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THE IMPACT OF ORGANIC COMPOST ON THE INFECTIVITY AND VIRULENCE OF ENTOMOPATHOGENIC NEMATODES AND THEIR BACTERIAL SYMBIONTS AS BIOCONTROL AGENTS

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Declaration

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Abstract

A safer and greener alternative to chemical pesticides includes the use of entomopathogenic nematodes (EPNs) and their endosymbiotic bacterial pathogens as biocontrol agents. Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* cause disease in insect pests, killing them within 48 hours, with the help of their highly virulent insect pathogenic bacterial symbionts belonging to the genera *Xenorhabdus* and *Photorhabdus,* respectively. The efficacy of EPNs in the field is affected by abiotic and biotic factors, thus understanding the soil ecosystem and its interaction with EPNs will aid in improving the application of EPNs in many agricultural soils post application. The research project focused on isolating and identifying locally adapted EPNs and their bacterial symbiont isolated from the North West province in the Magaliesburg region. Investigating factors affecting the survival, virulence, and persistence of EPNs in the soil, by the use of adding soil amendments such as organic compost namely mulch, organic compost, professional potting mix, organic lawn dressing mixed with loamy soil and coarse river in soils undergoing progressive dehydration. The effects of different soil composition on the behaviour and host-seeking efficiency within the soil profile and EPN response to desiccation tolerance was also investigated. EPNs were retrieved and harvested using the soil baiting technique with *Galleria mellonella* and White trap method respectively. PCR amplification of the 18S rDNA and 16S rDNA sequencing of the PCR amplicon was used to establish the phylogenetic affinities of EPN isolates and their bacterial symbiont using GenBank sequence database respectively. The presumptive *Heterorhabditis* species and *Steinernema* species isolated were found to show high affinity to *H. bacteriophora* (MG334257.1) and *S. khoisanae* (MH697401.1) respectively. The symbiont bacteria only isolated of *H. bacteriophora* was identified as *Photorhabdus luminescence* subsp. *laumondi* (KT963833.1). The use of organic compost did not have any detrimental effect on EPNs. The Mulch soil amendment resulted in the highest insect mortality rate across different application concentration (mass: mass basis) when mixed with loamy soil. It was observed that virulence and efficacy decreased over 7 days of progressive dehydration. In soil profile larvae locating behavioural studies, *H. bacteriophora* was found to be a cruise predator (search and forage) in search of a new host. Soil composition affected host locating ability of *H. bacteriophora* infective juveniles (IJs). *H. bacteriophora* IJs were able to survive desiccation tolerance with resuscitation by rehydration with water for a period of 8 weeks however infectivity and survival of IJs to locate the host were affected. Overall the type of compost used based on its feedstock

composition, the concentration of nutrients and organic matter mixed with a specific type of soil texture had a significant effect on the infectivity and survival of *H. bacteriophora* IJs in the soil. In conclusion soil physical structures affects EPNs infectivity post application and the knowledge obtained will be used to improve soil application strategies and formulation products so that EPNs can be used as commercially viable biocontrol agents.

Dedications

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This work is dedicated to my beloved late mother Zamantungwa Khumalo (1972 – 2007). Truly a blessing and forever loved and remembered, your spirit lives on. Special gratitude to my grandmother Nonhlanhla Hlatshwayo for the love, support throughout my academic career.

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Research Output

Conference 1: 9th University of Witwatersrand Cross Faculty Postgraduate Symposium (2018)

Output: 2nd Price for best flash talk and poster presentation cross-faculty

Poster title: The effect of using organic compost on the infectivity and survivorship of entomopathogenic nematodes as biocontrol agent

Authors: Khumalo N, Lephoto T and Gray V

Conference 2: Molecular Bioscience Research Thrust (MBRT) research day at the University of Witwatersrand (2018)

Output: Poster presentation

Poster title: The effect of using organic compost on the infectivity and survivorship of entomopathogenic nematodes as biocontrol agent

Authors: Khumalo N, Lephoto T and Gray V

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BLAST- Basic Local Alignment Search Tool

EPNs - Entomopathogenic Nematodes

ITS – Internal Transcribed Spacer

rDNA – Ribosomal Deoxyribonucleic acid

NBTA – Nutrient Bromothymol blue-triphenyl tetrazolium chloride- Agar

LS – Loamy Soil

RS- Coarse Riversand

TTC – Triphenyltetrazolium chloride

PCR – Polymerase Chain Reaction

PPM – Professional Potting Mix

OLD – Organic Lawn Dressing

OC – Organic Compost

IJs – Infective Juveniles

IPM – Integrated Pest Management

Chapter 1: Literature review

1.1Introduction

1.1.1 The use of chemical pesticides

The agricultural sector is one of the largest consumers of pesticides making up 85% of the world production. Pesticides are used as a quick, easy and inexpensive method to improve the quality of agricultural and horticultural crops, protection of crop losses, yield reduction against weeds, diseases and insect pest which have provided economic and labour benefits over the years (Aktar *et al.* 2009; Mostafalou and Abdollahi 2013; Kim *et al.* 2017). However, due to the major demand for food as the population increases, food security remaining high due to the unemployment rate and means of increasing supply and income in the agricultural sector, farmers have resorted to misusing chemical pesticides and fertilisers to mitigate the problem (Aktar *et al.* 2009; Mostafalou and Abdollahi 2013). However, over the past years, the extensive use of pesticides has caused target pest to develop resistance, resulted in pest outbreaks, affected non-target organisms and detrimental effects on the environment leading to contamination of water, soil, air. Lastly, health risk associated with animals and humans noticeable in Asia and Africa (Tisdell and Wilson 2001; Aktar *et al*. 2009; Mostafalou and Abdollahi 2013; Fan *et al.* 2015; Kim *et al*. 2017).

The most exposed group to suffer from mild poisoning of pesticides such as headaches, nausea, vomiting, and irritation of skin and eyes has been production workers, since they handle various toxic, raw materials and agricultural workers from developing countries in rural areas (Aktar *et al*. 2009). The most exposed group to suffer from mild poisoning of pesticides are from rural areas in most developing countries. The symptoms that have been noticeable include headaches, nausea, vomiting and irritation of the skin and eyes from production workers since they handle various toxic raw material and agricultural workers (Aktar *et al.* 2009). Mild poisoning has resulted in approximately 180000 mortalities among agricultural workers annually (Fan *et al*. 2015).

Long term health effects include developing neurodegenerative diseases such as Parkinson, Alzheimer, human chronic diseases including cancer, kidney, cardiovascular, reproductive and developmental toxicity affecting 3 million farmers annually (Aktar *et al.* 2009; Mostafalou and Abdollahi 2013; Kim *et al.* 2017). Due to incorrect perception, lack of knowledge (retailers providing little information about the proper usage of the products), regulation and education among farmers, farmers still continue to use pesticides despite these adverse effects (Tisdell and Wilson 2001; Fan *et al.* 2015; Kim *et al*. 2017)

1.1.2 Biocontrol Agents

A safer and eco-friendly alternative to chemical pesticides includes the use of natural biocontrol agents (Sharma *et al.* 2009; Kim *et al*. 2017). Biological control is a strategy or an approach which uses natural biological organism (microbial biopeptides) such as entomopathogenic fungi, bacteria, fungi, nematodes, viruses and protozoa, to eradicate specific insect pests causing detrimental effects in agricultural crops (Lacey *et al.* 2001; Thakore 2006; Sharma *et al*. 2009; Lacey *et al*. 2015; Kim *et al.* 2017; Hatting *et al.* 2018). On a global scale, microbial pesticides only account for 1-2% of all the pesticides sold of which *Bacillus thuringiensis* has been the most widely used and applied biocontrol agent to date (Ehlers 1996; Lacey *et al*. 2001; Thakore 2006; Lacey *et al.* 2015).

Biocontrol agents have gained their popularity due to the modes of action they take to cause infection (Sharma *et al*. 2009). To successfully suppress the activity of many pathogens, biocontrol agents produce antibiotics and lytic enzymes responsible for cell wall degradation of the pathogen, induce resistance, grow faster than the pathogen and have the ability to survive under conditions that are unfavourable to the pathogen (Sharma *et al.* 2009). Biocontrol agents are safer to human and non-target organisms, specific to target insects (Lacey *et al.* 2001), can be easily mass produced, compatible with existing chemical pesticides and results in less pesticide residue in food (Ehlers 1996; Hatting *et al.* 2018).

On the contrary, entomopathogenic nematodes (EPNs) have received the most attention because they possess many attributes of effective biological control agents of both insect predator and microbial pathogens (Kaya and Gaugler 1993; Kaya *et al.* 2006). EPNs have been used to control insect pests in high-value crops since the early 1930s (Kaya *et al.* 2006; Lacey *et al*. 2015; Hatting *et al.* 2018). EPNs of the families Steinernematidae and

Heterorhabditidae of the order Rhabditida share an obligate mutualistic relationship with members of the Enterobacteriaceae family (Kaya and Stock 1997; Lacey and Georgis 2012). EPNs can be potentially used in large scale integrated pest management (IPM), sustainable agriculture systems and organic farming (Neves *et al.* 2001) to control soil-borne insects pest causing serious damages to agricultural crops (Ehlers 1996; Thakore 2006; Shapiro-Ilan *et al.* 2006; Fan *et al*. 2015).

1.2 Entomopathogenic Nematodes

Entomopathogenic nematodes are non-segmented, colourless roundworms which lack circular and respiratory systems (Gaugler 2002), the only nematodes which have attained the ability to carry and introduce their symbiotic bacteria into the body cavity of insects (Boemare *et al*. 1996; Gaugler 2002; Lewis *et al*. 2006). EPNs of the genera *Heterorhabditis* and *Steinernema* are obligate parasites of insect pests which kill their host by the help of their highly virulent symbiotic bacteria *Photorhabdus* and *Xenorhabdus* respectively (Ehlers 1996; Shapiro-Ilan *et al*. 2006; Grewal *et al.* 2006; Malan *et al.* 2006; Dillman *et al.* 2012). EPNs have been isolated from various insects and soil habitats worldwide and to date, one-hundred and twelve species of EPN have been described of which ninety-five species belong to *Steinernematids*, sixteen belonging to *Heterorhabditis* and one species of *Neosteinernema* (Hunt 2016). A recently described nematode species that have shown to use bacteria to parasitize insect host include the genus *Oscheius* with *Serratia* as its symbiotic bacteria (Zhang *et al.* 2009; Dillman *et al.* 2012). The genera include O*scheius chongimingensis* (Liu *et al*. 2012) and *Oscheius carolinensis* (Torres-Barragan *et al.* 2011) with its symbiont bacteria *Serratia* shares similar characteristics with the two known EPN species (Dillman *et al.* 2012).

1.2.1 The life cycle of entomopathogenic nematodes

EPNs exist as free non-feeding Infective Juveniles (IJs), the only 3rd stage in the life cycle (Liu and Glazer 2000) that exist outside the host, persist in the soil for longer periods, seek and kill the host very quickly (Kaya and Gaugler 1993; Gruner *et al.* 2007; Dillman and Sternberg 2012). To infect the insect host, IJs first exhibit different types of foraging behaviour (cruiser or ambusher) to find a suitable host followed by penetration into the host haemocoel through natural openings (Kaya and Gaugler 1993; Gaugler 2002; Lacey *et al.*

2015). IJs of *Steinernema* enter the insect host through the respiratory spiracle, the anus or mouth then travel to the haemocoel (Bird and Akhurst 1983), while *Heterorhabditis* IJs have a tooth-like appendage near the mouth that helps to penetrate the cuticle through the host integuments (Kaya and Gaugler 1993; Forst and Clarke 2002; Gaugler 2002; Adams *et al*. 2006). The nematodes then invade the potential hosts and release their pathogenic bacteria into the nutrient-rich haemolymph (Dillman and Sternberg 2012). *Steinernema* IJs and *Heterorhabditis* IJs carry their symbiotic bacteria in specialised intestinal vesicles and in the midgut of the intestine respectively (Thomas and Poinar 1979; Boemare *et al.* 1996; Gaugler 2002).

The bacterial proliferate and suppress the host immune system, thus killing the host via septicaemia and the insect dies within 24-48 h (Kaya and Gaugler 1993; Neves *et al.* 2001; Dillman *et al.* 2012). The nematode then feeds on the bacterial symbiont and host tissues until nutrients are depleted (Dillman and Sternberg 2012; Lacey *et al*. 2015). Nematodes then develop into juvenile stages and reproduce to 1-3 generations (Kaya and Gaugler 1993). The 1st generation adults of *Heterorhabditis* develop to self-fertilising hermaphrodites, while the 1st generation of *Steinernema* develops into amphimictic males and females (Ehlers 1996; Lewis and Gaugler 1994; Forst and Clarke 2002; Gaugler 2002). A new generation of IJs then emerges into the environment from the dead host in search of a new host to infect (Neves *et al*. 2001).

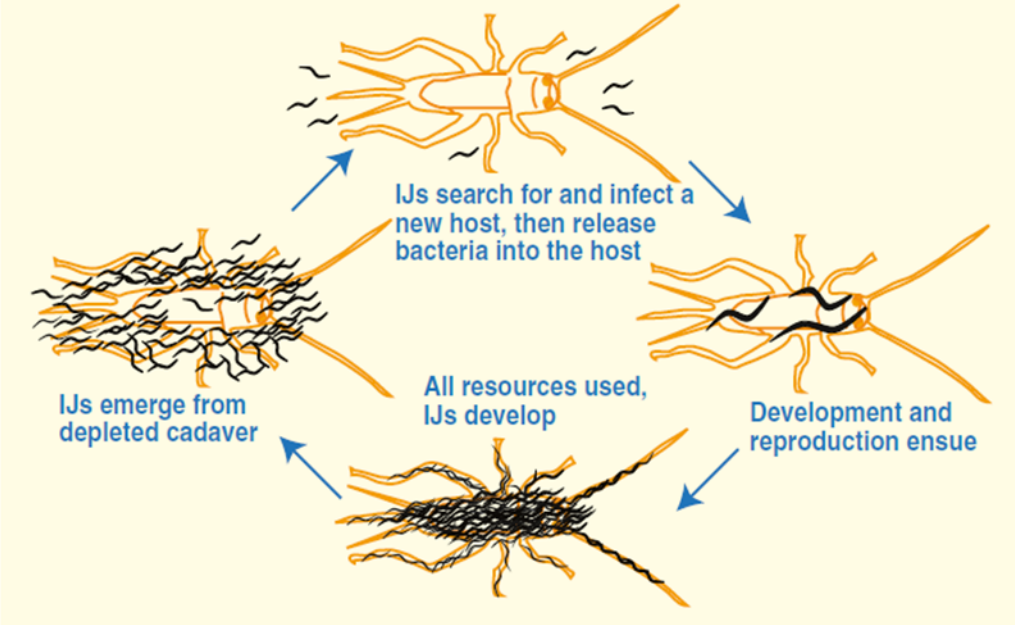

Figure 1. 1 Shows the general life cycle of entomopathogenic nematodes (EPNs). Adapted from Dillman and Sternberg 1992.

1.2.2 Mutualistic relationship of EPNs and their bacterial symbionts

The mutualistic relationship formed between EPNs and their bacterial symbionts begins when the nematodes act as an insect locating vector, providing protection from the external environment in the soil (Forst and Clarke 2002; Ferreira *et al.* 2016). Nematodes also inhibit host antibacterial proteins and transport the bacteria to the nutrient-rich haemolymph of the insect resulting in the death of the host (Kaya and Gaugler 1993; Gaugler 2002; Forst and Clarke 2002). In turn, the bacteria symbiont produces toxic secondary metabolites that not only are lethal to the insect host but also prevent opportunistic bacteria and fungi from utilizing the nutrient-rich cadaver (Boemare *et al.* 1996; Forst and Nealson 1996; Forst and Clarke 2002; Dillman and Sternberg 2012). The bacterial symbiont produces virulence factors such as degradative enzymes including lipases, phospholipids, proteases, lipopolysaccharides and toxins that suppress the host immune system and break down the insect tissue and thereby providing a rich nutrient supply to the developing nematodes (Kaya and Gaugler 1993; Forst and Nealson 1996; Forst and Clarke 2002; Webster *et al.* 2002).

During late infection, *Xenorhabdus* and *Photorhabdus* produce antimicrobial compounds (Webster *et al.* 2002) such as antibiotics that are active against a variety of microbial species (Paul *et al.* 1981; Gaugler 2002; Adams *et al.* 2006). The antibiotics produced inhibit the

growth of other organisms inside the host cadaver and provide protection against completing fungal, bacterial and yeast organisms (Forst and Nealson 1996; Forst and Clarke 2002). Antibiotics produced by the bacterial symbiont include bacteriocins such as xenorhabdins, xenocoumacins, hydroxystilbenes and lumicins of which many have gained interest in pharmaceutical and agricultural sectors (Paul *et al*. 1981; Forst and Clarke 2002; Webster *et al.* 2002; Adams *et al.* 2006). Bacteriocins prevent the growth of species or strains of closely related *Xenorhabdus* and *Photorhabdus* spp. (Webster *et al.* 2002). Lastly, the bacteria then supply nutrients to the nematode important for growth and development (Adams *et al*. 2006).

1.2.3 Symbiotic bacteria

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are associated with EPNs of the genera *Steinernema* and *Heterorhabditis.* The bacteria symbionts belongs to the family Enterobacteriaceae, members of the γ-Proteobacteria which are characterized by gram-negative, facultative anaerobes, negative for oxidase and non-spore forming (Thomas and Poinar 1979; Forst and Clarke 2002; Gaugler 2002; Adams *et al* 2006). To distinguish between the two bacteria symbionts, *Photorhabdus* spp. are capable of bioluminescence and positive for catalase, while *Xenorhabdus* spp. are negative for both traits however both bacteria are negative for nitrate reductase, which is a major positive characteristic of Enterobacteriaceae (Boemare *et al*. 1996; Forst and Clarke 2002; Gaugler 2002). Both bacterial species occupy similar habitats however the difference is based on their interactions with the nematode (Boemare *et al.* 1996). Both symbionts produce phenotypic variants forms, Phase 1 and Phase 2 variants which both differ in dye adsorption, response to biochemical test and antibiotic production (Forst and Nealson 1996). The primary Phase 1 cells are characterized by the presence of numerous phenotypic traits, providing essential nutrients and antimicrobial agents responsible for the death of the host (Boemare *et al.* 1996; Gaugler 2002; Forst and Clarke 2002).

1.3 Behavioural ecology of EPNs

Entomopathogenic nematodes exhibit different foraging strategies (cruiser and ambusher) to increase their chances of encountering and recognising a suitable host (Kaya and Gaugler 1993). Ambusher foragers such as most *Steinernema* spp. search for the host at or near the

soil surfaces. Ambushers take an energy conserving tactic remaining nearly sedentary, while waiting for a mobile surface insects such as mole crickets in cryptic and soil surface environment (Lewis *et al*. 1992; Kaya and Gaugler 1993; Campbell *et al.* 2003; Shapiro-Ilan *et al*. 2006; Divya and Sankar 2009; Lacey and Georgis 2012). Ambush foragers exhibit standing (nictation) and jumping behaviour to attach to a mobile host. When standing, nematodes become straight and immobile and can maintain this posture with interspersed periods of waving for an extended period of time (Campbell and Kaya 2002). Nictation allows ambushers to reduce the surface tension holding the nematode to the insect (Campbell and Gaugler 1993) and to scan the soil environment for host associate cues thereby increasing their chances of encountering a host (Campbell and Kaya 2002; Campbell *et al*. 2003; Lewis *et al*. 2006).

Cruise foragers which include most *Heterorhabditis* spp. prefer to search deeper in the soil profiles to locate a suitable host (Campbell *et al*. 2003; Lewis *et al.* 2006). Cruisers are very mobile and respond strongly to chemo attractants released by the host and are better adapted to infect sedentary subterranean hosts at certain above ground habitats (foliar and epigeal) (Lewis *et al.* 1992; Kaya and Gaugler 1993; Campbell *et al*. 2003; Lacey and Georgis 2012). Cruise foraging hardly ever exhibits standing behaviour, however, spend most of their time crawling on the insect (Lewis *et al.* 2006). Intermediate foragers such as *S. riobrave* use both characteristics of being an ambusher and cruiser. They are attracted to host volatile cues but also exhibit standing and jumping behaviour to attach to a suitable host (Campbell *et al*. 2003; Lewis *et al*. 2006).

1.4 EPNs as Biocontrol agents

The main advantages of using EPNs as biological control in agricultural soil is that they are effective at identifying, infecting and killing the insect host within 48 h (Kaya and Gaugler 1993 and Boemare *et al*. 1996). Nematodes have a broad host range infecting the majority of insect orders and families, *Steinernema carpocapsae* was found to infect more than 250 species of insect from over 75 families in 11 orders (Boemare *et al.* 1996; Kaya and Gaugler 1993; Divya and Sankar 2009; Lacey and Georgis 2012). Nematodes are environmentally friendly, have no negative effects on humans, plants, mammals and non-target organisms and can persist in the field for about 2-3 weeks (Kaya and Gaugler 1993; Boemare *et al*. 1996; Lacey and Georgis 2012; Hatting *et al*. 2018). Nematodes are They easily mass produced on huge scales using artificial solid and liquid media and are compatible with a wide range of

biological pesticides and fertilisers used in many IPM practices (Boemare *et al.* 1996; Grewal 2000a). Nematodes can survive longer periods of desiccation for many years and can be applied using conventional sprayers however it's very expensive to produce EPNs compared to chemical pesticides (Lacey and Georgis 2012).

There are more than 12 companies found in Asia (China, India), North America and Europe that currently manufacturers EPNs (Kaya *et al*. 2006) and of to date more than 13 different species have been commercialised (Kaya *et al.* 2006; Lacey *et al*. 2015). This includes *Heterorhabditis bacteriophora, Heterorhabditis indica, Heterorhabditis megidis, Heterorhabditis zealandica, Heterorhabditis marelata, Steinernema carpocapsae, Steinernema krausseii, Steinernema glaseri, Steinernema longicaundum, Steinernema feltiae, Steinernema scapterisci, Steinernema kushidae,* and *Steinernema riobrave* (Kaya *et al*. 2006; Lacey *et al.* 2015). Furthermore, EPNs have caused significant field reduction of about 75-100% on the Mediterranean flat-headed root, borer *Capnodis tenebrionis* (L) (Coleoptera: Buprestidae) and the peach tree borer *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae), these are root boring pest of stone fruits (Lacey *et al*. 2015). In China, *S. carpocapsae* species are used to control the peach fruit moth *Carposina niponensis* Walsingham (Lepidoptera: Carposinidae) serious pest of apples (Kaya *et al.* 2006) and so far, *S. carpocapsae* is the most applied species for control of foliar and above ground pests.

In the African continent, only a few countries such as Kenya, Egypt, Ethiopia and South Africa (SA) have exploited the use of EPNs as biocontrol agents (Kaya *et al*. 2006; Malan *et al.* 2006). New species of EPNs described in Africa as endemic include *Steinernema karii* and *Heterorhabditis taysaerae* isolated in Kenya and Egypt respectively and 9 species have been isolated in SA (Malan *et al.* 2011; Kaya *et al*. 2006). In Namibia, various trains of *S. carpocapsae* and *H. bacteriophora* have been used to target different developmental stages of African ticks (Kaya *et al*. 2006). Currently, research in Africa is mainly based on the efficacy of EPNs as investigated in laboratory or field studies, to date, there are no reports on the commercialization of EPNs as biocontrol insect pest products in the African continent (Kaya *et al*. 2006; Hatting *et al*. 2018).

1.5 Factors affecting the efficacy of EPNs in the field

The efficacy of EPNs in agricultural soils is depended on the ability of the IJs to disperse in the environment and persist until a suitable host is found (Koppenhöfer and Fuzy 2007). Numerous environmental factors such as temperature, desiccation, moisture, soil type (small pore size), ultraviolet light (UV), plant roots and biotic factors all affect the survival and persistence of EPNs in many agricultural soils (Gaugler *et al.* 1992; Kaya and Gaugler 1993; Koppenhöfer and Fuzy 2007; Susurluk and Ehlers 2008; Lacey and Georgis 2012; Lacey *et al.* 2015).

1.5.1 Desiccation tolerance

Anhydrobiosis is a process that occurs naturally in EPNs as well as other invertebrates and is used as a strategy to survive extended periods of soil dehydration (Womersley 1990; Grewal 2000a; 2000b; 2002). True anhydrobiosis can lose up to 95-98% of their body water and as dehydration continues (Liu and Glazer 2000; Grewal 2002), they lower their metabolism to levels that are not detectable thus entering a state of cryptobiosis (Womersely 1990; Grewal 2000a; 2000b). Such species can survive cryptobiosis anhydrobiosis for longer period of time under unfavourable circumstances, however (Womersley 1990; Grewal *et al*. 2006), following seasonal rehydration of the soil by the availability of water, they able to recover and resume their metabolic process. Conditions that may be unfavourable for EPNs include extreme temperature, absence of water, lack of oxygen and osmotic stress (Gaugler 2002; Grewal *et al*. 2006). Thereby to achieve successful anhydrobiosis, the target organisms can be dried at a rate that simulates the drying conditions of its natural environment (Womersley 1990; Gaugler 2002).

On the contrary, EPNs are aerobic, they require a water film for effective movement thus they are not are true anhydrobiosis since they cannot survive or persist in the complete absence of water cause their survival and mortality is greatly affected (Kaya and Gaugler 1993; Glazer 1996; Grewal *et al*. 2006). To survive periods of host absence and desiccation, EPNs enter a state of partial anhydrobiosis (Womersley and Smith 1981; Liu and Glazer 2000; Grewal 2000a; 2000b; Koppenhöfer and Fuzy 2007). At this state, EPNs are able to

survive and persist longer at ambient temperatures, they are resistance to environmental extremes, radiation, hypoxia and metabolic poisons lethal to active organism (Liu and Glazer 2000; Grewal 2000a). Desiccation tolerance of EPNs is an important factor affecting commercialization and can be used to improve storage stability and increased shelf life (Grewal 2000b).

The physiological mechanisms involved in desiccation tolerance of nematodes is not fully understood (Glazer 1996; Grewal *et al*. 2006). It has been found that nematodes accumulate sugars and polyols such as alcohol, which act as protectants of intracellular proteins and biological membranes during dehydration (Womersley and Smith 1981; Gaugler 2002; Grewal *et al.* 2006). Also, it has been demonstrated that following a slow water loss, levels of trehalose and glycerol increased significantly while glycogen and lipids levels decreased (Glazer 1996; Grewal *et al*. 2006). The rate-limiting enzyme in the synthesis of glycogen, glycogen synthase (Sf-gsy-1), was found to play a significant role in the survival of EPNs during desiccation (Grewal *et al.* 2006).

1.5.2 Soil ecosystem

The soil environment shields nematodes from environmental factors, which greatly enhances their survival and persistence in the soil (Kaya and Gaugler 1993). Clay soils which have small pores and contain high organic matter results in oxygen deprivation which restrict the movement of EPNs in the soil and reduce their survival and persistence (Barbercheck 1992; Kaya and Gaugler 1993; Glazer 1996; Shapiro-Ilan *et al*. 2006). EPNs are exposed to various soil organisms including potentially antagonistic bacteria, fungi, predators and invertebrates in agricultural soils (Glazer 1996; Shapiro-Ilan *et al*. 2006). *Steinernema* and *Heterorhabditis* can survive exposure to various kinds of chemicals pesticides and fertilisers however IJs are highly susceptible to nematicides which are likely to be found in agroecosystems (Glazer 1996; Shapiro-Ilan *et al*. 2006).

1.5.3 Temperature and pH

The pH of the soil is likely to affect most nematodes. Soil pH ranging from 4-8 does not have any negative effects on nematodes, however, a pH ranging from 10 and above is likely to have a negative effect on EPNs (Shapiro-Ilan *et al.* 2006). Most arid and semiarid regions are

characterized by alkaline soils while acids soils are moistly found in humid regions (Barbercheck 1992). Temperature range for survival and infectivity of EPNs is always depended on the type of nematode species and where it was isolated (native habitat) (Shapiro-Ilan *et al.* 2006; Lacey and Georgis 2012). Temperatures above 40˚C and below 0˚C are lethal to EPNs but temperature ranging from 15-25˚C increases the rate of metabolism and shorten the life span of EPNs which reduces their efficacy (Susurluk and Ehlers 2008). Most *Heterorhabditis* spp. can infect the host at temperatures ranging from 7-35˚C (Lacey and Georgis 2012). EPNs that are heat tolerance and can maintain efficacy in temperatures 29˚C and above includes *H. indica*, and *S. glaseri* and cold tolerant EPNs such as *H. megidis* and *S. feltiae* are effective at temperatures 15˚C and below (Shapiro-Ilan *et al*. 2006). UV radiation studies concluded by Gaugler *et al.* 1992 found out that 60 min of exposure to direct sunlight would inactive *S. carpocapsae* and *H. bacteriophora* will takes 20 min of direct sunlight to be inactivated (Gaugler *et al*. 1992).

1.6 Methods of improving the efficacy of EPNs in the field

EPNs have developed survival mechanisms to overcome unfavourable conditions in many agricultural soils, which plays an essential role in their ability to persist and infect in the soil (Glazer 1996). EPNs have been isolated from cold regions (sub-zero temperature), in temperate, Arctic and Sub-Artic regions and are able to survive such climate conditions through supercooling and freezing (Glazer 1996; 2002). New *Heterorhabditis* species isolated from the arid region of Israel have shown tolerance to heat due to the presence of heat shock proteins which plays an important role in the survival of many organisms found in high temperatures (Glazer 1996; 2002).

Nematode host range, pathogenicity for the targeted insect and foraging strategy are all determining factors to successful parasitism. Persistence in the field can be improved by matching nematode species or strains against those insects for which they are best adapted to infect and kill (Kaya and Gaugler 1993; Susurluk and Enhlers 2008). To improve efficacy, EPNs should be applied at first sign of infection at the target site in the morning and evenings since nematodes are highly sensitive to UV radiation and desiccation (Gaugler *et al.* 1992; Ehlers 1996; Shapiro-Ilan *et al*. 2006; Divya and Sankar 2009; Sharma *et al.* 2009; Lacey and Georgis 2012; Lacey *et al*. 2015).

Isolating species or strains of nematodes that can infect and persist over a wide range of soil moisture conditions is crucial (Koppenhöfer and Fuzy 2007). To achieve optimum results when using nematodes in the field, the temperature should neither be extremely cold or hot, relative humidity should be high, soil temperature should be between 10-35˚C, with minimal direct sunlight can help increase survival and virulence (Divya and Sankar 2009). EPNs are generally applied at a rate of 25IJs/cm^2 in soils ecosystems and are capable of remaining effective in the soil for more than 8 weeks however re-application of nematodes is important in many cropping systems (Kaya and Gaugler 1993; Shapiro-Ilan *et al*. 2006). Also, irrigation is important in maintaining soil moisture and promoting the existence of nematodes in the soil sub-surfaces (Shapiro-Ilan *et al.* 2006).

1.7 Study Rationale

SA has become one of the biggest importers of chemical pesticides in sub-Saharan Africa and this is due to the fact that more than 700 insect pests have caused serious damages to agricultural crops (Hatting *et al.* 2018; Agri-Intel 2018). More than 500 pesticides have been registered under the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 36 of 1947 (Hatting *et al.* 2018), of which 31 biopesticides products are locally manufactured. *Bacillus thuringiensis* (Bt) (Bacillales: Bacillaceae) based products were among the 1st biological insecticides to be produced in SA (Hatting *et al.* 2018; Agri-Intel 2018). However, over the years the SA department of Agriculture, forestry, and fisheries (DAFF) has prohibited the import of numerous biopesticides since the late 1970s, thus the use of biological pest control in organic agriculture has sparked an interest in the scientific community (Malan *et al.* 2006; 2011; Hatting *et al.* 2018). The need to find new biological control agents within our agricultural systems, that protects and merges the biodiversity and can possibly benefit the agricultural sector and the environment would include the use of EPNs (Malan *et al.* 2006).

Famers are now under a lot of pressure to reduce the use of chemical pesticide and implement sustainable crop protection practices so to achieve optimal crop yield and quality due to increasing human population (Litterick *et al.* 2004). This can be done through organic agriculture, which requires knowledge about the complexity of the agroecosystem (Litterick *et al*. 2004). Entomopathogenic related research and bioproduct development have increased

over the past, showing effective against SA insect pests under laboratory conditions. However, the only nematode based product registered in SA is an imported formulation Cryptonem^{TM} based on the exploitation of *H. bacteriophora*. Cryptonem^{TM} is a wettable power formulation that is used to control insects of the false codling moth, codling moth and banded fruit weevil affecting agricultural crops such as citrus, apples, and strawberries respectively (Hatting *et al.* 2018).

Understanding nematode biology such as EPN foraging behaviour or insect host-seeking behaviour in agricultural soils, how EPNs respond to desiccation by artificially inducing partial anhydrobiosis to identify factors that enable EPNs to survive during dehydration conditions and understanding how EPNs exist and interact with a variety of organisms in agricultural soil is crucial. Such factors will aid in understanding the parasitism and persistence of EPNs in the soil crucial for successful application as biocontrol agents and improving soil application and formulation strategies necessary for ensuring good storage and long shelf life.

Eventually, EPNs can be used as commercially viable biocontrol agents for the 1st time in SA. This can help commercial and subsistence farmers to improve the quality of crops produced, over time generates more money and creates employment in many disadvantaged communities. Therefore, the need to isolate and identify endemic species of EPNs found in SA and their bacterial symbionts with better efficiency against specific target insects and better adapted to local climate is of importance. Thus, this study aims at understanding the effect of adding soil amendments such as organic compost on the infectivity, survivorship and host location of EPNs in the soil.

1.8 Aims and Objectives

1.8.1 Aim 1: Isolation and identification of EPNs collected from soil samples

Objectives:

- Collection of soil samples in the North West province
- Isolation of EPNs from soil using the baiting techniques

- Harvest IJs using the modified White trap method and reinfections for confirmation by Koch's postulate
- Molecular characterization based on the 18s rDNA
- Establish the phylogenetic affinities using NCBI BLAST algorithm, Finch TV and MEGA 7

1.8.2 Aim 2: Isolation and identification of the symbiont bacteria associated with EPNs

Objectives:

- Isolation the bacteria symbiont by targeting the endotokia matricida stage
- Culture of Phase 1 phenotypic variants of the bacteria through using selective media and confirmation studies through lipid agar studies
- Molecular characterization based on the 16s rDNA
- Establish the phylogenetic affinities using NCBI BLAST algorithm, Finch TV and MEGA 7

1.8.3 Aim 3: To investigate factors affecting the efficacy of EPNs in agricultural soils

Objectives:

- To investigate the use of organic compost on the infectivity, survival, and virulence in the soil undergoing progressive dehydration through Petri dish assay
- To test for factors that infect nematode infectivity in host location profiles using the sand column assay in amended soils
- To test for desiccation tolerance of EPNs

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Chapter 2: Isolation and identification of entomopathogenic nematode

2.1 Introduction

2.1.1 The use of EPNs as biocontrol in SA

South African insect pests such as the codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae), the vine mealybugs *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), false codling moth (L) *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera Tortricidae) and sugar cane stalk borer (*Eldana saccharina* Walker) (Lepidoptera: Pyralidae) (Malan *et al*. 2011; De Waal *et al*. 2010; Dayne and Vieux 2013), have caused substantial financial loses in major export-based industries such as in citrus and grape production. These sectors represent huge investments in foreign exchange earnings and employment opportunities especially in rural areas (CGA 2010). Agricultural crops that have been affected include pome fruits, citrus, grapes, and sugar cane and the majority of these crops are planted in Limpopo, Western Cape, Eastern Cape, Northern Cape, Mpumalanga, Northern Cape and Free State (CGA 2010).

The use of locally adapted EPNs as biocontrol agents have shown effectiveness against SA insect pests, under laboratory conditions, however, EPNs are not yet available as commercially formulated biological pest control products in South Africa (Malan *et al*. 2011). Due to strict South African regulation concerning the importation of commercially available exotic EPNs (Amendment of Act 28 of 1989 under Agricultural Pest Act 36 of 1947) (Malan *et al.* 2011), since the introduction of exotic EPNs may displace native nematodes, affect non-target organisms and not adapted to our local climate conditions (Grewal *et al.* 2001; Hazir *et al*. 2003; Ehlers 2005; Malan *et al*. 2006; 2011). Hence, the need to find indigenous ENPs with better efficiency against specific target insects, better adapted to local climate conditions is crucial (Grewal *et al*. 2001; Dolinski *et al*. 2008; Nikdel and Niknam 2015).

Currently, twelve EPNs have been described in SA of which nine are indigenous (Malan *et al*. 2016). Two belongs to Heterorhabditidae family and include *Heterorhabditis safricana* (Malan *et al*. 2008) and *Heterorhabditis. noenieputensis* (Malan *et al*. 2014). The remaining

seven Steinernematidae include *Steinernema khoisanae* (Nguyen *et al*. 2006), *Steinernema citrae* (Stokwe *et al*. 2011), *Steinernema sacchari* (Nthenga *et al.* 2014), *Steinernema tophus* (Çimen *et al* 2014), *Steinernema innovationi* (Çimen *et al*. 2015), *Steinernema jeffreyense* (Malan *et al*. 2016), *Steinernema fabii* (Abate *et al*. 2016), *Steinernema beitlechemi* (Çimen *et al*. 2016*).* Three EPNs that have reported 1st in the country include *Heterorhabditis bacteriophora* (Poinar 1967), *Heterhorhabditis zealandica* (Poinar 1990) and *Steinernema yirgalemense* (Malan *et al*. 2011).

2.1.2 Molecular characterization of EPNs

Accurate identification of EPNs is important in understanding the geographical distribution, biodiversity, habitat specificity and genetic relatedness (Alexander *et al.* 1997). A number of molecular techniques have been used for nematode identification (Hominick *et al*. 1996) which were based on isoenzyme patterns, total protein patterns immunological techniques, random amplification of polymorphic DNA (RAPDs) and restriction fragments length polymorphism (RFLP) (Akhurst 1980; Hominick *et al.* 1996; Alexander *et al*. 1997).

Some of the most powerful operational taxonomic units for species identification include the region of the mitochondrial genome which separates the cytochrome oxidases subunit (COII) (Hominick *et al.* 1996) and the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) repeat unit located between the 18S and 28S rDNA gene are used as genetic markers for EPNs (Hominick *et al*. 1996; Alexander *et al*. 1997; Reid *et al*. 1997; Powers *et al*. 1997; Liu *et al*. 2000). The ITS region is highly conserved across nematodes species thus allowing the designing of universal primers used for polymerase chain reaction (PCR) amplification of the DNA sequence (Alexander *et al*. 1997; Liu *et al.* 2000). The DNA sequence of the ITS region is then used to determine genetic diversity among closely related EPN families, genera and species (Reid *et al* 1997; Liu *et al*. 2000). To show the relatedness of these species and coevolution, a phylogenetic tree is created (Alexander *et al*. 1997; Powers *et al*. 1997).

Isolation and identification of indigenous EPN species based on morphological measures and molecular characterization will definitely provide initial screening for useful EPN strains or species and will contribute to documenting the existing diversity, geographical and distribution of EPNs (Liu *et al*. 2000). Therefore, the aim of the present study was to isolate,

identify locally adapted EPNs from soils found in North West province, at the Magaliesberg region.

2.2 Methods and Materials

2.2.1 Breeding and maintenance of insect host

Abiotic factors such as humidity, aeration, temperature and light and were taken into consideration since they affect the growth, development, and behaviour of the insect host (Kaya and Stock 1997). The most widely used susceptible host for *in vivo* maintenance of EPNs was *Galleria mellonella* (greater wax moth), since its cheap, easy to rear and relatively large, 2cm and weight 250 mg (Kaya and Stock 1997). The larvae were kept in 3 L Consol glass bottles incubated at 25°C to increase mating. The lids had a stainless mesh screen on the underside for ventilation of the larvae as shown in Figure 2. 1. Artificial media adapted from Woodring and Kaya (1988) was prepared (Appendix I) on a weekly basis to maintain larval growth and development. Wax paper which mimics the natural habitat of the host was added to facilitate the female moth to lay eggs within 3-5 days (oviposition). Eggs hatched 4-5 days after being laid and the larvae stage lasted between 20-40 days after the larvae pupated and developed into moths (life cycle starts over) in a period of 7 weeks. The cultures of *G. mellonella* were reared in the laboratory.

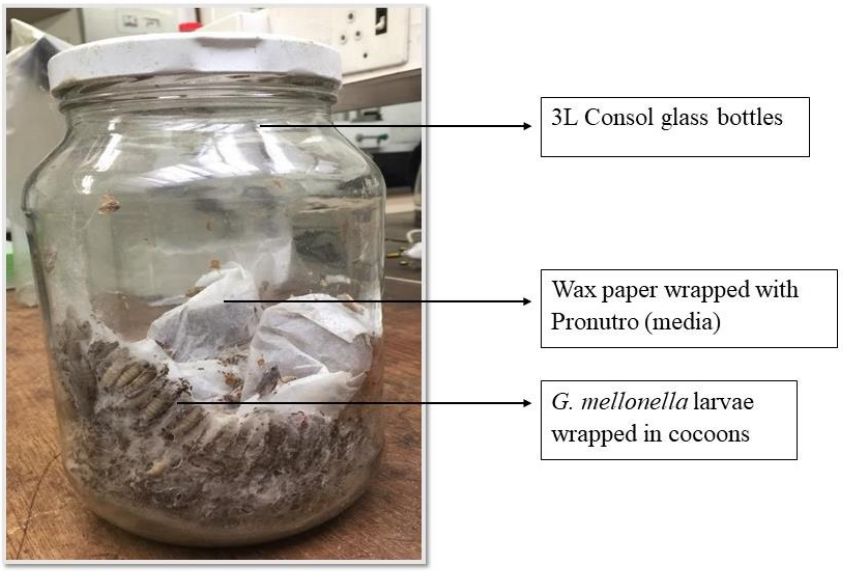

Figure 2. 1 *In vivo* rearing of *Galleria mellonella* larvae at the laboratory

2.2.2 Collection of soil samples

Soil samples were collected at 8 different sites in the North West province, Hartbeespoort Town, Magaliesberg Nature Reserve (26°00′31″S 27°32′46″E) as shown by the map in Figure 2. 2. At each specific site about 2-3 soil samples were collected and dug up using a hand shovel at a depth of 15cm randomly over an area of 2-4 m^2 (Kaya and Stock 1997). Soil samples from undisturbed (non-agricultural) soils were then transferred into 1 L ice-cream plastic containers (Figure 2. 3) and transported to the laboratory at Wits University and stored at 22˚C-25 °C, the optimal temperature for nematodes according to Kaya and Stock (1997).

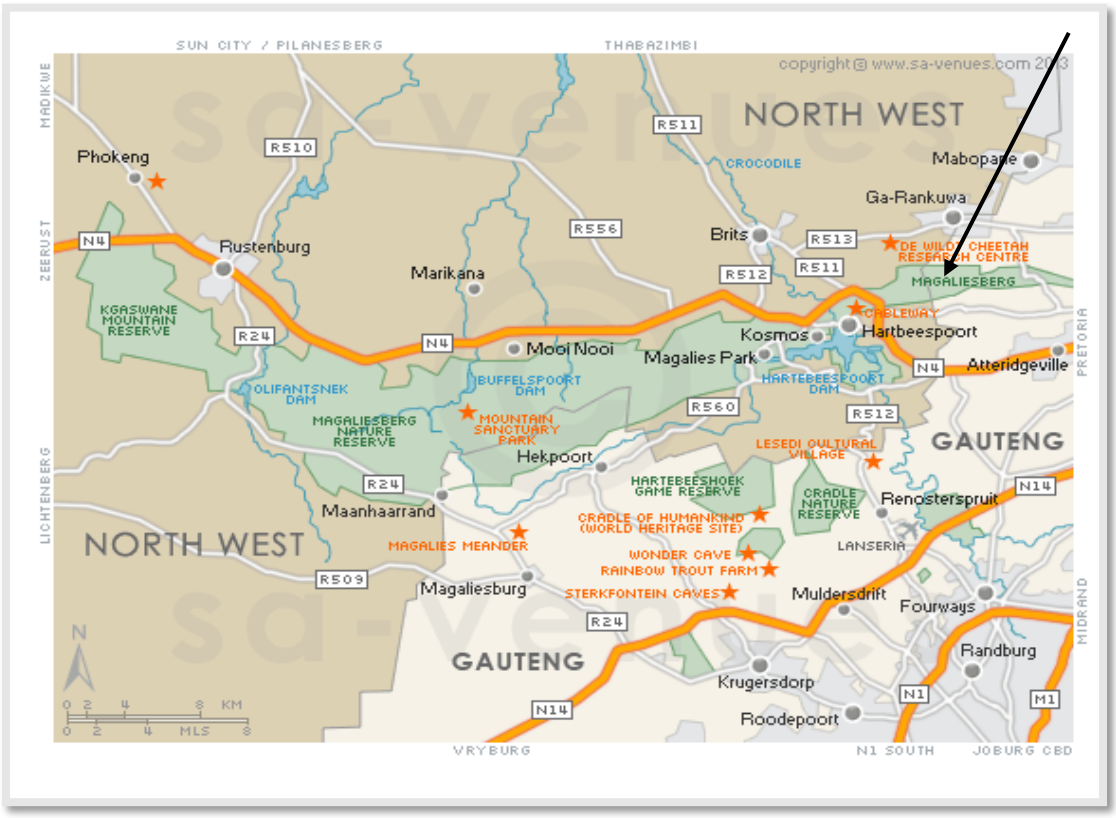

Figure 2. 2 Map of Magaliesburg region, North West Province where the collection of soil samples took place (ttps://www.sa-venues.com/maps/northwestprovince/brits.php).

Figure 2. 3 Soil samples collected in the different areas in the Magaliesberg region were stored in 1L plastic ice-cream containers and stored at room temperature 22-25˚C.

2.2.3 Soil baiting technique

To obtain a better quantitative estimation of the IJs, soil baiting technique adapted from Bedding and Akhurst (1975) was used. Prior to baiting, about 200ml of water was added to each container to bring the moisture content to 8% (v/w) so to facilitate the movement of nematodes. *Galleria mellonella* as the most susceptible host was used as bait to retrieve nematodes from the soil, about 10-15 larvae were placed on the soil surface of each container and stored at 22˚C-25˚C. Observations were done daily to monitor cadavers showing signs of EPN infection by looking at a combination of biology and morphological features, such as a change in colour of the cadaver. Presumptive *Heterorhabditis* spp turn dark maroon or red and *Steinernema,* brown or black within 3-4 days post baiting (Kaya and Stock 1997). For each round of baiting, infected dead larvae were removed, and fresh larvae were added so to increase EPN extraction efficiency.

Figure 2. 4 *Galleria mellonella* larvae, used as a bait insect was placed on top of the soil to retrieve the nematodes from the soil.

2.2.4 Recovery of IJs (The modified White trap method)

The method involves the natural migration of IJs from dead cadaver originally infected with EPNs from within the soil into the surrounding water in which they are collected in high quantity (Kaya and Stock 1997). The apparatus consists of two different sizes of Petri dishes, 60mm diameter Tissue culture dish which is inverted and placed inside a 90mm Petri dish covered with moist Whatman™ 1 filter paper disks (55mm) and filled with water to a depth of 0.5mm. EPN infected cadavers were surface sterilized with 70% ethanol to prevent contamination by fungi and mites and placed individually on the modified White traps. The emergence of IJs occurred after 48 hours at 22-25˚C.

2.2.5 Testing Koch's postulate

To prove or disprove parasitism by Koch's postulate (Kaya 1997), pure cultures of IJs from modified White traps were re-exposed to freshly reared *G. mellonella*. Coarse river sand was mixed with IJs in a sterile 90mm Petri dish, about 5 reared last instar *G. mellonella* were placed on the surface of the sand and the Petri dish was sealed, inverted and incubated at 22-25˚C. Coarse river sand was used since it does not restrict the movement of IJs in the soil and it allows some degree of aeration. Insect mortality was observed after 3-4 days by the dead cadaver showing signs of infections (colour) due to exposure of IJs found in the soil. The

emergence of IJs from infected EPN larvae placed on modified White trap confirmed Koch's postulate.

2.2.6 Extraction of genomic DNA

IJs isolated from the modified White traps were collected in a 50ml Falcon tube and allowed to sediment at the bottom followed by the removal of the supernatant consisting of water under a laminar flow. Surface sterilization of IJs was done for 3 h using 0.1% sodium hypochlorite (Appendix I) according to Kaya and Stock (1997). The solution was removed and IJs were washed 3 times with autoclaved distilled water. The IJs were then transferred into a 1.5 ml microfuge tube for genomic DNA extraction using the protocol from Puregene DNA Purification kit, Gentra System 2003 (Appendix II). The following modifications were done: All samples were centrifuged at 14000 rpm. Samples were incubated overnight at 55 °C instead of 3 h and 100 % Propa-2-nol was used instead of 100 % Isopropanol.

2.2.7 PCR amplification and DNA Sequencing

Characterization of EPNs was based on using the ITS region of the rDNA repeat unit, which flanks a conserved gene coding for 5.8S rDNA and 28.5s rDNA in nematodes (Hominick *et al*. 1996; Reid *et al*. 1997). About 20 µl of the DNA samples stored at 4 °C was sent to Inqaba Biotechnical Industries (Pty) Ltd South Africa for PCR amplification and DNA sequencing of the ITS region found between the 18S and 28S DNA region, using universal primers which are conserved throughout EPNs. Sequencing machine used by Inqaba Biotech company was carried out by using the ABI PRISM^{TM} 3500XL Genetic Analyser. The PCR conditions used by Inqaba biotech are as follows:

Forward Primer (TW81): 5' GTTTCCGTAGGTGAACCTGC 3' Tm = 62.45 °C
Reverse Primer (AB28): 5' ATATGCTTAAGTTCAGCGGGT 3' Tm = 58.66 °C

The PCR cycling profile consisted of the initial step at 94˚C for 5 min before cycling followed by the denaturing step at 95˚C for 60 sec, annealing at 60˚C for 60 sec, extension at 72˚C for 120 sec and the final extension at 72˚C for 10 min for a 25 cycle amplification series.

2.2.8 Molecular phylogenetic analysis by Maximum Likelihood method

Finch TV V.1.4.0 was used to read the chromatogram files (DNA sequences) obtained from Inqaba Biotech (see Appendix III). The sequences were trimmed by removing the 3' and 5' end followed by proofreading the sequence to check for ambiguous sites where N was replaced with respective nucleotide bases (C, G, T, A) using Finch TV (Geospiza, Inc. 2004). NCBI BLASTN (mega blast) sequence alignment algorithm was used to establish taxonomic affinities among the EPN species, the entire ITS region 18S-ITS1-5.8S-ITS2-28S rDNA nucleotide homology was used. The output sequence with the highest hit, highest percentage coverage and lowest E value (0) was chosen as the sequence similar to the query (isolated species in the study).

Molecular Evolutionary Genetics Analysis (MEGA) 7.0 was used for conducting statistical analysis of molecular evolution and constructing phylogenetic trees. MUSCLE was used to perform multiple sequence alignment because it results in high accuracy and high throughput compared to T Coffee and Clustal W. Since Neighbour-joining trees establish relationships between sequences according to their genetic distance without considering the evolutionary relationship (Kumar *et al*. 2016) Maximum Likelihood tree using 1000 replicates (bootstrap number) which makes the tree more reliable was used (Felsenstein 1985). *Caenorhabditis elegans,* which is a free-living nematode was chosen as an outgroup to root the tree.

2.3 Results

Two species of EPNs were recovered from 4 (20%) of the 20 soil samples isolated from 8 different locations in the North West province from different vegetation types including Kalahari thornveld, shrub bushveld and Cymbopogon-Themeda veld moistly from loamy soil type as shown in Table 2. 1. Preliminary identification was based on symptoms variation of infected larvae, with brick red or dark maroon associated with *Heterorhabditis* (Figure 2. 5) and beige brown and black associated with *Steinernema* infection (Figure 2. 6) after successful reinfections which favoured Koch's postulate. IJs for *Heterorhabditis* spp. was recovered after 48 hours and for *Steinernema* spp. after 72 hours on the modified White trap

method. *Steinernema* infected cadaver would disintegrate, collapse and would appear flat after emergence (Kaya and Stock 1997).

Table 2.1 Twenty samples were isolated in eight different locations from the Magaliesburg region. From a total of twenty samples, seven (35%) were positive for EPN identification. However, due to contamination scavengers of EPNs after 3rd round of baiting with *Galleria mellonella* only four (20%) out of 20 samples were successfully recovered. Two samples belonged to steinernematids and two samples belonged to heterorhabditids.

Location	Number of samples	Soil type	Isolated EPN strain
L1	3	Loamy soil	Unsuccessful
L2	2	Loamy soil	Consumed by EPN scavengers
L3	2	Loamy soil	Unsuccessful
L4	2	Loamy soil	Consumed by EPN scavengers
L5	5	Loamy soil	*S. khoisanae*
L6	2	Loamy soil	Consumed by EPN scavengers
L7	2	Loamy soil	Consumed by EPN scavengers
L8	2	Loamy soil	*H. bacteriophora*

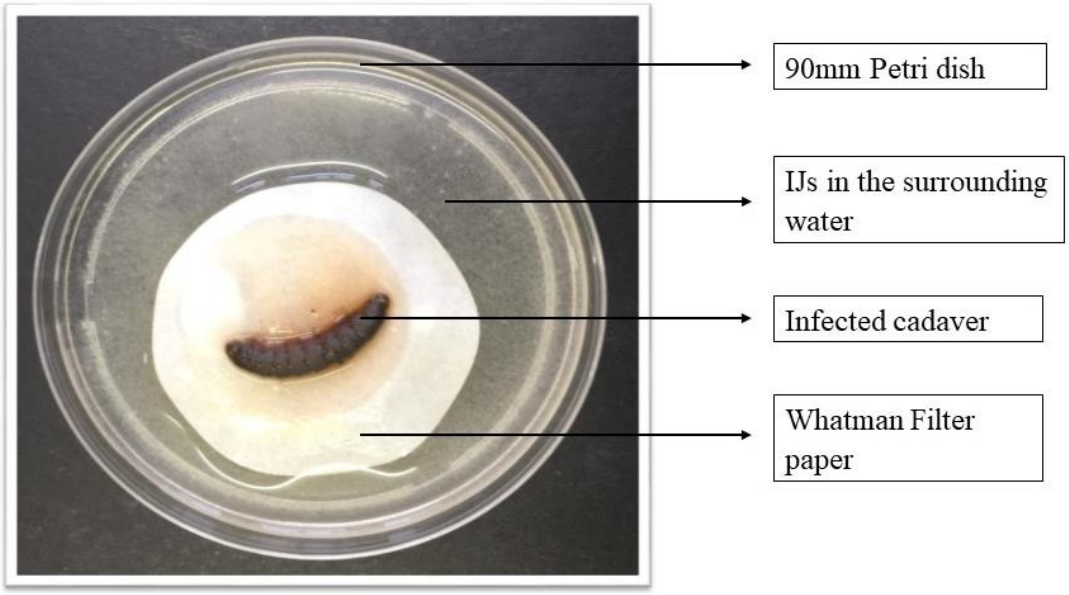

Figure 2. 5 IJs recovered from the modified White trap after 48 hours post infection of larvae. *Heterorhabditis* IJs found on the surrounding water film.

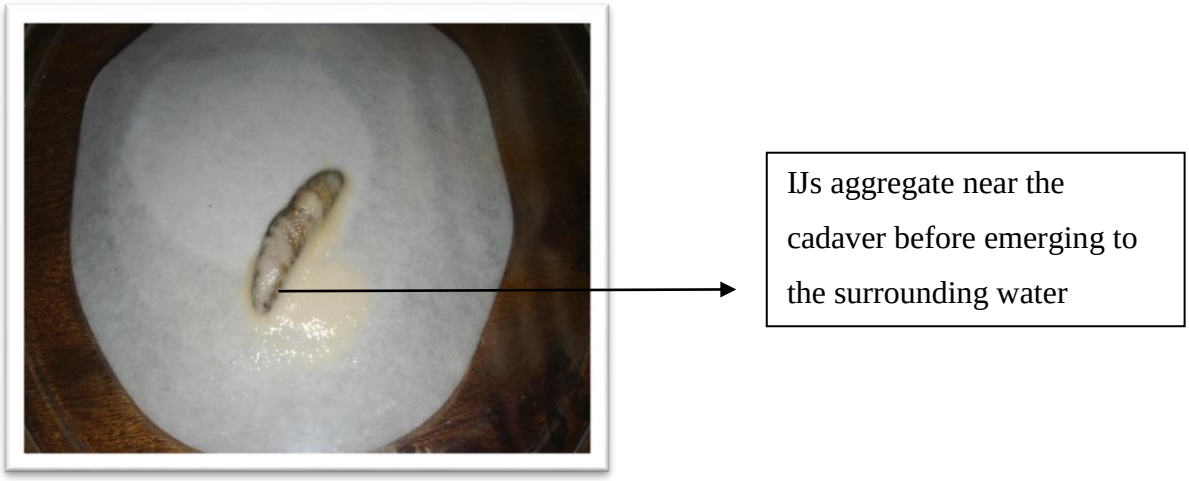

Figure 2. 6 IJs recovered from the modified white traps after 72 hours post infection of the cadaver from the baiting technique. *Steinernema* IJs aggregate near the cadaver before moving to the surrounding water film.

Figure 2. 7 To confirm pathogenicity from Koch postulates, the collected IJs from White traps were re-exposed to *Galleria mellonella* and mixed with coarse river sand. Insect mortality was observed after 72 h. EPN infected cadavers turned maroon/ red for the presumptive *Heterorhabditis* spp. and brown for *Steinernema* spp. The associate bacterial symbiont was responsible for the pigment observed.

Molecular characterization

The NCBI BLASTN algorithm which searches the query sequence against the GenBank's nucleotide databased based on pairwise sequence comparison to find the best matches using scores based on a matrix was used. The amplified DNA sequence of *Heterorhabditis* spp. was identified as *H. bacteriophora* which was identical to the sequence MG334257.1 from Genbank, the identity score was 100%, query coverage 100% and E value 0. The 2nd sequence of *Steinernema* spp. had the highest affinity to *S. khoisanae* strain LTV (MH697401.1) with an identify score of 99%, query coverage of 96% and E value 0.

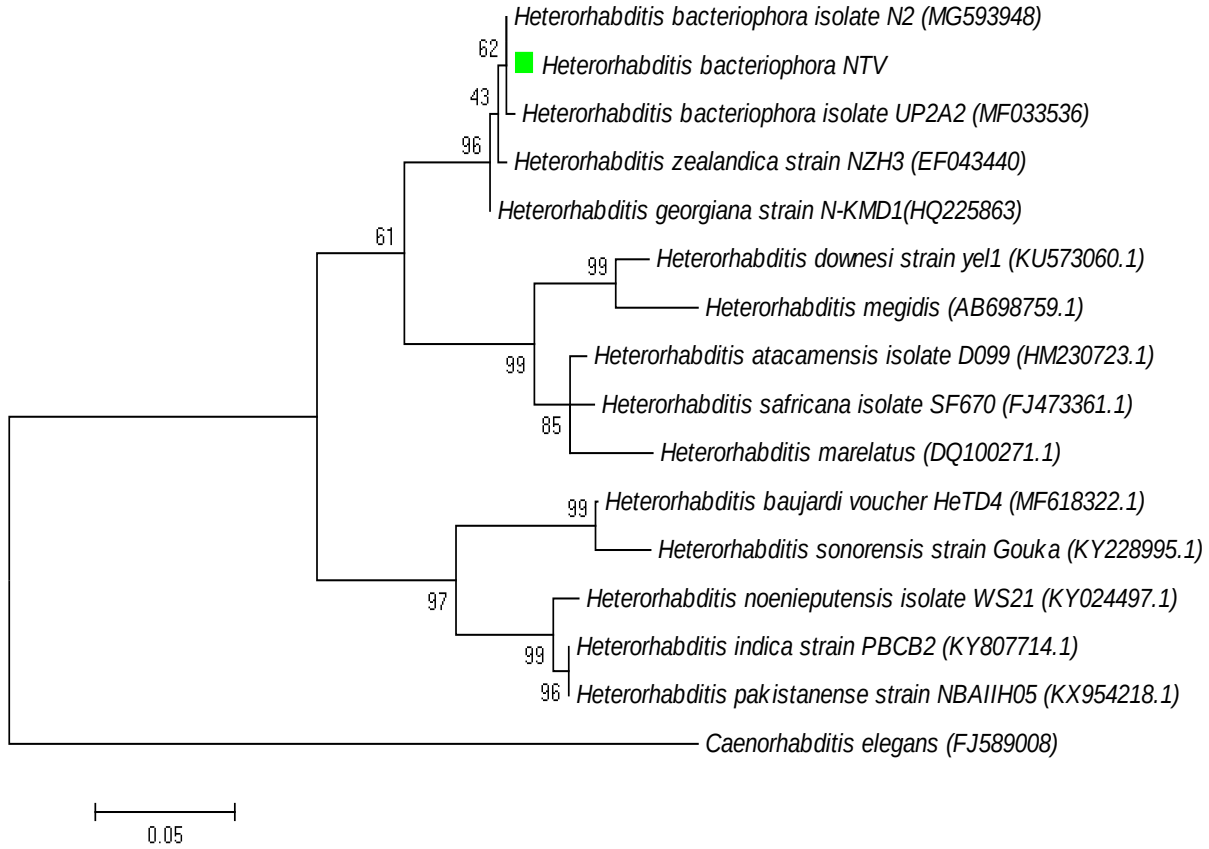

Figure 2. 8 Phylogenetic relationship of the isolated species, *H. bacteriophora* NTV with 14 species of *Heterorhabditis* based on the ITS region sequences obtained from GenBank. The phylogenetic tree was conducted using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Neil 1993). *Caenorhabditis elegans* was used to root the tree. MEGA7 was used to conduct evolutionary analysis (Kumar *et al.* 2016).

p

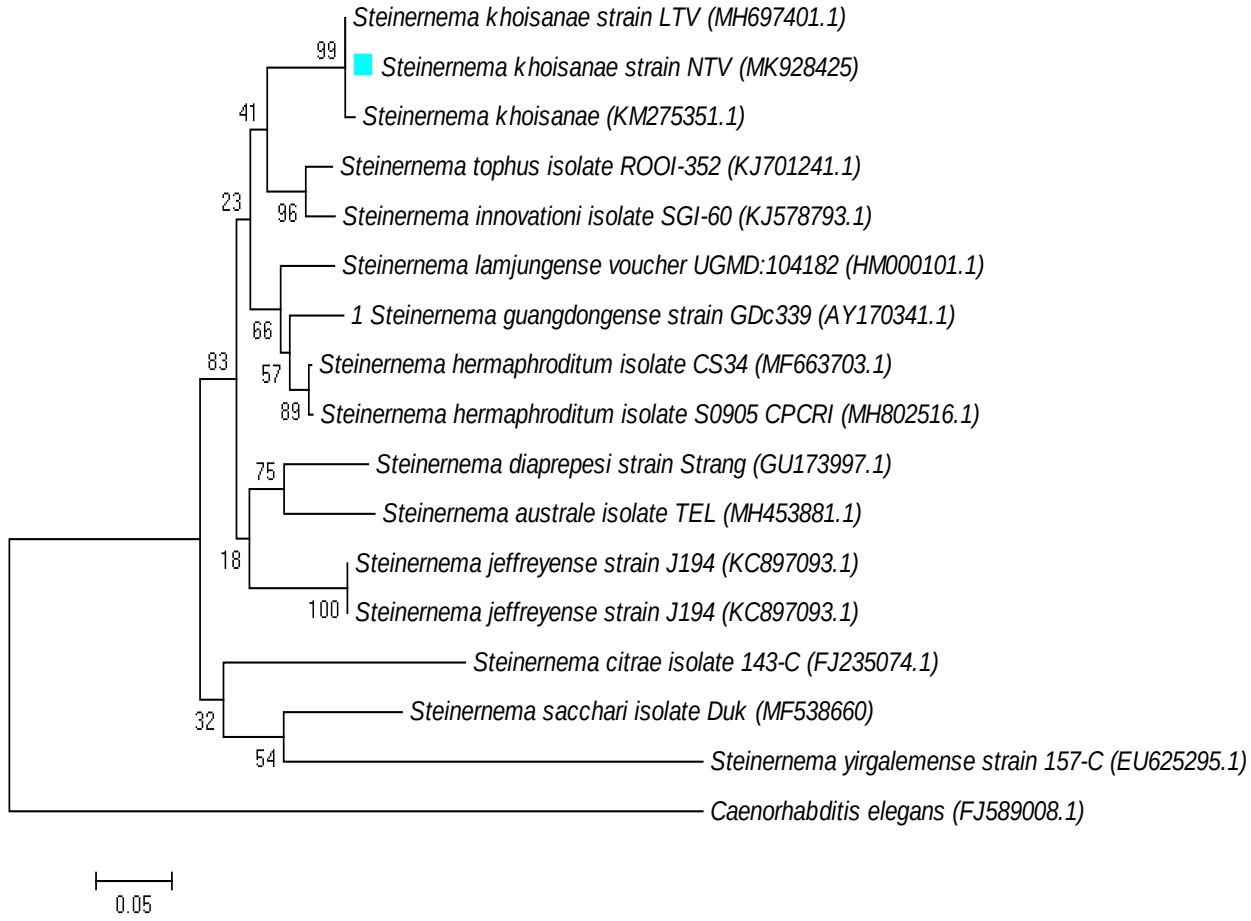

Figure 2. 9 Phylogenetic relationship of the isolated species, *S. khoisanae* strain NTV with 16 species of *Steinernema* based on the ITS region sequences from GenBank. The phylogenetic tree was conducted using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Neil 1993). *Caenorhabditis elegans* was used to root the tree. MEGA 7 was used to conduct evolutionary (Kumar et al. 2016).

2.4 Discussion

2.4.1 Collection of soil samples

The percentage recovery of EPNs that was successfully isolated in the study was 20% of which two samples (10%) were identified as *S. khoisanae* and two samples (10%) were identified as *H. bacteriophora,* through the insect baiting technique and White trap method. Preliminary identification was based on symptom variation of infected larvae in which *H. bacteriophora* turned dark maroon or brick red and those infected by *S. khoisanae* turned brown. The different colour pigments produced results from anthraquinone pigments

produced by the bacteria associated with the symbiotic EPN isolates (Kuwata *et al*. 2008). It was observed with more successive baiting of *Heterorhabditis*, the pigment changed from maroon to yellow.

EPNs can infect more than 100 different insect hosts in the laboratory (Poinar 1990), however, most studies have used the *G. mellonella* larval soil baiting technique, which leaves the identity of the natural host unknown (Bedding and Akhurst 1975). The use of *G. mellonella* may have only isolated selected species that have a relatively broad host range for Lepidopteran larvae, making it difficult to find EPNs in the soil in the absence of a susceptible non-Lepidopteran host which may be more suitable for isolating other EPN species (Hazir *et al.* 2003; Hominick *et al*. 1996). Thus, integrating other Coleopteran and Dipteran bait insects may increase the isolation, diversity, and distribution of EPNs as natural control agents (Hazir *et al*. 2003). Various factors such as geographical location such as collection of soil samples at the chosen site with distinct vegetation types, soil texture, moisture, pH and climate conditions may have also increased the prevalence rate for isolating more diverse species of nematodes (Hominick *et al.* 1996; Stock *et al.* 1999; Hazir *et al.* 2003).

The reduced EPN recovery in the study was based on the presence of natural enemies of nematodes. These include invertebrate predators, omnivores and scavengers that consume free-living IJs and feed on the nematode killed insects thus, killing the developing nematode within the host cadaver (Baur *et al.* 1998; Ulug *et al.* 2014). Potential enemies of EPNs include invertebrate predators such as turbellarians, predatory nematodes, tardigrades, oligochaetes, (Ulug *et al.* 2014), and omnivorous and scavengers insects such as crickets, ants, mites, and springtails (Kaya 2002). Usually, the cadaver is at risk of consumption by these scavengers in the soil, or soil surface for 7-15 days or longer, depending on the nematode species and the environmental conditions (Kaya 2002). Once the nematode killed cadaver integuments are slowly consumed, the developing nematodes die because of desiccation and or invasion of opportunistic organisms such as fungi and mites that reproduce on the cadaver (Ulug *et al* 2014). This was observed in the study since 8 out of 20 samples (40%) were contaminated or consumed by scavengers, which resulted in no emergence of IJs, when infected or dead cadavers were placed on the White trap. The presence of natural

enemies reduces the EPN population by either consuming the free-living IJs, nematode killed insects or causing unfavorable conditions for nematode development (Ulug *et al.* 2014).

A Study done by Zhou *et al.* (2002) showed that the symbiotic bacteria of some species of bacteria of EPNs produce a compound(s) that deters ants or any scavengers thus protecting the cadaver from being consumed during their reproduction within the insect cadavers. These compounds are known as scavengers deterrent factors (SDF). Production of the SDF by the symbiotic bacteria plays a significant role in protecting a two-day or older nematode killed insects from scavengers (Zhou *et al*. 2012). A study conducted by Timper and Kaya (1992) found out that the presence of the second stage J2 cuticle surrounding IJs of *H. bacteriophora* and *S. carpocapsae* prevented nematode parasitic fungi (*Hirsutella rhossilieusis*) from infecting them. Conidia of the fungus *Hirsutella rhossiliensis* attach and penetrate the J2 cuticle but the hyphae cannot penetrate the intact IJ cuticle (Timper and Kiya 1992; Kaya and Gaugler 1993).

2.4.2 Species abundant is SA

In South Africa, only a few surveys have been done in which EPNs were identified to species level (Malan 2006, 2011; Hatting *et al.* 2009). A study conducted by Hatting *et al* (2009) involved isolating nematode species in five provinces (Kwazulu Natal, Mpumalanga, Gauteng, Western Cape, and Free State) from different habitats which include the Fynbos, Nama Karoo, Grassland and Savanna. An important indicator determining whether EPNs occur in the environment was soil type. Total of 1506 soil samples were collected 79 (5.2%) were EPN positive, 55.7% were identified as steinernematids and 44.5% belonged to heterorhabditids. About 87% of EPNs were recovered from subtropical zones and 13% from semi-arid zones characterized by sandy acidic soils (Hatting e*t al*. 2009). The low recovery rate was also observed in Malan (2011) study, in which, only 35 (17%) of the 202 samples tested positive for the presence of EPNs (Malan 2011). Out of the 498 soil samples that were collected by Malan 2006, only 36 (7%) soil samples tested positive for EPN (Malan 2006).

Heterorhabditis bacteriophora isolate is by far the most abundant EPN species found in SA 32Misolated from distinct biogeography and different habitats (Hatting *et al*. 2009; Malan *et al* 2006; 2011). Soil types with high sand content and low clay content (sandy to sandy loam soils) favoured mobility and survival of most EPNs including *H. bacteriophora* commonly

found in tropical and subtropical areas (Stock *et al*. 1999; Hazir *et al*. 2003). Steinernematids dominates in cooler and temperate soils hence *S. khoisanae* was found in winter rainfall regions and fynbos vegetation type (Hatting *et al.* 2009). In the present study, *H. bacteriophora* and *S. khoisanae* were successfully isolated in North West province consisting mainly of the savanna grassland biome with a climate characterized by hot summer temperature (34˚C) and dry winter temperature (22˚C) and an average annual rainfall of 300mm – 700mm annually. Few studies have tested the efficacy of SA isolated EPNs such as *S. citrae* and *S. yirgalemense* against insect pest which have showed promise to be effective against false codling moth, *H. zealandica* against *Cydia pomonella* under laboratory conditions (De Waal *et al*. 2010; Malan *et al*. 2011). These EPN species can soon be implemented in successful agricultural pest management programs (Çimen *et al*. 2016).

2.4.3 Phylogenetic Analysis

The isolated *H. bacteriophora* NTV spp. was 100% identical with *H. bacteriophora* N2 (MGS93948) and *H. bacteriophora* (MF033536) by the presence of a monophyletic group with 62 % bootstrap support as illustrated in Figure 2. 7. Evolutionary divergence based on the slight branch length difference between the isolated *H. bacteriophora* NTV spp. and *H. bacteriophora* N2 (MGS93948) was 0.003 (Appendix III) and *H. bacteriophora* (MF033536) to unknown A was 0.000 indicating a close relation (same species). Hence, the difference may indicate the strains might differ in virulence, survival, host finding abilities and efficacy against an insect pest (Liu *et al.* 2000). The isolated *S. khoisanae* spp. shares a common ancestry with *S. khoisanae* LTV (MU697401.1) (species identified and reported in the lab) and *S. khoisanae* (KM275351.1) supported by 99% bootstrap value as shown in Figure 2. 8. The evolutionary divergence between *S. khoisanae NTV* and *S. khoisanae* LTV was 0.000 (Appendix III) indicating that it's the same species. The slight branch difference (evolutionary divergence) was 0.006 between *S. khoisanae* NTV and *S. khoisanae* (KM275351.1) which may indicate genetic variation, which might have happened through genetic drift and natural selection by exposure from a host and local environment (Liu *et al*. 2000). As shown in both phylogenetic tree (figure 2. 7 and 2. 8). EPNs can exist as strains, which differ biologically even when isolated in the same region (Hominick *et al.* 1996; Liu *et al*. 2000).

A phylogenetic tree constructed from rDNA repeat units suggested that *Heterorhabditis* species are more closely related than *Steinernema* species which show a greater biological diversity (Hominick *et al*. 1996). Both families are polyphyletic, have evolved from different ancestry however similarities in their life cycle can be attributed to convergence (Poinar 1990; Liu *et al*. 1997). The difference in biogeography in both families is based on their reproduction strategies. The 1st generation of *Heterorhabditis* nematodes are self-fertile hermaphrodite leading to the production of clones, while *Steinernema* nematodes involves both male and females (amphimictic) which favor greater genetic diversity and speciation based on molecular data (Hominick *et al*. 1996). The presence of satellite DNA sequences which are species-specific because of the DNA evolving at a high rate represents 5% or 10% of the EPN genome could attribute to the genome differences both in size and complexity (Liu *et al.* 2000).

2.5 Conclusion

Species identification is an important step during the selection of appropriate isolates for the control of insect pests. Isolating species from different regions that are climatically adapted have a greater potential to be used as biocontrol agents so that they can be available as commercially formulated products. In the present study *H. bacteriophora* and *S. khoisanae* strain NTV (MK928425) were successfully isolated in the Magaliesburg region, North West province However *H. bacteriophora* was chosen as an ideal EPN species for successive objectives.

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Chapter 3: Isolation and molecular characterization of the bacterial symbiont

3.1 Introduction

3.1.1 Taxonomic identification of the bacterial symbiont

Enteric bacteria of the genus *Photorhabdus* are associated symbiotically with entomopathogenic nematodes of the genus *Heterorhabditis*, both responsible for the death of the insect host (Kaya and Stock 1997; Lee and Stock 2010; Ferreira *et al*. 2013). *Photorhabdus* is a gram-positive bacterium, motile, facultatively anaerobic, bioluminescent which belongs to the Enterobacteriaceae family (Boemare *et al.* 1993; Forst and Nealson 1996; Bowen *et al.* 1998; Gerrard *et al*. 2004; Adams *et al.* 2006; Kuwata *et al*. 2008). So far three species of *Photorhabdus* have been isolated and includes *Photorhabdus luminescens, Photorhabdus temperata* and *Photorhabdus asymbiotica* (Gerrard *et al.* 2004; Ferreira *et al.* 2013). There are nine subspecies of *P. luminescens* and three subspecies of *P. temperata* of which form mutualistic relationships with *Heterorhabditis* species and are used as biocontrol agents (Akhurst *et al*. 2004; Adams *et al*. 2006; Kuwata *et al*. 2008; Ferreira *et al*. 2013). *Photorhabdus asymbiotica* is an opportunistic human pathogen bacterium that used to be a symbiont of *Heterorhabditis* species. It was isolated in the USA and Australia and consist of two subspecies (Akhurst et al. 2004; Gerrard *et al*. 2004; Kuwata *et al*. 2008).

3.1.2 Targeting the endotokia matricida stage for the isolation of bacterial symbiont

Heterorhabditis IJs develop into self-fertilising hermaphrodites feeding on their symbiont bacterium *P. luminescens*. *Heterorhabditis* gravid females reproduce by laying eggs in which juveniles either hatch outside into the surrounding media or develop inside the maternal body then feed on the maternal body causing its death, the above process is known as endotokia matricida (Liu *et al*. 1997; Johnigk and Ehlers 1999; Baliadi *et al*. 2004, 2009; Ulug *et al*. 2015). Inside the maternal hermaphrodite female, eggs are fertilised by the sperm located in the area between ovary and uterus then fertilised eggs develop inside the uterus and begin to hatch inside the maternal uterus (intra-uterine hatching) (Ehlers and Johnigk 1999; Burnell

and Stock 2000). The developing IJs start to move around and cause destruction of maternal reproductive systems (gonad tissues) and maternal intestine (Johnigk and Ehlers 1999). Developing IJs then feed on the intestine content cells (intracellular material and storage granules), unfertilised eggs and sperm for a food source (Ehlers and Johnigk 1999). The maternal pharynx which is still intact provides the developing IJs with cells of bacterial symbiont which are later stored in the intestinal lumen of the IJs (Ciche *et al.* 2008). Juveniles further develop and migrate out causing death to the maternal hermaphrodite female. Endotokia matricide provides optimal conditions for the developing IJs when the environment is unfavourable (Johnigk and Ehlers 1999; Burnell and Stock 2000; Baliadi *et al.* 2004; 2009).

3.1.3 In vitro culture of the bacterial symbiont

Photorhabdus luminescence can exist and be grown as a free-living bacterium in laboratory conditions (Bowen *et al.* 1998). When cultured the bacterium exists as different phase variants that differ in biochemical and physiological properties (Forst and Nealson 1996). Phase I cells are larger than Phase II produces lecithinase, absorb dye, contain paracrystalline inclusion and produce greater amounts of antibiotics, proteases, exoenzymes and toxins whereas Phase II occur spontaneously after prolonged cultures in vitro (Boemare *et al.* 1993; Forst and Nealson 1996; Burnell and Stock 2000). In vitro growth of the bacterium is favoured by the absence of competition which would occur naturally in the soil and a rich nutrients supply which promotes the growth of the bacterium in laboratory conditions however the bacterium cannot exist in the soil in the absence of nematode association (Bowen *et al.* 1998; Burnell and Stock 2000).

3.1.4 Molecular characterization of the bacterial symbiont

Characterization of the bacterial symbiont has involved the sequencing of the 16s and 28s rRNA gene which has been used for determining taxonomy, phylogenetic relationships, identification and characterization of bacterial species (Brunel *et al*. 1997; Liu *et al*. 1997; Janda and Abbott 2007; Lee and Stock 2010). The 16s rRNA gene is highly conserved and present in all bacterial species thus been used as the most common housekeeping genetic marker (Janda and Abbott 2007). Sequencing only a part of the 16s rRNA gene can be

sufficient to establish phylogenetic relationships but not species to subspecies identification (Liu *et al*. 1997; Adams *et al.* 2006).

The genomes of bacteria are very dynamic and vulnerable to many changes through the process of gene duplication, divergence, genome reduction, and lateral gene transfer, all of which occur due to pressure in the environment, competition and mutation (Chaston *et al*. 2011). To resolve the intra-generic relationships of the bacterial symbiont, concatenation of several partial sequences of five protein-coding gene fragments has been used to improve the robustness of the phylogenetic trees (Kuwata *et al*. 2008; Adams *et al.* 2006; Tailliez *et al.* 2010; 2012). The multigene approach uses 5 universal conserved protein-coding sequences (recA, dnaN, gyrB, gltX, and rpiB) (Lee and Stock 2010; Tailliez *et al.* 2010) these are all single copy of orthologous genes resistant to horizontal gene transfer (Tailliez *et al*. 2012). The recA gene encodes for a DNA recombination protein, the dnaN gene encodes for a DNA polymerase III beta chain involved in DNA metabolism, gyrB encodes a DNA gyrase beta subunit, the gltX gene encodes a glutamyl-tRNA sythetase involved in protein synthesis and rpiB gene encodes for 50S ribosomal protein L2 (Tailliez *et al*. 2010). To date, all the protein-coding genes are now used in many bacterial phylogeny studies (Akhurst *et al*. 2004; Tailliez *et al.* 2010, 2012; Ferreira *et al*. 2013).

The mutualistic relationship between nematodes and bacterial symbiont play a significant role in infectivity, mass production (large scale bioreactors) and as biocontrol agents (Liu *et al*. 2001). Antibiotics produced by the bacterium may possibly be used in drug development which may assist many pharmaceutical companies. Therefore, it is very important to verify which species of bacteria are found in association with different EPN species in nature (Liu *et al.* 2001). The aim of the study was to isolate and identity the *Photorhabdus* species isolated from infected *H. bacteriophora* spp.

3.2 Methods and Materials

3.2.1 Isolation of bacterial symbionts

Targeting the mature female stage or the endotokia matricida stage for isolation of bacterial symbiont was used to improve the efficacy of bacterial isolation since at this point, the

production of *P. luminescence* reaches its highest (Ulug *et al*. 2015; Ciche *et al*. 2008). *Galleria mellonella* infected larvae from Koch's postulate was dissected on the (5^{th} – 8^{th} day) before and during matricide to obtain mature female nematodes which were picked up using an L-shaped handling needle and transferred into a watch glass containing sterile Ringer's solution pH 7.3 (Appendix IV). Hermaphrodites of *H. bacteriophora* nematodes were surface sterilized with sodium hypochlorite for 60 min under the laminar flow then dipped in autoclaved distilled water for 10-15 sec (Ulug *et al.* 2015).

3.2.2 Characterization of the bacterial symbiont

Isolation of Phase 1 colonies showing morphological difference was done through using selective media such as Nutrient Agar Bromothymol Blue Triphenyltetrazolium Chloride (NBTA) and McConkey plates (Appendix IV). To release the bacterial symbiont, nematodes were crushed into 2-3 pieces with a sterile loop where the resulting macerate was streaked on the NBTA surface and incubated at 25˚C for 72 hours. Greenish colonies surrounded by clear zones indicative of Phase I bacterial symbiont based on the ability to adsorb bromomethyl blue and reduce triphenyl tetrazolium chloride (TTC) were subcultured 7-8 times to obtain pure cultures. Phase II bacterial symbionts were able to reduce TTC but could not adsorb bromomethyl blue thus producing reddish colonies with no clear zones (Forst and Nealson 1996). MacConkey plates were used as an indicator to confirm the Phase 1 variant based on the ability of the bacterium to absorb neutral red. Single greenish colonies from NBTA plates were then transferred and streaked on MacConkey plates. The plates were incubated at 25˚C, wrapped with foil and placed on the dark for 72 h. The resulting colonies were reddish, pinkish indicative of *Photorhabdus* species.

3.2.3 In vitro culturing of *H. bacteriophora* and *Photorhabdus spp.*

To confirm whether the isolated *Photorhabdus* species is associated with *H. bacteriophora*, the growth of nematodes was observed in lipid agar plates (Appendix IV) inoculated with the bacteria. Lipid agar plates were used since they mimic the natural environment of EPNs. Greenish colonies isolated from NBTA were grown in 500µl of nutrient broth (Appendix IV) and incubated at 25˚C for 72 h. About 100µl of the broth was spread on the lipid agar to form a bacterial lawn and left to grow for (3-4 days) at 25˚C. *Heterorhabdtis bacteriophora* IJs recovered from modified White traps were resuspended in 0.1% sodium hypochlorite in a

Falcon tube and left for 30 minutes for surface sterilization and washed 3 times with autoclaved distilled water. About 100µl of IJs were spread on lipid agar plates and left to grow for 5-6 days. *Heterorhabdtis bacteriophora* IJs were able to feed on the bacteria, grow and survive on the lipid agar confirming the correct isolation of the presumptive *Photorhabdus* species.

3.2.4 Extraction of genomic 16s rDNA of *Photorhabdus* spp.

Characterization was based on the 16s rRNA gene sequence, which has been used extensively to distinguish between groups of *Photorhabdus* bacteria and to study bacterial phylogeny and taxonomy (Adams *et al*. 2006). Genomic DNA of the bacterial isolate was extracted according to the protocol ZR Fungal/Bacterial DNA Kit™ Catalog No. D6005 (Appendix V) by isolating pure cultures of Phase 1 colonies from NBTA plates under laminar flow. The DNA was stored at 4˚C and sent to Inqaba Biotechnical Industries (Pty) Ltd for PCR amplification and Sequencing of the 16s rDNA gene which was carried out using the ABI PRISM™ 3500XL Genetic Analyser.

3.2.5 PCR amplification and DNA sequencing

The 16s rDNA was amplified using two oligonucleotide universal primers (Brunel *et al*, 1997). The PCR cycling profile consisted of the initial step at 95˚C for 3 min before cycling followed by the denaturing step at 95˚C for 60 sec, annealing at 60˚C for 60 sec, extension at 72˚C for 2 minutes and the final extension was carried out at 72˚C for 3 min, for a 35 cycle amplification series.

Forward primer (EUB968) (5’ -ACGGGCGGTGTGTRC- 3’) Tm= 62˚C
Reverse primer (UNIV1382) (5’ -AACGCGAAGAACCTTAC- 3’) Tm= 66˚C

3.2.6 Phylogenetic Analysis

Finch TV was used to read of the chromatogram files for the 16s rDNA sequences obtained from Inqaba Biotechnical Industries (Appendix VI). The sequences were trimmed by removing the 3’ and 5’ end of the sequence followed by proofreading the sequence to check

for ambiguous sites where N was replaced with respective nucleotide bases (C, G, T, A) using Finch TV (Geospiza, Inc.2004). To establish taxonomic affinities NCBI BLASTN (mega blast) sequence alignment was used. The sequence with the highest hit, highest % coverage and E value of 0 was chosen as the sequence similar to the query sequence. MEGA 7.0 was used for conducting statistical analysis of molecular evolution and constructing a phylogenetic tree. Multiple sequence alignment was performed using MUSCLE (Kumar *et al.* 2016). Maximum Likelihood tree using 1000 replicates (bootstrap number) was used to make the tree more reliable (Felsenstein 1985). *Escherichia coli* was chosen as an out-group to root the tree since it is one of the 1st prokaryote to be sequenced and mapped.

3.3 Results

Photorhabdus species was successfully isolated by targeting the endotokia matricida stage and was subsequently characterized on NBTA and MacConkey plates. The Phase 1 bacterial symbiont were able to adsorb bromomethyl blue and reduce TTC, thus producing greenish colonies surrounded by clear zones and having a sticky consistency on NBTA agar plates. The colony morphology for Phase 1 bacterial symbionts were granulated, opaque, convex, and circular with irregular margins (Boemare *et al.* 1993; Kaya and Stock 1997). The cells were small to middle sized in both NBTA and MacConkey agar plates as shown in Figure 3. 1. The colonies were pink, reddish for presumptive *Photorhabdus* spp. on MacConkey plates. Furthermore, the lipid agar study confirmed that the presumptive *Photorhabdus* spp. were a native endosymbiotic partner of *H. bacteriophora* NTV. The amplified 16s rDNA (Appendix VI) sequence of *Photorhabdus* spp. showed high affinity to *Photorhabdus luminescens* subsp. *laumondi* strain SF 281 (KT963833.1), the identify score was 99%, query coverage 100% and E value of 0 on the NCBI BLASTN algorithm.

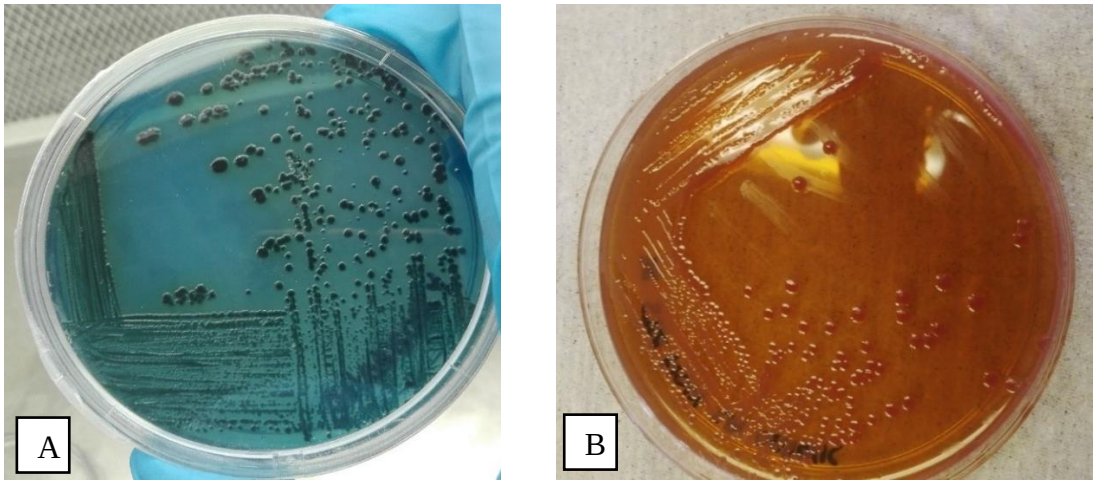

Figure 3. 1 Isolated Phase 1 symbiotic bacteria on NBTA and MacConkey plates. A) Presumptive *Photorhabdus* spp. isolated produced greenish colonies with sticky consistency on the plates after 48 hours at 25˚C. B) *Heterorhabditis* associated bacteria formed red colonies on MacConkey agar plates after 48 h at 25˚C.

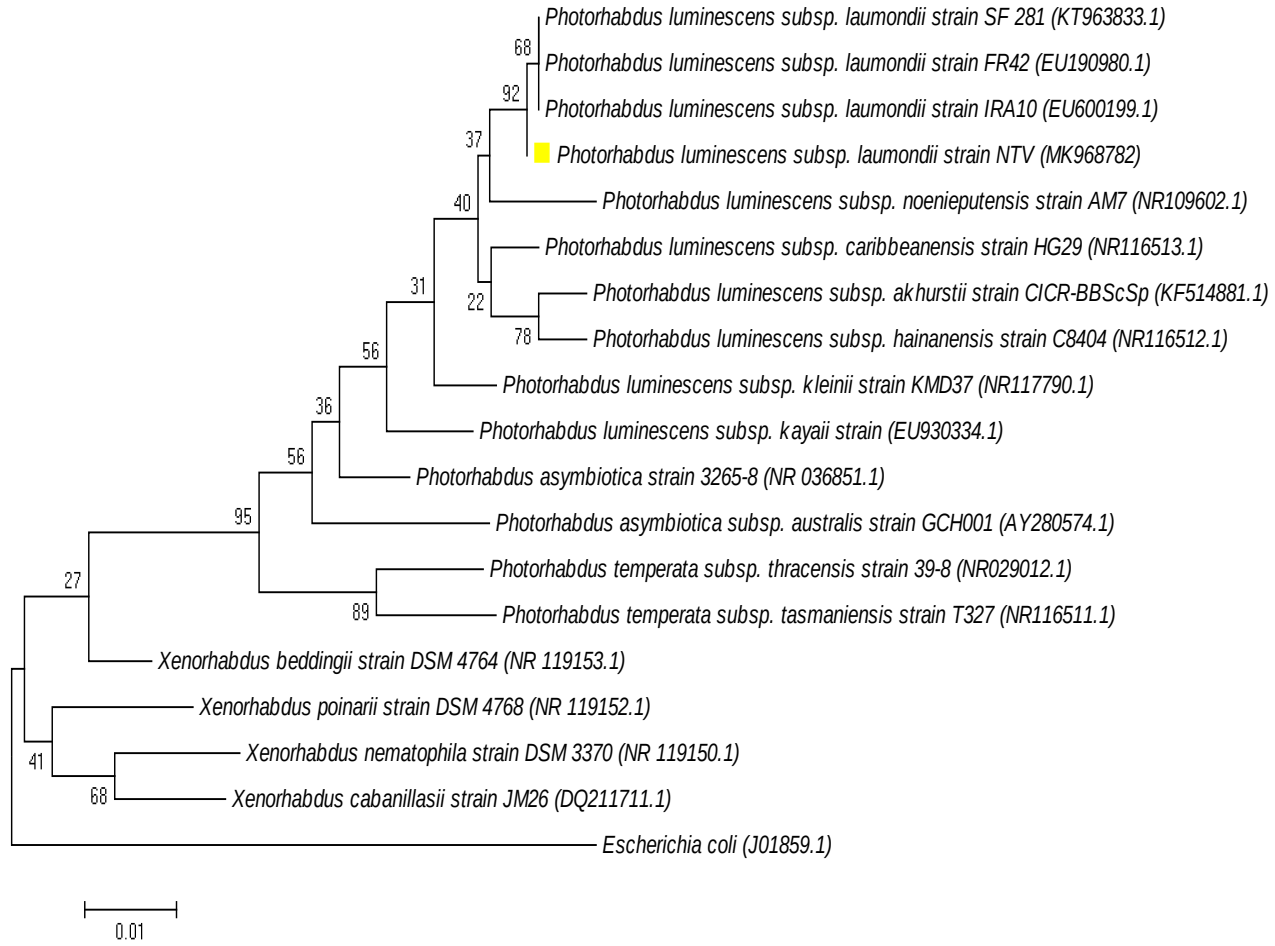

Figure 3. 2 Phylogenetic relationship of the isolated *Photorhabdus* species NTV (MK968782) with seven *P. luminescens* subsp, two *P. temperata* subsp, two *P. asymbiotica* subsp and the closely related enteric group *Xenorhabdus* based on the 16s rDNA sequences from GenBank. The phylogenetic tree was conducted using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Neil 1993). MEGA 7 was used to conduct evolutionary analyses (Kumar *et al*. 2016).

3.4 Discussion

Ciche *et al* (2008) reported that endotokia matricida in *Heterorhabditis* spp. results in the transmission of the symbiont from the parent to the progeny making it easy to target and isolate the bacteria. *P. luminescens* subsp *laumondii* strain symbiont of *H. bacteriophora* was therefore successfully isolated by targeting the endotokia matricida stage. This method did not produce mixed cultures of bacterial contamination from the intestinal microflora of the insect (Ulug *et al*. 2015). A study conducted by Ferreira *et al* (2013) showed phenotypic characteristics of most *P. luminescens* strains which tested positive for catalase activity,

negative for lecithinase, oxidase and nitrate reductase activity and were able to assimilate glycerol, fructose, maltose, glucose, D-mannose and ribose (Boemare *et al.* 1993; Ferreira *et al.* 2013). *Photorhabdus luminescens* strains also have the ability to emit light at stationary phase (bioluminescence) compared to Phase II bacteria which emit 1% of the light emitted by Phase I cells (Forst and Nealson 1996). Phase I *Photorhabdus* spp. are able to secrete high levels of lipases, proteases and huge amounts of antibiotics that are active against other bacteria, fungi and yeast (Burnell and Stock 2000; Adams *et al*. 2006).

The 16s rDNA sequence of unknown A isolate was compared with sequences and strains of different species and subspecies of the genus *Photorhabdus* (Ferreira *et al.* 2013). Unknown A formed a monophyletic group and clustered with *P. luminescens* subsp. *laumondi* strains supported by a bootstrap of 92% given the species were all isolated from *H. bacteriophora* as shown in figure 3. 2. The evolutionary divergent between the isolated *Photorhabdus* spp. (NTV) with three other *P. luminescens laumondi* strains was (0.001) see Appendix VI, however unknown A may be of a different strain of *P. luminescens*. The difference in most of the *Photorhabdus* subspecies is based on the type of *Heterorhabditis* species it was isolated from. *P. luminescence* subsp. *kleinii, P. luminescence* subsp. *noenieputensis* and *P. temperate* subsp. *tasmanensis* have been isolated from *H. georgiana*, *H. indica,* and *H. zealandica* respectively. *Xenorhabdus* bacterial isolates were used as closely related species given that the 16s rRNA genes in both bacterial symbionts are over 94% identical (Chaston *et al.* 2011).

The use of 16s rRNA sequence for phylogenetic analyses has its limitations and fails to determine the phylogenetic relationship at the level of the deepest nodes of the tree (subspecies level) (Adams *et al.* 2006; Tailliez *et al*. 2010). These genes undergo lateral gene transfer across taxonomic groups which may result in incorrect analysis. Therefore, bacterial phylogeny based only on the 16s rDNA gene should be regarded as a preliminary step (Adams *et al.* 2006). A multigene approach using the five protein-coding genes such as glnA and gyrB gene has been proven to be very useful in deducing intrageneric phylogenetic relations of *Photorhabdus* species to subspecies (Akhurst *et al*. 2004; Kuwata *et al*. 2008; Tailliez *et al*. 2010; Ferreira *et al.* 2013).

EPNs forms an exclusive mutualistic relationship with its symbiont bacteria and no cross association is known. However other bacteria have been isolated from an infected host and from the cuticle of the nematode which includes *Pseudomonas aeruginosa* (Schroeta),

Acinetobacter spp. and *Ochrobactrum* spp. which were found to naturally associate with *P. luminescens* subsp. *akhurstii* (Lysenko and Weiser 1974; Adams *et al*. 2006). *Paenibacillus nematophilus* (Enright) attach to the surface of *Heterorhabditis* spp. and is able to reproduce inside the infected host by suppressing antibiotics produced by the symbiont *Photorhabdus* spp (Enright and Griffin 2004). Therefore, it is likely that nematodes can transport non-symbiotic bacteria however these association may be detrimental to the symbiotic relationship of the nematode and its symbiotic bacteria (Lysenko and Weiser 1974; Enright and Griffin 2004; Adams *et al*. 2006).

3.5 Conclusion

Entomopathogenic bacterium *P. luminescens* subsp. *laumondi* strains was found to exclusively form a mutualistic relationship with *H. bacteriophora* strain NTV isolated in the North West province. The knowledge acquired from the chapter can ensure that EPNs will become even more effective and their symbionts are regarded as important and fundamental in biocontrol agents.

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Chapter 4: The effect of using organic compost on the infectivity and survival of EPNs in the Soil

4.1 Introduction

4.1.1 Factors affecting EPNs in an agroecosystem

The efficacy of EPNs in field application is affected by biotic environmental factors such as host population and predators and abiotic edaphic factors such as desiccation, temperature but most importantly soil texture and moisture (Kung *et al.* 1990; Barbercheck 1992; Lawrence *et al.* 2006; Bal *et al*. 2017; Helmberger *et al*. 2017). EPNs are exposed to a broad biodiversity of soil organisms, potential insect host, predators in the form of mites and microbial pathogens in the form of nematophagous fungi which affect the survival of EPNs in the soil (Kaya 2002; Ulug *et al.* 2014; Helmberger *et al.* 2017). In addition phoretic hosts (earthworms), competitors (free-living nematodes) and finally chemical signallers (plants) affects the reproduction, movement and host location of EPNs in the soil respectively (Kaya and Koppenhöfer 1996; Helmberger *et al*. 2017). EPNs are best adapted for organic agriculture since they commonly found in agricultural soils, are environmentally friendly, safe to non-target organisms and easy to mass produce on large scales (Adams *et al.* 2006). To develop optimal EPN application strategies in the field, knowledge about the kinds of impact which the physical nature of the agricultural soil environment may have on nematode survival, persistence and host location is fundamental (Bal *et al*. 2017).

Soil moisture and texture affects the movement, pathogenicity, and survival of EPNs the soil (Kung *et al.* 1990; Koppenhöfer and Fuzy 2007; Campos-Herrera *et al.* 2015). Survival under low moisture conditions is very important for the persistence of EPNs in the soil. When water becomes a limiting factor, EPNs enter a state of partial anhydrobiosis, in which their metabolism is partly suppressed, enabling them to survive long periods of desiccation (Womersley 1990; Crowe *et al.* 1992; O'leary *et al* 2001; Koppenhöfer and Fuzy 2007), but when water becomes available again, nematodes are able to rehydrate and resume active metabolism (Crowe *et al.* 1992). The slow rate of water loss during desiccation enables

biochemical adaptation necessary for anhydrobiosis which results in an increase in glycerol and trehalose, these act as membrane protectants (replace water) allowing nematodes to gradually dehydrate which reduces their mortality (Womersley 1990; Crowe *et al.* 1992; O'leary *et al.* 2001; Strauch *et al*. 2004). At this state, EPNs use less energy and are more resistant to environmental extremes (Grewal 2002; Strauch *et al.* 2004; Yan *et al.* 2010). So far desiccation tolerance has been an important factor affecting the efficacy of EPNs in the field and has been identified to a play a significant role in the storage and shelf life of EPNs prior application (O'leary *et al*. 2001; Yan *et al.* 2010).

4.1.2 Application of soil amendments

Many agricultural practices such as irrigation, tillage, fertiliser and pesticide usage all influence nematodes infectivity and survivorship in the soil (Barbercheck 1992). Farmers add soil amendments such as compost in field soil to increase organic matter content, improves soil aeration, porosity, water holding capacity which over time improves the quality of crops by providing nutrients to the plants (Bailey and Lazarovits 2003; Steel *et al.* 2010). Compost has been shown to suppress, prevent and control some soil borne plant diseases (Bailey and Lazarovits 2003; Steel *et al.* 2010; 2012). Compost differs in physical, chemical and biological properties dues to its feedstock composition (wood/bark), the concentration of nutrients and organic matter content (Steel *et al*. 2012). It was observed that the accumulation of compost in field soil can affect the presents and abundance of the nematode community (Bulluck *et al*. 2002; Nahar *et al.* 2006; Leroy *et al*. 2009) when applied regularly over time (Steel *et al.* 2012).

Soil is a complex system that responds to environmental changes and energy supply (Barbercheck 1992). Soil water holding capacity is one of the most vital soil factors in crop production (Minasny and McBratney 2018). Water capacity in the soil can be enhanced by increasing organic matter content which is assessed from soil texture and bulk density (Manns *et al.* 2016). Soil organic content or soil organic matter has been shown to be strongly correlated with soil water content (Manns *et al.* 2016). However, review on the impact of soil organic matter on soil water retention has reported mixed results (Minasny and McBratney 2018). If soil organic content does, in fact, increase both water holding capacity and water retention then it should also extend the period of time over which an initial application of IJs would be efficacious as a biocontrol agent.

Thus, considering how these abiotic and biotic soil factors affect the efficacy of EPNs in field soils is mandatory for successful application against target insect since EPNs studies done under laboratory conditions normally use treated soil to reduce any of these interactions. The aim of the study was to illustrate the effect of soil physical factors on EPNs infectivity, thus investigating the use of adding soil amendments such as organic compost on the duration of infectivity and survivorship of *Heterorhabditis bacteriophora* IJs in soil sample undergoing progressive moisture lost due to evaporation.

Objective: A comparative study of the effects of using a wide range of percentage organic compost with respect to different composts, and soil textures on the continued infectivity of *H. bacteriophora* in soil sample undergoing progressive dehydration. The key question includes:

- 1a. Do the combined effects of % organic compost amendments and specific compost composition have a significant effect on increasing the duration of EPN infectivity in soil undergoing progressive moisture loss? In other does compost amendments increase the persistence of IJs capacity to continue infecting larvae in the soil undergoing dehydration?
- 1b. How does soil texture influence EPN survivorship and infectivity in compost-amended soils?
- 1c. Does the organic compost amendment affect the number of IJs released from infected larvae?

4.2 Methods and Materials

4.2.1 Petri dish assay

Two types of soil texture coarse river sand and loamy soil classes were mixed with four different kinds of commercially available organic compost which for the sake of this study have been labelled as follows: organic compost (OC), mulch, organic lawn dressing (OLD) and professional potting mix (PPM). The composts used were different with respect to organic matter content, the concentration of nutrients, feedstock composition, and

commercial usage. Each soil type and compost used were sieved and sterilised by autoclaving for 120 hours. Samples were left to dry in the oven for 14 days at 65^{o}C. The two soil texture classes were then amended with different organic compost by mixing the soil with on a mass: mass bases with the composts to give the following organic matter contents 0%, 5%, 10%, 15%, 20% as illustrated in Table 4.1 using the Petri dish assay. Plastic Petri dishes (90mm) were filled with a fixed volume (20g) of different percentage of compost-soil treatment. Water content for coarse river sand: compost was brought to 10% (v/w) and loamy soil: compost was 15% (v/w) using distilled water. The differences in percentage moisture content for sand versus loamy soil was due to soil particle size and consequently soil pore space volume and thus different moisture saturation volumes. Thereafter about 500 IJs per ml were dispersed into each Petri dish and closed with the lid.

Table 4. 1 Concentration of each soil type: organic compost measured in Petri dishes on a mass; mass basis

Organic compost concentration (%)
0 (Sand)
5 (5% Compost + 15% Sand)
10 (10% Compost + 10% Sand)
15 (15% Compost + 5% Sand)
20 (Compost)

Each row, of a total of four rows, consisted of four control plates and four Petri dishes for each of the 0, 5, 10, 15 and 20% compost concentration for coarse river sand and loamy soil, with a total of 20 Petri dishes per row (n = 100 Petri dishes). To assay IJs viability and infectivity following soil moisture loss, five *Galleria mellonella* larvae were placed on each row of plates at different dehydration intervals (0, 24 h, 48 h, and 64 h). The plates were inverted to prevent the larvae from escaping. This was done in order to evaluate both dehydration tolerance and the IJ capacity to infect larvae under conditions of diminishing moisture levels as illustrated in Figure 4.1. At each of the above time intervals, moisture loss was measured for each row of Petri dishes (see Appendix VII). Mortality of larvae placed in the Petri dishes after each dehydration interval was recorded for a period of 7 days at 23-25^{o}C. Infected larvae were put on White traps to confirm EPN induced mortality.

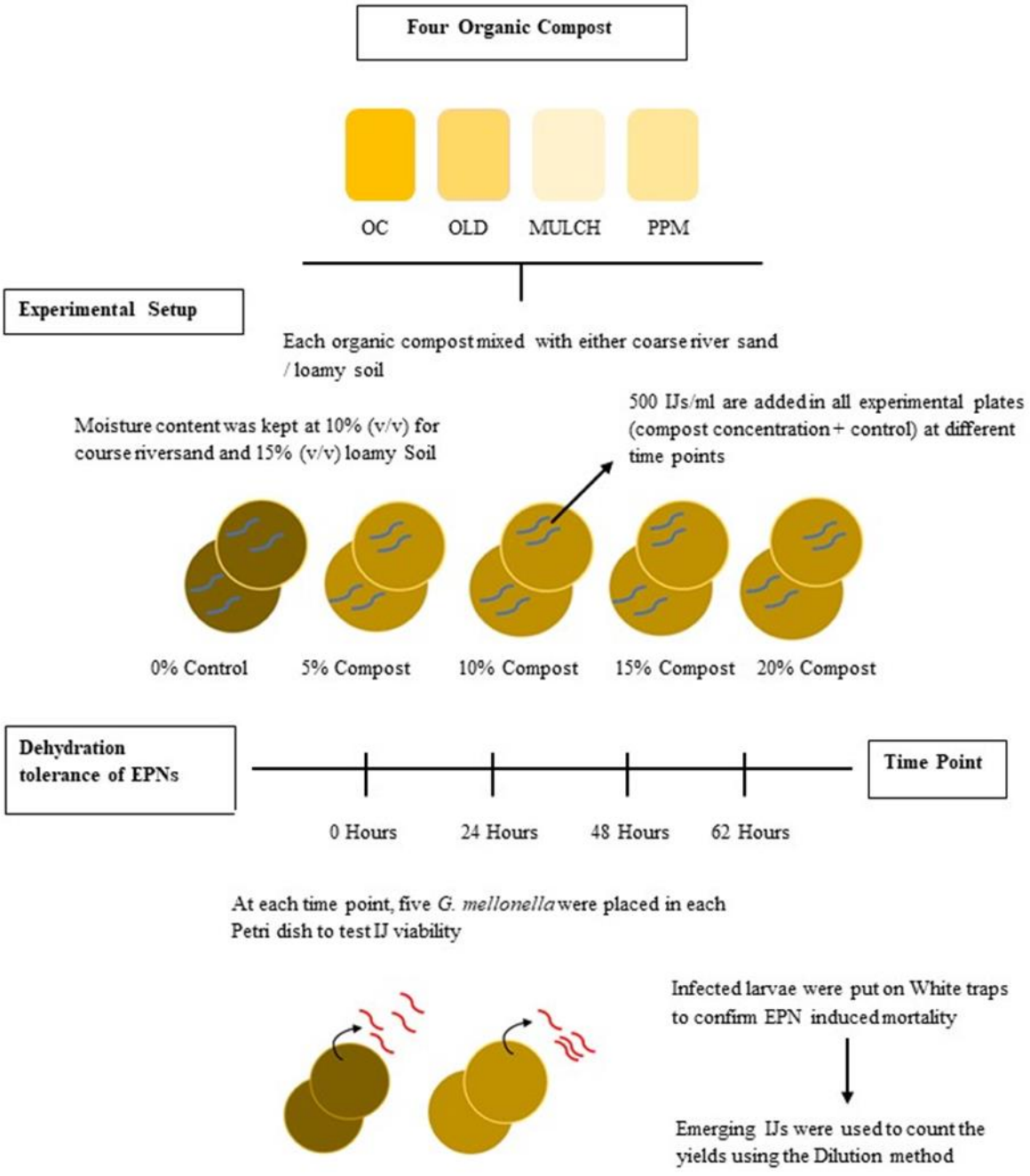

Figure 4. 1 Graphical representation of the Petri dish assay experimental setup (graphics developed by NN Khumalo).

4.2.2 Yield of EPN population

The total number of IJs emerging from a single cadaver for each treatment was enumerated by collecting all the IJs from the White traps into 50ml Falcon tubes. This was done to determine whether the addition of organic compost affects the population of IJs in the soil thus their ability to still infect. The IJs were allowed to sediment after which they were resuspended in a fixed volume of 10ml distilled water. To calculate the number of IJs produced per *G. mellonella* cadaver, the dilution method described by Kaya and Stock (1997) was used. From the dilution samples, aliquots of 10µl were used, the process was repeated four times for each recovered population of IJs for the 1st count followed by the 2nd count which was done when the number of emerging IJs was less than 50% on the White trap method.

4.2.3 Statistical Analyses

The means of each compost concentration (MULCH, OC, OLD, PPM) and soil type classes (controls) at different days of dehydration (0, 24 h, 48 h, 62 h) were analysed using 2-way ANOVA with replication using Microsoft Excel, 2016 Software. For further analyses, Tukey's HSD at 5% significant level, was used to find out which composts at each specific concentration (5%, 10%, 15%, 20%) were significant when compared with each other. Statistical analysis was completed using Microsoft Excel, 2016 Software.

4.3 Results

The type of compost used based on its feedstock composition (wood/bark), concentration of nutrients and organic matter content mixed with a specific type of soil texture class had a significant effect on the tolerance of EPNs in response to different dehydration periods ($P<0.05$; $F=33$; $Df=48$) based on 2-way ANOVA (see Appendix VIII). The differences in the persistence of infectivity under conditions of progressive moisture loss can be attributed to the possible effects of the two different soil texture classes. Compost mixed with loamy soil was more compact, had a high bulk density which increased water holding capacity however the movement of *H. bacteriophora* IJs was restricted. Coarse river sand has larger pores and tends to dry up quickly however when mixed with compost, allowed better aeration and movement of EPNs in the soil.

In histograms in Figure 4. 2 and 4. 3, almost 100% insect mortality was achieved at different soil amendments (MULCH, OC, OLD, PPM), which can be treated as a stage when moisture levels were optimum for IJ infectivity. Levels of larval infection and mortality by IJs subjected to the different durations of substrate moisture deficits were measured relative to the initial levels at zero time. The different types of compost concentration mixed with loamy soil had a significant effect on IJs in the soil ($P<0,05$; $F=44$; $Df = 16$) (see Appendix VIII). MULCH amendment had the highest insect mortality rate at all concentration (5%, 10%,15%,20%) compared to OLD, OC based on the Tukey's test, (P,0.05) (See Appendix IX). Most effective compost for protecting IJ against the stress effects of moisture loss and also for facilitating infection of larvae after substrate moisture loss as shown in histograms in Figure 4. 2 and 4. 3. Prolonged periods of dehydration exposure of IJs in the soil (0, 24hours, 48 hours, 62 hours) had a significant effect on the infectivity and persistence of H. bacteriophora in the soil ($P<0.05$; $F=17778$; $Df=3$). Virulence and efficacy decreased over 7 days of progressive dehydration. The implication of using different compost concentration influenced how long H. bacteriophora IJs survive dehydration exposure.

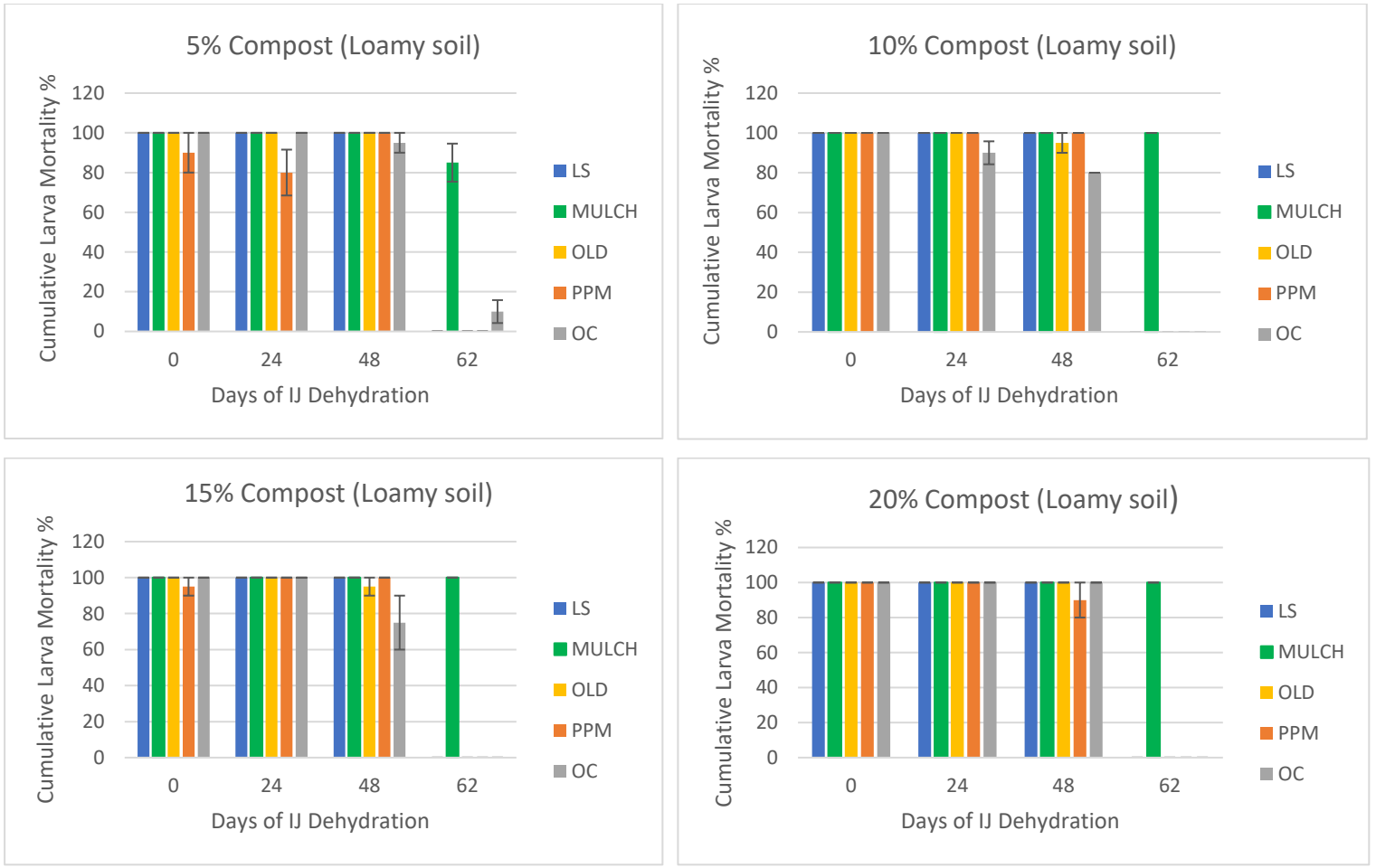

Figure 4. 2 The bar graphs show cumulative larval mortality of larvae placed on the different soil amendment treatments (MULCH, OLD, PPM, OC) mixed with loamy soil. The MULCH treatment was associated with the highest insect mortality at 5%, 10%, 15% and 20% ($p<0,005$) based on the Tukey's test. The overall trend was that prolonged dehydration exposure of EPNs to moisture loss (0, 24 hours, 48 hours, 62 hours) reduced the infectivity and survivorship of IJs, as measured in terms of their capacity to cause larval mortality (mean ± SEM).

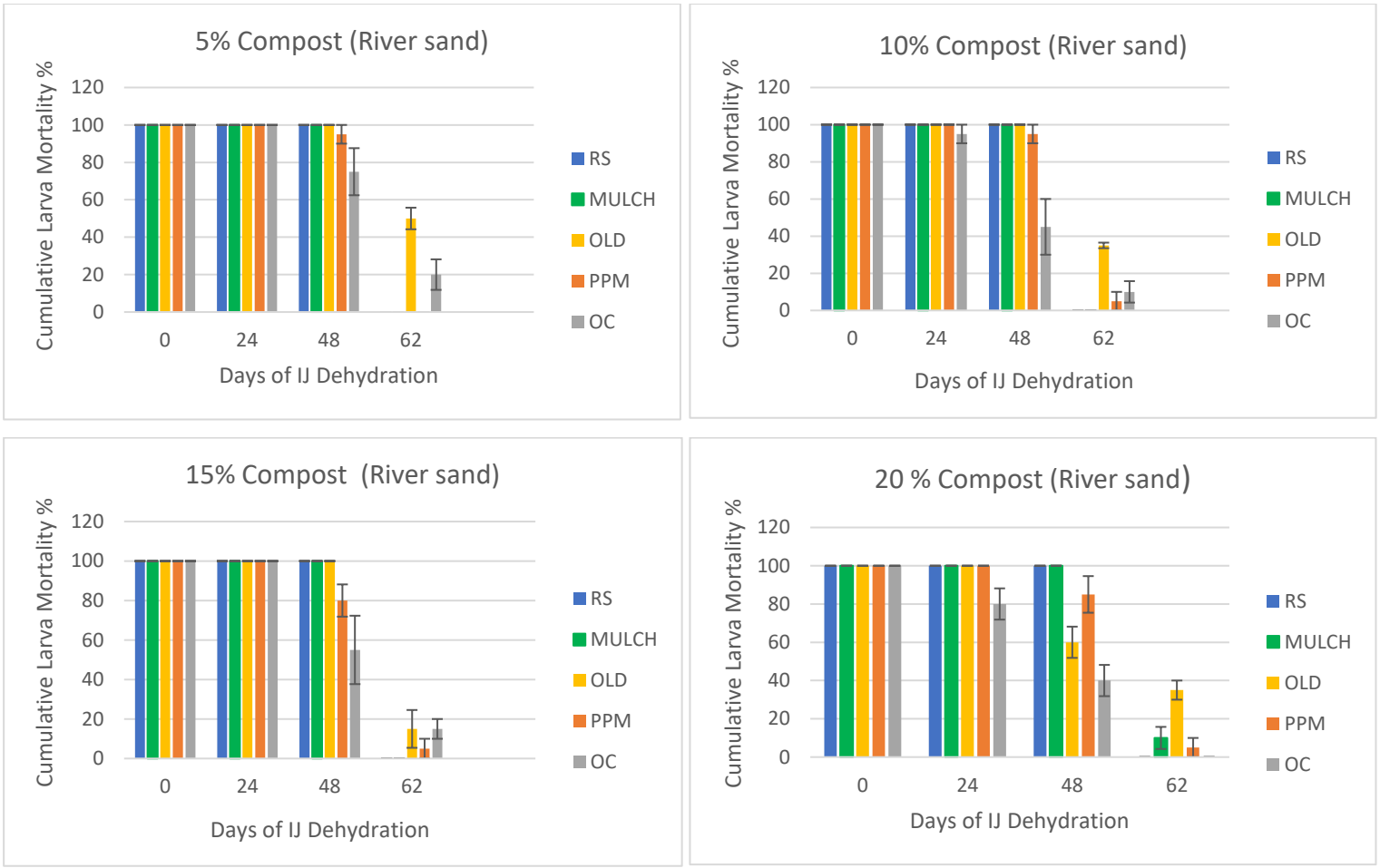

Figure 4. 3 The bar graphs show cumulative larval mortality of larvae placed on the different soil amendment treatments (MULCH, OLD, PPM, OC) mixed with coarse river sand. OLD treatment was associated with the highest insect mortality at 5%, 10% and for OC treatment at 20% ($p<0.005$) based on the Tukey's test. The overall trend was that prolonged dehydration exposure of EPNs to moisture loss at 48 hours and 62 hours reduced the infectivity and survivorship of IJs, as measured in terms of their capacity to cause larval mortality (mean ± SEM).

The type of compost concentration mixed with coarse river sand had an effect on how long IJs can survive and still be effective at different days of dehydration ($P<0.05$; $F=6$; $Df=48$) (see Appendix VIII). Moisture loss increased with progressive dehydration in each compost amendment (see Appendix VII). Insect mortality decreased as IJs were exposed to longer periods of dehydration which affected the infectivity and persistence in the soil ($P<0.05$; $F=952$; $Df=3$). Noticeable at 48 hours and 62 hours dehydration point. Based on Tukey's test 5%, 10% OLD and 20% OC had the highest infection rate compared to OC and PPM, however, there was no significant difference amongst the compost at 15% based on Tukey's test (P,0.05) (See Appendix X).

Loamy soil produced (120000-850000) IJs per insect cadaver followed by OLD (40000-780000), PPM (80000-900000), MULCH (20000-620000) and OC (30000-70000) as shown in Table 4. 2. IJs produced for coarse river sand ranged from (80000-800000), followed by OLD (4000-700000), PPM (6000-700000), MULCH (6000-620000) and OC (4000-700000) when exposed to 7 days of desiccation tolerance as shown in Table 4. 3. Regarding moisture from the different treatments, all closed Petri dishes were exposed to the same vapour pressure deficits at room temperature. The close Petri dishes helped to create a still air boundary layer above the soil-compost substrate thereby retaining the rate of moisture loss from the substrates over the 62 hours dehydration period (cumulative). See rates of water loss in appendix VII since the moisture loss was less than 0.5% in all the treatments.

Table 4. 2 The yields of IJs recovered from a single infected larval cadaver after the IJs had been exposed to different periods of dehydration in loamy soil with different organic compost amendments and % concentration (OC, OLD, MULCH, LS, PPM). Yields were obtained 24-48 hours post infection on White traps and when IJs were less than 50% using the dilution method illustrated by Kaya and Stock (1997).

	Days of IJs dehydration			
Compost concentration %	0	24 Hours	48 Hours	62 Hours
Loamy soil	720000 - 200000	850000 - 220000	410000 – 120000	
OLD (5)	400000 – 120000	620000 - 120000	840000 -40000	
OLD (10)	540000 – 200000	520000 - 100000	600000 - 100000	
OLD (15)	640000 – 140000	760000 - 100000	680000 - 80000	
OLD (20)	500000 – 120000	720000 - 100000	780000 - 80000	
PPM (5)	460000 – 140000	520000 - 80000	660000 - 120000	
PPM (10)	620000 – 120000	600000 - 140000	780000 - 100000	
PPM (15)	480000 – 180000	840000 - 140000	760000 - 120000	
PPM (20)	320000 – 60000	900000 - 60000	740000 - 80000	
MULCH (5)	620000 – 40000	520000 - 60000	600000 - 100000	220000 - 80000
MULCH (10)	440000 – 60000	400000 - 100000	380000 - 120000	240000 - 60000
MULCH (15)	420000 – 180000	460000 - 140000	600000 - 160000	320000 - 20000
MULCH (20)	400000 – 60000	360000 - 180000	280000 - 60000	300000 - 40000
OC (5)	400000 – 40000	700000 - 120000	580000 - 120000	100000 - 30000
OC (10)	460000 – 140000	480000 - 80000	660000 - 80000	
OC (15)	520000 – 60000	740000 - 140000	700000 - 100000	
OC (20)	440000 – 40000		580000 - 120000	

Table 4. 3 The yields of IJs recovered from a single infected larval cadaver after the IJs had been exposed to different periods of dehydration in coarse river sand with different organic compost amendments and % concentrations (OC, OLD, MULCH, LS, PPM). Yields were obtained 24-48 hours post infection on White traps and when IJs were less than 50% on White traps using the dilution method (Kaya and Stock 1997).

	Days of IJs Dehydration			
Compost concentration %	0	24 Hours	48 Hours	62 hours
River sand	800000 – 100000	640000 - 80000	420000 - 80000	
OLD (5)	380000 - 120000	700000 - 80000	440000 - 80000	440000 - 80000
OLD (10)	260000 - 120000	500000 - 120000	550000 - 100000	460000 - 80000
OLD (15)	320000 - 40000	680000 - 80000	480000 - 100000	420000 - 90000
OLD (20)	380000 - 60000	580000 - 220000	500000 - 90000	400000 - 40000
PPM (5)	500000 - 240000	700000 - 120000	360000 - 60000	420000 - 80000
PPM (10)	400000 - 180000	620000 - 120000	420000 - 80000	380000 - 100000
PPM (15)	520000 - 120000	640000 - 140000	380000 - 100000	380000 - 60000
PPM (20)	500000 - 180000	280000 - 80000	380000 -60000	
MULCH (5)	620000 - 140000	460000 - 80000	420000 - 20000	
MULCH (10)	460000 - 140000	580000 - 80000	320000 -60000	
MULCH (15)	420000 - 140000	420000 -80000	380000 - 20000	
MULCH (20)	440000 - 140000	500000 - 100000	360000 - 80000	120000 - 60000
OC (5)	440000 - 120000	600000 - 40000	300000 - 80000	100000 - 60000
OC (10)	300000 - 40000	480000 - 80000	360000 - 80000	120000 - 80000
OC (15)	400000 - 100000	620000 - 160000	340000 - 80000	100000 - 40000
OC (20)	700000 - 120000	540000 - 100000	340000 - 60000	

4.4 Discussion

4.4.1 Soil structure

The type of compost concentration used had a significant effect on the tolerance of EPNs in different dehydration periods. Firstly, the difference in infectivity was attributed by the two types of soil textures used namely coarse river sand and loamy soil. Georgis and Gaugler (1991) hypothesized that movement of IJs is restricted in fine soils. Finer soils which have high clay content and high organic matter are highly saturated, generally have small pore sizes which retain soil moisture longer however limit oxygen, resulting in poor aeration (Kung *et al.* 1990; Georgis and Gaugler 1991; Barbercheck 1992; Stuart *et al*. 2006; Koppenhöfer and Fuzy 2007). EPNs in such soils use stored carbohydrates in food reserves inadequately thus reducing the efficacy of finding a host and survivorship in the soil (Croll and Matthews 1977; Kung *et al.* 1990). A lot of studies have shown reduced infectivity of *H. bacteriophora* in finer textured soils. Portillo-Aguilar *et al* (1999) showed that *H. bacteriophora* moved better in sandy loam than in loam silt clay and the movement generally decrease as bulk density increases (Portillo-Aguilar *et al.* 1999).

4.4.2 Addition of organic compost

EPNs are applied in soils mixed with chemicals, pesticides, soil amendments and fertilisers (Ulug *et al*. 2014) and are compatible with existing *Bacilllus thuringiensis* (Bt) products, nematicides, fungicides and herbicides (Kaya *et al.*, 1994; Hazir *et al*. 2004) which has been shown to produce higher mortality when combined (Rovesti and Deseo 1990; Glazer 1996). Most fertilisers, when applied in many agricultural soils at recommended rates, have a minute effect on the efficiency of EPNs in the field (Bednarek and Gaugler 1997; Shapiro-Ilan *et al.* 2006).

As shown in figure 4. 2 and 4. 3 all the different compost concentration used (5%, 10%, 15%, 20%) had an effect on infectivity and persistence of EPNs in the soil when mixed with different classes of soil texture ($p<0.05$) using 2-way ANOVA with replication. The difference in infectivity was attributed by the type of compost concentration which was based on different moisture saturation volumes (water holding capacity), organic matter content and

type of soil classes used. Soil texture and structure affected moisture holding capacity for each soil type (Barbercheck 1992). In the present study, loamy soil mixed with finely textured organic compost such as OC and OLD affected the movement and infectivity of EPNs. MULCH and PPM contained more bark and wood chips when mixed with loamy soil offered better aeration thus enabled IJs to move and still infect as shown by the histograms in Figure 4. 2. The MULCH soil amendment resulted in the highest insect mortality rate across different application concentration (mass; mass basis) when mixed with loamy soil as illustrated in Figure 4. 2.

In contrast, coarse river sand mixed with OLD and OC was able to hold more moisture and provided good aeration environment for *H. bacteriophora* IJs, thus resulted in the highest insect mortality and survivorship in the soil as shown in Figure 4. 3. The observed difference in the use of organic compost on the infectivity of EPNs is based on its composition, organic matter content and commercial usage (Bulluck *et al*. 2002; Nahar *et al*. 2006). Coarse river sand alone dried up faster compared to course river sand mixed with organic compost which restricted the movement and affected the infectivity of *H. bacteriophora* IJs. The organic compost used were commercially available thus information about the chemical and physical properties could not be obtained.

Inorganic fertilisers and chemical pesticides have a detrimental effect on the population, survivorship, and efficacy of EPNs in many soils (Shapiro-Ilan *et al.* 2006). Bednarek and Gaugler (1997) demonstrated that EPNs respond to inorganic fertilisers according to concentration and composition. When EPNs are exposed to a high concentration of fertilisers for longer periods, EPN infectivity and reproduction is reduced. Similarly, fresh manure and urea have been shown to be detrimental to EPNs survivorship and efficiency (Bednarek and Gaugler 1997; Shapiro-Ilan *et al*. 2006). Some chemical pesticides such as methomyl are toxic, chlorpyrifos are compatible and tefluthrin combined act synergistically with EPNs (Bednarek and Gaugler 1997; Alumai and Grewal 2004; Shapiro-Ilan *et al.* 2006).

The addition of organic compost did not affect the population of IJs produced. Under laboratory condition, a single insect cadaver produces (30000-400000IJs) (Stuart *et al*. 2006). This was supported by the present data which showed the lowest produced IJs at 62 hours post desiccation to be 20000 IJs and highest 800000 IJs per insect cadaver shown in table 4. 2 and 4. 3. The population of IJs was not affected since soil texture, moisture was favourable

for the infectivity of EPNs in the soil. The slight gradual decline in the number of IJs produced was affected by longer periods of desiccation. In field soil, about (7400 – 1500000 IJs/square metre) or 2.5 $x10^9$ IJs/ha or higher. The recommended rate for commercial application for EPNs is 250 000 IJs per square meter (Shapiro-Ilan *et al*. 2006; Susurluk and Ehlers 2008; Helmberger *et al*. 2017).

4.4.3 Desiccation and survival strategies

Lastly, *H. bacteriophora* IJs were able to survive slow periods of desiccation. This was observed by a gradual moisture loss as days of dehydration increase for both types of soil texture (see Appendix VII). It was observed that virulence and efficacy decreased over 7 days of progressive dehydration specifically at 48 h and 62 h. The addition of organic matter hypothetically provided a suitable environment, shielded *H. bacteriophora* IJs from extreme desiccation which enabled better survival and longevity to still infect larvae in the soil.

To survive longer periods of desiccation, EPNs slow down the rate of moisture loss by forming tight coils which decrease the surface area of the J2 cuticle directly exposed to the air (Womersley, 1990; Kaya and Gaugler, 1993; Glazer, 1996), aggregate to form large clumps and also remain inside the cadaver of the insect until moisture conditions improve (Koppenhöfer *et al*. 1997; Hazir *et al.* 2004), which enables *Heterorhabditis* spp. to persist for 2-3 weeks in dry condition (Kaya and Gaugler, 1993; Glazer, 1996). *H. bacteriophora* IJs can tolerate moderate slow desiccation by accumulating glycerol and trehalose. In addition, antibiotic compounds produced by the symbiotic bacteria provides protections which enables EPNs to persist in the soil (Timper and Kaya 1989; Glazer 1996; O'Leary *et al*. 2001). Desiccation induces EPN quiescence anhydrobiosis leading to longer shelf life and longevity in the soil (Kung *et al.* 1990; Koppenhöfer *et al.* 1997; Lu *et al.* 2016).

4. 5 Conclusion

To accomplish successful application in agroecosystems a variety of biotic and abiotic factors must be considered. Chemical pesticides and fertilisers can either have positive or negative effects on EPNs and soil texture, size composition and organic matter all influence the efficacy of EPNs in soil. The study demonstrated that the addition of organic amended in the

soil does not have any detrimental effect on EPNs. It increased the persistence of IJs in soils undergoing progressive dehydration up to 4 days, important for application in regions experiencing drought condition. Knowledge obtained will be used to improve soil application strategies and formulation products so that EPNs can be used as commercially viable biocontrol agents.

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Chapter 5: Factors affecting EPNs survival and infectivity in host location profiles

5.1 Introduction

5.1.1 What type of foraging behavior does EPNs exhibit?

The process of host infection is influenced by a number of factors including host habitat, host location, host acceptance and suitability (Selvan *et al*. 1993; Lewis *et al*. 2006). EPNs exhibit different foraging strategies to increase their chances of encountering and recognizing a suitable host (Kaya and Gaugler 1993). Cruise foragers such as *Heterorhabditis bacteriophora* allocate most of their time moving and searching in the environment, foraging deeper in the soil profile to find a suitable insect host (Campbell *et al*. 1996; Gaugler 2002). Searching for a host for cruise foragers is more energetically expensive thus they store more lipids and tend to be larger and longer in size >801µl (Selvan *et al*. 1993; Campbell and Kaya 2002). Cruise foragers switch to movement patterns that focus on localized search (reduced speed and increase turning rate biases) and are most effective at finding and infecting sedentary and cryptic insects (Lewis *et al*. 1993; Campbell and Gaugler 1993; Campbell *et al*. 2003; Lewis *et al*. 2006).

5.1.2 How do EPNs recognize their host?

Entomopathogenic nematodes are restricted to chemosensation, mechanosensation and thermosensation to obtain information about their environment (Halloran and Burnell 2003; Lewis *et al.* 2006). To locate a potential host in the soil ecosystem, cruise foragers are attracted to host volatile cues, host contact cues, cues dissolved in water film, chemical cues, vibrations (Lewis *et al*. 1993; Jaffuel *et al.* 2016) and cues from damaged plants caused by insects (Van Tol *et al*. 2001). Host contact cues such as CO_2, cuticle and feces, are short range and provide specific information about the host (Lewis *et al.* 1993), while volatile cues are long range and give directional information for host location (Kaya and Gaugler 1993; Lortkipanidze *et al*. 2016). However, in the absence of host-associated cues, cruise foragers move using a linear movement pattern (Lewis *et al*. 1993).

5.1.3 How do EPNs gain entry?

Entomopathogenic nematodes use their chemosensory system to locate routes of entry into the haemocoel. This is through the physical structures and opening associated with the insect integuments, which enables nematodes to detects the passages through which the products of the insect metabolism are released (Lewis *et al.* 2006). EPNs are thus able to penetrate the host hemocoel through natural openings such as the cuticle, through the tracheal cuticle using the spiracles and using the mouth or anus through the wall of the gut (Bedding and Molyneux 1982; Lewis *et al*. 2006). IJs of *H. bacteriophora* use their proximal tooth to rupture the insect body wall through head thrusting movement (Lewis *et al.* 2006), a process which is believed to result in successful parasitism (Bedding and Molyneux 1982; Lewis *et al*. 2006)

5.1.4 Factors affecting the efficacy of EPNs in the field

Desiccation tolerance is the most important factor affecting not only the survival of IJs of all EPN species under natural conditions but also the commercial use of nematodes from mass production, formulation (shelf life) and application in the field (post application) (Gaugler 2002). EPNs can survive desiccation through partial anhydrobiosis (Womersely 1990; Grewal *et al*. 2006), an important adaptation for places that may experience dry conditions. Hence, it is important to identify strains that can tolerate desiccation once applied in the field. The effectiveness of EPNs then depends on a combination of foraging strategies, host location abilities and soil habitat quality (soil texture, structure, moisture), however knowledge about how these different factors interact is very limited (Shapiro-Ilan *et al.* 2006; Lortkipanidze *et al*. 2016). Therefore, understanding search behavior will help to predict what might happen in the soil ecosystem post application in many agricultural fields (Barbercheck 1992). In line with this, the study aimed to test how certain factors could affect the success of the foraging strategy employed by *H. bacteriophora* IJs and testing the host location abilities within soil profiles. Factors investigated were: 1) foraging efficiencies within different soil composition and texture, and 2) the soil moisture content effects on desiccation tolerance.

Objectives 1: Investigate the type of foraging strategy used by *H. bacteriophora* IJs and their ability to locate the insect host at different depths in the soil profiles. Key questions were:

- 1a. Does soil composition (mixture of organic compost to loamy soil) influence the behavior and survivorship in soil and host location in soil profiles?
- 1b. Does an increase in EPN population affect infectivity?

Objective 2: Investigate desiccation tolerance of *H. bacteriophora* at 0, 7, 14 days dehydration in soil profiles

- Does soil composition affect how long *H. bacteriophora* persist in dry conditions?
- How long can *H. bacteriophora* IJs survive in dry conditions?

5.2 Methods and Materials

5.2.1 Soil column bioassay

The sand columns bioassay was used as an effective tool for predicting EPN efficacy in field trials (Lortkipanidze *et al*. 2016). Four different organic compost (OLD, OC, MULCH, PPM) were mixed with loamy soil. Each mixture contained 50% (mass; mass) of organic compost; loamy soil and loamy soil alone (control) which were sieved and then sterilised by autoclaving for 120 hours for 20 min. Soil samples were dried using the oven for 14 days at 65^{o}C. Moisture content was bought to 20% (v/w) with distilled water and poured in each of the four vertical columns as shown in Figure 5.1.

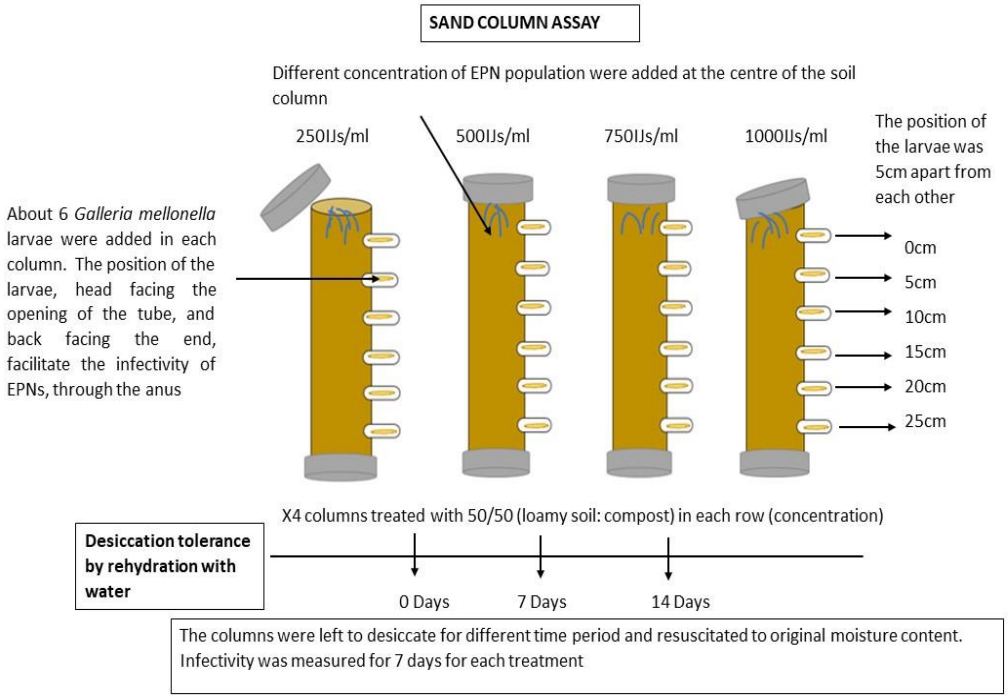

Figure 5. 1 Shows the experimental setup for soil columns bioassay (graphics developed by NN Khumalo).

To investigate the role EPN population on infectivity, different concentrations of *H. bacteriophora* IJs (250, 500, 750, 1000IJs/ml) were added at the top of each column. The IJs used were obtained in the early stages of the infection process, about 3-4 days post-infection, since IJs produced at this point benefit from insect cadaver (nutrients) and are able to survive for an extended period of time (O'leary *et al*. 2001). The top lid was loosely closed so as to allow some degree of aeration and also limit high rates loss of moisture. To facilitate the movement of IJs from the soil columns to the side tube holding the larva, a drop of water was added at the front end of the side tubes followed by placing the insect larvae (*Galleria. mellonella*). The larvae were placed tail facing the front-end tube, so to facilitate the infection process. The tubes were closed with cotton wool and prestick so as to prevent the larvae from escaping. Each treatment (soil composition) had a total of 16 columns with 4 replicates for each concentration.

To induce partial anhydrobiosis by desiccation, the survival and infectivity of *H. bacteriophora* in different soil compositions were monitored at 0, 7, 14 days of exposure to declining soil moisture content. All the columns were weighed and bought to 20% (v/w) soil

moisture content on Day 0. To determine the amount of moisture loss at each desiccation point, at day 7 and 14 the columns were weighed followed by rehydrating the soil back to its original water content (rehydration by water was equivalent to irrigation), about two days were left to allow IJs to slowly hydrate and adapt to the environment. Larval mortality was recorded for a period of 7 days with the position of the cadaver measured for each treatment. Dead larvae showing signs of *H. bacteriophora* infection were collected and placed on modified White traps to confirm EPN infection. The experiment was done over a period of 8 weeks for each treatment.

5.3 Results

Heterorhabditis bacteriophora IJs demonstrated host-seeking behaviour of being a cruise forager. IJs were able to travel different depth (0 - 25cm) to locate the insect host in soil profiles. Overall the different soil composition and texture used affected the behaviour of *H. bacteriophora* IJs to locate and infect the host at different days of dehydration tolerance (0, 7 days, 14 days) ($p<0.05$), using Two-way ANOVA (see Appendix XII). The type of soil substrate (LS, MULCH, OC, OLD, PPM) used based on difference in concentration of nutrients, organic matter and commercial usage had a significant effect on the behavior of EPNs in host location profiles (0, 5, 10, 15, 20cm) when infectivity was measured immediately ($P<0,05$; $F=12.62$; $DF=4$). The type of soil composition used (PPM, OC, OLD, MULCH, Loamy Soil) had a significant effect on the tolerance of *H. bacteriophora* to withstand longer periods of desiccation ($P<0.05$; $F=4$; $DF=14$) however infectivity and survivorship reduced with increased exposure to desiccation.

The observed difference in infectivity was attributed to soil composition texture. Finely textured compost (OLD and OC) mixed with loamy soil were denser, had higher bulk density compared to PPM and MULCH, which were better aerated and allowed movement of IJs to locate the host as illustrated by histograms in Figure 5. 2, 5. 3, and 5. 4. The increase in EPN population resulted in an increase in infectivity and persistence of *H. bacteriophora* IJs (direct dose-response). Lowest insect mortality was observed on the lowest concentration of IJs (250IJs/ml) and highest insect mortality increased with an increase in EPN population.

H. bacteriophora IJs were able to survive desiccation tolerance by resuscitation for a period of 8 weeks however infectivity and survivorship of IJs to locate the host was greatly affected as illustrated in Figure 5. 5. At Day 0, point in which we assume that the conditions were favourable for IJs to infect and locate the host, there was no significant difference observed based on 2-way ANOVA (see Appendix XII). At 7 and 14 days, post desiccation exposure followed by resuscitation, loamy soil (control) achieved almost 100% insect mortality at different soil depths compared to when loamy soil was mixed with different organic compost. The addition of compost hypothetically reduced the amount of evaporation during desiccation which increased the survival of IJs to still infect for a period of 8 weeks (see Appendix XI).

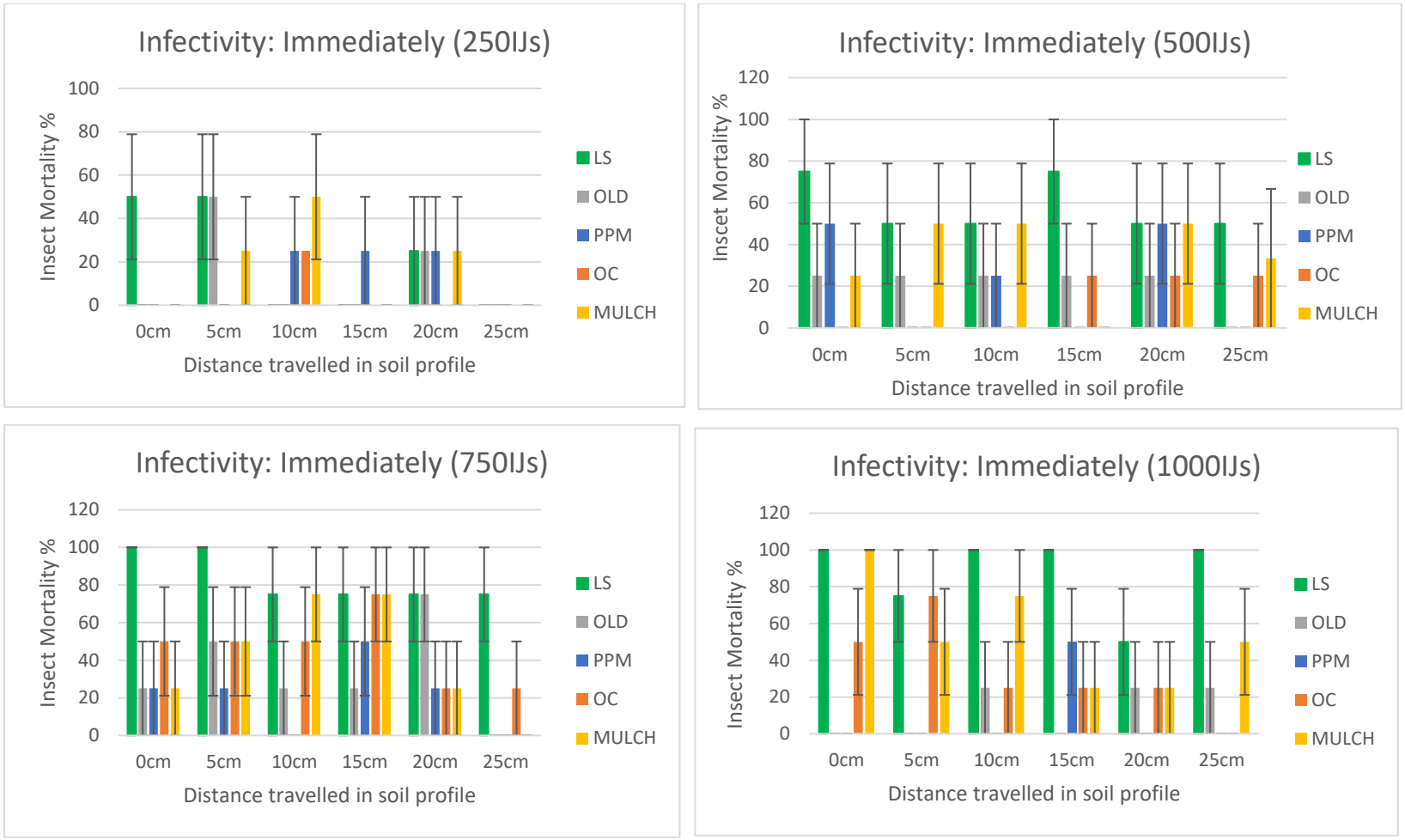

Figure 5. 2 The bar graphs show the type of soil substrate used (LS, OLD, PPM, OC, MULCH) had a significant effect on the behavior of *H. bacteriophora* IJs in host location profiles (mean ± SEM) (p<0,05*). H. bacteriophora* proved to be a cruiser forager, by moving randomly in the soil column to search for the host (0cm, 5cm, 10cm, 15cm, 20cm). An increase in EPN population (250µl, 500µl, 750µl, 1000µl) had a significant influence in the infectivity of *H. bacteriophora* in the soil column. Mortality was recorded for 7 days.

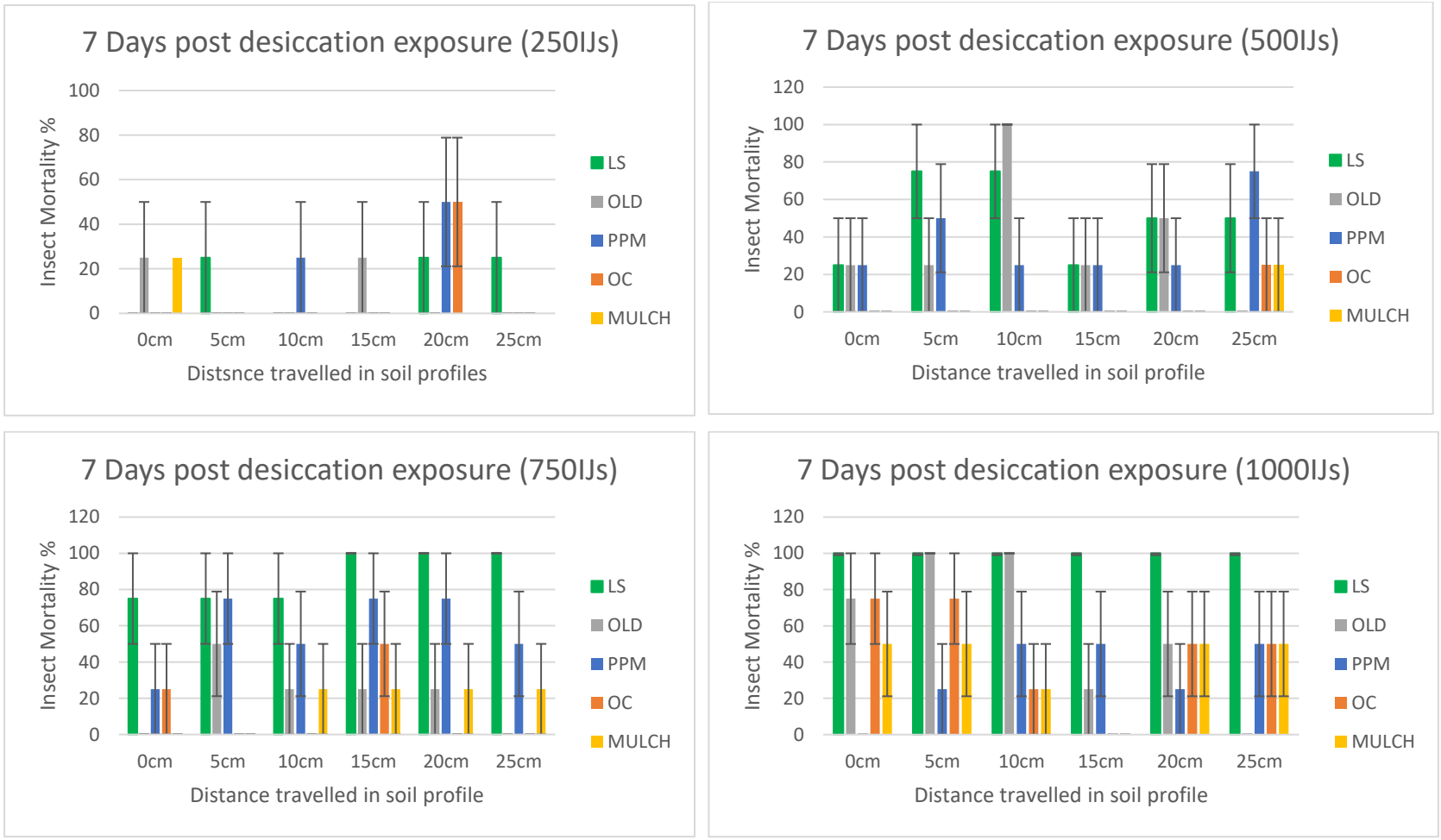

Figure 5. 3 The bar graph shows the type of soil substrate used (LS, OLD, PPM, OC, MULCH) had a significant effect on the behavior of *H. bacteriophora* IJs in host location profiles (mean ± SEM) (p<0,05*). H. bacteriophora* proved to be a cruiser forager, by moving randomly in the soil column to search for the host (0cm, 5cm, 10cm, 15cm, 20cm). 7 days of dehydration exposure with resuscitation by rehydration affected the infectivity, survivorship, and population of *H. bacteriophora* in the soil column (p<0.05). Mortality was recorded for 7 days.

Figure 5. 4 The bar graphs show the type of soil substrate used (LS, OLD, PPM, OC, MULCH) had a significant effect on the behavior of *H. bacteriophora* in host location profiles (mean ± SEM) (p<0,05*). H. bacteriophora* proved to be a cruiser forager, by moving randomly in the soil column to search for the host (0cm, 5cm, 10cm, 15cm, 20cm). 14 days of dehydration exposure with resuscitation by rehydration affected the infectivity, survivorship, and population of *H. bacteriophora* in the soil column (p<0.05). Mortality was recorded for 7 days.

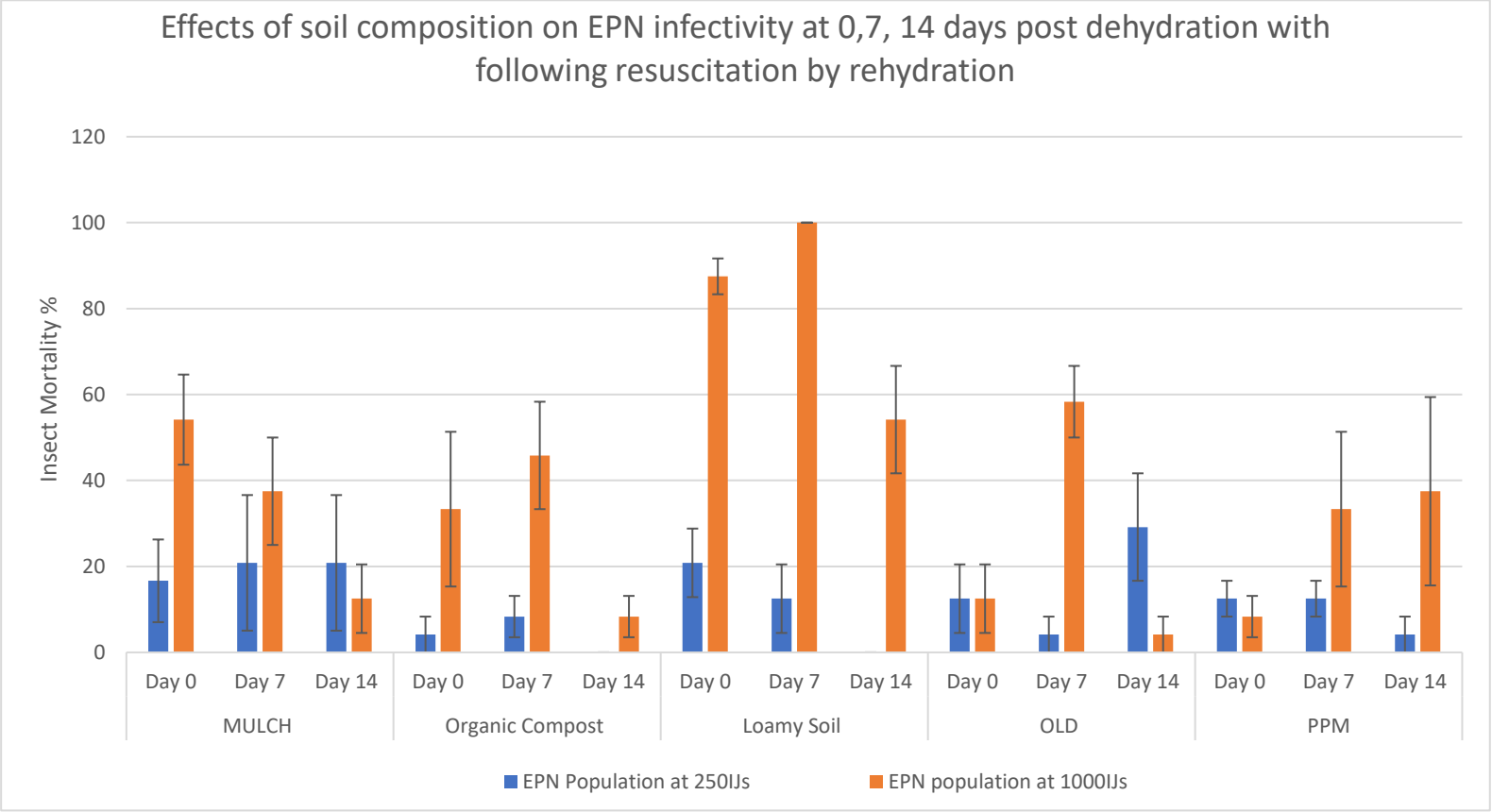

Figure 5. 5 The bar graph shows the desiccation tolerance of *H. bacteriophora*. The type of soil substrate used (LS, OLD, PPM, OC, MULCH) had a significant effect on the infectivity, survivorship of EPNs on the behavior of *H. bacteriophora* in host location profiles (mean ± SEM) (p<0,05). *H. bacteriophora* IJs were exposed to different dehydration conditions (0, 7, 14 days) and survived longer exposure of desiccation for 8 weeks however, infectivity and survivorship were greatly reduced. Two EPN populations used were at 250IJs and 1000IJs.

5.4 Discussion

5.4.1 Soil composition and foraging strategy

In the present study, the type of soil composition (physical soil structure) with the addition of organic compost (MULCH, PPM, OC, OLD, LS) had a significant effect on the movement of *H. bacteriophora* with respect to locating the host in sand columns. Factors such as high bulk density and the presence of organic matter in soil which has high sorption capacity for chemical cues both restricted and interfered with the movement and locating the host (Kung *et al.* 1990; Lewis *et al.* 1993; Portillo-Aguilar *et al*. 1999; Torr *et al.* 2004). This was observed in the study since loamy soil mixed with organic compost had the lowest insect mortality compared to loamy soil alone. Loamy soil alone (control) was less dense, better aerated compared to all soil composition, achieving almost 100% insect mortality at 0, 7, 14 days post desiccation as illustrated in Figure 5.2, 5.3 and 5.4. The reduced pore size and aeration observed in finely and heavy textured soils such as OLD and OC when sieved interfered with the transmission of host contact /volatile cues affecting the host location and infectivity of *H. bacteriophora* which declined with depth of soil (Gruner *et al*. 2007; Griffin 2012). In contrast, MULCH and PPM contained more bark and wood chips when mixed with loamy soil had better aeration and was less compact which resulted in increased infectivity and survivorship of *H. bacteriophora*. As observed in the study, the infectivity of *H. bacteriophora* IJs in soil columns increased with the concentration of IJ population. Thus, the efficacy of EPNs in agricultural soils depends on foraging strategy, insect host, host location and soil parameters such as soil texture, organic matter content, bulk density and soil water potential, which affects the behavior, survival and infectivity (Portillo-Aguilar *et al.* 1999; Shapiro-Ilan *et al.* 2006; Koppenhöfer and Fuzy 2006; Gruner *et al*. 2007; Lortkipanidze *et al.* 2016).

A study that was done by Lortkipanidze *et al*. (2016) showed that *S. carpocapsae* had difficulties locating insect larvae placed in the bottom of the soil column, based on weak reaction to chemical cues emitted by the host which limited the ability to assess the quality of host (Christen *et al*. 2007; Lortkipanidze *et al.* 2016). On the contrary, the reduced infectivity

of *H. bacteriophora* in the sand columns when OC and OLD were used, based on soil texture could have caused the same effect.

In soils with different organic matter compositions, IJs are exposed to different chemical cues which influence their behavior and infection rate (Christen *et al*. 2007). However, when chemical gradients are compromised, IJs are also able to follow vibration cues because these signals are less prone to any interference from the soil matrix which may hinder chemical cues (Torr *et al.* 2004). The decision to invade an insect depends not only on external factors such as cues from the potential host but also on the internal factors of the IJs such as aging and physiological conditions (Griffin 2012). The isolated *H. bacteriophora* demonstrated the attributes of being cruise foragers and therefore were dependent on various cures and sensory capacities to make contact with hosts using olfactory, volatile cues and environmental cues to find and locate the host in soil columns (O' Halloran and Burnell 2003; Hallem *et al*. 2011; Lortkipanidze *et al.* 2016). As illustrated in figure 5. 2, 5. 3 and 5. 4 *H. bacteriophora* moved from 0cm up to 25cm to locate the host. Another study showed that IJs can enter a host that is already occupied by conspecifics even to a point of overcrowding in either dead cadaver or healthy larvae (Griffin 2012; Lewis *et al*. 2006). This might have occurred in soil columns that had infectivity at 0cm, 5cm, 10cm and not at 15cm, 20cm and 25cm. Another contributing factor would be lack of oxygen since the level of oxygen decreases with depth, that affected infectivity of *H. bacteriophora* IJs.

A study done by O'Halloran and Burnell (2003) showed that *H. bacteriophora* respond chemotactically to long chains alcohol (1-hexanol, 1-heptanol, and 1-octanol) to direct them to infected damaged plants. Use various thiazoles (2-acetylthiazole, 2-Isobutythiazoles), aromatic compounds (Methyl Salicilate) and host metabolites (Uric acid and CO_2) as chemical cues and odorants (Haxanal and α-pinene) released by *G. mellonella* (O' Halloran and Burnell 2003; Hallem *et al.* 2011). Symbiotic bacteria also produces CO_2 and other compounds from the infected hosts, however, EPNs are not attracted to the volatiles byproducts of bacterial metabolism hence they are weakly attracted to water-soluble products released from their bacterial symbionts or from digested insect host tissue (O' Halloran and Burnell 2003; Christen *et al*. 2007).

5.4.2 Desiccation tolerance

In most agricultural fields, moisture levels are kept high to support crop grown and slow down desiccation. Steinernematids and Heterorhabditis can survive relative humidity, RH<99% in an inactive state if desiccation occurs at a slow rate (Wormersley 1990; Barbercheck 1992). EPNs have been reported to tolerate desiccation through the process of anhydrobiosis which enables EPNs to adapt and persist longer in dry conditions (Womersely 1990; O'leary *et al.* 2001). To survive dry conditions EPNs form aggregate (clumping together) which improves the survival of individual nematodes (Womersely 1990; Lewis e*t al.* 1993; Shapiro-Ilan *et al*. 2014) and form coils which reduces the amount of moisture loss. The presence of the sheath which is retailed in the 2nd stage juveniles cuticle act as water loss barrier which results in a very slow rate of water loss during desiccation (Crowe *et al*. 1992; Patel *et al*. 1997; O'leary *et al*. 2001). It allows EPNs enough time for the activation and expression of metabolic, biochemical and morphological adaptations to desiccation stress (Womersley 1990; Patel *et al*. 1997; O'leary *et al.* 2001). Previous studies have indicated the accumulation of trehalose and glycerol as one of the major biochemical adaptations which occur during desiccation (Womersely 1990; Crowe *et al*. 1992; O'leary *et al*. 2001). The distribution of *H. bacteriophora* population showed that they were found most abundantly in deeper soil layer (15-30cm) experiencing gradual desiccation than in upper ones (5-10cm) were rapid desiccation occurs (Liu and Glazer 2000). Thus *H. bacteriophora* may be adapted to environmental conditions in which a certain minimum level of humidity is maintained all year round. On the contrary, EPNs are known to be poor anhydrobiosis (Liu and Glazer 2000; O'leary *et al*. 2001).

Desiccation had a significant effect on the total EPN population as illustrated in Figure 5.5. Several studies have shown that *H. bacteriophora* can tolerate slow desiccation since they are partial anhydrobiosis and requires a more gradual and longer period of adaptations (Crowe *et al*. 1992). When organic compost was mixed with loamy soil, it had the capacity to hold more moisture thus based on observation there was gradual moisture loss at each soil composition which played an important role in desiccation (see Appendix VIII). In the present study, *H. bacteriophora* was able to survive longer periods (8 weeks) of desiccation tolerance. Resuscitation with rehydration by water triggered a new cycle of *H. bacteriophora* IJs after different periods of dehydration (0, 7,14 days) which enabled *H. bacteriophora* to survive

long periods of desiccations as illustrated in Figure 5.4, however, infectivity, virulence, and persistence were reduced with time. Under such conditions IJs are no longer motile, decrease in size and lose water content which affects the infectivity and survivorship in the soil (Liu and Glazer 2002).

5.5 Conclusion

Desiccation had detrimental effects on *H. bacteriophora* infection success and IJ population. Our results suggest that *H. bacteriophora* IJs can survive a slow rate of moisture loss for a period of 8 weeks post application in the field. The ability to tolerate desiccation is important for field application especially in regions that experience drought. It was shown that soil characteristics and the use of soil amendments such as organic compost had a significant impact on EPN foraging efficacy and therefore on the ability of EPNs to be used as a successful biocontrol agent.

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Chapter 6: Conclusion and Future recommendation

Two EPN species were successfully isolated from undisturbed soils in the Magaliesburg region, North West Province and were identified as *H. bacteriophora* (MG) and *S. khoisanae* (MH697401.1) based on phylogenetic analysis. *S. khoisanae* is indigenous to the country and both species are the most abundant EPN isolates found in Free State, Western Cape, KwaZulu Natal, and Mpumalanga. Complete characterization of the two isolated EPN species was not concluded but nonetheless would have included doing morphological studies. This is important for taxonomic identification and interpretation of EPNs structural features using Light Microscopy and Scanning Electron Microscope. The symbiont bacterium was successfully isolated by targeting the mature female stage (endotokia matricida stage) of *H. bacteriophora*. Based on phenotypic and molecular characterization the symbiont bacterium was identified as *Photorhabuds luminescens laumondi* strain NTV (MK928425). However, to identify the isolate at the subspecies level, more molecular makers such as targeting the five protein-coding genes besides the 16s rDNA would improve the robustness of the phylogenetic analysis.

Most studies use treated soils which eliminate the microflora of which does not mimic the actual type of environment or soil ecosystems found in many agricultural soils. To understand the effects of soil amendments such as organic compost has on the efficacy of EPNs to infect, the soils were treated with different organic compost (MULCH, OLD, OC, PPM) that were commercially available. The study showed that the addition of organic compost did not have any detrimental effect on the efficacy, survival of *H. bacteriophora*. The addition of increasing organic compost concentration using two types of soil texture, loamy soil and coarse river sand based on organic matter content, increased the water holding capacity and water retention which increased the duration of EPN infectivity. Slow moisture loss in each treatment prolonged the infectivity of *H. bacteriophora*, enabling survival for up seven days in soils undergoing progressive dehydration.

H. bacteriophora was chosen as in ideal EPNs species based on the type of foraging behavior. Since cruiser foragers rely on host contact cues and environmental cues, the addition of organic compost affected the behavior of EPNs to locate and cause infection. Important factors such as soil physical structures including texture, aeration, organic matter

content and rate of evaporation played a significant role in this. Also, EPNs were able to survive desiccation by resuscitation by rehydration with water for 8 weeks however infectivity and virulence decreased implicating that they are capable of partial anhydrobiotes.

The study gives an insight into what can affect the efficacy of EPNs species in many agricultural soils post application. Research on general soil ecology and EPNs in the field is important. This can be used to improve soil application strategies in many field trials. Overall *H. bacteriophora* can be used as a potential biocontrol agent for the control of insect pest affecting agricultural crops in SA.

Future Recommendations

Sequencing the whole genome of EPN and studying the genes related to infectivity, persistence and storage stability can help in improving the efficacy of EPNs in field soils (Strauch *et al*. 2004; Dillman *et al.* 2015; Lu *et al*. 2016). This can be done by exposing EPNs to biotic and biotic factors under laboratory conditions, greenhouse and field studies to see which genes influence field efficacy and when altered leads to improved application of EPNs in the field. Next-generation sequencing platforms, genetic manipulation and genetic engineering (Somvanshi *et al.* 2008) can be used to identify and screen genes to be used as molecular markers to aid in selective and breeding studies (Segal and Glazer 2000; Strauch *et al*. 2004). Knowledge obtained from such findings can be used to genetically engineer EPNs to better withstand environmental factors thus improving their efficacy in the field (Dillman *et al.* 2015; Lu *et al*. 2016).

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Appendix I

***Galleria mellonella* media** (adapted from Woodring and Kaya, 1988)

The following modifications are made

- Calcium propionate substituted with benzoate
- Multivitamin bran substituted Pronator (Banana Flavour)

Recipe

- 500 Pronutro (Banana Flavour)
- 600ml pure natural honey
- 600ml of boiled distilled water
- 15 teaspoon of yeast extract'
- teaspoons of benzoate

Mix all the ingredients thoroughly. Place in the foil and sealed properly. Autoclave at 121ºC 15 psi for 35min to sterilize the food.

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 then place the tube on ice for at least 30 seconds and discard the supernatant.

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

6) Add 3µl Proteinase K solution (from the 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 the pellet is not visible repeat step 10, followed by incubation on ice for 5 minutes, then 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 the 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.

0.1% Jik solution for infective juvenile sterilization

- 34ml distilled water
- 1ml 3.5% sodium hypochlorite

Autoclave distilled water and mix with jik in bottles and autoclave at 121˚C for 15 min.

Appendix III

DNA Sequences

The sequence obtained from Inqaba Biotech company after editing with Finch TV was used on the BLASTN algorithm to identify the isolated species which showed to have high affinity to *Heterorhabditis* spp.

>*Heterorhabditis bacteriophora* NTV

CTTTGATCACGAGAGATCGGTACCAATGGAATCAGGCTTGTTCTTGATTTCAATCGGTTT
CTCACCCCATCTAAGCTCATGGAGAGGTGTCTAGTCCCAATTGGAGTCGCTTTGAGTGAC
GGCTATGAAAATTGGGTATGTTCCCCGTGAGGGTCGAGCATAGACTTTATGAACAGTGCT
GGAGCTGTCGCCTCACCAAAAAATCATCGATAACTGGTGGCTATGTGTGACATTAGTCAC
ATAGGTATCTGCTGATGCAGAGAGCCTTAATGAGTTGTTCGTGTCATCTGACCTACAACC
GCCACTATCGGTAAATCAACCCAATTAACTTGTTTCTTGTGTCGTGTTAATACATACTGGC
AAAGTGTATTAGCTTTAGCGATGGATCGGTTGATTCGCGTATCGATGAAAAACGCAGCA
AGCTGCGTTATTTACCACGAATTGCAGACGCTTAGAGTGGTGAAGTTTTGAACGCACAGC
GCCGTTGGGTTTTCCCTTCGGCACGTCTGGCTCAGGGTTGTTTAATAAGCGAAAGTGTTG
AAAGTTCATTAAACGAGAGTTCGGTGATACTGACAACACTGCGTCGATCGGTGTACTGTT
GAAAGTACCCCGTTCAAGTATCTTTATGGGGCAACATGTCTTCTATACGGAGACATGAAA
GATATTAAGAGTATATACCTGTGGATGCCCACGTATGAAATATGACGTGTCGTATACACG
GCTAGGAGGTATGTCTCAGATGAATTTGTTTATGCAACCTGAGCTCAGTCGTGATTACCC
GT

The sequence obtained from Inqaba Biotech Company after editing with Finch TV was used on the BLASTN algorithm to identify the isolated species which showed to have high affinity to *Steinernema* spp.

>*Steinernema khoisanae* NTV (MK928425)

TTATGTGCTTTTGATAGACTGAAACGGCGCAGGTTGCGTTTCTAAGTGTCGATTTCGGTC
ATGAACGGCTTTTAATGGTTTCTATAGGTGTCTGGAGCAGCTGTATGAGCGTGGCTGTTA
TGATGGACATTTAACATCATTTTACCGTGCGTTTGCGCAGTTTCTAGAACGTTCGGTGATG
AGAATTAAAGAGGTCAGTCGGAGACCCGCCATTGACAAACCCTACTATTAACATTTTTTA
CATTGATTATGCTCCATGCGTATGGTAGCGTAACCAACAATCTTTATCAAGTCTTATCGGT
GGATCACTCGGTTCGTAGTTCGATGAAAAACGGGGCAAAAACCGTTATTTGGCGTGAATT
GCAGACATATTGAACGCTAAAATTTTGAACGCAAATGGCACTATCAGGTTTATATCTGAT
AGTATGTTTGGTTGAGGGTCGATTAACTAGTTACTTGCAGTCAGCTTGGACTGTTTTTTCG
ATGAGCTACTCTGCAAAGAGTACCTTTTCGGTGTGATTGCGCTTTTGTGCGATAGTTTAAT
GGCGCGCAGTTTCATTCTTGCGAGCGTTTCTTTCGCGAGATTGCTCTCTGTGTCGCTGCTA
TCATATCGGTTCGGTGCGTTAGTGGTTTTGGCGTGTCTCTTGCCAGCTGACTTGTACTAAG
CTTCTGCTTTGTGCGTAATCGTTTCTTGAAGAGTCGGTAACCATGTCATTGATTTAACACG
TTTCGTTGATCAGCGGACGCATTGTGAACTTTAATCGATGTTTTCGATTACGACCTCAACT
CAAGCAAGACTAC

Phylogenetic Analysis

Estimates of Evolutionary Divergence (pairwise distance) between sequences of *Heterorhabditis* spp and the isolated *Heterorhabditis bacteriophora* NTV, denoted as Unknown A. The number of base substitutions per site from the 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 Tajima-Nei model (Tajima and Neil 1984). The analysis involved 16 nucleotide sequences. There was a total of 346 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*. 2016).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Heterorhabditis bacteriophora isolate N2 (MG593948)		0.003	0.004	0.004	0.016	0.019	0.022	0.022	0.023	0.022	0.022	0.019	0.024	0.016	0.000	0.043
2. Heterorhabditis bacteriophora isolate UP2A2 (MF033536)	0.003		0.005	0.005	0.017	0.020	0.022	0.022	0.023	0.022	0.022	0.019	0.024	0.017	0.003	0.043
3. Heterorhabditis georgiana strain N-KMD1(HQ225863)	0.006	0.009		0.004	0.016	0.019	0.022	0.022	0.023	0.022	0.022	0.019	0.024	0.016	0.004	0.043
4. Heterorhabditis zealandica strain NZH3 (EF043440)	0.006	0.009	0.006		0.017	0.020	0.022	0.022	0.023	0.022	0.022	0.019	0.024	0.017	0.004	0.043
5. Heterorhabditis safricana isolate SF670 (FJ473361.1)	0.093	0.097	0.093	0.100		0.013	0.026	0.025	0.027	0.025	0.016	0.011	0.027	0.006	0.016	0.044
6. Heterorhabditis downesi strain yel1 (KU573060.1)	0.117	0.120	0.116	0.123	0.061		0.028	0.028	0.028	0.028	0.011	0.017	0.029	0.013	0.019	0.047
7. Heterorhabditis noenieputensis isolate WS21 (KY024497.1)	0.141	0.145	0.141	0.141	0.187	0.209		0.006	0.018	0.006	0.030	0.027	0.019	0.025	0.022	0.043
8. Heterorhabditis indica strain PBCB2 (KY807714.1)	0.141	0.145	0.141	0.141	0.179	0.201	0.015		0.017	0.000	0.030	0.026	0.018	0.024	0.022	0.042
9. Heterorhabditis baujardi voucher HeTD4 (MF618322.1)	0.160	0.156	0.156	0.157	0.196	0.214	0.097	0.090		0.017	0.029	0.027	0.007	0.025	0.023	0.045
10. Heterorhabditis pakistanense strain NBAIIH05 (KX954218.1)	0.141	0.145	0.141	0.141	0.179	0.201	0.015	0.000	0.090		0.030	0.026	0.018	0.024	0.022	0.042
11. Heterorhabditis megidis (AB698759.1)	0.135	0.139	0.135	0.142	0.080	0.042	0.226	0.218	0.223	0.218		0.019	0.029	0.017	0.022	0.047
12. Heterorhabditis marelatus (DQ100271.1)	0.120	0.124	0.120	0.127	0.039	0.087	0.210	0.202	0.206	0.202	0.108		0.028	0.010	0.019	0.044
13. Heterorhabditis sonorensis strain Gouka (KY228995.1)	0.179	0.175	0.174	0.175	0.207	0.225	0.110	0.103	0.021	0.103	0.230	0.217		0.026	0.024	0.044
14. Heterorhabditis atacamensis isolate D099 (HM230723.1)	0.090	0.094	0.090	0.097	0.015	0.058	0.168	0.160	0.181	0.160	0.081	0.036	0.187		0.016	0.044
15. Unknown A	0.000	0.003	0.006	0.006	0.093	0.117	0.141	0.141	0.160	0.141	0.135	0.120	0.179	0.090		0.043
16. Caenorhabditis elegans (FJ589008)	0.406	0.406	0.406	0.406	0.423	0.448	0.422	0.416	0.428	0.416	0.450	0.422	0.427	0.414	0.406	

Estimates of Evolutionary Divergence (pairwise distance) between sequences of *Steinernematids* spp and *Steinernema khoisanae* strain NTV (MK928425), denoted as Unknown B. 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 Tajima-Nei model (Tajima and Neil 1984). The analysis involved 17 nucleotide sequences. There was a total of 474 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. Steinernema khoisanae (KM275351.1)		0.004	0.016	0.016	0.015	0.017	0.017	0.018	0.015	0.014	0.024	0.004	0.060	0.036	0.018	0.018	0.027
2. Steinernema khoisanae strain LTV (MH697401.1)	0.006		0.015	0.015	0.015	0.016	0.016	0.017	0.014	0.014	0.024	0.000	0.060	0.036	0.017	0.018	0.026
3. Steinernema hermaphroditum isolate CS34 (MF663703.1)	0.103	0.095		0.003	0.011	0.015	0.010	0.015	0.013	0.013	0.021	0.015	0.060	0.035	0.015	0.015	0.024
4. Steinernema hermaphroditum isolate S0905 CPCRI (MH802516.1)	0.108	0.100	0.004		0.011	0.015	0.010	0.015	0.013	0.013	0.021	0.015	0.059	0.035	0.015	0.015	0.024
5. Steinernema lamjungense voucher UGMD:104182(HM000101.1)	0.095	0.090	0.055	0.055		0.017	0.014	0.016	0.014	0.014	0.023	0.015	0.058	0.036	0.016	0.018	0.024
6. Steinernema diaprepesi strain Strang 8(GU173997.1)	0.123	0.116	0.101	0.101	0.126		0.016	0.017	0.017	0.017	0.025	0.016	0.065	0.037	0.017	0.015	0.029
7. Steinernema guangdongense strain GDc339 (AY170341.1)	0.113	0.105	0.044	0.048	0.076	0.116		0.017	0.015	0.016	0.020	0.016	0.061	0.034	0.017	0.016	0.025
8. Steinernema jeffreyense strain J194(KC897093.1)	0.121	0.113	0.098	0.096	0.108	0.123	0.110		0.015	0.017	0.024	0.017	0.059	0.034	0.000	0.019	0.026
9. Steinernema tophus isolate ROOI-352(KJ701241.1)	0.098	0.091	0.079	0.079	0.091	0.127	0.098	0.098		0.009	0.022	0.014	0.065	0.035	0.015	0.017	0.025
10. Steinernema innovationi isolate SGI-60 (KJ578793.1)	0.090	0.083	0.083	0.083	0.093	0.119	0.110	0.115	0.037		0.023	0.014	0.061	0.036	0.017	0.017	0.026
11. Steinernema sacchari isolate Duk(MF538660.1)	0.205	0.196	0.173	0.178	0.191	0.213	0.180	0.207	0.184	0.189		0.024	0.063	0.035	0.024	0.024	0.027
12. Unknown B	0.006	0.000	0.095	0.100	0.090	0.116	0.105	0.113	0.091	0.083	0.196		0.060	0.036	0.017	0.018	0.026
13. Caenorhabditis elegans (FJ589008.1)	0.654	0.648	0.668	0.660	0.652	0.714	0.688	0.661	0.701	0.665	0.701	0.648		0.070	0.059	0.061	0.065
14. Steinernema yirgalemense strain 157-C(EU625295.1)	0.365	0.365	0.355	0.355	0.387	0.370	0.354	0.352	0.358	0.375	0.351	0.365	0.804		0.034	0.035	0.037
15. Steinernema jeffreyense strain J194 (KC897093.1)	0.121	0.113	0.098	0.096	0.108	0.123	0.110	0.000	0.098	0.115	0.207	0.113	0.661	0.352		0.019	0.026
16. Steinernema australe isolate TEL(MH453881.1)	0.134	0.126	0.098	0.103	0.136	0.110	0.115	0.134	0.131	0.128	0.204	0.126	0.674	0.363	0.134		0.027
17. Steinernema citrae isolate 143-C (FJ235074.1)	0.245	0.236	0.214	0.213	0.223	0.269	0.227	0.237	0.220	0.237	0.252	0.236	0.735	0.400	0.237	0.259	

Appendix IV

Media for symbiont bacteria

MacConkey agar (commercially available)

- 17 g peptone
- 3 g protease peptone
- 10 g lactose
- 1.5 g bile salts
- 5 g NaC1
- 13.5 g agar
- 0.03 g neutral red
- 0.001 g crystal violet

Recipe

Weight out MacConkey agar powder and suspend in 1000 ml of distilled water and stir until completely dissolved. Autoclave at 121 ºC and 15psi for 15min. Cool to 45-50 ºC aseptically transfer the agar into petri dishes before its solidifies.

NBTA agar (adapted from Akhurst, 1980)

- 1 litre nutrient agar
- 0.04 g triphenyl tetrazolium chloride (TTC)
- 0.025 g Bromothymol Blue (BTB)

Recipe

Mix nutrient agar and BTB and melt agar in the water bath followed by autoclave at 121ºCand 25 psi for 15min. TTC will be poured to the agar at a temperature less than 50 50 ºC

Swirl to mix. Dispense into sterile petri dishes.

Lipid agar plates (adapted from Woodring and Kaya, 1997)

- 10 g honey instead of Corn Syrup
- 5 g yeast extract
- 25 g nutrient agar

- 2.5 ml cod liver oil
- 2 g $MgCl_2.6H_20$
- 1 litre distilled H_20

Autoclave at 121˚C and 15 psi for 20 min. Pour aseptically into Petri dishes at a the temperature of less than 50 ºC.

Nutrient broth

- Nutrient Broth
- 4.0% (W/V) Canola oil
- 25mg/ml glucose

Recipe

Weigh out nutrient broth powder and suspend in the desired volume of distilled water. Add glucose. Mix well and dispense adequate amounts into volumetric flasks. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth. Autoclave at 121˚C for 15 min.

Appendix V

Isolation of genomic DNA from bacterial cells (Protocol from ZR Fungal/Bacterial DNA Kit, catalog # D6005)

1) Pick an isolated bacterial colony from a previously streaked NBTA plate and suspend in a ZR BashingBead™ Lysis Tube.
2) Vortex at maximum speed for 5 minutes.
3) Centrifuge the ZR BashingBead™ 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-Spin™ 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-Spin™ II Column in a new Collection Tube and centrifuge at 10000rpm for 1 minute.
30
9) Add 500µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ II Column and centrifuge at 10000rpm for 1 minute.
10) Transfer the Zymo-Spin™ 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 VI

The amplified 16s rDNA of *Photorhabdus luminescense* associated with *H. bacteriophora* edited using Finch TV and was used on the BLASTN algorithm.

>*Photorhabdus luminescens* subsp. *laumondii* strain NTV (MK968782)

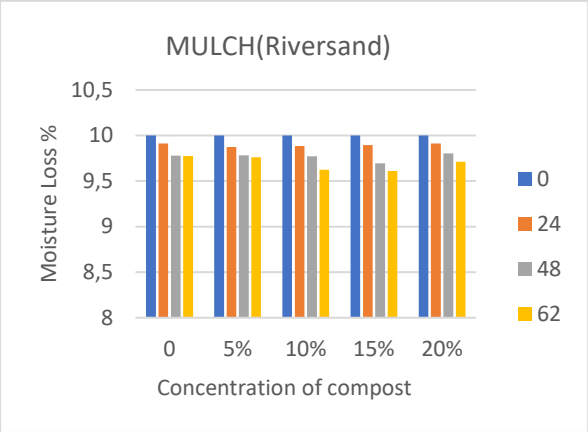
ATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGGGCGGTAACA
GGAAAAGTGCTTGCACTTTTGCTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGGATC
TGCCCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATGTCGCGAG
ACCAAAGTGGGGGACCTGAAAGGGCCTCACGCCGTCGGATGAACCCAGATGGGATTAGC
TGGTAGGTAGGGTAATGGCCTACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
TTGTAAAGTACTTTCAGCGGGGAGGAAGGGTTTAGCCTGAAGAGGGCTGGACTTTGACG
TTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGA
TGTGAAATCCCCGGGCTCAACCTGGGAATGGCATCTAAGACTGGTTGACTGGAGTCTCGT
AGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCG
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGCG
GCCTTGAACGGTGGCTTCCGAAGCTAACGCGTTA

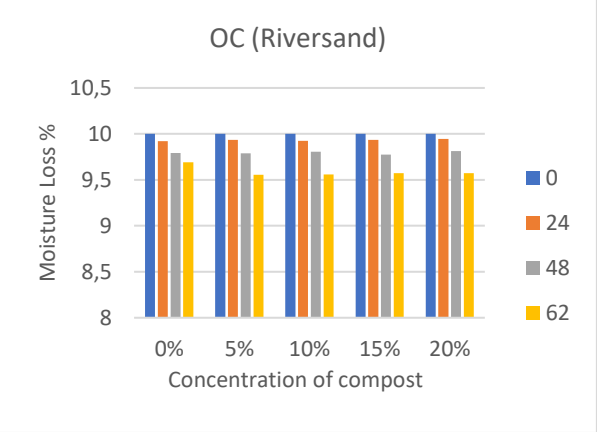
Estimates of Evolutionary Divergence (pairwise distance) between sequences of *Photorhabdus* and *Xenorhabdus* bacterial symbiont associated with EPNs. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model (Tajima and Neil 1984). The analysis involved 19 nucleotide sequences. There was a total of 774 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

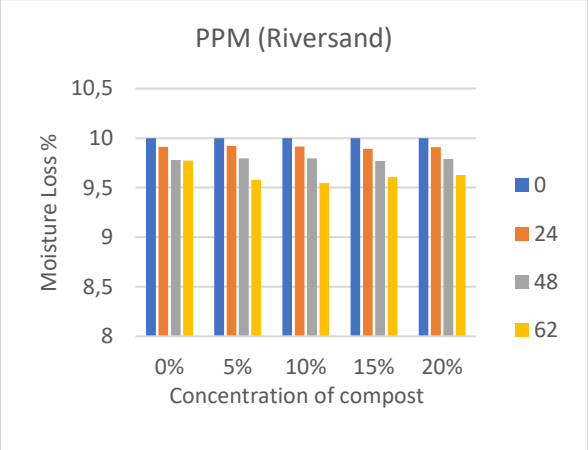
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Photorhabdus luminescens subsp. laumondii strain SF 281 (KT963833.1)		0.000	0.004	0.005	0.008	0.009	0.008	0.009	0.000	0.005	0.001	0.005	0.005	0.004	0.004	0.007	0.008	0.011	0.007
2. Photorhabdus luminescens subsp. laumondii strain FR42 (EU190980.1)	0.000		0.004	0.005	0.008	0.009	0.008	0.009	0.000	0.005	0.001	0.005	0.005	0.004	0.004	0.007	0.008	0.011	0.007
3. Photorhabdus luminescens subsp. akhurstii strain CICR-BBScSp (KF514881.1)	0.016	0.016		0.006	0.008	0.009	0.008	0.008	0.004	0.006	0.004	0.005	0.004	0.005	0.005	0.007	0.007	0.010	0.007
4. Photorhabdus asymbiotica strain 3265-8 (NR_036851.1)	0.024	0.024	0.029		0.007	0.008	0.007	0.009	0.005	0.005	0.005	0.005	0.006	0.005	0.005	0.006	0.006	0.010	0.006
5. Xenorhabdus beddingii strain DSM 4764 (NR_119153.1)	0.048	0.048	0.046	0.040		0.006	0.006	0.006	0.008	0.007	0.008	0.008	0.008	0.007	0.008	0.007	0.007	0.010	0.008
6. Xenorhabdus nematophila strain DSM 3370 (NR_119150.1)	0.058	0.058	0.057	0.053	0.029		0.006	0.006	0.009	0.008	0.009	0.009	0.009	0.008	0.009	0.008	0.008	0.010	0.008
7. Xenorhabdus poinarii strain DSM 4768 (NR_119152.1)	0.048	0.048	0.045	0.043	0.029	0.033		0.006	0.008	0.008	0.007	0.007	0.008	0.007	0.007	0.008	0.008	0.010	0.008
8. Xenorhabdus cabanillasii strain JM26 (DQ211711.1)	0.063	0.063	0.051	0.061	0.028	0.025	0.032		0.009	0.008	0.009	0.009	0.008	0.008	0.009	0.008	0.008	0.010	0.009
9. Photorhabdus luminescens subsp. laumondii strain IRA10 (EU600199.1)	0.000	0.000	0.016	0.024	0.048	0.058	0.048	0.063		0.005	0.001	0.005	0.005	0.004	0.004	0.007	0.008	0.011	0.007
10. Photorhabdus luminescens subsp. kayaii strain (EU930334.1)	0.024	0.024	0.031	0.022	0.043	0.059	0.047	0.060	0.024		0.005	0.005	0.005	0.005	0.006	0.007	0.007	0.010	0.007
11. Unknown A	0.001	0.001	0.014	0.025	0.047	0.057	0.047	0.061	0.001	0.022		0.004	0.005	0.004	0.004	0.007	0.008	0.010	0.007
12. Photorhabdus luminescens subsp. kleinii strain KMD37 (NR117790.1)	0.018	0.018	0.021	0.021	0.048	0.061	0.044	0.063	0.018	0.016	0.017		0.005	0.005	0.005	0.007	0.007	0.010	0.007
13. Photorhabdus luminescens subsp. hainanensis strain C8404 (NR116512.1)	0.018	0.018	0.010	0.029	0.048	0.060	0.047	0.057	0.018	0.024	0.017	0.018		0.004	0.005	0.006	0.007	0.010	0.006
14. Photorhabdus luminescens subsp. caribbeanensis strain HG29 (NR116513.1)	0.013	0.013	0.016	0.022	0.040	0.053	0.039	0.050	0.013	0.024	0.012	0.018	0.016		0.005	0.006	0.007	0.010	0.006
15. Photorhabdus luminescens subsp. noenieputensis strain AM7 (NR109602.1)	0.014	0.014	0.022	0.021	0.048	0.063	0.045	0.063	0.014	0.028	0.016	0.021	0.021	0.017		0.007	0.007	0.010	0.006
16. Photorhabdus temperata subsp. thracensis strain 39-8 (NR029012.1)	0.037	0.037	0.034	0.033	0.044	0.058	0.051	0.058	0.037	0.037	0.036	0.036	0.026	0.029	0.034		0.005	0.010	0.007
17. Photorhabdus temperata subsp. tasmaniensis strain T327 (NR116511.1)	0.049	0.049	0.043	0.039	0.046	0.050	0.051	0.053	0.049	0.039	0.047	0.047	0.041	0.040	0.043	0.024		0.010	0.007
18. Escherichia coli (J01859.1)	0.089	0.089	0.086	0.089	0.077	0.079	0.077	0.077	0.089	0.081	0.087	0.088	0.086	0.080	0.087	0.086	0.081		0.010
19. Photorhabdus asymbiotica subsp. australis strain GCH001 (AY280574.1)	0.040	0.040	0.036	0.028	0.045	0.053	0.049	0.060	0.040	0.037	0.039	0.035	0.036	0.032	0.036	0.047	0.050	0.089	

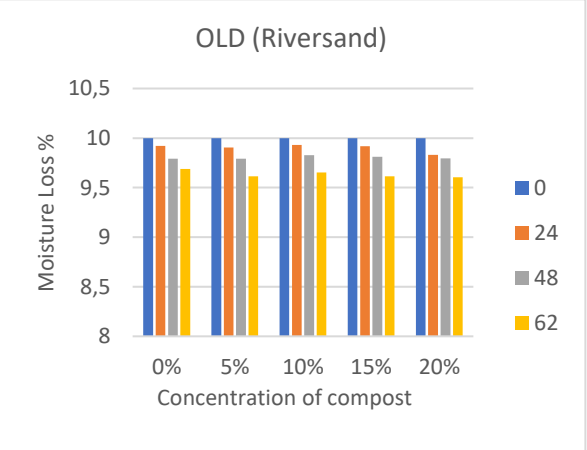
Appendix VII

Moisture loss measured at each day of dehydration (0, 24, 48, 62 hours) for each compost (OC, OLD, PPM, MULCH) at different concentration (0%, 5%, 10%, 15%, 20%). Original moisture content for coarse river sand was 10% (v/w).

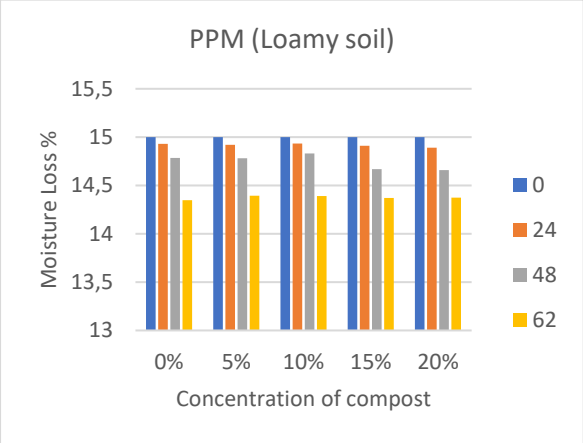


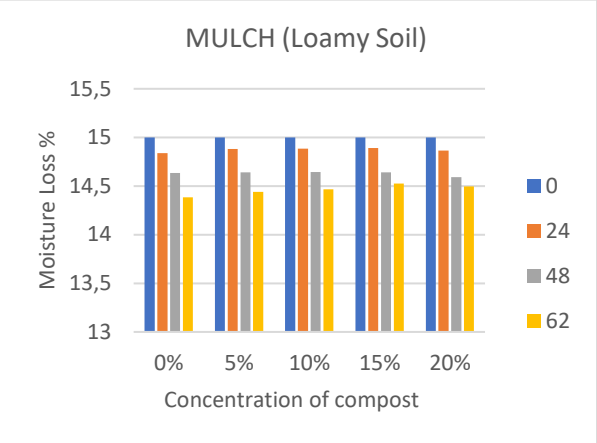


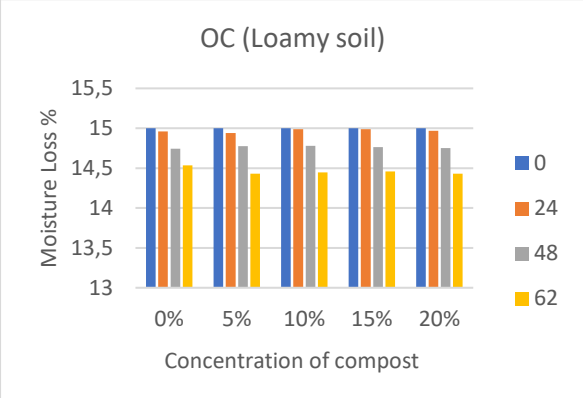


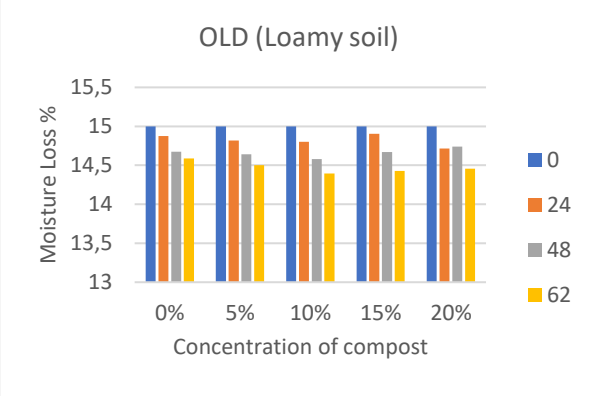


Shows the moisture loss of each compost (OC, OLD, PPM, MULCH) at different concentration (0%, 5%, 10%, 15%, 20%) during (0, 24, 48, 62hours) days of dehydration exposure. Original moisture content for loamy soil: compost was 15% (v/w).









Appendix VIII

Statistical Analysis for Organic Compost mixed with Loamy soil

2-way ANOVA results

Row Factor: Treatment (Compost concentration + Soil texture)

H_0: The type of compost concentration used does not affect the infectivity and persistence of EPNs in the soil

$H_{1:}$ Reject H_0 if $F \geq F_{crit}$

Column Factor: Days of Dehydration

H_0: Increase in dehydration exposure of IJs at 0, 24 hours, 48 hours, 62 hours does not affect insect mortality and IJ infectivity

$H_{1:}$ Reject H_0 if $F \geq F_{crit}$

Interaction (Treatment + Days of Dehydration)

H_0: The type of compost concentration used based on using a specific type of soil texture does not affect the tolerance of EPNs in dry conditions

$H_{1:}$ Reject H_0 if $F \geq F_{crit}$

Two-way ANOVA with replication. Loamy soil mixed with each type of compost concentration had a significant effect on the tolerance of EPNs in different dehydration periods.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Compost concentration)	36627.94	16	2289.246	43.747658	5.758E-57	1.69329572
Columns (Days of dehydration)	279262.9	3	93087.62	1778.911	6.15E-146	2.64886341
Interaction	83030.88	48	1729.81	33.056792	2.865E-73	1.42036726
Within	10675	204	52.32843			
Total	409596.7	271				

The different types of compost concentration mixed with loamy soil had a significant effect on IJs in the soil ($P<0,05$; $F=44$; $Df = 16$). Prolonged periods of dehydration exposure of IJs in the soil (0, 24hours, 48 hours, 62 hours) had a significant effect on the infectivity and persistence of *H. bacteriophora* in the soil ($P<0.05$; $F=17778$; $Df=3$). Virulence and efficacy decreased over 7 days of progressive dehydration. The implication of using different compost concentration influenced how long *H. bacteriophora* IJs survive dehydration exposure ($P<0.05$; $F=33$; $Df=48$).

Two-way ANOVA with Replication. Coarse river sand mixed with different types of compost concentrations had a significant effect on the tolerance of EPNs in different dehydration periods.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Compost concentration)	13802.94	16	862.6838	7.34045	2.11E-13	1.69329
Columns (Days of dehydration)	335665.8	3	111888.6	952.0448	1.2E-119	2.64886
Interaction	34802.94	48	725.0613	6.16944	9.82E-21	1.42036
Within	23975	204	117.5245			
Total	408246.7	271				

Supplementing the soil with organic compost did not have any detrimental effect on *H. bacteriophora* infectivity in compost mixed with coarse river sand. They type of compost concentration used had a significant effect on the infectivity and survivorship of IJs in the soil ($P<0.05$; $F=7$; $Df=16$). Insect mortality decreased as IJs were exposed to longer periods of dehydration which affected the infectivity and persistence in the soil ($P<0.05$; $F=952$; $Df=3$). The type of compost concentration used had an effect on how long IJs can survive and still be effective at different days of dehydration ($P<0.05$; $F=6$; $Df=48$).

Appendix IX

Tukey's Test Result for Loamy soil mixed with Organic Compost

	Tukey Test
df	204
MS	52.32843137
# of treatments	17
# of samples	16
Q	4.998

5% Concentration			
Combination	Absolute Difference	Critical Value	Results
PPM: OC	5	9.038682236	not significantly different
PPM: OLD	7.5	9.038682236	not significantly different
PPM: MULCH	28.75	9.038682236	means are significantly different
PPM: LS	3.125	9.038682236	not significantly different
OC: OLD	2.5	9.038682236	not significantly different
OC: MULCH	23.75	9.038682236	means are significantly different
OC:LS	1.875	9.038682236	not significantly different
OLD: MULCH	21.25	9.038682236	means are significantly different
OLD: LS	4.375	9.038682236	not significantly different
MULCH: LS	25.625	9.038682236	means are significantly different

10% Concentration			
Combination	Absolute Difference	Critical Value	Results
PPM: OC	7.5	9.038682236	not significantly different
PPM: OLD	1.25	9.038682236	not significantly different
PPM: MULCH	25	9.038682236	means are significantly different
PPM: LS	4.375	9.038682236	not significantly different
OC: OLD	6.25	9.038682236	not significantly different
OC: MULCH	32.5	9.038682236	means are significantly different
OC:LS	3.125	9.038682236	not significantly different
OLD: MULCH	26.25	9.038682236	means are significantly different
OLD: LS	3.125	9.038682236	not significantly different
MULCH: LS	29.375	9.038682236	means are significantly different

15% Concentration			
Combination	Absolute Difference	Critical Value	Results
PPM: OC	5	9.038682236	not significantly different
PPM: OLD	0	9.038682236	not significantly different
PPM: MULCH	26.25	9.038682236	means are significantly different

PPM: LS	3.125	9.038682236	not significantly different
OC: OLD	5	9.038682236	not significantly different
OC: MULCH	31.25	9.038682236	means are significantly different
OC:LS	1.875	9.038682236	not significantly different
OLD: MULCH	26.25	9.038682236	means are significantly different
OLD: LS	3.125	9.038682236	not significantly different
MULCH: LS	29.375	9.038682236	means are significantly different

	20% Concentration		
Combinations	Absolute Difference	Critical Value	Results
PPM: OC	2.5	9.038682236	not significantly different
PPM: OLD	2.5	9.038682236	not significantly different
PPM: MULCH	27.5	9.038682236	means are significantly different
PPM: LS	1.875	9.038682236	not significantly different
OC:OLD	0	9.038682236	not significantly different
OC: MULCH	25	9.038682236	means are significantly different
OC: LS	4.375	9.038682236	not significantly different
OLD: MULCH	25	9.038682236	means are significantly different
OLD: LS	4.375	9.038682236	not significantly different
MULCH: LS	29.375	9.038682236	means are significantly different

Appendix X

Tukey's Test Result for Coarse river sand mixed with organic compost

TUKEY TEST

DF	204
MS	117.5245098
# of treatments	17
# of samples	16
Q	4.998

5% Concentration

Combinations	Absolute Difference	Critical Value	Results
OC:PPM	0	13.5456698	not significantly different
OC: OLD	13.75	13.5456698	means are significantly different
OC:MULCH	1.25	13.5456698	not significantly different
OC:RS	3.125	13.5456698	not significantly different
PPM: OLD	13.75	13.5456698	means are significantly different
PPM: MULCH	1.25	13.5456698	not significantly different
PPM: RS	3.125	13.5456698	not significantly different
OLD: MULCH	12.5	13.5456698	not significantly different
OLD: RS	16.875	13.5456698	means are significantly different
MULCH: RS	4.375	13.5456698	not significantly different

10% Concentration

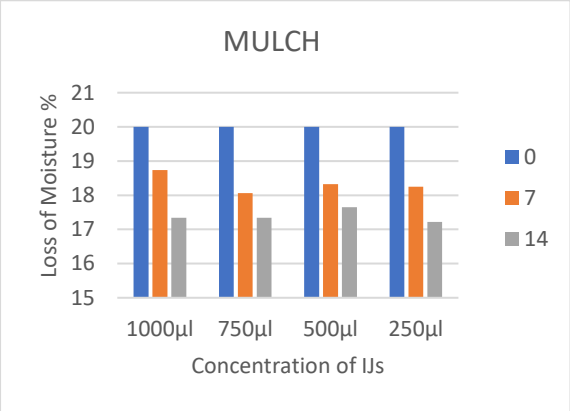
Combinations	Absolute Difference	Critical Value	Results
OC:PPM	12.5	13.5456698	not significantly different
OC: OLD	21.25	13.5456698	means are significantly different
OC:MULCH	12.5	13.5456698	not significantly different
OC: RS	8.125	13.5456698	not significantly different
PPM: OLD	8.75	13.5456698	not significantly different
PPM: MULCH	0	13.5456698	not significantly different
PPM: RS	4.375	13.5456698	not significantly different
OLD: MULCH	8.75	13.5456698	not significantly different
OLD: RS	13.125	13.5456698	not significantly different
MULCH: RS	4.375	13.5456698	not significantly different

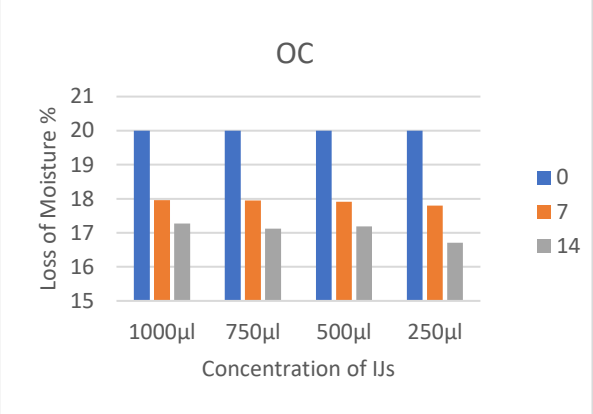
15% Concentration			
Combination	Absolute Difference	Critical Value	Results
OC:PPM	1.25	13.5456698	not significantly different
OC: OLD	8.75	13.5456698	not significantly different
OC:MULCH	2.5	13.5456698	not significantly different
OC:RS	1.875	13.5456698	not significantly different
PPM: OLD	10	13.5456698	not significantly different
PPM: MULCH	3.75	13.5456698	not significantly different
PPM:RS	0.625	13.5456698	not significantly different
OLD: MULCH	7.25	13.5456698	not significantly different
OLD: RS	10.625	13.5456698	not significantly different
MULCH: RS	4.375	13.5456698	not significantly different

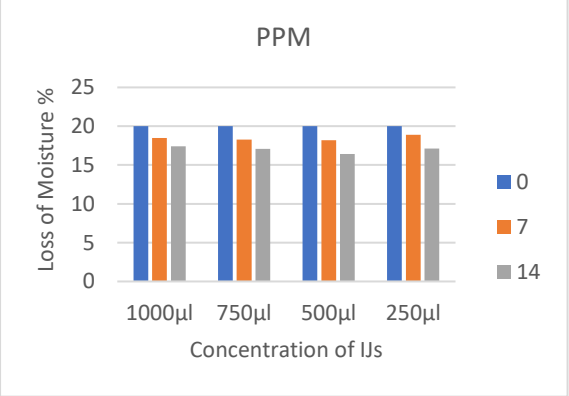
20% Concentration			
Combinations	Absolute Difference	Critical Value	Results
OC:PPM	17.5	13.5456698	means are significantly different
OC: OLD	18.75	13.5456698	means are significantly different
OC: MULCH	22.5	13.5456698	means are significantly different
OC: RS	15.625	13.5456698	means are significantly different
PPM: OLD	1.25	13.5456698	not significantly different
PPM: MULCH	5	13.5456698	not significantly different
PPM: RS	1.875	13.5456698	not significantly different
OLD: MULCH	3.75	13.5456698	not significantly different
OLD: RS	3.125	13.5456698	not significantly different
MULCH: RS	6.875	13.5456698	not significantly different

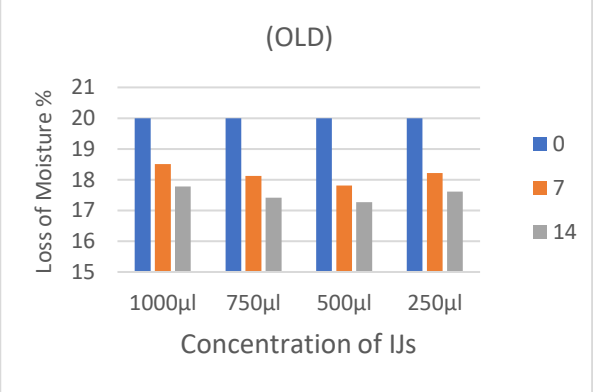
Appendix XI

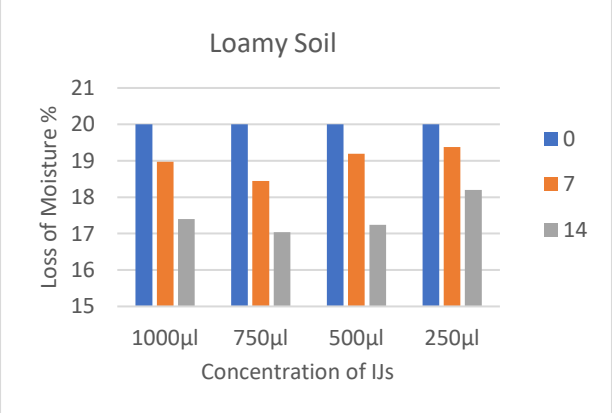
Represents the moisture lost before rehydration at 0,7,14 days desiccation tolerance for each soil composition at different EPN population











Appendix XII

2-way ANOVA results

Row Factor: Different types of soil substrates

H_0: They type of soil substrate (PPM, OLD, MULCH, OC, LS) used does not affect the behavior of EPNs in host location profiles (0, 5, 10, 15, 20, 25cm)

H_1: Reject H_0 if $F \geq Fcrit$

Column Factor: Concentration of EPN population

H_0: An increase in EPN population (250, 500, 750, 1000IJs) does not influence the infectivity of EPNs in host location profiles

H_1: Reject H_0 if $F \geq Fcrit$

Interaction: Soil Substrates and EPN population

H_0: The type of soil substrate used at different EPN population does not affect the behavior of EPNs in host location profiles

H_1: Reject H0 if $F \geq Fcrit$

Two-way ANOVA with replication: The effect of using different soil substrate on the infectivity of EPNs in soil location profiles. Infectivity was measured at Day 0, 1st day of infection.

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Soil Substrates)	23752.73	4	5938.182	12.62858	1.62E-07	2.525215
Columns (EPN population)	12059.18	3	4019.727	8.548651	8.19E-05	2.758078
Interaction	9105.545	12	758.7954	1.613711	0.111999	1.917396
Within	28213.06	60	470.2177			
Total	73130.51	79				

The type of soil substrate (LS, MULCH, OC, OLD, PPM) used had a significant effect on the behavior of EPNs in host location profiles (0cm, 5cm, 10cm, 15cm, 20cm) when infectivity was measured immediately ($P<0,05$; $F=12.62$; $DF=4$). An increase in EPN population had a significant effect on the infectivity ($P<0.05$; $F=8.54$; $DF=3$) however there was no significant effect on the type of substrate used and EPN population when infectivity of G. mellonella was measured immediately ($P>0.05$; F1.61; $DF=12$).

Two-way ANOVA with replication. Desiccation tolerance of *H. bacteriophora* at 7 Days post-resuscitation by rehydration.

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Soil substrates)	22672.14	4	5668.035	13.49108	6.66E-08	2.525215
Columns (EPN population)	23370.99	3	7790.331	18.54258	1.25E-08	2.758078
Interaction	13381.89	12	1115.157	2.654303	0.006401	1.917396
Within	25207.92	60	420.1319			
Total	84632.94	79				

At 7 days desiccation tolerance, the type of soil substrate used had a significant effect on the behavior of *H. bacteriophora*, cruiser forager in soil location profiles ($P<0.05$; $F=13$; $DF=4$). An increase in EPN population influenced the infectivity in host location profiles ($P<0.05$; $F=18$; $DF=3$). The type of substrate used at different EPN population had a significant effect on the infectivity and behavior in host location profiles ($P<0.05$; $F=2$; $DF=12$).

Two-way ANOVA with replication. 14 days post-resuscitation by rehydration of *H. bacteriophora* in host location profiles.

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Soil Substrates)	9257.051	4	2314.263	2.988867	0.025683	2.525215
Columns (EPN Population)	7093	3	2364.333	3.053533	0.035191	2.758078
Interaction	11188.19	12	932.349	1.204127	0.301558	1.917396
Within	46457.67	60	774.2944			
Total	73995.91	79				

At 14 days of desiccation tolerance, the type of substrate used had a significant effect on the behavior of EPNs in host location profiles ($P<0.05$; $F=3$; $DF=4$). An increase in EPN population influenced the infectivity ($P<0.05$; $F=1$; $DF=12$). However, the type of substrate used at different EPN population did not have any significant effect on the infectivity and behavior ($P>0.05$, $F=1$; $DF:12$).

Desiccation Tolerance of *H. bacteriophora*

Row Factor: Soil Composition

H_0: The use of different soil composition in host location profiles does not increase the tolerance of EPNs in dry conditions

H_1: Reject H_0 if $F \geq Fcrit$

Column Factor: EPN Population

H_0: An increase in EPN population (250 and 1000IJs) does not increase the tolerance of EPNs in different soil compositions

H_1: Reject H_0 if $F \geq Fcrit$

Interactions: Soil Composition and EPN Population

H_0: The type of soil composition used at different EPN population does not affect the tolerance of EPN in desiccation

H_1: Reject H_0 if $F \geq Fcrit$

Two-way ANOVA with replication. Desiccation tolerance of *H. bacteriophora* at different soil compositions

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Soil substrate)	23988,65	14	1713,475	4,237968	1,11E-05	1,803206
Columns (EPN Population)	22230,57	1	22230,57	54,98329	6,43E-11	3,946876
Interaction	26241,41	14	1874,387	4,635955	2,88E-06	1,803206
Within	36388,36	90	404,3151			
Total	108849	119				

An increase in EPN population, comparing the lowest and highest concentration (250IJs/ml and 1000IJs/ml) played a significant role in the infectivity and tolerance of *H. bacteriophora* IJs in different soil compositions ($P<0.05$; $F=54$; $DF=1$). The type of soil composition used at different EPN population had a significant effect on the desiccation tolerance of *H. bacteriophora* IJs ($P<0.05$; $F=5$; $DF=14$). The type of soil composition used (PPM, OC, OLD, MULCH, Loamy Soil) had a significant effect on the tolerance of *H. bacteriophora* to withstand longer periods of desiccation ($P<0.05$; $F=4$; $DF=14$) however infectivity and survivorship reduced with increased exposure to desiccation.