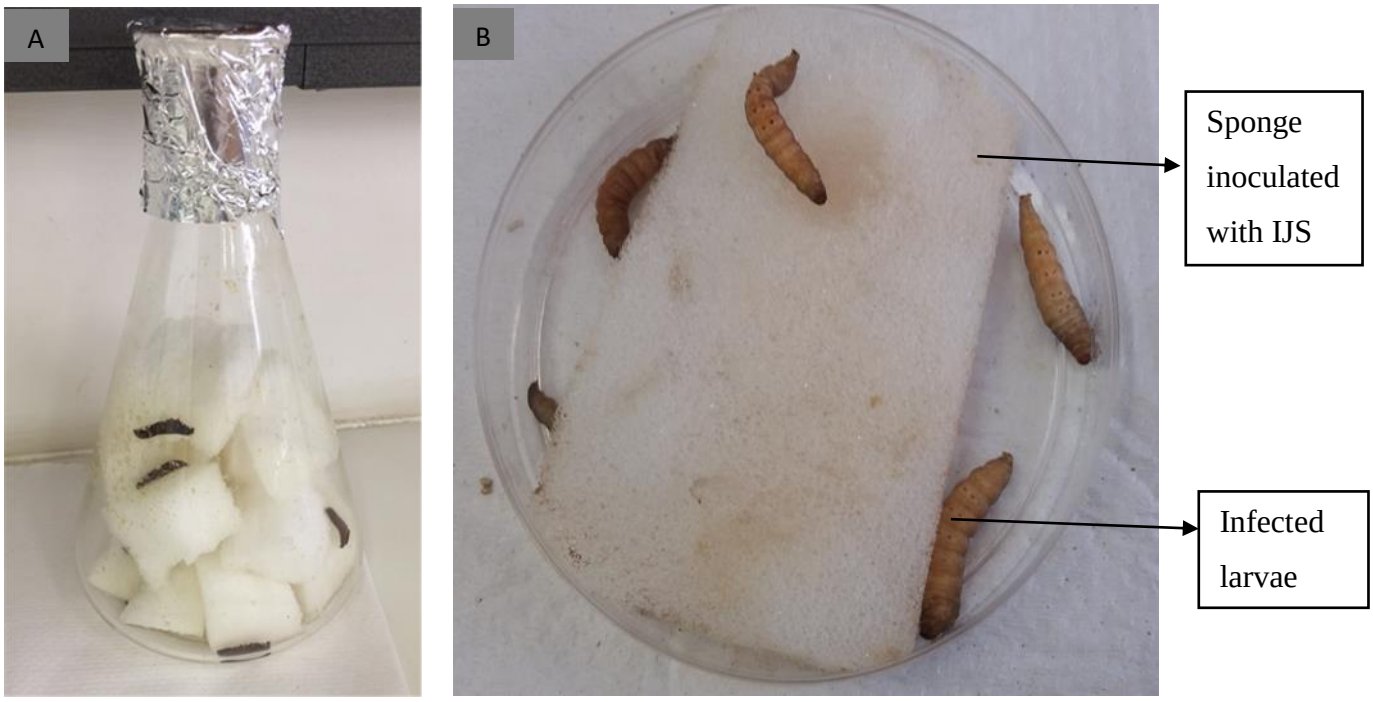

Figure 4.2: A) Polyutherane sponge cubes and B) sponge sheet infested with aqueous IJs baited with *Galleria mellonella* larvae showing symptoms

4.2.2. Infectivity of EPN formulated in cadavers

Previously infected *Galleria mellonella* larvae from soil were stored in enclosed Petri dishes. Petri dishes containing infected larvae were incubated in different storage temperatures (16 ºC, 25 ºC and 37 ºC) for 31 days. Post the storage period, infected larvae were placed in small disc plates and hydrated with sterile water for varying time intervals; 1hr, 6hrs, 8hrs and 24 hrs. Following this, hydrated larvae were exposed to live larvae in soil with 10% moisture content in Petri dishes. In each Petri dish, 3 EPN formulated cadavers were exposed to 3 live larvae. Three replicates for each temperature corresponding to each rehydration time were performed and mortality was assessed daily for 48 hours after exposing the larvae to EPN formulated cadavers.

4.2.3. Statistical analysis

One way ANOVA statistical analysis was used for infectivity of EPN aqueous suspensions formulated in inert carriers' experiments and two way ANOVA without replication statistical analysis under the hypothesis that there is no interaction between the variables was conducted for infectivity of EPN formulated in cadavers and stored at varying temperatures experimental investigations. The alpha value selected for statistical analyses of all experiments was 0.05 and Microsoft excel 2013 program was utilised to perform the ANOVA.We hypothesized that incorporating desiccated nematodes in formulation increases their shelf life and maintains nematode viability.

4.3. Results

4.3.1. Infectivity of EPN aqueous suspensions mixed in inert carriers

Infectivity of EPN IJs, recorded as mortality of the larvae, increased from day 3 till 6 for all formulation materials. Infective juveniles in vermiculite and loamy soil alternated on the high levels of infectivity, on day 3 EPNs in vermiculite induced 50% larval mortality, whereas EPN IJs in loamy soil induced 20% larval mortality. Entomopathogenic nematode (EPN) IJs embedded in vermiculite were 30% more infective than EPN IJs in loamy soil. On days 5 and 6, EPN IJs embedded in both formulations; vermiculite and loamy soil induced 100% larval mortality. Mortality induced by EPN IJs in sponge increased slightly from day 3 to day 4 with larval mortalities of 17% and 21% respectively. A gradual increase was observed from 21% to 61% on days 4 and 5 until the EPNs were able to completely kill all the larvae on day 6 reaching 100% cumulative mortality. One-way ANOVA analysis table in Appendix VIIII

revealed no statistical difference (p-value =0.75>0.05) in formulating IJs in different formulation medium on infectivity against *Galleria mellonella* larvae.

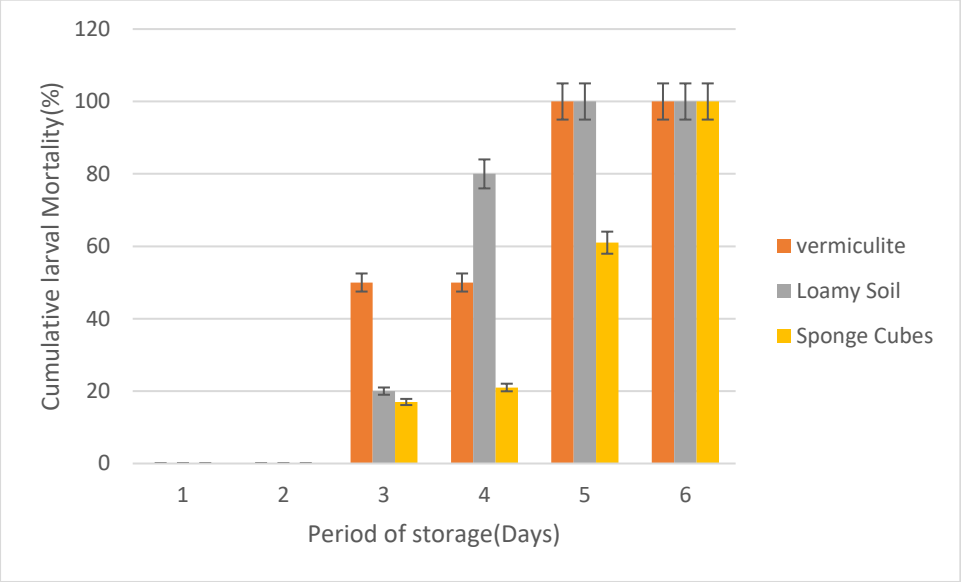

Figure 4.3: Percentage cumulative mortality of larvae exposed to nematode IJs. Entomopathogenic nematode IJs were formulated in different formulation media (sponge, vermiculite, loam soil) over a period of 6 days under controlled room temperature. Three replicates were performed in the study. P>0.05 based on one-way ANOVA.

4.3.2. Infectivity of EPN formulated in cadavers and stored at varying temperatures

Dehydrated host cadavers revealed differences in infectivity at different rehydration incubation times. EPN formulated cadavers which were stored at 16ºC and hydrated for 1 hour induced 66% larval mortality whereas EPN formulated host cadavers which were incubated at 25ºC and 37 ºC induced 33% larval mortality. Rehydrating the cadavers for 6 hours before application induced 100% larval mortality at the time of exposure for all storage temperatures that EPN IJs were incubated in, a similar pattern resulted for 8 hours and 24 hours rehydration time, particularly for host cadavers which were previously stored under 25 ºC and 37 ºC. Entomopathogenic nematodes formulated cadavers stored at 16 ºC produced different results for both 8 and 24 hours rehydration time interval. Rehydrating formulated nematodes for 8 hours induced 33% larval mortality, furthermore larval mortality increased two fold from 33% to 66% under 24 hour rehydration period. Two way ANOVA statistical test analyses tables in

Appendix VIII indicated a significant difference (P=0.07<0.05) in storing EPN formulated cadavers in varying temperatures on infectivity, however no significant difference (p=0.39>0.05) was observed on infectivity induced by hydrating EPN formulated cadavers at varying hydration intervals.

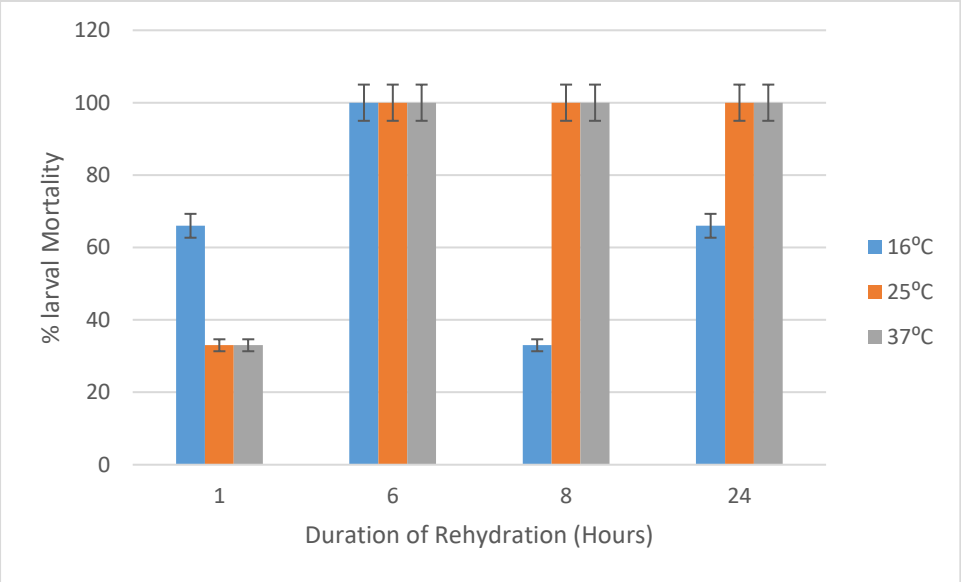

Figure 4.4: Percentage mortality of larvae exposed to desiccated EPN IJs formulated in host cadavers stored at varying temperatures(16 ºC, 25 ºC, 37 ºC) and rehydrated at different incubation times (1hr, 6hrs, 8hrs, 24hrs) before application. Three replicates for each temperature corresponding to each rehydration time were performed. P<0.05 under storage temperature, p>0.05 under duration of rehydration from two way ANOVA analysis. 3:3 ratio was used for live larvae and host cadavers carrying desiccated EPN IJs.

4.4. Discussion

The efficacy of formulated nematodes against key susceptible insect hosts has shown improvement in field applications (Lacey et al., 2010). Formulation of EPNs could range from applying anti desiccant agents to host cadavers (Lacey et al., 2010), formulating nematode IJs suspension or host cadavers in different inert carriers like vermiculite amongst others (Georgis, 1990) as well as directly dehydrating nematodes as formulations in wetteable dispersible granules (Bauer et al., 1997). Results in the study reveal an increase in infectivity of the identified nematode species formulated in the different substrates (figure 4.3), which is measured by insect (Galleria mellonella) mortality. Substrates; sponge, vermiculite and loamy

soil (figure 4.3) used in the study allow active movement of EPN IJs (Grewal, 2002) therefore, a gradual increment of mortality of the larvae over time is expected.

Heterorhabditis spp. are regarded as temperate species, which survive in temperatures ranging from 15ºC and above (Grewal and Gaugler, 1994). This statement confirms the results obtained from the study, the experiments were conducted at room temperature and according to figure 4.3, and there was an increment in larval mortality induced by nematode IJs formulated in inert carriers. Moreover, based on the one way ANOVA which was used to assess variation in formulating EPN IJs in different inert carriers on infectivity, it revealed no significant difference ($P>0.05$) rejecting the null hypothesis, indicating that the infectivity of the identified EPN species is not affected by the carriers used, these carriers offer advantages on application overall (Gaugler, 2002), they provide the same effects on the EPN ability to survive and therefore infect the host larvae.

The location that the EPN species are isolated from directly influences their thermal adaptation niche, species which are isolated from temperate regions are more likely to survive and infect their host at temperate temperatures, and this was proven by a study conducted by Chung et al., (2010). The study proved that the different Heterorhabditis bacteriophora strains were isolated from different locations with specific thermal conditions. Heterorhabditis bacteriophora isolate B1 in this study was isolated from a thermal location, Brits and based on what Chung et al, (2010) stated, it can survive thermal conditions.

Our study was conducted in laboratory settings where the natural habitat climate conditions are mimicked and thus the room temperature of the laboratory might possibly be different from the untampered natural environment, Heterorhabditis bacteriophora isolate B1 survived and infected the host larvae under these conditions however there is still some uncertainty about survival outside of the laboratory by the same species. Hiltpold, (2010) addressed the possibility of getting different results in the field under the same treatments and conditions, in their study, they tested the effect of coated encapsulated EPNs on the control of the widely known western corn root larvae, although great results were obtained in the laboratory experiments, under field conditions, the factor of coating the capsule did not improve western corn root (WCR) control.

Storage temperature, amongst other abiotic factors such as oxygen and pH affect the survival of EPNs. In particular to formulation, the formulated cadavers have to be stored in temperatures

optimum for EPNs survival, temperature also has to be optimum for EPNs to be active at the time of application in the field. The effect of storage temperature on the EPN infectivity was investigated in this study and based on our results (figure 4.4), *Heterorhabditis bacteriophora* isolate B1 survived for 31 days in host cadavers that were stored at temperatures; 16 ºC, 25 ºC and 37 ºC and furthermore infected the host larvae within 48 hours following rehydration. Although the survival of the EPN, which we inferred as the ability of the EPN to infect the larvae post exposure, was observed in different storage temperatures, the effect of infectivity was superior from formulated cadavers stored at temperatures 25 ºC and 37 ºC.

The cause of survival and infectivity of Heterorhabditis bacteriophora isolate B1 specie in the study at 25-37ºC is because EPNs, particularly *Steinernema* and *Heterorhabditis* genera generally have the ability to withstand these temperature niches and this further explains the wide range of geographic locations associated with varying temperatures that these EPNs are isolated from. Host cadavers retaining EPN IJs were stored in Petri dishes without the addition of water or liquids to moisten them, this induced partial anhydrobiosis and according to (Womersley, 1990; Glazer, 1996) their investigation on survival reveal that quiescent EPNs are able to withstand environmental extremes.

In this study, temperatures 25-37 ºC could possibly be regarded as extreme temperatures by other researchers and this then justifies the results obtained which reveal EPN survival in the extreme temperatures. In the study conducted by Strauch *et al.*, (2000), results revealed that *H.bacteriophora* was stable and survived for 128 days under temperatures between 15-20 ºC furthermore, EPNs used in the study were non-quiescent and this explains the contrasting results they obtained compared to ours, because at 25 ºC, EPN IJs were unable to survive.

Entomopathogenic nematodes (EPNs) require a film of water to infect their insect host, both quiescent and non-quiescent EPNs. Adequate moisture content enables EPNs to actively search for their host, most Heterorhabditis and some *Steinernema* species because of the cruiser foraging behaviour (Bal and Grewal 2015). In the study, host cadavers were incubated in distilled water for a range of hours (1, 6, 8, and 24) before applying them in soil with live larvae. The effect of rehydrating host cadavers on the mortality of the larvae was investigated. Our results revealed that the longer the formulated cadavers were hydrated for (figure 4.4), the higher the infectivity of the formulated EPN although infectivity was still observed in shorter hydration periods ($p>0.05$). Bauer et al., (1997) reported that EPNs in wettable granules were effective when humidity was high and were rehydrated after desiccating them in granules for

at least 48 hours. Both results confirm the importance of rehydrating EPNs before application because the process actives EPNs to resume with their metabolic activities and therefore be in a state to infect its host (Bauer et al., 1997).

Selected substrates in the study offer a convenient method of application, where carriers (vermiculite and loamy soil) will be homogeneously mixed with water and transferred into tanks or small spray bottles and the EPN suspension applied to the field. The same procedure as the latter can also be applied with sponge, although, the EPN IJs will firstly be collected by soaking the sponge in water (Gaugler, 2002). These carriers, especially vermiculite and sponge offer the advantage of water retention and absorption, they have furthermore been able to retain EPNs in storage for 0.3 -6 months (Gaugler, 2002). The shelf life of the EPNs in the different formulations vary based on the storage temperature, ideally Steinernema species; S carpocapsae at room temperature has a shelf life of 0.03-0.1 months, in contrast, the same species in low temperatures has a shelf life of 2.0-3.0 months. Heterorhabditis bacteriophora, however, in relation to the latter results, experience difficulties to survive up to a month at room temperature, but survives for 1-2 months at low temperatures (2-10ºC). In vermiculite formulation, H. megidis survives for 2-3 months (Gaugler, 2002). The selected media are also cost effective and easily accessible to consumers.

The study reveals that formulation strategies improve survival and efficacy of entomopathogenic nematodes. Heterorhabditis bacteriophora isolate B1 species is able to infect host larvae under the exposure of different formulation media.

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5. CHAPTER 5: DESSICATION TOLERANCE OF AN ENTOMOPATHOGENIC NEMATODE

5.1. Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are effective biological control agents (Gaugler and Kaya, 1990). Their potential is attributed to the specialized third living stage referred to as the infective juvenile (IJ). This stage is non-feeding and has the ability to thrive and survive in the soil environment, outside of its insect host. It is attributed by a thick cuticle (Gaugler and Kaya, 1990; Grewal and Georgis, 1998) and the carriage of an endosymbiont bacterial species in its gut. The latter characteristics enable EPNs to infect and kill its host within 24-48 hours and survive extreme environmental conditions (Grewal, 2000).

Infectivity and survival of EPNs are affected by biotic and abiotic factors. Soil which is a natural habitat for EPNs, is believed to be an important abiotic factor influencing and affecting the ability of EPNs to persist in the field. Abiotic factors such as temperature, texture, chemical composition and moisture content of the different soil types affect EPNs ability to survive (Glazer, 2000). Entomopathogenic nematodes employ different survival strategies to survive under environmental stress (heat, cold, and desiccation). Mechanisms they induce include morphological, biochemical and behavioural mechanisms, however the mechanisms vary among the different population and species of entomopathogenic nematodes (Womersley, 1990; Kung *et al*., 1991; Glazer, 2002).

Desiccation is one of the extreme environmental stresses that entomopathogenic nematodes get exposed to. It threatens the survival, infectivity of entomopathogenic nematodes (Kaya and Gaugler, 1993). Members of *Steinernema* and *Heterorhabditis* genera are able to survive desiccation by suspending their metabolic activities as well as movement. This response to desiccation is referred to as anhydrobiosis, a reversible physiologically and metabolically state of dormancy which is caused by the loss of water in the body (Shannon *et al*., 2005) when soil dwelling entomopathogenic nematodes (EPNs) are exposed to desiccation (Crowe *et al*., 1992). Entomopathogenic nematodes are referred to as quiescent anhydrobiotes because of their partial anhydrobiosis trait. Anhydrobiosis is believed to increase storage stability of EPNs which is important for their mass production and commercialization (Bedding, 1998).

Entomopathogenic nematodes form clumps and aggregate in one place and coil as a behavioural response to dessication (Womersley *et al*., 1990). Moreover, as a behavioural response to desiccation, some EPNs forage beneath the soil surface (cruiser EPNs) in search of insect host while some EPNs stay on the soil surface and actively search for its insect host (ambusher EPNs) (Lewis, 2002). An induction of certain genes as a response to desiccation has also been reported, the induction of the genes enables the survival of EPNs under desiccation (Somvanshi *et al,* 2008).

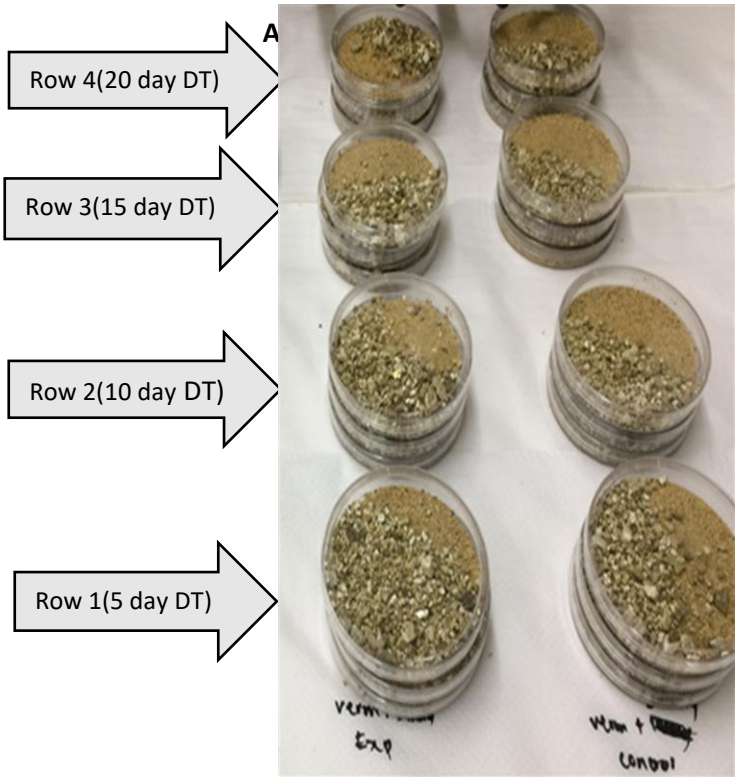
The study investigated the desiccation tolerance of *Heterorhabditis bacteriophora* isolate B1 embedded in various soil matrices associated with different soil texture and chemical composition.

5.2. Materials and Methods

5.2.1. Desiccation studies

The ability of *Heterorhabditis bacteriophora* isolate B1 to survive desiccation was assessed. The different soil matrices were autoclaved at 121ºC and furthermore baked in an oven for three days to remove contaminants. A mass of 40g of each soil matrice was weighed and transferred into Petri dishes. All soil matrices in respective dishes were hydrated with sterile water to 10% moisture content. Infective juveniles harvested 2 days post emergence in White traps were transferred into Falcon tubes and allowed to sediment. The settled IJs were further surface sterilized with 0.1% hypochlorite for elimination of possible contamination for 1 hour and thereafter rinsed three times. Hydrated plates were each inoculated with 1000IJs/ml. Control plates were kept at 10% moisture throughout the study and experimental plates were allowed to undergo desiccation for different days (5, 10, 15, and 20). Five *Galleria mellonella* were placed in control plates and 4 *Galleria mellonella* placed in experimental plates. On the day of dehydration, experimental plates were rehydrated back to 10% moisture by weighing the dried plates with the respective substrates and subtracting the dried substrates from the hydrated substrates and working out the amount of water needed to bring the plates containing soil matrices back to 10%. Larval cumulative mortality post different dehydration days following resuscitation/recovery was recorded for 24, 48, 72 and 96 hours. Three replicates per soil matrice were used for both control and experimental plates, corresponding to days of dehydration exposure. *Galleria mellonella* larvae were added in Petri dishes every third day interval and in plates were 100% larval mortality was induced. Infected cadavers which were

recovered from plates were placed on White traps to confirm if the cause of death was as a result of EPN pathogenicity.



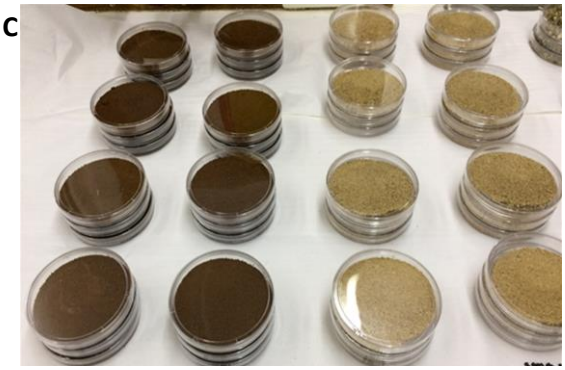


Figure 5.1: Experimental design for desiccation studies.

Rows represent dehydration times (5, 10, 15, and 20) with each soil matrice replicated three times.

Key: from left to right **A** (Vermiculite mixed with sand), **B** (Vermiculite; Vermiculite with loamy soil), **C** (Loamy soil; sand), DT=Dehydration time.

5.2.2. Rate of Infective juveniles' emergence studies

The rate of infective juveniles' emergence from previously EPN infected desiccated larvae and EPN infected undesiccated larvae was assessed. Infected *Galleria mellonella* previously desiccated in soil matrices; loam, vermiculite, loam mixed with vermiculite, sand mixed with

vermiculite and sand were placed on saturated White traps. Similarly, infected *Galleria mellonella* previously exposed to aqueous suspensions of IJ populations inoculated in sponge, loam soil and vermiculite were placed on White traps. The rate at which infective juveniles emerged from non-formulated (undesiccated) and formulated cadavers (desiccated) was observed for 9 days. Four replicates for each formulation were performed.

5.2.3. Statistical analysis

Two way ANOVA statistical analysis was conducted to assess the variation on dehydrating EPN IJs in different soil matrices on infectivity against *Galleria mellonella* larvae experiments. One way ANOVA statistical analysis was used to determine the variation in the rate of IJ emergence from formulated and unformulated cadavers' experiments. The p-value selected was 0.05 and Microsoft excel 2013 program was utilised to perform the ANOVA. The null hypothesis was stated as follows; exposing nematodes to desiccation for long period of time increases larval cumulative

5.3. Results

The capacity of each soil matrices to lose water was reported, all five matrices gradually lost water from day 1 of dehydration until the 20th day of dehydration. Moisture content was lost the fastest in vermiculite mixed with sand and vermiculite was the slowest in losing water (figure 5.2). *Heterorhabditis bacteriophora* isolate B1 could tolerate desiccation up to 15 days by inducing 100% larval mortality post 96 hours of resuscitation, in all the five soil matrices (figure 5.3).There was no statistical difference in larval mortality induced by IJs desiccated in different soil matrices as $p=0.117>0.05$ according to the ANOVA table in Appendix VIII. An increase in larval mortality induced by *Heterorhabditis bacteriophora* isolate B1 post resuscitation after 24, 48,72 and 96 hours was observed with average larval mortalities ranging at 80-100% at most. The more days the EPN specie was exposed to desiccation, it took more hours to induce larval mortality following rehydration, more larval mortality was induced by the EPN IJs embedded in loam soil and vermiculite mixed with loam soil (table 5.1).

The rate of IJ emergence from desiccated and undesiccated host cadavers were recorded. Undesiccated IJs emerged at a slower mean rate of 10 IJs/day at most contrasting results obtained in desiccated IJs from EPN infected cadavers which emerged at a mean rate of approximately 16.67 IJs/day at most (table 5.2). One way ANOVA statistical analysis revealed

no statistical difference (p=0.97>005), see Appendix VIII, in IJ emergence from desiccated and undesiccated host larvae.

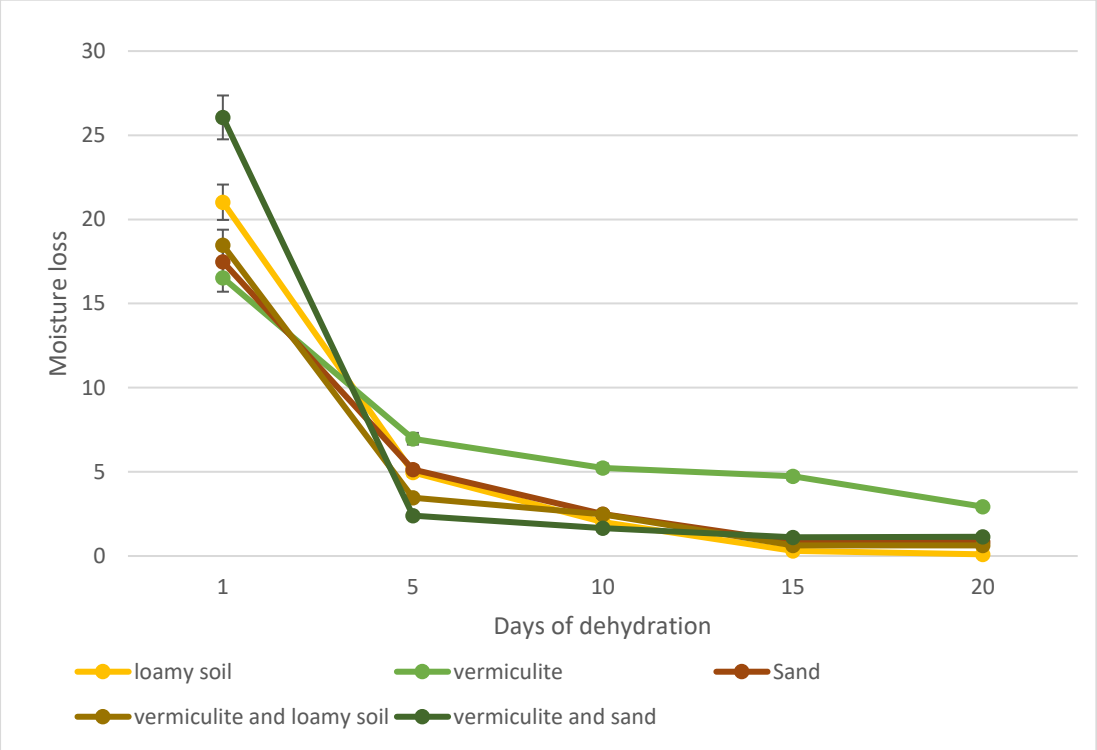


Figure 5.2: Water loss reported as moisture content in soil matrices (loam soil, vermiculite, vermiculite mixed with loam soil, sand and vermiculite mixed with sand) allowed to dehydrate for 20 days.

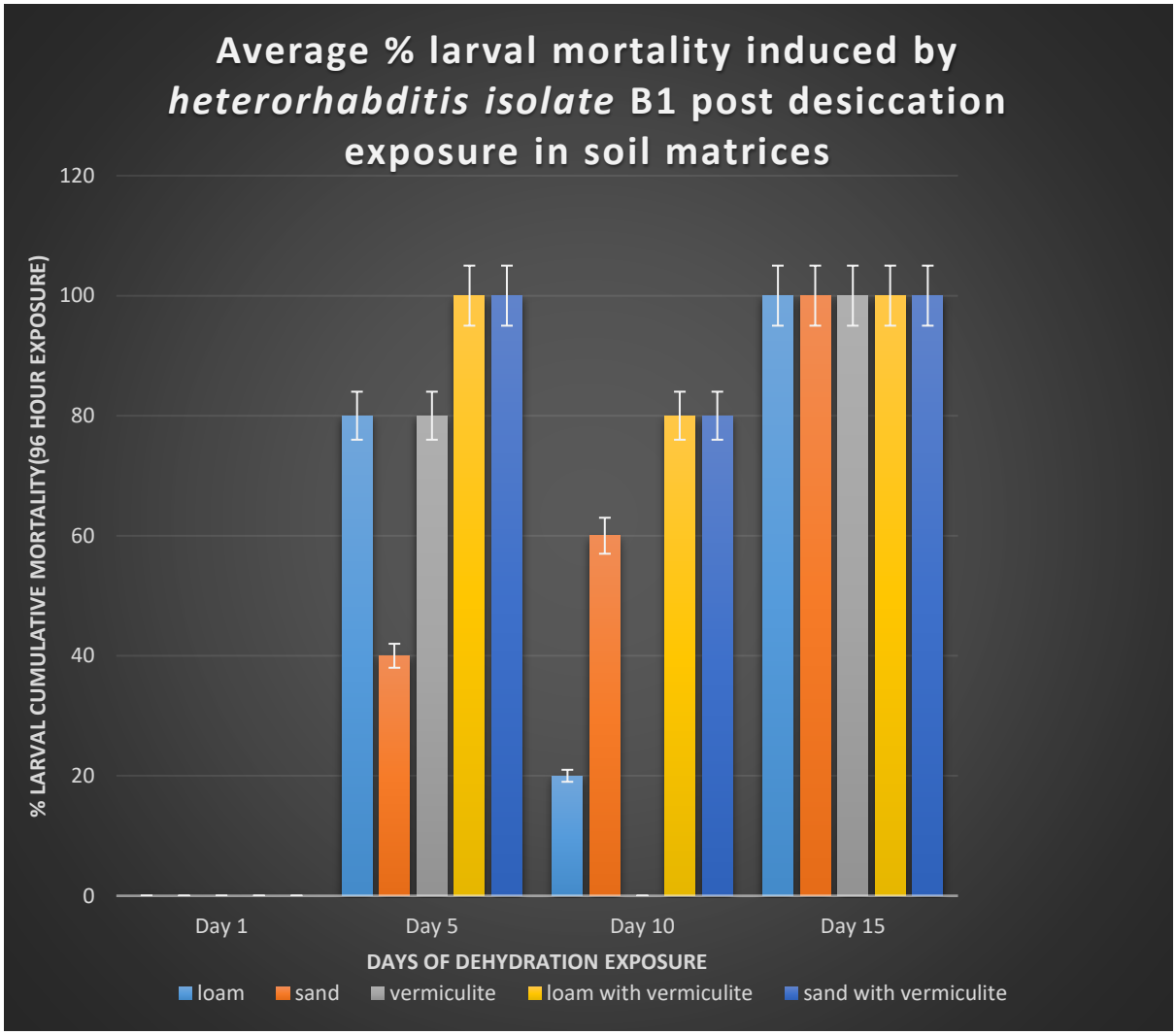


Figure 5.3: Average larval cumulative mortality induced by *Heterorhabditis bacteriophora* isolate B1 formulated in different soil matrices (loam soil, sand, vermiculite, loam mixed with vermiculite and sand mixed with vermiculite) in 96 hours post dehydration days(1,5,10,15).

Table 5.1: Average larval cumulative mortalities induced by *Heterorhabditis bacteriophora* isolate B1 formulated in different soil matrices in 24, 48, 72 and 96 hours post dehydration days (1, 5, 10, and 15) in experimental and control plates which were kept at constant moisture content (10%).

	Average % Cumulative mortality							
Loam	Experiment				Control			
Days of dehydration	24	48	72	96	24	48	72	96
1	100	100	80	0	100	100	100	100
5	100	100	100	80	100	100	50	50
10	20	100	40	100	100	100	100	100
15	80	80	100	100	100	100	100	100
Sand	Experiment				Control			
Days of dehydration								
1	100	100	100	0	75	75	100	100
5	40	80	0	40	100	100	100	100
10	60	80	80	60	50	20	50	50
15	80	100	100	100	100	100	100	100
Vermiculite	Experiment				Control			
Days of dehydration								
1	100	40	40	0	100	100	100	100
5	100	100	100	80	100	100	100	100
10	0	20	40	80	100	50	50	100
15	100	100	100	100	100	100	100	100
sand mixed with vermiculite	Experiment				Control			
Days of dehydration								
1	80	80	60	0	100	50	50	50
5	80	100	60	100	75	25	100	100
10	80	40	60	80	100	100	100	100
15	80	100	100	100	100	100	100	100
Loam mixed with vermiculite	Experiment				Control			
Days of dehydration								

1	100	100	60	0	100	100	100	100
5	100	100	60	100	100	100	100	100
10	80	100	60	80	100	50	100	100
15	100	100	100	100	100	100	100	100

Table 5.2: The rate of infective juveniles emergence from EPN infected larvae previously exposed to desiccation in various substrate matrices(A) and undesiccated aqueous suspensions of IJs in various substrate matrices(B)

Substrate matrice	**Mean% ±SEM**
Loam soil	16,67 ±13,08
Sand	16,67±13,08
Loam and Vermiculite	14,28±8,41
Sand and Vermiculite	14,28±7,19
Vermiculite	14,28±9,47

Substrate matrice	**Mean% ±SEM**
Sponge	3,67±3,67
Vermiculite	10±10
Loam	10±10

5.4. Discussion

Soil matrices lost water at different rates, vermiculite, loam, and loam mixed with vermiculite lost water at a slower rate while sand and sand mixed with vermiculite lost water at a faster rate. The ability of the above mentioned soil matrices to lose water at different rates affected their ability to promote survival of EPN IJs embedded in the substrates. High larval mortality was induced by IJ populations which were embedded in soil matrices that retained water thus slowly lost water (figure 5.2 and table 5.1).

Entomopathogenic nematodes, amongst other invertebrates have been reported to survive under extreme environmental stresses such as hot and cold temperatures, osmotic stresses as well as desiccation (Womersley and Ching, 1989). To survive desiccation, EPNs have been reported to undergo anhydrobiosis, which is the temporary suspension of metabolic processes

by EPNs until conditions improve for metabolic processes to resume (Serwe-Rodriguez *et al*., 2004). In the current study it was hypothesized that desiccated host cadavers influence the survival and infectivity of the desiccated EPN IJs positively, desiccating host cadavers significantly increases the infectivity of nematodes. Desiccated *Heterorhabditis bacteriophora* isolate B1 IJs were able to induce mortality in 96 hours post exposure to different dehydration days following resuscitation. The nematode IJs infectivity increased with more exposure to desiccation. A similar trend as the former and latter was also observed in 24, 48 and 72 hours in table 5.1 for the experimental plates which were allowed to dehydrate.

Larval desiccation has been shown to have an effect on IJ survival (Shapiro-Ilan *et al*., 2001). In a previous study, this effect was investigated and it was reported based on the experiments, that desiccation of a larval host which ultimately desiccates IJ populations hosted inside significantly increased their infectivity levels post desiccation following resuscitation (Serwe-Rodriguez *et al*., 2004). The host enables desiccated IJs to acquire cross protection against other stresses, for example temperature and pH (Serwe-Rodriguez *et al*., 2004). Desiccated hosts are therefore considered an important means of functioning as vectors in EPN application and formulation, for controlling and eradicating agricultural crop insect pests due to the positive influence they pose on EPN infectivity as well as protecting EPN IJs from exposure to UV radiation (Shapiro-Ilan *et al.*, 2001). From this evidence and other studies, we can deduce that *Heterorhabditis bacteriophora* isolate B1 has the ability to develop cross protection against other environmental stresses based on the reasoning that the species is desiccation tolerant.

Control plates were kept hydrated at 10% moisture content throughout. Infective juveniles (IJs) exposed to these conditions demonstrated increased larval mortality inducing 100% cumulative mortality at most (table 5.1). Entomopathogenic nematodes (EPNs) are aquatic in nature, and therefore need slight volumes of water, to infect and kills its host (Womersley and Ching, 1988) therefore, more infectivity under hydrated conditions is expected. Our results are in contrary to Serwe-Rodriguez *et al*., (2004) who found from their investigation that EPNs from desiccated hosts demonstrated higher levels of infectivity in comparison to the controls, where induced larval mortality reached 97% on day 24. However, in our study the EPN species under investigation was a *Heterorhabditis* sp. whereas in their study, the species under investigation was *Steinernema carpocapscae*. The two EPNs are from different families, genera and

therefore it would be expected that the behaviour of the two species in response to desiccation would be different.

Most *Heterorhabditis* species have been classified as cruisers (Dillion *et al*, 2006) foraging beneath the soil profile to find insect hosts (Alatorric-Rosas and Kaya, 1990). The identified species in this study was a *Heterorhabditis* species and therefore we could classify it as a cruiser EPN. Studies have not proven that all cruiser EPNs are unable to survive under desiccation or are poor anhydrobiotes (Grewal, 2000), therefore the ability of the identified species to tolerate desiccation and furthermore induce larval mortality post desiccation exposure was still exhibited, however in minimal amounts as compared to the fully hydrated host and undesiccated IJs (table 5.1) and because more larval mortality was induced in substrates which lost water at a slower rate, we could deduce that it is a slow dehydration strategist.

Moreover, the substrate moisture content, plus the ambient surroundings' relative humidity, applied in this study were low in comparison to other studies where the ambient humidity used was in the range of 40-90% and EPN survival was observed (Womersley and Ching, 1989). In this study, exposure of the EPN to low ambient humidity and low substrate moisture contents (10%) revealed that *Heterorhabditis bacteriophora* isolate B1 was able to survive and infect its host.

The genetic ability of the EPNs to tolerate desiccation could possibly be caused by their evolutionary adaptation to a wide range geographical distribution in different habitats (Salme and Glazer, 2015). The identified species was isolated from Brits in the North West province. Based on the holistic environmental services report, (2014) this area which can be classified as an open bushveld savannah, has an abundance of trees, shrubs and grasses that have become established on a mixture of rock outcrop, Mispan and Aracadia soils. With regards to climate, the area has a significant variability in temperature and rainfall in that there are cold seasons where the temperature can go as low as 0.9 ºC, in the summer season, the temperatures are around 32 ºC. The area has arid and semi-arid conditions as well as low relative humidity. The desiccation tolerance of the identified species is justified by the fact that it could have genetically evolved to be desiccation tolerant because of the following reasons, 1) Arcadia soil consists of clay with minerals and one of the properties of clay is that it has a low infiltration rate (Franzmeir *et al*., 1989) thus this promotes a desiccation environment, 2) The low humidity of the area explains the evidence that our results reveal that the species is able to tolerate desiccation under low relative humidity, the last point is the very low and high temperatures of

the area which conferred the EPN to induce a cross-protective responses towards secondary stresses (Serwe-Rodriguez *et al.,* 2004).

Two way ANOVA without replication was conducted to assess the variation in exposing *Heterorhabditis bacteriophora* isolate B1 IJ populations in different soil matrices on their infectivity against host larvae. The analyses revealed no statistical difference ($p=0.117>0.05$; $F=2.26<Fcrit=3.24$). Soil texture and chemical composition of soil matrices do not play a vital role in the survival and infectivity of desiccated EPNs and this is mainly because, given that an EPN species is desiccation tolerant, survival is attainable regardless of the extrinsic physical differences that the EPN is placed or formulated in (Gruner *et al.,* 2007). The genetic makeup of EPNs (Tyson *et al*., 2006) selected under the pressure of an arid environment; which shapes its behaviour (Solomon *et al.,* 2003) is the building block of the ability for EPNs to survive desiccation. Furthermore the survival of IJs in desiccation is directly and mainly influenced by the EPN species original geographic location, host cadaver as well as the relative humidity and moisture content used (Shapiro-Ilan *et al.,* 2001). These claims are supporting our reported results which in overall showed successful infectivity of the desiccated IJs in all the different substrate types.

The rate of IJ emergence on saturated White traps from the formulated desiccated and unformulated undesiccated cadavers was also investigated in the study, results obtained (table 5.2) showed that the emergence of IJs from formulated cadaver were superior from the emergence of IJs from unformulated cadaver previously exposed to aqueous suspensions. These obtained results are supported by Shapiro ad Glazer, (1996) where assays performed in the laboratory proved that dispersal of *H.bacteriophora* improved from cadavers relative to those applied in aqueous suspensions. This claim could possibly give us more reason to suspect that desiccation tolerant EPN IJs are capable of emerging from the host cadavers at a faster rate compared to undesiccated IJs. One way ANOVA analysis revealed no statistical difference ($p=0.97$; F (df between 7, df within 55) = 0, 246981) in the rate of IJ emergence from desiccated and undesiccated cadavers.

As a biochemical response to desiccation, EPNs have been shown to synthesise trehalose sugar which functions in protecting the cell membrane and proteins from damage by replacing structural water in the membranes (Crowe *et al*., 1984). The species under investigation was able to tolerate desiccation for 15 days and furthermore infect its host larvae for a maximum

of 96 hours following resuscitation and from this we could conclude that *Heterorhabdtidis bacteriophora* isolate B1 possibly synthesized trehalose as a mechanism to survive desiccation.

5.5. References

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6. CHAPTER 6: RECOMMENDATIONS

Chapter 1: Literature review

This focused on literature review which covered the discovery of entomopathogenic nematodes and their successful use in biological control agents as an alternative method in eradicating key insect pests damaging some of the most important crops in agriculture. It further addressed the different types of media which can have an effect on EPN infectivity.

Chapter 2: Isolation and molecular characterization of entomopathogenic nematodes

Heterorhabditis bacteriophora isolate B1 was successfully isolated from soil samples in Brits, North-West province, South Africa.

Heterorhabditis bacteriophora was identified using the sequencing of the 18S rDNA

The wide distribution of *Heterorhabditis* species was furthermore emphasised with the isolated specie in the study being isolated from a different environment and province to *Heterorhabditis UP2A2* which was a specie in close relation to the identified specie in the study. This specie (*Heterorhabdits bacteriophora* UP2A2) was isolated from grapevine soil.

Heterorhabditis species are rarely identified and characterized in South Africa. Complete characterization of the isolated *Heterorhabditis bacteriophora* isolate B1 was not conducted. Full characterization can be performed as a study on its own by the employment of morphometrics -taking measurements of different anatomical structures of the isolated EPN, the use of scanning electron microscopy (SEM) and light microscopy taking into account all the life stages-hermaphrodites, females and males of the EPN species. More molecular techniques can also be explored.

Experiments undertaken in the study were conducted under controlled conditions where the microflora associated with soil matrices were eliminated through the process of autoclaving and only one susceptible host larvae, *Galleria mellonella* used. Because of mimicking the environment in the laboratory, there might be a possibility of obtaining different results in the field and it is therefore recommended that field trials are conducted within South Africa for further investigations and validity of results. Furthermore, whole genome sequencing of

Heterorhabditis bacteriophora isolate B1 is recommended for future studies in order to identify and report on genes which enabled the specie under investigation desiccation tolerant. Investigating the former will contribute to science in nematology knowledge and further expand on knowledge that is already reported on desiccation tolerance of EPNs.

Chapter 3: Isolation and molecular characterization of an EPN associated bacterial symbiont

Partial identification of *Photorhabdus* isolate 1492 was conducted in the study based on phenotypic characterization. However unresolved polytomy could possibly indicate that the species is a new species hence *Photorhabdus* isolate 1492 bacterial symbiont associated with Heterorhabditis bacteriophora isolate B1 was not fully characterized.

Future studies need to be undertaken to further characterize an EPN associated bacterial symbiont to fully understand the coevolution between *Photorhabdus* and *Heterorhabditis bacteriophora* isolate B1 complex. Genome annotation can possibly give insight in genes associated with the pathogenicity of the bacterial symbiont. Further molecular techniques are also advised to resolve the taxonomy of the isolated *Photorhabdus* sp. in the study.

Chapter 4 &5: The influence of formulation media on EPN infectivity (chp 4), Desiccation tolerance of an entomopathogenic nematode (chp 5)

Heterorhabditis bacteriophora isolate B1 identified in the study is desiccation tolerant and is able to infect host larvae within a period of four days following recovery from desiccated conditions.

Heterorhabditis bacteriophora isolate B1 formulated in different formulation medium was infective against *Galleria mellonella* larvae. Desiccating entomopathogenic nematodes is another form of formulation. Furthermore formulating desiccated IJs in host cadavers is even better because the host acts as vector to transport EPN IJ population in the field as well as protecting IJs from UV radiation. Moreover, further investigations need to be conducted to improve formulations which will enhance storage and increase shelf life of the EPNs without losing pathogenicity.

Advances still need to be created which will not require a lot of water being used to moisten the soil to create a favourable environment for EPNs to infect and kills its host. Ease of application by the user needs to be considered when creating formulation materials. A more

significant note to take is that, now that it has been proven that EPNs have the ability to tolerate desiccation- by entering into a quiescent state, this state can be induced in EPNs and used as a formulation strategy by either desiccating EPNs in host cadavers or in adjuvants such as granules amongst others, to prolong shelf life until application season.

Soil which is a natural habitat of entomopathogenic nematodes undergoes gradual desiccation, promoting desiccation of EPNs. Other soil matrices which were tested in the study showed the capacity to promote desiccation of *Heterorhabditis bacteriophora* isolate B1 and so from this, induction of anhydrobiosis in EPNs can be conducted in various soil matrices.

Desiccation tolerance of entomopathogenic nematodes plays a pivotal role in application technology. Due to the changing climate and sometimes, drought seasons, dessication tolerant EPNs are important mainly due to the fact that their infectivity, survival and storage stability are not affected during drought seasons and that when environmental conditions become favourable, mortality of problematic insect pests will still be exhibited.

Appendix I

***Galleria mellonella* media**

(Adapted from Woodring and Kaya, 1988)

The following modifications were made:

Calcium propionate substituted with benzoate

Multivitamin bran substituted with ProNutro (banana flavour)

Recipe:

500g ProNutro

200ml pure natural honey

200ml glycerol

5 teaspoon yeast extract

200ml boiled distilled water

1 teaspoon benzoate

Protocol:

1. Mix honey, glycerol and ProNutro together.

2. Add yeast extract, boiling water and benzoate to ProNutro mixture.

3. Mix contents thoroughly.

4. Place mixture in tin foil and seal adequately.

5. Autoclave at 121˚C and 15 psi for 25 min.

Appendix II

Nematode Genomic DNA extraction (Protocol from Puregene® DNA Purification Kit, Gentra systems 2003)

1) Rinse infective juveniles three times using approximately 4ml distilled water per wash.

2) Pellet nematodes in a microfuge tube by spinning at 14000rpm for 10 minutes. Place on ice for 30 seconds. Remove excess water.

3) Re-suspend nematode pellet in 1 ml distilled water and transfer the nematode suspension to a 1.5 ml microfuge tube on ice.

4) Centrifuge at 13000-16000 rpm for 3 minutes than place the tube on ice for atleast 30 seconds and discard the supernatant.

5) Add 600µl Cell Lysis Solution (from kit) and invert several times.

6) Add 3µl Proteinase K solution (from kit) and invert 25 times. Incubate at 55˚C for 3 hours to overnight, until the tissue particulates have dissolved. Invert periodically.

7) Add 3µl RNase A Solution (from kit) to the cell lysate, invert 25 times and incubate at 37˚C for 15-30 minutes.

8) Cool the sample to room temperature.

9) Add 200µl Protein Precipitation Solution (from kit) to the RNase A treated cell lysate.

10) Vortex at high speed for 20 seconds.

11) Centrifuge at 13000-16000 rpm for 3 minutes. A tight protein pellet should form. If this pellet is not visible repeat step 10, followed by incubation on ice for 5 minutes, than repeat step 11.

12) Pour the supernatant containing the DNA into a 1.5ml centrifuge tube containing 600µl 100% Isopropanol.

13) Invert gently 50 times.

14) Centrifuge at 13000-16000 rpm for 1 minute, the DNA will be visible as a white pellet.

15) Pour off the supernatant and drain the tube on clean absorbent paper.

16) Add 600µl 70% Ethanol and invert the tube to wash the pellet.

17) Centrifuge at 13000-16000 rpm for 1 minute and carefully pour off the ethanol. Pour slowly as the pellet may be loose.

18) Invert and drain the tube on absorbent paper again and allow to air dry for 10-15 minutes.

19) Add 100µl DNA hydration Solution (from kit).

20) Rehydrate the DNA by incubating the sample 1 hour at 65˚C. Tap the tube to aid dispersing the DNA.

21) Store DNA at 4˚C.

Appendix III

Isolation of bacterial symbionts from the haemolymph of larvae infected by EPNs

1) Place 5 instar *G. mellonella* larvae in a Petri dish plate with river sand inoculated with EPN infective juveniles (IJs).

2) At 48 hours post infection, collect infected and dead larvae.

3) Surface sterilize infected *G. mellonella* larvae by spraying with 70% ethanol.

4) Secondary surface sterilization: dip the larvae in 70% ethanol followed by slight heating of the larval surface for 2-3 seconds to avoid heat-killing the bacteria.

5) Cut open or dissect sterilized larvae using sterile scissors and sculpt, working aseptically.

6) Use a syringe to draw the sticky fluid or haemolymph from the cadaver into an Eppendorf tube containing 200µl of nutrient broth or distilled water.

7) Streak on NBTA plate NBTA (nutrient bromothymol triphenyltetrazolium agar) and MacConkey agar and incubate for 48-72 hours at 25ºC.

8) Screen for green colonies in NBTA and red colonies in MacConkey agar plates.

Appendix IV

DNA isolation of bacterial cells associated with EPNs

Bacterial genomic DNA isolated using DNA extraction kit (ZR Fungal/Bacterial DNA Kit, catalog no: D6005)

1) Pick a colony of isolated bacteria from NBTA plate and suspend in a ZR BashingBeadTM Lysis Tube.

2) Secure in bead beater and process at maximum speed for 5 minutes.

3) Centrifuge the ZR BashingBeadTM Lysis Tube in a microcentrifuge at 10 000 x *g* (rpm) for 1 minute.

4) Transfer up to 400µl supernatant to a Zymo-Spin TM IV Spin Filter in a Collection Tube and centrifuge at 7000 rpm for 1 minute.

5) Add 1200µl of Fungal/ Bacterial DNA binding buffer to the filtrate in the Collection Tube from Step 4.

6) Transfer 800µl of the mixture from Step 5 to a Zymo-SpinTM II Column in a Collection Tube and centrifuge at 10000rpm for 1 minute.

7) Discard the flow through from the Collection Tube and Repeat Step 6.

8) Add 200µl DNA Pre-Wash Buffer to the Zymo-SpinTM II Column in a new Collection Tube and centrifuge at 10000rpm for 1 minute.

9) Add 500µl Fungal/Bacterial DNA Wash Buffer to the Zymo-SpinTM II Column and centrifuge at 10000rpm for 1 minute.

10) Transfer the Zymo-SpinTM II Column to a clean 1.5 ml microcentrifuge tube and add 100µl DNA Elution Buffer directly to the column matrix. Centrifuge at 100000rpm for 30 seconds to elute the DNA.

Appendix V

NBTA (adapted from Akhurst, 1980)

1 litre nutrient agar

0.04g triphenyltetrazolium chloride (TTC)

0.025g bromothymol blue (BTB)

Protocol:

1. Mix nutrient agar and BTB.

2. Autoclave at 121˚C and 15 psi for 15 min.

3. Add TTC, just before pouring into Petri dishes, however ensure the autoclaved medium is less than 50˚C. TTC will break down if added when medium is too hot.

4. Swirl to mix.

5. Dispense into sterile Petri dishes and leave to solidify.

3. 0.1% jik solution for infective juvenile sterilization

34ml distilled water

1ml 3.5% jik

Protocol:

1. Autoclave distilled water at 121˚C and 15 psi for 15 min.

2. Mix jik and autoclaved distilled water in bottles.

Appendix VI

Table 1: PCR reaction mixture for the amplification of the nematode associated bacteria 16S rDNA

	Experiment	Negative control
Type of Reagent	Volume	Volume
Master mix	25	25
Bacterial genomic DNA	2	0
Forward primer(EUB968)	3	3
Reverse primer(UNIV1382)	3	3
Nuclease free water	17	19
Total quantity of added reagents	50	50

The amplification cycle was as following

The total number of cycles in the amplification series were 25 with the mentioned steps, in an orderly pattern as described below

- Denaturation at 94°C for 30 seconds
- Annealing at 57°C for 45 seconds
- Extension at 72°C for 900 seconds
- Final extension after cycling: 72°C for 7 minutes

Table 2: PCR reaction mixture for the amplification of the entomopathogenic nematode 18S and 28S rDNA

	Experiment	Negative control
Type of Reagent	Volume	Volume
Master mix	25	25
EPN genomic DNA	3	0
Forward primer(TW81)	3	3
Reverse primer(AB28)	3	3
Nuclease free water	16	19
Total quantity of added reagents	50	50

The amplification cycle was as following

The total number of cycles in the amplification series were 25 with the mentioned steps, in an orderly pattern as described below

- 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

Appendix VII

18S rDNA *Heterorhabditis* sp. sequence

TCACGAGAGATCGGTACCAATGGAATCAGGCTTGTTCTTGATTTCAATCGGTTTCTCACCCCATCT
AAGCTCATGGAGAGGTGTCTAGTCCCAATTGGAGTCGCTTTGAGTGACGGCTATGAAAATTGGGT
ATGTTCCCCGTGAGGGTCGAGCATAGACTTTATGAACAGTGCTGGAGCTGTCGCCTCACCAAAAA
ATCATCGATAACTGGTGGCTATGTGTGACATTAGTCACATAGGTATCTGCTGATGCAGAGAGCCT
TAATGAGTTGTTCGTGTCATCTGACCTACAACCGCCACTATCGGTAAATCAACCCAATTAACTTGT
TTCTTGTGTCGTGTTAATACATACTGGCAAAGTGTATTAGCTTTAGCGATGGATCGGTTGATTCGC
GTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACGCTTAGAGTGGTGAA
GTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTGGCTCAGGGTTGTTTAATAAGC
GAAAGTGTTGAAAGTTCATTAAACGAGAGTTCGGTGATACTGACAACACTGCGTCGATCGGTGTA
CTGTTGAAAGTACCCCGTTCAAGTATCTTTATGGGGCAACATGTCTTCTATACGGAGACATGAAA
GATATTAAGAGTATATACCTGTGGATGCCCACGTATGAAATATGACGTGTCGTATACACGGCTAG

16S rDNA *Photorhabdus* sp. sequence

GCTACCGACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCGTAGCATGCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGC
AGACTCCAATCCGGACTACGACAGACTTTGTGTGTTCCGCTTGCTCTCGCGAGGTCGCTTCACTT
TGTATCCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATC
CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGCCATTACGCGCTGGCAACA
AAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACA
GCCATGCAGCACCTGTCTCTCAGGTCCCGAAGGCACTTCCTTGTCTCCGAGGAATTCTGAGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCG
GG

Appendix VIII

ANOVA statistical analysis

Anova: Single Factor(Infectivity of an EPN formulated in inert carriers)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Vermiculite	6	300	50	2000
Loamy Soil	6	300	50	2360
Sponge Cubes	6	199	33,16667	1570,167

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1133,444444	2	566,7222	0,286698	0,754761	3,68232
Within Groups	29650,83333	15	1976,722			
Total	30784,27778	17				

Anova: Two-Factor Without Replication (Infectivity of EPN formulated in cadavers)

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Time	3	78	26	111
1	3	132	44	363
6	3	300	100	0
			44,3333	2596,33
8	3	133	3	3
24	3	66	22	1452
temp	5	281	56,2	1066,2
	5	158	31,6	1680,3
	5	270	54	1969,5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	11672,27	4	2918,067	3,246023	0,073411	3,837853
Columns	1852,933	2	926,4667	1,030591	0,399728	4,458971
Error	7191,733	8	898,9667			
Total	20716,93	14				

Anova: Two-Factor Without Replication(Desiccation studies)

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
100	3	180	60	2800
100	3	180	60	2800
20	3	220	73,33333	933,3333
80	3	280	93,33333	133,3333
100	3	200	66,66667	3333,333
40	3	120	40	1600
60	3	220	73,33333	133,3333
80	3	300	100	0
100	3	80	26,66667	533,3333
100	3	280	93,33333	133,3333
0	3	140	46,66667	933,3333
100	3	300	100	0
80	3	140	46,66667	1733,333
20	3	220	73,33333	533,3333
80	3	180	60	400
80	3	280	93,33333	133,3333
100	3	160	53,33333	2533,333
100	3	260	86,66667	533,3333
80	3	240	80	400
100	3	300	100	0
48	20	1660	83	601,0526
72	20	1340	67	1022,105
96	20	1280	64	1667,368

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	27493,33	19	1447,018	1,569852	0,116414	1,867332
Columns	4173,333	2	2086,667	2,263799	0,117799	3,244818
Error	35026,67	38	921,7544			
Total	66693,33	59				

Anova: Single Factor (Rate of Infective Juveniles Emergence Test)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Loam soil	6	100	16,66667	1026,667
Sand	6	100	16,66667	1026,667
Vermiculite ijs	10	100	10	1000
Loam and Vermiculite	7	100	14,28571	495,2381
Sand and Vermiculite	7	100	14,28571	361,9048
verm cadavers	7	100	14,28571	628,5714
Sponge	10	33	3,3	108,9
Loam	10	100	10	1000

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1199,551	7	171,3644	0,246981	0,971081	2,181333
Within Groups	38161,05	55	693,8373			
Total	39360,6	62				