INVESTIGATING THE FORAGING BEHAVIOUR OF ENTOMOPATHOGENIC NEMATODES CULTURED *IN VITRO*

Dissertation by:

Aviwe Matsha

(1840595)

Supervisor:

Dr. Tiisetso Elizabeth Lephoto

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Declaration

I, Aviwe Matsha, declare this dissertation is my own and unaided work, and material obtained from other sources has been referenced and cited. It is submitted in fulfilment of the Master of Science degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Aviwe Matsha

1840595

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Abstract

Synthetic pesticides have provided a cost-effective approach for the control and protection of agricultural and horticultural crops against insect pests and diseases since their introduction and success in the mid-1940s. Despite the noticeable benefits of synthetic pesticides, an inconsiderable amount of evidence has shown that their overuse poses several adverse effects, including the development of resistance, crop contamination and the unconscious reduction of non-target organisms. Therefore, safer yet effective techniques for managing insect pests are required. Insect parasitic nematodes such as entomopathogenic nematodes (EPNs) are widely studied for their use as potential biological control agents (BCAs) against various economically important insect pests. EPNs are classified into three foraging groups namely cruise, ambush, and intermediate foragers which influence their soil depth distribution and host range. Upon their emergence from host cadavers or inundative release into soil, EPNs experience a variety of environmental and host-associated cues which significantly affect their foraging behaviour and, ultimately, their pathogenicity, survival, and persistence. Therefore, understanding the foraging behaviour of EPNs following their exposure to abiotic and chemotactic stimuli is essential for classifying EPNs into foraging groups and their assignment to specific hosts. As such, this study aimed to isolate EPNs from soil samples collected from Mahobong Leribe in Lesotho and investigate their foraging behaviour by exposing the nematodes to abiotic factors and chemical cues that influence their host-seeking behaviour. Nematodes were recovered from collected soil samples and infected *Tenebrio molitor* cadavers using the soil baiting technique and White trap method, respectively. PCR amplification and Sanger sequencing of the 18S and 16S rDNA regions were used to identify the phylogenetic relationships of the isolated nematode and its potential bacterial symbiont using the GenBank sequence database, respectively. The isolated nematode had a high affinity to *Cruznema* sp. isolate 734C-1 (OR606776.1), whereas the potential bacterial symbiont had a high affinity to *Enterobacter ludwigii* strain EN-119 (NR 042349.1). The foraging behaviour of *Cruznema* sp. NTM-2021 was studied by investigating the influence of soil moisture and the nematode's response to a distal chemotactic cue. After 7 days, insect mortality observed in the 2D sand substrate assays was 0%, 23.3%, 40%, 46.7% and 66.7% for 10%, 12.5%, 15%, 17.5% and 20% soil moisture levels, respectively. This overall trend suggests that insect mortality

increases with an increase in soil moisture. After 7 days, the vertical sand column assay revealed that at 10% soil moisture levels, the IJs travelled to a maximum depth of 8 cm and caused <12% insect mortality at this depth. At 12.5% soil moisture, the IJs travelled to a maximum depth of 14 cm and caused >85% insect mortality at this depth. In soil moisture levels ranging between 15-17.5%, IJs reached depths of 32 cm, causing >85% insect mortality at all depths along the column. Interestingly, at a soil moisture level of 20%, IJs reached lengths of 26 cm; however, they could only parasitise <15% of the insect larvae, suggesting that higher moisture levels may restrict the vertical dispersal of nematodes. The PF127 gel assay revealed that the IJs of *Cruznema* sp. NTM-2021 were strongly repelled by prenol, suggesting its use as a distal chemotactic cue. Based on the IJs ability to move to depths of 32 cm and its response to prenol, *Cruznema* sp. NTM-2021 may be classified as a cruise forager. Overall, the results of this study not only classified the foraging behaviour of a potential entomopathogenic nematode but also characterised the nematode according to its moisture requirements to optimise its performance in field applications since it showed great promise as a biological control agent.

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Lastly, I thank God for giving me the strength to finish my research.

1 Samuel 7:12

"Thus far, the LORD has helped us."

Dedication

This dissertation is dedicated to my mother, Lindelwa Faith Matsha and grandmother, Alicia Tembeka Matsha. Thank you for your love and selflessness in providing me with all I need to reach significant life milestones such as this. No words could ever express my gratitude to you, and I pray that the Lord blesses you exceedingly and abundantly above all you may ask or imagine.

Research Output

Conference 1: Molecular Bioscience Research Thrust (MBRT) research day at the University of Witwatersrand (2023)

Output: Poster presentation

Poster title: Investigating the foraging behaviour of entomopathogenic nematodes cultured *in vitro*.

Authors: Aviwe Matsha and Tiisetso E. Lephoto

Table of contents

Declaration ii

Abstract iii

Acknowledgements v

Dedication vi

Research Output vii

List of Figures xiii

List of tables xiv

Chapter 1: Literature Review 15

1. Introduction 15

1.1. Chemical pesticides 15

1.2. Integrated Pest Management 17

1.3. An introduction to biological control 17

1.4. An introduction to nematodes 18

1.4.1. Nematode taxonomy and diversity 18

1.4.2. Entomopathogenic nematodes 20

1.4.3. The basic body plan of EPNs 21

1.4.4. The life cycle of EPNs 22

1.4.5. The effectiveness of EPNs as biological control agents 23

1.4.6. The use of EPNs in biological control 24

1.4.7. Foraging behaviour of EPNs 25

1.4.8. Factors affecting the foraging behaviour of EPNs 26

1.4.9. Changes in the foraging behaviour and classification of EPNs into foraging groups 27

1.5. Research Motivation 27

1.6. Aim and Objectives 29

1.6.1. Aim 29

1.6.2. Objectives 29

1.7. References 30

Chapter 2: Isolation and molecular identification of a potential entomopathogenic nematode 43

2.1. Introduction 43

2.2. Materials and Methods 46

2.2.1. Breeding and maintenance of *Tenebrio molitor* larvae 46

2.2.2. Isolation of nematodes 47

2.2.2.1. Collection of soil samples 47

2.2.2.2. Soil baiting 47

2.2.2.3. Recovery of nematodes from infected larvae using White Traps 48

2.2.2.4. Confirmation of pathogenicity using Koch's Postulates 48

2.2.2.5. In vivo maintenance of nematodes 49

2.2.2.6. Morphological identification of the unknown nematode isolate 49

2.2.3. Molecular identification of nematodes 49

2.2.3.1. Extraction of genomic DNA from nematodes 49

2.2.3.2. Polymerase Chain Reaction (PCR) amplification of the 18S rDNA region 50

2.2.3.3. Agarose gel analysis and amplicon purification 51

2.2.3.4. Sanger sequencing of the 18S rDNA region 51

2.2.4. Bioinformatic analysis 51

2.2.4.1. Sequence analysis 51

2.2.4.2. Molecular phylogenetic analysis 52

2.3. Results and discussion 53

2.3.1. Isolation of nematodes 53

2.3.2. Bioinformatics analysis 56

2.3.2.1. Sequence analysis 56

2.3.2.2. Molecular phylogenetic analysis 57

2.4. Conclusion 59

2.5. References 60

Chapter 3: Isolation and identification of a potential bacterial symbiont from *Cruznema* sp. NTM-2021 64

3.1. Introduction 64

3.2. Materials and Methods 67

3.2.1. Isolation of potential bacterial symbionts from *Cruznema* sp. NTM-2021 67

3.2.2. Morphological analysis 68

3.2.2.1. Gram Stain 68

3.2.3. Molecular identification 68

3.2.3.1. Bacterial DNA extraction 68

3.2.3.2. Polymerase Chain Reaction (PCR) amplification of the 16S rDNA region 69

3.2.3.3. Agarose gel analysis and amplicon purification 69

3.2.3.4. Sanger sequencing of the 16S rDNA region 70

3.2.4. Bioinformatics analysis 70

3.2.4.1. Sequence analysis 70

3.2.4.2. Molecular phylogenetic analysis 70

3.2.5. Bacterial pathogenicity assay 71

3.2.5.1. Bacterial serial dilution and cell enumeration 71

3.2.5.2. Intra-haemocoelic injection assay 72

3.2.6. Confirmation of dependency using the lipid agar test 73

3.3. Results 73

3.3.1. Isolation of a potential bacterial symbiont from *Cruznema* sp. NTM-2021 73

3.3.2. Morphological identification of potential bacterial symbiont 74

3.3.3. Bioinformatics analysis 75

3.3.3.1. Sequence analysis 75

3.3.3.2. Molecular phylogenetic analysis 76

3.3.4. Bacterial pathogenicity assay 77

3.3.5. Confirmation of dependency using the lipid agar test 78

3.4. Discussion 79

3.5. Conclusion 82

3.6. References 84

Chapter 4: Investigating the foraging behaviour of a *Cruznema* sp. NTM-2021 89

4.1. Introduction 89

4.2. Materials and Methods 92

4.2.1. 2D Sand substrate assay 92

4.2.2. Vertical sand columns 93

4.2.3. Chemotaxis assay 94

4.3. Results 95

4.3.1. The effects of soil moisture on foraging behaviour using 2D Sand substrates. 96

4.3.2. The effects of soil moisture on foraging behaviour using vertical sand columns . 97

4.3.3. Prenol chemotaxis assay using PF127 gel 98

4.4. Discussion 99

4.5. Conclusion 102

4.6. References 104

Appendix I 107

Appendix II 109

Appendix III 110

Appendix IV 112

List of Figures

Figure 1. The life cycle of entomopathogenic nematodes.

Figure 2.1. The basic body plan of nematodes.

Figure 2.2. Insect baiting technique used for the isolation of nematodes from soil samples using *T. molitor* larvae.

Figure 2.3. Symptom variations observed in insect cadavers.

Figure 2.4. Pathogenicity test confirming Koch's Postulates.

Figure 2.5. The White trap method showing the emergence of nematodes from infected insect cadavers into water.

Figure 2.6. Morphology of the unknown nematode isolate under a light microscope.

Figure 2.7. The 18S rDNA sequence of the unknown nematode isolate.

Figure 2.8. Maximum-likelihood tree, based on the analysis of the 18S rDNA region, showing the phylogenetic relationships of *Cruznema* spp. and several species of EPNs.

Figure 3.1. Bacterial species isolated from *Cruznema* sp. NTM-2021 and grown on MacConkey and NBTA media at 25 °C for 24 hours.

Figure 3.2. Gram stain of the unknown bacterial isolate at 1000X magnification.

Figure 3.3. The 16S rDNA sequence of the unknown bacterial isolate.

Figure 3.4. Molecular phylogenetic analysis of *Enterobacter* spp. based on the analysis of the 16S rDNA region.

Figure 3.5. Bacterial standard curve.

Figure 3.6. Intra-haemocoelic injection assay of *Enterobacter ludwigii* strain NTM-2021 into *T. molitor* larvae assessed over 72 hours at 25 °C.

Figure 3.7. Confirmation of dependency using the lipid agar test.

Figure 4.1. Jumping behaviour observed in ambush foraging nematodes.

Figure 4.2. The column design for the vertical sand column assay.

Figure 4.3. Assay arenas of the prenol chemotaxis assay on PF127 gel.

Figure 4.4. Average cumulative percentage mortality of *T. molitor* observed on 2D sand substrates in response to varying moisture levels (10%, 12,5%, 15%,17.5%, 20%).

Figure 4.5. Average cumulative percentage mortality of *T. molitor* observed at different distances (2 cm, 8 cm, 14 cm, 20 cm, 26 cm, 32 cm) along vertical sand columns in response to different moisture levels (10%, 12,5%, 15%,17.5%, 20%).

Figure 4.6. The response of *Cruznema* sp. NTM-2021 to different doses of prenol on PF127 media.

List of tables

Table 3.1. The bacterial CFU approximations administered into each *T. molitor* larvae at 25 °C

Chapter 1: Literature Review

1. Introduction

1.1. Chemical pesticides

According to estimates compiled by the Food and Agriculture Organization (FAO) (2017), the global population is expected to grow to approximately 10 billion people by 2050, with developing countries such as Africa being the biggest contributors. This growth places a significant demand on global food production, which is expected to increase by at least 70% from the current levels to support the growing population (Food and Agriculture Organization of the United Nations, 2017). Annually, food loss and waste occurring during pre-harvest (field) and post-harvest (storage) account for a significant proportion of the global agricultural outputs and result in substantial economic losses globally, thus posing a serious risk to global food security (Parsa *et al.*, 2014; Skendžić *et al.*, 2021). Over the years, insect pests have been recognised as the main competitors of humans for agricultural produce and are favoured by large-scale monocultures and extensive use of fertilisers (Oerke and Dehne, 2004). Annually, 18-20% of the global crop production losses (accounting for more than US$ 470 billion) are attributable to insect pests (Sharma *et al.*, 2017; Rakesh *et al.*, 2023). According to Mendoza-Fernández *et al*. (2022), this number is expected to increase due to the projected increase in species as a result of climate change. Furthermore, insect pests can directly propagate or encourage secondary infections caused by bacteria, fungi, and viruses in addition to causing direct harm (Mendoza-Fernández *et al.*, 2022).

Since their introduction and success in the mid-1940s, synthetic chemical insecticides have provided a fast, easy and cost-effective approach for controlling several insect pests affecting both agricultural and horticultural crops (Bruce, 2010). The application of these substances has aided in the reduction of crop damage and losses as well as the improvement of crop quality.

They have also contributed to protecting humans against vector-borne diseases such as malaria by targeting mosquitoes, the insect vectors that facilitate disease transmission. This is achieved by applying neurotoxic insecticides such as pyrethroids which bind to sodium channels, resulting in excitatory paralysis of the mosquito followed by death (Soderlund, 2012). Despite several advantages of using chemical insecticides, as the scale of agriculture expands, concerns regarding their safety towards humans, animals, and the environment have risen. This is because several studies have shown that insecticides can penetrate the environment upon their application to target crops (Park *et al.*, 2020; Tudi *et al.*, 2021; Maggi *et al.*, 2023). This results in the movement of the insecticide from the application site to additional environmental media by processes such as adsorption, leaching, and volatilisation (Robinson *et al*., 1999; Scholtz and Bidleman, 2007). The type of insecticide applied then determines its impact on animals, humans, and the environment. For instance, the insecticide Dichlorodiphenyltrichloroethane (DDT) has low acute toxicity; however, it persists and accumulates in the fatty tissues of animals and humans (Tudi *et al.*, 2021). During periods of starvation fat reserves are used and the breakdown products of DDT are released into the blood where they may be toxic to the liver and the nervous system. Upon application, insecticides also undergo a process of degradation by chemical reactions, light, and microbes (Abian *et al*., 1993). This process limits their permanence in the environment and yields several metabolites that can pose additional safety concerns (Tariq and Nisar, 2018). For instance, the primary metabolite of the insecticide chlorpyrifos, known as 3, 5, 6-trichloro-2-pyridinol (TCP) is significantly more dangerous and mobile than the original pesticide (Zhao *et al.*, 2017). Chlorpyrifos and its metabolites are found regularly in groundwater, sediment, and soil in numerous locations (Wołejko *et al.*, 2022). These substances are considered endocrine disruptors and may harm human health (Yue *et al.*, 2017). Some of the known adverse effects caused by insecticides in humans include several neurological, pulmonary, reproductive, carcinogenic, and gastrointestinal diseases (Aktar *et al*., 2009; Nicolopoulou-Stamati *et al.*, 2016). Additionally, insecticides have been shown to kill non-target organisms and trigger resistance in the target pests, which can cause a resurgence of the pest or an outbreak of secondary pests (Hawkins *et al.*, 2019).

However, due to a lack of knowledge (retailers providing little information about the proper usage of the products), incorrect perception, and regulation, farmers continue to use chemical insecticides despite their adverse effects on biodiversity. Therefore, sustainable and durable solutions are required to manage economically devastating insect pests.

1.2. Integrated Pest Management

Integrated pest management (IPM) is a science-based decision-making process that uses different control and management-associated strategies to successfully identify and reduce damage caused by pests (Prokopy and Kogan, 2009). It combines environmental data, pest biology, and current technology to prevent unacceptably high levels of pest damage while lowering the risk to the economy, public health, and the environment (Radcliffe *et al*., 2009). This definition is one of many; however, the main idea of each definition is that IPM focuses on minimising or eliminating the reliance on chemical control options by adopting a variety of alternatives that consider environmental and human health (Ehi-Eromosele *et al*., 2013). As opposed to insecticides, IPM aims to manage pests at low numbers below economically damaging levels rather than eradicate them (Karlsson-Green *et al*., 2020). IPM uses a variety of techniques to manage insects, including biological, cultural (manipulation of agronomic practices), physical (use of physical methods or mechanical devices), and chemical control (includes the use of biopesticides) (Karuppuchamy and Venugopal, 2016). Among the mentioned management strategies, biological control is the most beneficial as it is sustainable, energy-self-sufficient, environmentally safe, and cost-effective (Dara, 2019).

1.3. An introduction to biological control

Biological control is the suppression and management of insect pest populations below economically injurious levels through the exploitation of natural enemies such as bacteria, fungi and nematodes (Köhl *et al*., 2019). These organisms, i.e., natural enemies, act against insect pests through several mechanisms, including parasitism, antibiosis, competition, and enhancing host plant defences (Pandit *et al.*, 2022). Biological control consists of four main categories, namely

natural, conservation, classical and augmentative biological control (Lahlali *et al.*, 2022). Natural and conservation biological control focus mainly on existing indigenous natural enemies in an ecosystem. In contrast, classical and augmentative biological control focuses on introducing exotic natural enemies into an ecosystem (Stenberg *et al*., 2021). Natural biological control occurs when indigenous natural enemies reduce indigenous pest populations independent of targeted interventions by humans (Lahlali *et al.*, 2022). However, conservation biological control involves increasing the abundance and efficacy of indigenous natural enemies by implementing several practices (Bale *et al*., 2008). These practices include manipulating the crop environment, constructing overwintering shelters (e.g. beetle banks), and supplying essential food resources (Bale *et al*., 2008). Contrastingly, classical biological control refers to importing and inoculating co-evolved natural enemies to control exotic pest populations that have spread to new geographical areas (van Driesche and Abell, 2008). This establishes natural enemies in the new environment and permanently controls the targeted pest populations. Augmentative biological control is the reinforcement of existing natural enemies through periodic releases to increase their effectiveness and usually requires mass production of the released agents (Hoy, 2008). It comprises two main strategies: inundative and inoculative augmentative releases (Bale *et al*., 2008). Inundative releases involve releasing large quantities of the natural enemy to achieve immediate control of insect pest populations (Hoy, 2008). However, inoculative releases rely on the inoculation of relatively small quantities of the natural enemy to establish reproducing populations of the natural enemies (dos Anjos and Costa, 2016).

1.4. An introduction to nematodes

1.4.1. Nematode taxonomy and diversity

Nematodes are multicellular, non-segmented, triploblastic, pseudocoelomic, and bilaterally symmetrical thin worms constituting the phylum Nematoda (Basyoni and Rizk, 2016). Along with the phyla Arthropoda and Mollusca, Nematoda is the third most species-rich phylum within the kingdom Animalia (Ahmed *et al.*, 2015). Nematodes are ubiquitous and abundant across marine, freshwater and terrestrial ecosystems and exist as free-living and/or parasitic organisms (Iqbal

and Jones, 2017). Free-living nematodes are classified into several trophic (feeding) groups based on their diet and include bacterivorous (bacterial-feeding), fungivorous (fungi-feeding), herbivorous (plant-feeding), omnivorous, and predatory nematode species (Yeates *et al.*, 1993). Taxonomically, the families Rhabditidae, Cephalobidae, and Panagrolaimidae in the order Rhabditida contain bacterial-feeding nematodes, while the families Aphelenchidae and Aphelenchoididae in the order Aphelenchida and Ditylenchus and Tylenchus in the order Tylenchida include fungal-feeding nematodes (Yeates and Bongers, 1999; Zhang *et al.*, 2020). Both bacterivorous and fungivorous nematodes can be classified as saprophytic as they decompose organic matter and improve the soil structure, water-holding capacity, and drainage. Predatory nematodes belong to four main orders, Aphelenchid, Mononchid, Diplogasterid, and Dorylaimida (Devi and George, 2018). In soil, these nematodes feed indiscriminately on soil microorganisms including parasitic nematodes such as plant parasitic nematodes (PPNs) therefore reducing the nematode population and releasing nutrients in forms accessible to plants so that they can withstand the stress placed by PPNs on their roots (Khan and Kim, 2007). The omnivorous nematodes are found within the order Dorylaimida and, like predaceous nematodes, feed on soil microorganisms; however, their diet varies due to several factors, including environmental conditions, food availability, and life cycle stages.

Parasitic nematodes encompass nematode species that are parasitic to plants and animals. PPNs, also known as herbivorous nematodes, feed on plant tissues such as the roots, stem, seeds, flowers, and leaves (Poveda *et al*., 2020). Functionally, PPNs are divided into groups based on their feeding techniques, with nematodes of the order Tylenchida using a stomatostylet while nematodes of the order Dorylaimda use an odontostylet (Khan and Khan, 2021). These nematodes are then further categorised as ectoparasitic or endoparasitic (Shah *et al.*, 2017). The ectoparasitic nematodes feed on the outermost layer of plants without piercing the host plant root and include *Tricodorus* species (spp.) and *Xiphinema* spp. Contrastingly, endoparasitic nematodes enter plants through the cortical or vascular region of the root and include *Pratylenchus* spp., *Anguina* spp., *Globodera* spp., and *Heterodera* spp. (Khan and Khan, 2021).

Animal parasitic nematodes include nematode species, parasitic to both vertebrates and invertebrates (Riddle *et al*., 1997). Vertebrate parasitic nematodes include nematodes that are parasitic to humans, such as those belonging to the genera *Ascaris*, *Trichinella, Strongyloides, and Ancylostoma* (Al-Banna and Gardner, 2022). These nematodes attack several body tissues (muscles, digestive tract, eyes, etc.), resulting in morbidity and mortality. As parasites to agricultural livestock, parasitic nematodes reduce economic value and profitability and affect animal health and reproduction (Strydom *et al.*, 2023). These include nematodes from the genera *Haemonchus* and *Ostertagia*. The invertebrate parasitic nematodes belong to the orders Mermithida, Tylenchida, and Rhabditida and include nematodes from the families Mermithidae, Allantonematidae, Tetradonematidae, Steinernematidae, and Heterorhabditidae. These insect parasitic families have been intensely studied, particularly nematodes of the genera *Steinernema, Heterorhabditis* and *Oscheius*, which can be used as biological control agents (BCAs) against economically devastating insect pests.

1.4.2. Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are microscopic, soil-dwelling roundworms that are obligate insect parasites (van der Linden *et al*., 2022). Currently, three genera of EPNs exist, namely, *Steinernema, Heterorhabditis,* and *Oscheius,* which have evolved a mutualistic symbiotic relationship with the insect pathogenic bacteria of the genera *Xenorhabdus, Photorhabdus,* and *Serratia*, respectively (Lephoto and Gray, 2019). Over the years, it has been speculated that the mutualistic relationship between EPNs and their bacterial symbionts evolves separately and that similarities in their life cycle and behaviour occur through convergent evolution from separate bacterivorous or microbivorous ancestors (Griffin, 2012). According to empirical data, the pathogenicity of EPNs to insect hosts is exclusively due to the activity of the bacterial symbionts, with the nematodes functioning only as mobile vectors (Ogier *et al.*, 2020). In *Steinernema* spp. the bacterial symbionts are contained within a specialised vesicle called the receptacle, whereas *Heterorhabditis* spp. and *Oscheius* spp. lack this specialised structure and harbour the bacteria in the intestinal lumen of their non-feeding and developmentally arrested state, known as the third-stage infective juveniles (IJs) (Stock and Goodrich-Blair, 2012). The IJs of *Oscheius*

spp. and *Steinernema* spp. (except *Steinernema hermaphroditum)* develop into male and female adults, with subsequent generations producing male and female adults (Koppenhöfer *et al*., 2020; Kumar *et al*., 2019). However, *Heterorhabditis* spp. IJs develop into hermaphroditic females in the first generation; however, subsequent generations consist of males, females, and hermaphroditic adults (Stock and Goodrich-Blair, 2012). To date, approximately 100 species of *Steinernema*, 21 species of *Heterorhabditis* and at least 13 species of *Oscheius* have been identified globally (Bhat *et al*., 2020).

1.4.3. The basic body plan of EPNs

The overall body structure of EPNs is cylindrical and tapers at the anterior and posterior ends (Barrón-Bravo *et al.*, 2021). The body is covered by the cuticle, a transparent multi-layered matrix of chitin and protein polymers, which is synthesised by the hypodermis. The cuticle is enveloped by a carbohydrate-rich surface coat epicuticle, which plays a vital role in evasive immune systems (Basyoni and Rizk, 2016). Functionally, the cuticle maintains a positive hydrostatic pressure necessary for nematode motility, enables growth through moulting, and protects against harsh environmental conditions. Underlying the cuticle along the hypodermis is a longitudinal strap-like muscular layer that forms the outer tube (Poinar, 2015). Along with the inner alimentary tract, the muscular layer encloses the fluid-filled pseudocoelom which functions as a hydrostatic skeleton (Basyoni and Rizk, 2016). The movement of the longitudinal muscles against the hydrostatic skeleton produces the whip-lash motion that facilitates nematode motility during host-seeking (Riddle *et al*., 1997). In EPNs, the alimentary tract extends from the mouth to the anus. It is divided into three sections, namely the foregut (mouth, stoma, and oesophagus), midgut (intestine), and hindgut (rectum and anus) (Barrón-Bravo *et al.*, 2021). The nervous system of the nematodes consists of a circumpharyngeal nerve ring with ventral and dorsal nerve cords that span the entire length of the nematode, thus enervating the mouth, lips and anterior sense organs (Schafer, 2016). Functionally, the nervous system of nematodes is designed to integrate a variety of host and environmentally associated cues to produce an appropriate response. EPNs also possess well-developed reproductive systems, which enable their classification as either males or females, with different species of EPNs employing diverse sexual

reproductive strategies at different life stages (Daramola *et al*., 2022). Some nematode species can be hermaphroditic, while others are amphimictic. EPNs, however, lack discrete circulatory and respiratory systems; therefore, gaseous exchange occurs through the cuticle (Basyoni and Rizk, 2016). Regarding osmoregulation and excretion, the nematodes possess a simple excretory system that consists of two lateral longitudinal tubes that open into two excretory pores at the anterior end.

1.4.4. The life cycle of EPNs

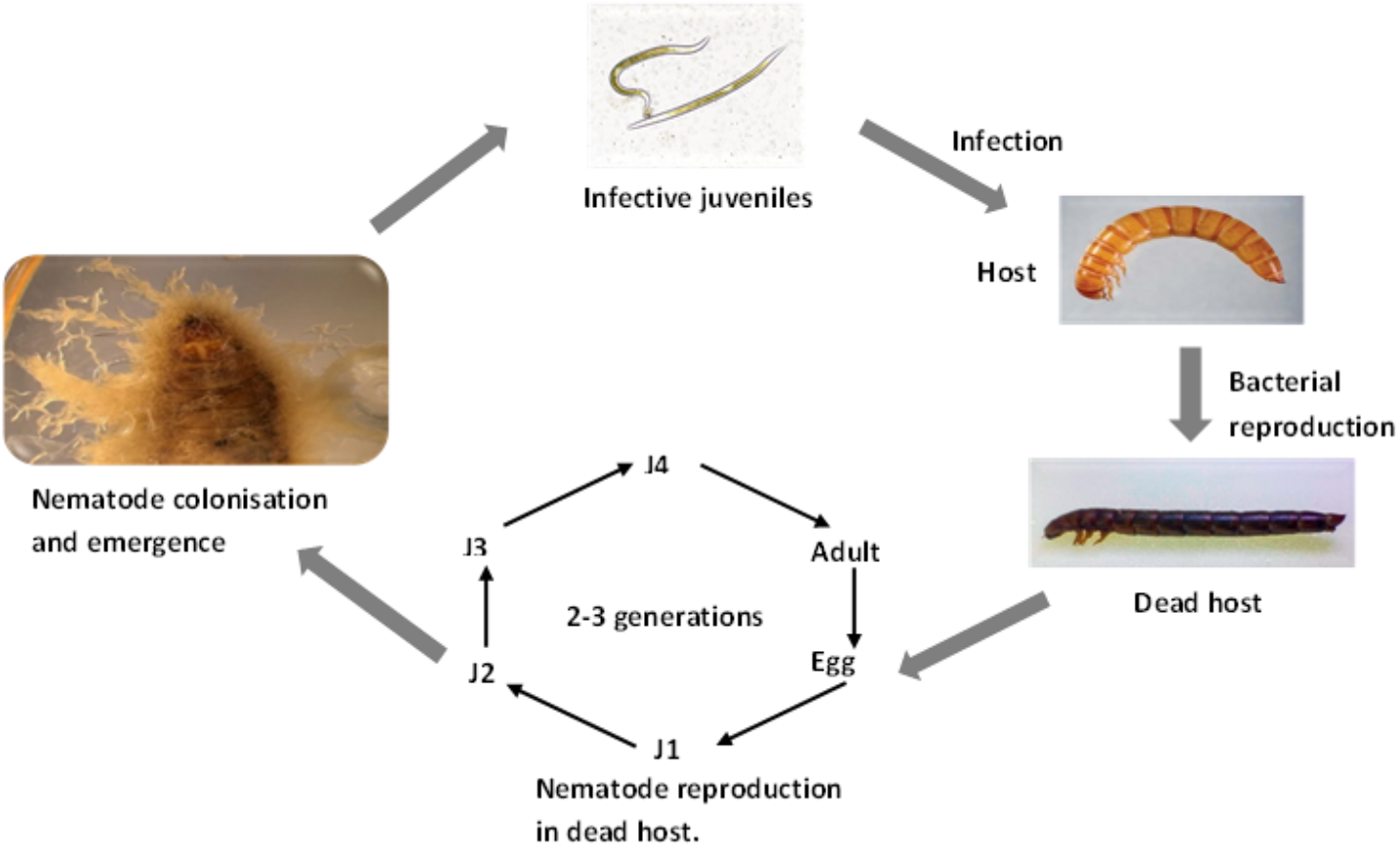

Figure 1. The life cycle of entomopathogenic nematodes (Image adapted from Lephoto *et al*., 2016).

The life cycle of EPNs comprises three developmental stages: an egg stage, four juvenile stages, and the adult stage (Feyisa, 2021). The third stage IJs, represent the only soil-dwelling and non-feeding stage of EPNs and are physiologically and morphologically modified to withstand harsh soil conditions. IJs initiate pathogenicity in insect hosts through a three-step process *(*Alonso *et al*., 2018). Firstly, IJs seek and locate susceptible insect hosts within the environment using chemosensory and mechanosensory neurons on their cuticle, which facilitate their response to several environmental and host-associated cues (Basyoni and Rizk, 2016). Secondly, upon identification and location of susceptible insect hosts, IJs initiate infection by entering the insect hosts through natural openings such as the mouth, anus, and spiracles or by piercing thin cuticle areas (Koppenhöfer *et al*., 2020). Finally, the nematode-bacterial complex overcomes the host defences and multiplies, producing new generations of IJs. This step occurs in the host gut, where IJs regurgitate their associated pathogenic bacteria into the haemocoel (Kushwah *et al.*, 2017). During infection, the insect haemolymph provides nutrients for the bacteria to grow and release toxins and exoenzymes that weaken the host response, resulting in insect death by septicaemia within 24 to 48 hours (Manochaya *et al.*, 2022). This leads to the conversion of the cadaver into nematode biomass, in which the bacteria digest the host tissues to provide nutrients to the reproducing nematodes (Fodor *et al.*, 2022). The bacteria also release bacteriocins, antimicrobials, and other antibiotics which prevent the contamination of the host cadaver by secondary invaders, therefore allowing the nematodes to develop into adults which reproduce for 2-3 successive generations (Labaude and Griffin, 2018). Once the available nutrients are exhausted, the new IJs emerge and disperse from cadavers to locate and parasitise new insect hosts to continue their life cycle.

1.4.5. The effectiveness of EPNs as biological control agents

The effectiveness of EPNs as biological control agents is primarily due to their high virulence and pathogenicity towards their target host (Yağcı *et al.*, 2021). In soil, EPNs can identify, locate, and infect susceptible insect hosts, therefore releasing the bacterial symbionts which produce insecticidal compounds that cause septicaemia and death within 24-48 hours. EPNs also possess a wide host range and infect over 250 insect species across 75 families in 11 orders (Lacey and

Georgis, 2012). Moreover, EPNs may be readily mass-produced on large scales employing both liquid and solid artificial media, and work well with a variety of biological pesticides and fertilisers used in several IPM techniques (Boemare *et al*., 1996). Another advantage of using EPNs as biological control agents lies within their life cycle. During the non-feeding and developmentally arrested stage of the life cycle, IJs possess the physiological and morphological capacity to persist in the soil for extended periods or in a formulated state and can be applied using conventional sprayers (Lacey and Georgis, 2012). This makes EPNs cost-effective as they reduce the need for reapplication due to the establishment of reproducing populations within the environment that actively respond to changing pest densities; an evident advantage over insecticides (Holmes *et al.*, 2015).

1.4.6. The use of EPNs in biological control

More than 13 species of EPNs have been commercialised by several manufacturers globally. Of these, *Steinernema carpocapsae*, *Steinernema feltiae*, *Steinernema glaseri, Steinernema riobrave, Heterorhabditis bacteriophora* and *Heterorhabditis megidis* have been identified as the most successful in suppressing several insect pest populations (Askary and Abd-Elgawad, 2021)**.** In a study, Askary and Abd-Elgawad (2021) observed that during field studies, both *H. bacteriophora* and *H*. *megidis* effectively controlled the black vine weevil, *Otiorhynchus sulcatus* which is considered a major insect pest of yew, and several other nursery plants. Using EPN-host matching, *S. feltiae* was shown to successfully control sciarid flies infesting mushrooms whereas, *S. glaseri* and *S. kraussei* provided effective control of grubs and spruce web-spinning sawfly, respectively (Lacey *et al*., 2015). In China, foliar and above-ground applications of *S. carpocapsae* species successfully suppressed the peach fruit moth *Carposina niponensis* Walsingham (Lepidoptera: Carposinidae) which is a serious insect pest of apples (Kaya *et al*., 2006). In addition, laboratory bioassays of *H. bacteriophora* showed effective control of *Ceratitis capitata* (Wiedemann), also known as the Mediterranean fruit fly, an important pest of citrus fruits in Southern Africa (Malan and Manrakhan, 2009). In Africa, only a few countries; such as South Africa (SA), Kenya, Egypt and Ethiopia; have exploited the use of EPNs as biological control agents

(Kaya *et al*., 2006; Malan *et al*., 2006). In SA, *Cryptonem*®, a powdery formulation of *H. bacteriophora* produced by River BioScience, is the only commercialised species of EPNs and is used to control the false codling moth, banded fruit weevil and other weevil species, codling moth, and sciarid (Hatting, Moore and Malan, 2019).

1.4.7. Foraging behaviour of EPNs

The foraging behaviour of an organism refers to a series of actions taken to obtain and utilise sources of energy and nutrition (Koy and Plotnick, 2007). This includes searching, locating, retrieving, consuming, and storing food sources. The foraging behaviour of EPNs varies among species and influences their environmental distribution and host range (Lortkipanidze *et al.*, 2016). EPNs are classified into a continuum between two extremities known as cruise and ambush foragers, with many species (intermediate foragers) falling between these endpoints (Ruan *et al.*, 2018). EPNs utilising a cruise foraging behaviour actively search the environment to locate insect hosts by responding to long-range host-associated and environmental cues (Griffin, 2012). Due to their motility, cruiser foragers are more likely to identify immobile hosts, such as those buried deep below the ground or in other cryptic habitats (Griffin, 2015). EPNs that exhibit this behaviour include all *Heterorhabditis* spp. and some *Steinernema* spp. such as *S. glaseri* and *S. Kraussei* (Campbell and Gaugler, 1997). Ambush foragers employ a "sit-and-wait" approach where the EPNs wait for their prey to cross their strike area boundary, enabling their attachment and invasion (Sivaramakrishnan and Razia, 2021). Ambush foragers are also capable of nictation, which is a process that allows the IJs to lift the anterior portion of their body and wave it while balancing using their tail to facilitate attachment to mobile hosts (Grewal *et al.*, 1994). Some EPNs also exhibit jumping behaviours in addition to sitting and waiting for the prey, which increases the likelihood of EPNs infecting the insect host (Yang *et al.*, 2020). In this process, the IJs stand on their tails and curl into a loop before propelling forward with their body stretched. Due to their low motility, ambush foragers successfully locate highly mobile insect hosts and include species such as *S. carpocapsae* and *S. siamkayai* (Campbell *et al*., 2003; Campbell and Gaugler, 1997). The intermediate foragers are species of EPNs which utilise behavioural characteristics common to both cruise and ambush foragers to locate hosts (Baiocchi *et al.*, 2017). These include

species such as *S. feitiae* and the infrequently nictating *S. riobravis,* which can identify and locate mobile and non-mobile insect hosts.

1.4.8. Factors affecting the foraging behaviour of EPNs

IJs experience a variety of environmental and host-associated cues upon their emergence from host cadavers or inundative release into soil, which significantly affects their foraging behaviour and, ultimately, their pathogenicity, survival, and persistence (Koppenhöfer *et al*., 2020). Over the years, several studies have revealed that various abiotic factors affect the foraging behaviour of EPNs; however, soil type and moisture are regarded as the two most important (Lacey *et al*., 2015). Soil is divided into six categories: clay, sand, silt, peat, loamy and chalky (Yu *et al.*, 2020). As such, soil characteristics such as particle size, pore size and organic matter are associated with soil texture and the ability of the soil to retain moisture (Koppenhöfer and Fuzy, 2006). Soil with smaller pore sizes, such as clay, is known to restrict the movement of EPNs; however, soil with larger pore sizes, such as loamy soil, facilitates the free movement of EPNs. Therefore, the efficacy of EPNs against soil-dwelling insects in finer-textured soils should typically decline. Additionally, in soil, EPNs move through thin films of water coating the interstitial spaces; therefore, adequate moisture is crucial (Koppenhöfer *et al*., 2020). However, since rapid declines in soil moisture occur in the upper soil layers, particularly in sandier soils which exhibit lower water retaining capabilities, the optimum soil moisture at which EPNs are active differs among different species of EPNs (Yadav and Lalramliana, 2012). This is partly due to variations in the sheaths of *Steinernema* spp. and *Heterorhabditis* spp., which enable them to respond differently to desiccation. *Steinernema* spp. have loose-fitting sheaths that are easily lost during foraging, while *Heterorhabditis* spp. have tight-fitting sheaths. As such, *Heterorhabditis* spp. are more adapted to surviving desiccation than *Steinernema* spp. (Grewal *et al*., 2005). The response of nematodes to chemical cues is also essential for predator-prey interactions. This is because the infected host cadaver releases chemical compounds such as insecticidal compounds, antimicrobials, and scavenging deterrents that affect the foraging behaviour of EPNs (Grunseich *et al.*, 2021). In a study, Baiocchi *et al*. (2019) revealed that 3-Methyl-2-buten-1-ol (prenol) and 3-Hydroxy-2-butanone (AMC), which were associated with larvae infected by EPNs, acted as a

repulsive agent for *S. glaseri* (cruiser forager), and *S. riobrave* (intermediate forager). Contrastingly, Hallem *et al*. (2011) revealed that exposing the ambush forager, *S. carpocapsae,* to propanoic acid, hexanal, 2,3-butanedione, α-pinene, and γ-terpinene which are host-associated chemical cues stimulated jumping, therefore, indicating attraction to the odorants. This suggests that ambush foragers are more sensitive and responsive to short-range cues, while intermediate and cruise foragers are primarily sensitive and responsive to long-range cues.

1.4.9. Changes in the foraging behaviour and classification of EPNs into foraging groups

Over the years, differences in the jumping rates of several *Steinernema* species have been observed. According to the general pattern, ambush foragers exhibit higher jumping rates, while cruiser foragers do not use this strategy (Campbell *et al*., 2003). However, exceptions to the general pattern have been made in the case of *S. ceratophorum.* This species of EPNs exhibits short-duration standing bouts and high jumping rates, which implies an ambush to intermediate foraging strategy; however, they were categorised as cruiser foragers due to their efficiency in locating stationary hosts rather than mobile hosts (Dillon *et al.*, 2006). Additionally, in a study, Ramakuwela *et al*. (2018) reported that *Steinernema innovation*, which utilised a cruiser-type behaviour, was also capable of nictation. This was the first report on a nematode species, particularly from the 'glaseri group' exhibiting this behaviour. Field application studies have also revealed that *S. carpocapsae* is potentially effective against distant and stationary hosts, which contradicts previous evidence suggesting that these EPNs are strictly ambush foragers. According to Dillon *et al*. (2006), *S. carpocapsae* can parasitise the larvae and pupae of the large pine weevil *Hylobius abietis* living beneath the bark of tree roots down to a depth of 40 cm.

1.5. Research Motivation

Foraging is an essential step in the infection success and survival of EPNs. As such, it is important to enhance the efficacy of EPNs and their success as BCAs. This is because behavioural adaptations such as foraging enable nematodes to successfully exploit diverse habitats to locate, recognise and accept a wide range of hosts. According to several studies, the performance of

EPNs in biological control differs significantly according to their biology and response to biotic and abiotic factors. Interestingly, over the years, several studies have shown that some species of EPNs deviate from their known and classified foraging behaviour during field applications. It has also been shown that although EPNs can attack over 200 insect hosts, significant declines in host range have been observed during field applications (Hazir *et al*., 2003). Based on this information, although EPNs have shown great promise as BCAs, significant gaps still exist in our knowledge regarding their foraging behaviour and factors affecting host-seeking and potentially reducing their efficacy. As such, this project aimed to investigate the different foraging behaviours employed by EPNs isolated from soil and further confirm these strategies by exposing the EPNs to various factors that affect their foraging behaviour. This would allow an appropriate classification of the EPNs into foraging groups, improve EPN-to-Host matching, and promote effective and permanent control of insect pests during field applications. Additionally, the application of target-specific EPNs results in a substantial increase in crop yield and quality while remaining harmless to humans, animals and the environment. Increased production yields increase food availability thus alleviating the issue of food insecurity which affects over 10 million South Africans annually (Ngarava, 2022). An increase in the annual production yield also improves the agricultural sector's contribution to the country’s GDP, resulting in economic growth due to productivity. This then leads to an increased demand for labour therefore contributing to job creation.

1.6. Aim and Objectives

1.6.1. Aim

To Investigate the foraging behaviour of EPNs obtained from soil samples.

1.6.2. Objectives

- To isolate EPNs from soil samples using the insect baiting and White trap methods
- To identify the morphology of the isolated EPNs using light microscopy
- To identify the isolated EPNs by analysis of the 18S rDNA region
- To isolate bacterial symbionts from EPNs using selective media
- To identify the bacterial symbionts by analysis of the 16S rDNA region
- To investigate the pathogenicity of the bacterial symbiont
- To investigate nematode-bacterial symbiosis
- To Investigate the foraging behaviours of the EPNs using the 2D sand substrate assay, vertical sand column assay and chemotaxis assay

1.7. References

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Chapter 2:

Isolation and molecular identification of a potential entomopathogenic nematode

Abstract

Nematodes are colourless, unsegmented roundworms which exist in free-living, predaceous, and/or parasitic forms. Over the years, insect-parasitic nematodes, particularly entomopathogenic nematodes (EPNs) have gained recognition as effective biological control agents against a wide range of insect pests due to their ability to seek out and penetrate insect hosts, subsequently releasing lethal bacteria and toxins. *Cruznema* sp. NTM-2021 was recovered from soil samples collected from Mahobong Leribe in Lesotho using the soil baiting technique and White trap method, respectively. Light microscopy was used to visualise the isolated nematode while PCR amplification and Sanger sequencing of the 18S rDNA region was used for molecular identification. The 18S rDNA sequence was deposited onto the National Centre for Biotechnological Information (NCBI) Genbank database and assigned the accession number, OQ408141. The Molecular Evolutionary Genetics Analysis (MEGA) software was then used to infer evolutionary relationships among closely related species. Nematode pathogenicity was confirmed against the model insect host *Tenebrio molitor* larvae. The infective juveniles (IJs) of these nematodes could invade the insect larvae and cause mortality within 120 hours after inoculation under laboratory conditions.

2.1. Introduction

The phylum Nematoda, also known as “roundworms”, is one of the largest within the kingdom Animalia (Zhang *et al.*, 2020). Over 27,000 nematode species have been described, of which 25% are found in freshwater and soil, while 50% are in marine saltwater (Shao *et al.*, 2023). In soil, nematode communities contribute to the food web by occupying primary, secondary, and tertiary positions in at least five trophic groups**:** predators, omnivores, fungivores, bacterivores, and herbivores (Ortiz *et al*., 2016). Based on these attributes, nematodes contribute significantly

to essential processes such as mineralisation, nutrient cycling, and decomposition; as such, changes in the nematode population structure may significantly affect ecosystem functioning (Kouser *et al*., 2021). Additionally, soil nematodes may be beneficial or detrimental to humans, animals, and crops. To date, over 4,100 nematode species have been identified as plant parasitic nematodes (PPNs), which parasitise a variety of plant species, while more than 20,000 species belong to animal-parasitic nematodes, which are known to parasitise several species of vertebrates (Al-Banna and Gardner, 2022; Shao *et al.*, 2023). While rarely fatal, annually soil-transmitted nematode infections account for approximately 125,000 human deaths, many of which were reported in tropical regions and developing countries where poverty and poor sanitation are high (Stepek *et al.*, 2006; Iqbal and Jones, 2017). This is because infection may cause anaemia through the damage of the intestinal mucosa which causes blood leakage as well as bowel obstruction which may result in death if left untreated (Hawdon, 2014; de Lima Corvino and Horrall, 2024). Therefore, parasitic nematodes significantly impact the global economy and play crucial roles in agriculture, medicine, and veterinary sciences.

In nature, nematode-bacterial associations are abundant and vary between beneficial and antagonistic. At least three categories have been established for these associations: 1) trophism, wherein nematodes consume bacteria; 2) parasitism, wherein, if unchecked, bacteria cause diseases in nematodes; and 3) mutualism, wherein nematodes and bacteria work together (Dillman *et al.*, 2012). The nematodes of the genera *Steinernema*, *Heterorhabditis,* and *Oscheius* are insect-parasitic nematodes which have evolved a mutualistic symbiotic relationship with insect pathogenic bacteria of the genera *Xenorhabdus, Photorhabdus,* and *Serratia*, respectively (Lephoto and Gray, 2019). These nematodes are termed “entomopathogenic” because of their ability to kill susceptible insect hosts within 120 hours with the assistance of their bacterial partners (Dillman *et al.*, 2012). Currently, more than 100 *Steinernema* spp., 21 *Heterorhabditis* spp*.,* and over 13 *Oscheius* spp. have been identified and may be used as biopesticides for a wide range of insect pests (Bhat *et al*., 2020; Loulou *et al.*, 2022).

To understand nematode biodiversity and biology, accurate identification is essential. Traditional identification methods use microscopic image analysis to observe morphological and anatomical differences among species (Bogale *et al*., 2020). The primary identification characteristics include the shape of the head, length of the stylet, mouth and tail region, structure of the reproductive organs, body length and other visible physical characteristics (as shown in **Figure 2.1**). Although morphological-based methods are cost-effective and may relate structure to function, the lack of diversity among closely related taxa makes their use challenging (Bhat *et al.*, 2022). As such, measuring these characteristics is time-consuming and requires highly trained taxonomists. Therefore, an integration of molecular-based methods is essential for accurate identification.

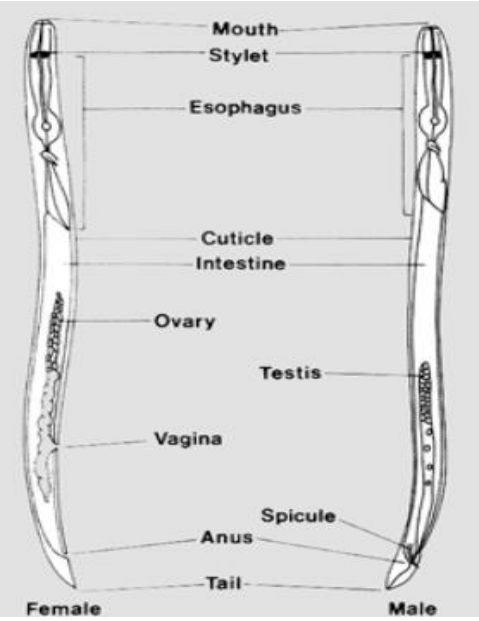

Figure 2.1. **The basic body plan of nematodes** (Lambert and Bekal, 2002).

Molecular-based techniques consist of two main categories: Fingerprint-based and Sequence-based (Bogale *et al*., 2020). Sequence-based molecular techniques enable the analysis of nucleotide sequences from nuclear DNA, mitochondrial DNA (mtDNA), and whole genomes to infer phylogenetic relationships (Lulamba and Serepa-Dlamini, 2020). Among these, ribosomal DNA (rDNA) genes have been widely used for characterisation as they possess multiple copy numbers and evolve at different rates. The sequences contain conserved and variable regions,

enabling nematode differentiation at taxonomic levels (Bhat *et al.*, 2022). The rDNA conserved regions include the small subunit (SSU) or 18S, 5.8S and the large subunit (LSU) or 28S, as well as variable non-coding regions including the external transcribed spacer (ETS) and internal transcribed spacer (ITS). The 5.8S coding region intersperses the ITS in the rDNA cistron into ITS-1 and ITS-2 and provides sequence variability, making it helpful in differentiating closely related species (Shao *et al.*, 2023).

Using these approaches has thus improved nematode taxonomy, which has enhanced our knowledge and understanding of nematode biodiversity, biology, geographic distribution, ecology, and co-evolution. Therefore, this study aimed to isolate potential EPNs and use phylogenetic analysis to identify and infer evolutionary relationships among species.

2.2. Materials and Methods

2.2.1. Breeding and maintenance of *Tenebrio molitor* larvae

Tenebrio molitor (Coleoptera: Tenebrionidae), also known as the yellow wheat mealworm, was bred and maintained as the host insect for isolating nematodes from the soil and the *in vivo* propagation of isolated nematodes. This is primarily because *T. molitor* is inexpensive, easily cultured, and retains its structural integrity upon nematode infection (Nthenga *et al*., 2021). Adult male and female beetles were transferred into 2 L plastic containers to facilitate mating and were fed oat bran and carrots, which provided sugar and water to support the developing larvae. The container lids were perforated to allow aeration, and the sealed containers were incubated in the dark at 25 °C and continuously sub-cultured to prevent overcrowding.

2.2.2. Isolation of nematodes

2.2.2.1. Collection of soil samples

Soil samples were collected from a farm in Mahobong Leribe, Lesotho (28°59'17.411" S, 28°8'25.027" E). Using a hand shovel, soil was collected at a depth of 15 cm and kept in separate 2 L plastic tubs (22.5 × 16 × 8.5 cm) during transportation to the laboratory (Kaya and Stock, 1997). Before soil baiting, the soil moisture of each soil sample was adjusted to 8% by adding the corresponding volume of distilled water and stored at room temperature (22-25 ℃) overnight (Lephoto and Gray, 2021).

2.2.2.2. Soil baiting

Nematodes were isolated from collected soil samples using the insect baiting technique described by Bedding and Akhurst (1975). A total of 10 *T. molitor* larvae were placed on top of each soil sample stored within the tubs. The tubs were closed with their compatible lids and inverted every 24 hours to enable free movement of the larvae and to increase the chances of infection. The tubs were incubated at 25 ℃ and observed daily for mortality and signs of infections. Dead insect larvae were collected from tubs and placed onto White traps to facilitate nematode emergence at room temperature (22-25 ℃).

Figure 2.2. Insect baiting technique used for the isolation of nematodes from soil samples using *T. molitor* larvae.

2.2.2.3. Recovery of nematodes from infected larvae using White Traps

Modified White traps, as described by White (1927), were used for the recovery and collection of nematodes from dead larvae suspected of nematode infection. This technique takes advantage of the nematodes' natural movement from resource-depleted cadavers in search of new insect hosts. The apparatus includes an inverted 60 mm diameter tissue culture Petri dish layered with moist filter paper (Whatman #1) and positioned centrally in a 100 mm diameter Petri dish containing +/-20 ml of sterile distilled water. The filter paper was kept moderately moist to prevent excess water from entering the insect cadaver and killing the nematodes before they could emerge. During summer, the White traps were kept on top of benches at room temperature (22-25 °C); however, heaters were used to maintain a temperature of approximately 25 °C during cold winter days (Lephoto and Gray, 2019). The emerged nematodes were collected into 50 ml Falcon tubes and were surface sterilised using a 0.1% sodium hypochlorite solution. Following surface sterilisation, the nematodes were rinsed with autoclaved distilled water and used for molecular and morphological identification.

2.2.2.4. Confirmation of pathogenicity using Koch's Postulates

Koch's Postulates were used to confirm nematode pathogenicity using the protocol described by Lulamba and Serepa-Dlamini (2020). Into 100 mm Petri dishes, 40 g of sterile coarse river sand was added and used as the substrate since it does not restrict nematode movement in the soil and allows some degree of aeration. The plates were hydrated to a moisture level of approximately 8% and inoculated with 100 IJs suspended in 100 µl of sterilised distilled water, followed by the addition of 10 *T. molitor* larvae. The plates were sealed with parafilm and incubated at 22-25 °C, and insect mortality was observed daily. Insect cadavers were retrieved from the plates and placed onto White traps to monitor the emergence of nematodes, therefore confirming nematodes as the causative agent.

2.2.2.5. *In vivo* maintenance of nematodes

The nematodes were maintained and cultured *in vivo* through the continuous reinfection of fresh *T. molitor* larvae (using the method described in 2.2.2.4) and recovered from insect cadavers using the White trap method (as described in 2.2.2.3).

2.2.2.6. Morphological identification of the unknown nematode isolate

The nematodes were harvested from the White traps and rinsed three times using sterile distilled water. A pasteur pipette was used to place a drop of water containing nematodes onto glass slides. The drop of water was warmed by passing the slide 6-8 times over a flame to reduce the nematodes' motility. The slides were then covered with a cover slip and sealed with nail polish to prevent the nematodes from drying out and the cover slip from sliding off. The slides were viewed using the Olympus BX63 light microscope at the 10X, 40X, 60X and 100X magnification.

2.2.3. Molecular identification of nematodes

2.2.3.1. Extraction of genomic DNA from nematodes

The E.Z.N.A Insect DNA kit (Omega Biotek, catalogue D0926-01) was used to extract genomic DNA from nematodes using the manufacturer's protocol. Approximately 50 mg of fresh nematodes were taken from White traps and transferred to sterile falcon tubes. Excess water was removed, and nematodes were immersed in 20 ml of 0.1% sodium hypochlorite and set aside for 1 hour. The nematodes were then rinsed using sterile deionised water, transferred into a 1.5 ml Eppendorf tube, and crushed using a disposable microtube pestle. Into the tube, 350 µl of CTL Buffer and 25 µl Proteinase K Solution were added and vortexed before incubating the tubes overnight at 37 °C. A volume of 350 µl of chloroform: isoamyl alcohol (24:1) was then added to the sample and mixed thoroughly before centrifugation at 10,000 x g for 2 minutes. The supernatant was carefully transferred into a 1.5 ml microcentrifuge tube, and one volume of BL Buffer and 2 µl RNase A was added. The tube was vortexed at maximum speed for 15 seconds and incubated at 70 °C for 10 minutes before adding one volume of 100% ethanol and vortexing

the sample at maximum speed for 15 seconds. A HiBind® DNA Mini Column was then inserted into a 2 ml Collection Tube, and 750 µl cleared lysate was transferred into the column, which was centrifuged at maximum speed for 1 minute. The filtrate was discarded, and the collection tube was reused. The HiBind® DNA Mini Column was then transferred into a 2 ml Collection Tube, and 500 µL HBC Buffer was added before centrifuging at maximum speed for 30 seconds. The filtrate was discarded, and the collection tube was reused. A volume of 700 µL of DNA Wash Buffer was added and centrifuged at maximum speed for 1 minute. The filtrate was discarded, and the collection tube was reused. A second DNA Wash Buffer step was performed before centrifuging the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix. The HiBind® DNA Mini Column was then transferred into a clean 1.5 ml microcentrifuge tube, and 50 µl Elution Buffer heated to 70 °C was added to the column membrane and set aside at room temperature for 2 minutes before centrifugation at maximum speed for 1 minute. The eluted DNA was stored at -20 °C until further use.

2.2.3.2. Polymerase Chain Reaction (PCR) amplification of the 18S rDNA region

The extracted DNA was sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa, for PCR amplification of the 18S rDNA region using the forward primer TW81 (5'-GCGGATCCGTTTCCGTAGGTGAACCTGC -3') and the reverse primer AB28 (5'-GCGGATCCATATGCTTAAGTTCAGCGGGT-3'). The reaction mixture contained NEB OneTaq 2X MasterMix with Standard Buffer, 10-30 ng/µl genomic DNA, 10 µM forward primer, 10 µM reverse primer and nuclease-free water until the desired reaction volume was reached. The PCR cycling conditions included an initial denaturation step at 94 °C for 5 minutes, followed by a 35-cycle amplification series with a denaturation step at 94 °C for 30 seconds, an annealing step at 50 °C for 30 seconds, an extension step at 68 °C for 60 seconds, and a final extension step at 68 °C for 10 minutes.

2.2.3.3. Agarose gel analysis and amplicon purification

Agarose gel electrophoresis was performed at Inqaba Biotechnical Industries (Pty) Ltd, South Africa using a 1% agarose gel stained with EZ-vision Bluelight DNA Dye to visualise the integrity of the PCR-generated amplicons with the NEB Fast Ladder used as a size standard. The amplicons were then enzymatically purified using the ExoSAP procedure for further applications.

2.2.3.4. Sanger sequencing of the 18S rDNA region

Sanger sequencing of the PCR-generated amplicons was performed at Inqaba Biotechnical Industries (Pty) Ltd, South Africa using the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 based on the manufacturer's protocol. The labelled products were then cleaned using the Zymo Research ZR-96 DNA Sequencing Clean-up Kit™ and sequenced on the ABI 3730xl Genetic Analyzer.

2.2.4. Bioinformatic analysis

2.2.4.1. Sequence analysis

The sequences from Inqaba Biotechnological Industries Pty (Ltd) were edited and error-corrected using the biological sequence alignment editor BioEdit version 5.0.9. The consensus sequence (query sequence) of the unknown nematode isolate was created and uploaded onto the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLAST) to search and align the sequence against known sequences in the GenBank nucleotide sequence database (Altschul, 1997). Sequences with the highest query coverage, percentage identity, and lowest E value were chosen as sequences similar to the query sequence (Lulamba and Serepa-Dlamini, 2020).

2.2.4.2. Molecular phylogenetic analysis

The Molecular Evolutionary Genetics Analysis (MEGA) version 11.0.13 was used for the comparative analysis of gene sequences to create phylogenetic trees and analyse molecular evolution statistically (Kumar et al., 1994). The sequences were aligned using the Multiple Sequence Alignment Method (MUSCLE) set at default parameters (Edgar, 2004). The evolutionary relationships between the aligned isolates were inferred using the Tamaru-Nei model based on the Maximum Likelihood method, which seeks the tree and related model parameters that maximise the likelihood of generating a specified collection of leaf genomes (Lin *et al.*, 2013; Tamaru *et al.,* 2021). Bootstrap replications of 1000 base substitution were used, which made the tree more reliable, and *Caenorhabditis elegans* isolate X5005 (FJ589008.1) was used as the outgroup and to root the tree.

The following taxa were selected for the construction of the phylogenetic tree for the unknown nematode isolate:

Cruznema sp. GD-1 (ON191475.1), *Cruznema* sp. CS50 (MW228469.1), *Cruznema* sp. isolate 734C-1 (OR606776.1), *Steinernema guangdongense* strain GDc339 (AY170341.1), *Steinernema lamjungense* voucher UGMD:104182 (HM000101.1), *Steinernema hermaphroditum* isolate CS34 (MF663703.1), *Steinernema hermaphroditum* isolate S0905 (MH802516.1), *Oscheius* sp. KAT-2015 (KR119081.1), *Oscheius* sp. Pak.S.N.10 (KT878513.1), *Oscheius* sp. AH13 (MF599577.1), *Oscheius* sp. MCB (KF684370.1), *Heterorhabditis atacamensis* isolate D099 (HM230723.1), *Heterorhabditis noenieputensis* isolate WS21 (KY024497.1), *Heterorhabditis indica* strain PBCB2 (KY807714.1), *Heterorhabditis indica* (AY321483.1) and *Caenorhabditis elegans* isolate X5005 (FJ589008.1).

2.3. Results and discussion

2.3.1. Isolation of nematodes

Preliminary identification of EPNs is based on observing and monitoring the colour change caused by the infecting genera within the insect larvae. In most infected larvae, the isolated nematode caused softening of the insect cadaver and no distinct colour change (**Figure 2.3A**). However, some insect cadavers displayed incomplete (**Figure 2.3B**) or complete blackening upon nematode infection (**Figure 2.3C**). The observed colour change in some of the larvae may be caused by the activation of the host's immune system, which resulted in melanisation (an immediate localised blackening reaction caused by phenoloxidase (PO) activity) in response to wounding which occurs during nematode infection (Nakhleh *et al*., 2017).

Figure 2.3. Symptom variation observed in insect cadavers. Figure 2.3A. Insect cadaver showing no colour change. **Figure 2.3B.** Insect cadaver showing incomplete blackening. **Figure 2.3C.** Insect cadaver showing complete blackening.

The same symptoms were observed in the pathogenicity tests, fulfilling Koch's Postulates which revealed that larvae mortality was caused by the isolated nematode species (**Figure 2.4**).

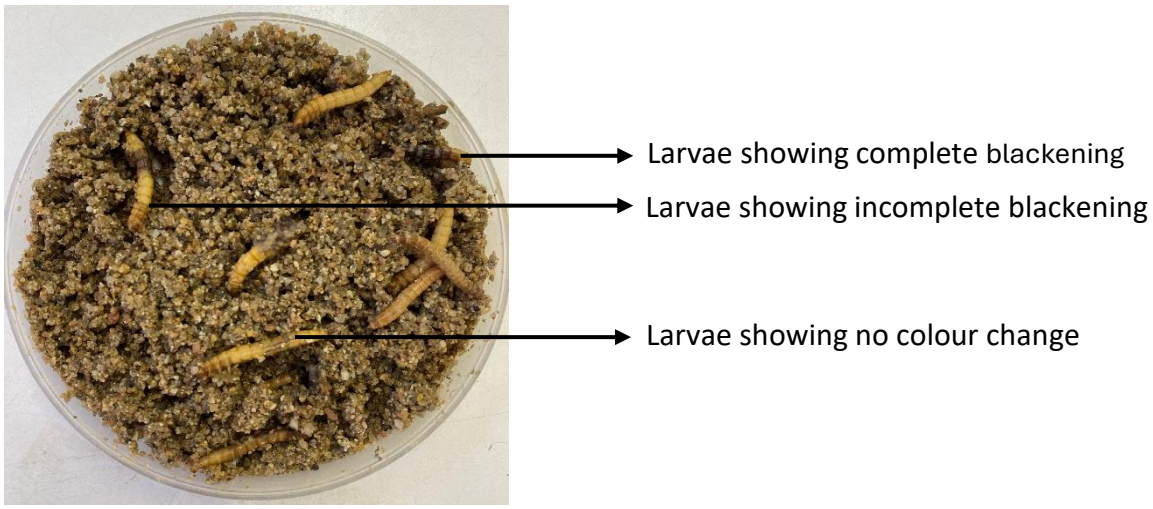

Figure 2.4. Pathogenicity test confirming Koch's Postulates.

The emergence of nematodes from insect cadavers was observed within 3-7 days, and the White trap method was used to recover the nematodes upon their emergence (**Figure 2.5**). The larvae infected with the isolated nematode disintegrated, collapsed, and appeared flat following emergence.

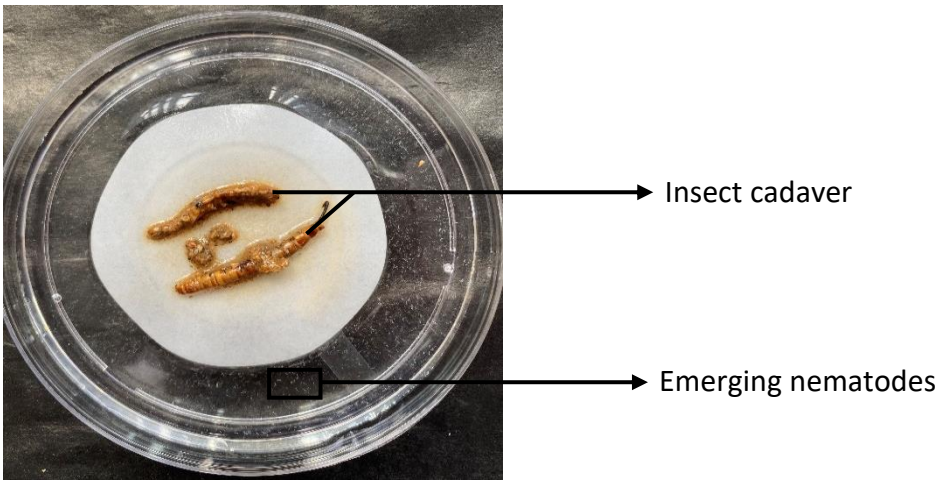

Figure 2.5. The White trap method showing the emergence of nematodes from infected insect cadavers into water.

The morphology of the adult male and female nematodes is shown in **Figure 2.6**. From the micrographs, anatomical structures such as the head, mouth, tail, and reproductive organs are visible.

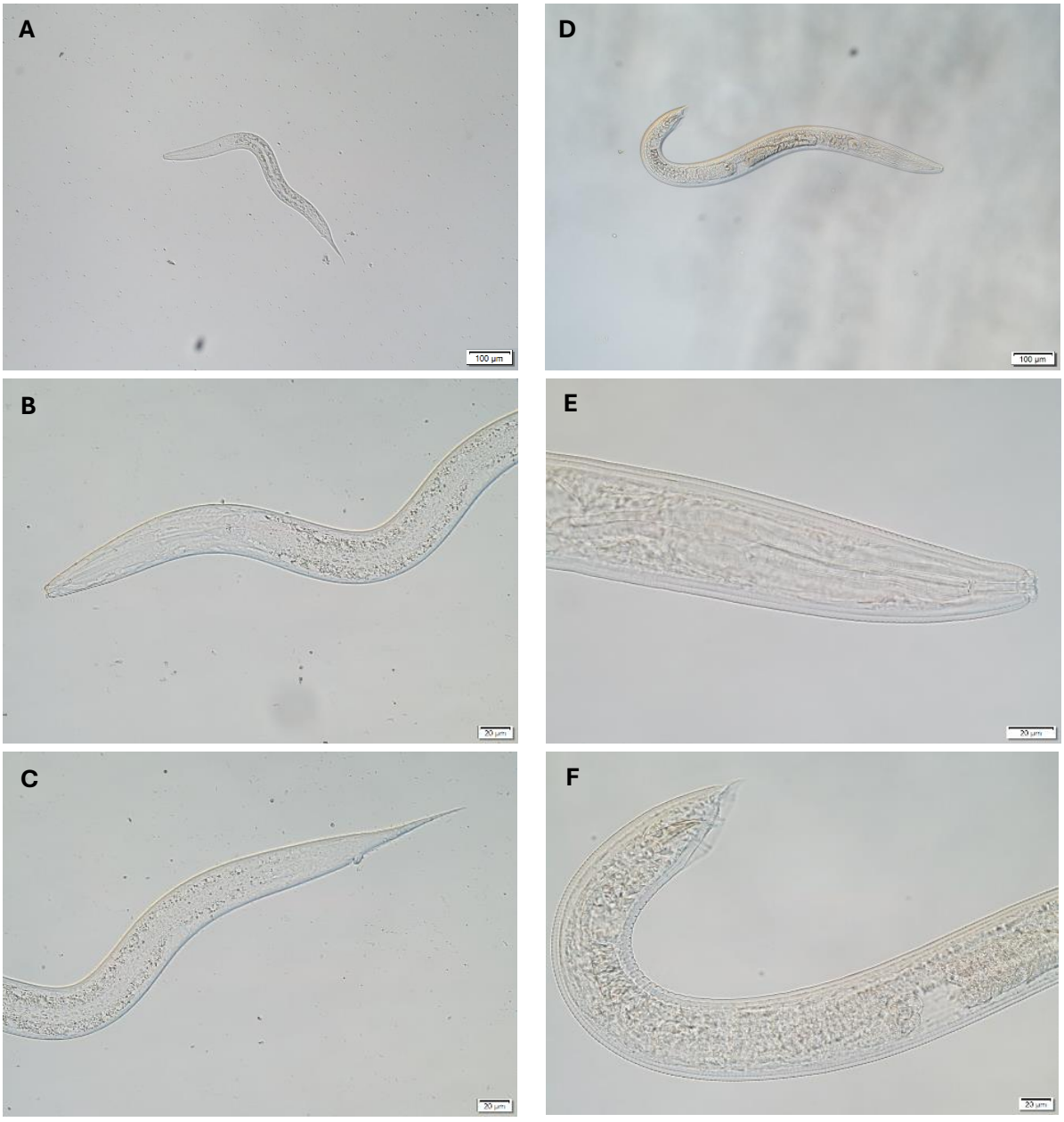

Figure 2.6. Morphology of the unknown nematode isolate under a light microscope. A. Adult female body. **B.** Female anterior end showing the mouth and pharynx. **C.** Female posterior end showing the tail **D.** J-shaped male body. **E.** Male anterior end showing the mouth region. **F.** Male posterior end showing the tail and spicule.

2.3.2. Bioinformatics analysis

2.3.2.1. Sequence analysis

Chromatograms containing the sequences for the PCR-generated amplicons of the 18S rDNA region were obtained from Inqaba Biotechnical Industries (Pty) Ltd. These chromatograms were edited and error-corrected using BioEdit version 5.0.9 and saved as FASTA formatted files. The 18S rDNA sequence of the unknown nematode isolate is shown in **Figure 2.7**. The NCBI BLAST results revealed that the unknown nematode isolate had the highest affinity to *Cruznema* sp. isolate 734C-1 (OR606776.1), a species that has not yet been described, with a percentage identity of 96.77%, query coverage of 99% and E value of 0 (see Appendix II). The sequence was deposited on NCBI GenBank and assigned the name *Cruznema* sp. NTM-2021 with the accession number OQ408141.

>AAAAATATCCGAAAACCTACCTAACTATCTCTTCACGAGGTGTAGTCGTGTCGATGAGCTGTTCCGG
GAGAGGATGGCGATGAATCGATGCACGTTGCTTTTGCGACGATACTTAATCGCTCTGCCTGAGAGTG
GCCTCGGATTGGTTTGGTGGCTTGGAAGCCTTGTGCTTCTAGTCTTAATGAGATAGCTTGCTATCCGC
CATCCAATCACTTCTTTCATAACTGAGTTGGTAATCGAAACGTGACAGGCTCACGCTAAAAAGCCTAC
TAACTAGCATTAGCGATGGATCGGTTGATTTGGTCATCGATGAAAAACGCAGCCAGCTGCGTTATTT
ACCACGAATCGCAAATTTGTAGAGTGGTAAAATTTTGAATGCATAGCGCCGAAGGGTATCATCCCTT
CGGCACGCCTGGTTCAGGGTTGTTTAATCGAATTCCGTCGACAGACAGTTGATGGATAGTCGAGGG
GCTGCGCTTCGGCGTACCCTCTCTGGTTTCGAGAACTATGTTCCTAGTGAACTGTGAATTCGCGTTTG
CGTAGCCTCCATGGCTCTGTGCTTGAATTAGTGCAGTGTCGATGGCGCTTATAAGGCAGCAACGTGC
ATTTGCAATCGCAAACT

Figure 2.7. The 18S rDNA sequence of the unknown nematode isolate. The NCBI BLAST results revealed a high affinity to *Cruznema* sp. isolate 734C-1 (OR606776.1).

2.3.2.2. Molecular phylogenetic analysis

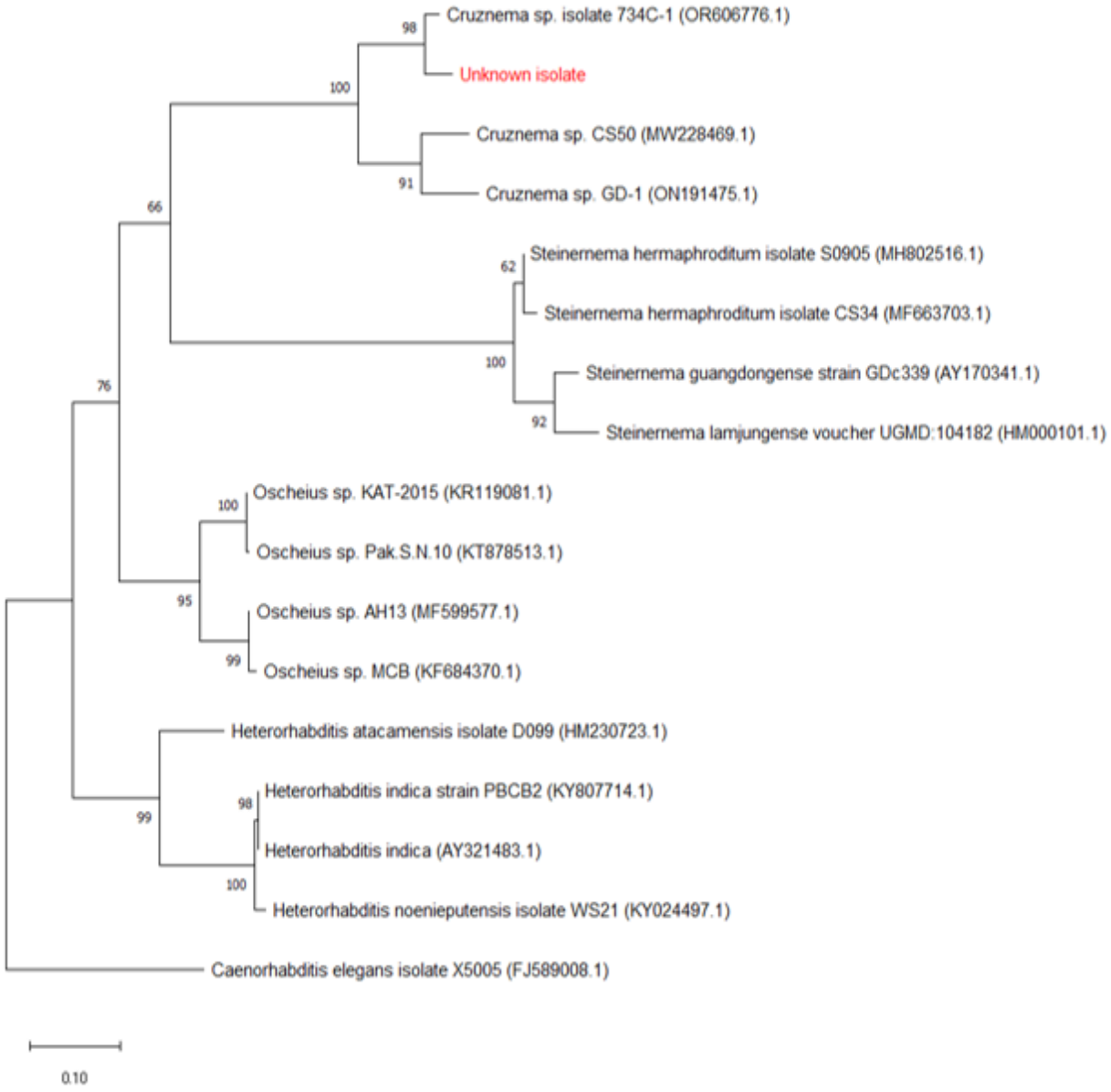

Figure 2.8. Maximum-likelihood tree, based on the analysis of the 18S rDNA region, showing the phylogenetic relationships of *Cruznema* spp. and several species of EPNs. Unknown nematode isolate is indicated in red, and *Caenorhabditis elegans* isolate X5005 (FJ589008.1) was used as the outgroup. The NCBI accession numbers are provided in brackets, and the numbers adjacent to the tree branches represent the bootstrap percentages. The tree was drawn to scale with the branch lengths measuring the number of substitutions per site.

Phylogenetic analysis of the unknown nematode isolate was conducted using MEGA version 11.0.13, and the Maximum Likelihood Method was used to demonstrate the phylogenetic relationships among the isolates. From the phylogenetic tree in **Figure 2.8**, the unknown nematode isolate (*Cruznema* sp. NTM-2021) clustered on the same clade with the undescribed species *Cruznema* sp. GD-1 (ON191475.1), *Cruznema* sp. CS50 (MW228469.1) and *Cruznema* sp. isolate 734C-1 (OR606776.1) with a bootstrap percentage of 100%, suggesting that these species are closely related. Currently, only seven valid species of *Cruznema* have been described of which many are free-living bacterivores besides *Cruznema tripartitum* which has been identified as an entomophilic nematode (Du *et al.*, 2022). From the phylogenetic tree, *Cruznema* sp. NTM-2021 is also shown as the sister taxon of *Cruznema* sp. isolate 734C-1 (OR606776.1) with a bootstrap percentage of 98%. However, the differences in the branch lengths of the two species suggest that *Cruznema* sp. NTM-2021 has undergone some degree of genetic variation. *Cruznema* sp. NTM-2021 is also part of a monophyletic group containing *Steinernema* and *Oscheius* species, supported by a bootstrap percentage of 76%. Species belonging to the genus *Oscheius* are classified as scavengers or bacteriophagous free-living nematodes, while others exhibit insecticidal properties, whereas species belonging to the genus *Steinernema* are strictly insect parasitic (Lephoto and Gray, 2019; Loulou *et al.*, 2022). Furthermore, species of the genera *Steinernema*, *Heterorhabditis,* and *Oscheius* are entomopathogenic nematodes (EPNs) and are used as biological control agents (BCAs) against several insect pests (Lephoto and Gray, 2019; Askary and Abd-Elgawad, 2021). From the results of the pathogenicity test, *Cruznema* sp. NTM-2021 may be characterised as an insect parasitic strain, and its shared ancestry with *Steinernema* spp. and *Oscheius* spp. may account for the shared pathogenicity. However, due to genetic variations, *Cruznema* sp. NTM-2021 may differ in virulence, survival, and host-seeking abilities from the undescribed *Cruznema* sp. isolate 734C-1 (OR606776.1). Additionally, *Cruznema* sp. NTM-2021 caused insect mortality within 120 hours and fulfilled one of the criteria to classify nematodes as EPNs. However, a clear bacterial symbiont and transferal of this symbiont from the infecting parental generation to the emerging generation in at least two successive insect infections is essential for differentiating true symbiosis from transitory cuticle hitchhiking (Dillman *et al.*, 2012).

2.4. Conclusion

The soil baiting and White trap method enabled the isolation and recovery of an insect parasitic nematode from soil samples collected in Mahobong Leribe, Lesotho. Using DNA extraction, PCR amplification and Sanger sequencing of the 18S rDNA region, the isolated nematode was identified as belonging to the genus *Cruznema*. The evolutionary relationships between the isolated species and other known species of EPNs were revealed by phylogenetic analysis. *Cruznema* sp. NTM-2021 caused insect mortality within 3 to 7 days; therefore, it may be used as a potential biological control agent against problematic insect pests. However, further studies are required to determine its host range, optimum temperature, desiccation potential and foraging behaviour.

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Chapter 3:

Isolation and identification of a potential bacterial symbiont from *Cruznema* sp. NTM-2021

Abstract

Entomopathogenic nematodes (EPNs) are unique parasites due to their symbiotic relationship with entomopathogenic bacteria which rapidly kill insect hosts following infection through the production of insecticidal and antimicrobial compounds. Recently, a potential entomopathogenic nematode, *Cruznema* sp. NTM-2021, was isolated from Mahobong Leribe in Lesotho. Selective media, MacConkey and Nutrient bromothymol blue agar (NBTA) was used to isolate a potential bacterial symbiont from the haemocoel of surface sterilized nematodes. Using light microscopy, the bacterial isolate was identified as Gram-negative and rod-shaped. Following PCR and Sanger sequencing of the 16S rDNA region the bacteria isolate was identified as *Enterobacter ludwigii* strain NTM-2021 and assigned the accession number PP495524. Using the intra-haemocoelic injection assay, *E. ludwigii* strain NTM-2021 was observed as pathogenic to *Tenebrio molitor* larvae while its growth and reproduction on lipid agar indicated potential symbiosis with *Cruznema* sp. NTM-2021.

3.1. Introduction

The entomopathogenic bacteria of the genera *Xenorhabdus, Photorhabdus,* and *Serratia* belong to the family Enterobacteriaceae and class Gammaproteobacteria (Sajnaga and Kazimierczak, 2020). In nature, to aid insect pathogenesis, the bacteria have established a mutualistic symbiotic relationship with soil nematodes of the genera *Steinernema*, *Heterorhabditis,* and *Oscheius*, respectively (Lephoto and Gray, 2019). *Steinernema* spp. harbour their bacterial symbionts in specialised vesicles known as the receptacle located in the anterior portion of the gut, whereas *Heterorhabditis* spp. and *Oscheius* spp. harbour their bacterial symbionts within the intestinal lumen (Sajnaga and Kazimierczak, 2020). To date, only 29 *Xenorhabdus* spp., 21 *Photorhabdus,* and 4 *Serratia* spp. have been identified in association with EPNs (Yimthin *et al.*, 2021; Awori, 2022). Phenotypically, the bacterial symbionts are characterised as motile, gram-

negative and non-spore forming rods (Askary and Abd-Elgawad, 2021). Furthermore, the bacterial species are facultative anaerobes with both fermentative and respiratory forms of metabolism. To differentiate between the bacterial symbionts, bioluminescence and catalase activity is essential. *Photorhabdus* spp. are positive for both traits, *Serratia* spp. are catalase positive only, while *Xenorhabdus* spp. are negative for both traits (Boemare and Akhurst, 2006; Rafii, 2014).

Despite their divergent evolutionary histories, the bacterial symbionts of EPNs have a similar life cycle divided into three physiological states corresponding to their two distinct ecological niches (Ciche *et al.*, 2006). The first state is a dormant, non-feeding phoretic state occurring within the infective juveniles (IJs). In this state, the bacterial population remains relatively constant within the developmentally arrested IJs. The second state is a metabolically active and pathogenic state that occurs within the insect host. In this state, the bacterial symbiont counteracts and overcomes the insect's immunity by producing and releasing several insecticidal compounds. The final state is a saprophytic state occurring within the insect cadaver wherein the bacterial symbionts and EPNs feed on host tissues to enable reproduction.

Within the insect host and cadaver, the bacterial symbionts also exhibit a unique ability to exist as two distinct phenotypic or phase variants known as Phase I (primary Phase) and Phase II (secondary Phase) variants, which occur during the stationary phase of the growth cycle (Sajnaga and Kazimierczak, 2020). Naturally, Phase I variants represent the bacterial isolates harboured by the IJs and are the only phase detected in nature. However, Phase II variants are a variable proportion of clones that appear spontaneously during *in vitro* culture or the production of EPNs on artificial diets (Boemare and Akhurst, 2006). It is unclear what stimulates the phase shifting and what function it serves in the life cycle of the bacteria. According to Owuama (2001), phase shifting is a response to environmental changes. Phase I variants are motile and produce mucoid colonies. According to Givaudan *et al*. (1996), the presence of peritrichous fimbriae and flagella in Phase I variants assists in swarming motility and potentially promotes the fast and coordinated migration of the bacteria during the colonisation of the nematode vector and insect body during

parasitism. Phase I variants can also be distinguished by two main properties: dye absorption and antibiotic production. The antimicrobials synthesised and released by *Xenorhabdus* spp. include benzylideneacetone, nematophin, xenocoumacins, xenortides, xenematide, and bicornutins (Dreyer *et al*., 2018). On the contrary, *Photorhabdus* spp. produce antimicrobial compounds such as 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3- diol, 3,5, - dihydroxy-4-isopropyl-stilbene and β-lactam carbapenem (Ullah *et al.*, 2014). *Oscheius* spp. have not been broadly studied; however, it is thought they share similar mechanisms regarding their pathogenicity towards insect hosts. A study by Serepa *et al*. (2015) revealed that the indole derivatives, tryptophan and indole-3-acetic acid (IAA), which are produced by *Xenorhabdus* spp. and *Photorhabdus* spp. are also produced by *Serratia marcescens* strain MCB, a bacterial symbiont of *Oscheius safricana*. IAA possesses antimicrobial properties, whereas tryptophan derivatives have insecticidal properties. Therefore, the high virulence and production of antimicrobials by Phase I variants supports nematode reproduction by converting the insect cadaver into nematode biomass (Boemare and Akhurst, 2006). During the phenotypic switch, the phenotypic traits displayed by Phase I variants are reduced or absent in Phase II variants. Therefore, Phase II variants may kill insect hosts and colonise IJs; however, they are unable to provide appropriate growth conditions for nematodes and do not associate with the nematode (Griffin *et al*., 2005). According to Akhurst *et al*. (1992), the respiratory activity, nutrient uptake and transmembrane proton motive force are higher in Phase II variants, which suggests that Phase II variants may represent a free-living form of the bacteria.

Over the years, morphological and biochemical tests have been used to identify and differentiate bacteria (Baron, 1996). The morphological characterisation provides information regarding the cellular structure, arrangement, motility, and endospore and capsule production (Ogodo *et al.*, 2022). Biochemical characterisation differentiates bacteria based on their biochemical activities and may reveal differences in enzyme production, compound utilisation, and protein, carbohydrate, and fat metabolism. However, these techniques enable bacterial identification at a genus level; therefore, molecular characterisation is required. Nuclear ribosomal DNA (rDNA) genes have been widely used to identify bacterial species and infer phylogenetic relationships

(Lulamba and Serepa-Dlamini, 2020). The 16S and 23S rDNA genes form the basis of bacterial taxonomy primarily because these DNA sequences are universal and consist of highly conserved and variable regions (Amit Roy, 2014). These genes contain characteristic oligonucleotide signature sequences unique to a specific phylogenetic group. *Photorhabdus* spp. contain the sequence TGAAG while *Xenorhabdus* spp. contain the sequence TTCG, which enables their differentiation and identification (Boemare and Akhurst, 2006).

Therefore, this study aimed to isolate potential bacterial symbionts of *Cruznema* sp. NTM-2021 and use phylogenetic analysis of the 16S rDNA region to identify and infer evolutionary relationships among species. Furthermore, the pathogenicity of the bacterial isolate/s was investigated using an intra-haemocoelic injection assay.

3.2. Materials and Methods

3.2.1. Isolation of potential bacterial symbionts from *Cruznema* sp. NTM-2021

Fresh nematodes were retrieved from White traps, transferred into sterile falcon tubes, and set aside to settle. The excess water was removed, and the nematodes were surface sterilised by immersing the sediment in 10 ml of 0.1% sodium hypochlorite solution for 3 hours (Lephoto and Gray, 2021). Under a laminar flow hood, the nematodes were rinsed three times using sterile distilled water. The sterile nematodes were then transferred into a 1.5 ml microcentrifuge tube and crushed using a disposable microtube pestle. 1 ml of sterile nutrient broth was added to the tube, and the bacteria were grown in the dark at 25 °C on a shaking incubator at 150 rpm for 24 hours. The bacterial culture was streaked on MacConkey agar and Nutrient bromothymol blue agar (NBTA) before growing the plates in the dark at 25 ℃ for 24 hours (Kgosiemang *et al.*, 2024). The bacteria were continuously sub-cultured by streaking the bacterial colonies on MacConkey agar and NBTA to maintain pure cultures, which were used for further analysis.

3.2.2. Morphological analysis

3.2.2.1. Gram Stain

Heat-fixed bacterial smears on microscope slides were prepared using colonies grown on both MacConkey agar and NBTA streak plates. For 60 seconds, the bacterial smears were flooded with the crystal violet stain before being gently rinsed with tap water. The slides were then exposed to the mordant gram's iodine for 60 seconds, after which they were rinsed and carefully drained. 95% alcohol was added to the slides for 10 seconds to decolourise the smears before rinsing them with water to stop the decolourisation process. The counterstain safranin was added to the smears for 45 seconds, rinsed and set aside to air-dry. The slides were viewed using the Olympus BX63 light microscope at the 100X oil immersion objective.

3.2.3. Molecular identification

3.2.3.1. Bacterial DNA extraction

Genomic DNA extraction from bacteria was performed using the E.Z.N.A bacterial DNA kit (Omega Biotek, catalogue D3350-01). Firstly, bacterial colonies were retrieved from MacConkey agar and NBTA plates and cultured in LB media overnight. In 2 ml microcentrifuge tubes, 2 ml of the bacterial culture was added and centrifuged at 4000 x g for 10 minutes. The supernatant was aspirated and discarded before repeating the process to increase the number of bacterial cells. To resuspend the pellet, 100 µl of TE Buffer was added and vortexed before adding 10 µl lysozyme resuspended in elution buffer. The mixture was incubated at 37 °C for 10 minutes, and 100 µl TL Buffer and 20 µl Proteinase K Solution was added before incubating the sample at 55 °C in a shaking water bath overnight. A volume of 5 µl of RNase A was added, and the tubes were mixed and set aside for 5 minutes. The tubes were centrifuged at 10,000 x g for 2 minutes to pellet any undigested material, and the supernatant was cautiously transferred into new 1.5 ml microcentrifuge tubes. Into each tube, 220 µl of BL Buffer was added and vortexed to mix thoroughly. The tubes were incubated at 65 °C for 10 minutes, and 220 µl 100% ethanol was added before vortexing the tubes for 20 seconds at maximum speed. A HiBind® DNA Mini Column was transferred in a 2 ml Collection Tube, and the entire sample was transferred into the column

and centrifuged at 10,000 x g for 1 minute. The filtrate and collection tube were discarded, and the HiBind® DNA Mini Column was inserted into a new 2 ml Collection Tube. Into each column, 500 µL of HBC Buffer was added and centrifuged at 10,000 x g for 1 minute. The filtrate was discarded, and the collection tube was reused. A volume of 700 µl of DNA Wash Buffer was added into each column and centrifuged at 10,000 x g for 1 minute. The filtrate was discarded, the collection tube was reused, and a second DNA Wash Buffer step was performed. The empty HiBind® DNA Mini Column was centrifuged at maximum speed for 2 minutes to dry the column. The HiBind® DNA Mini Column was inserted into a new nuclease-free 1.5 ml microcentrifuge tube, and 50 µl of Elution Buffer heated to 65 °C was added. The tubes were set aside at room temperature for 5 minutes and centrifuged at 10,000 x g for 1 minute to elute the DNA. The eluted DNA was stored at -20 °C until further analysis.

3.2.3.2. Polymerase Chain Reaction (PCR) amplification of the 16S rDNA region

The extracted DNA was sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa, for PCR and Sanger sequencing of the 16S rDNA region using the universal primers: 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-CGGTTACCTTGTTACGACTT-3'). The reaction mixture contained NEB OneTaq 2X MasterMix with Standard Buffer, 10-30 ng/µl genomic DNA, 10 µM forward primer, 10 µM reverse primer and nuclease-free water until the desired reaction volume was reached. The PCR cycling conditions included an initial denaturation step at 94 °C for 5 minutes, followed by a 35 cycle amplification series with a denaturation step at 94 °C for 30 seconds, an annealing step at 50 °C for 30 seconds, an extension step at 68 °C for 60 seconds, and a final extension step at 68 °C for 10 minutes.

3.2.3.3. Agarose gel analysis and amplicon purification

Agarose gel electrophoresis was performed at Inqaba Biotechnical Industries (Pty) Ltd, South Africa using a 1% agarose gel stained with EZ-vision Bluelight DNA Dye to visualise the integrity of the PCR-generated amplicons with the NEB Fast Ladder used as a size standard. The amplicons were then enzymatically purified using the ExoSAP procedure for further applications.

3.2.3.4. Sanger sequencing of the 16S rDNA region

Sanger sequencing of the PCR-generated amplicons was performed at Inqaba Biotechnical Industries (Pty) Ltd, South Africa using the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 based on the manufacturer's protocol. The labelled products were then cleaned using the Zymo Research ZR-96 DNA Sequencing Clean-up Kit™ and sequenced on the ABI 3730xl Genetic Analyzer.

3.2.4. Bioinformatics analysis

3.2.4.1. Sequence analysis

The sequences from Inqaba Biotechnological Industries Pty (Ltd) were edited and error-corrected using the biological sequence alignment editor BioEdit version 5.0.9. The consensus sequence (query sequence) of the unknown bacterial isolate was created and uploaded onto the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLAST) to search and align the sequence against known sequences in the GenBank nucleotide sequence database (Altschul, 1997). Sequences with the highest query coverage, percentage identity, and lowest E value were chosen as sequences similar to the query sequence (Lulamba and Serepa-Dlamini, 2020).

3.2.4.2. Molecular phylogenetic analysis

MEGA version 11.0.13 was used for the comparative analysis of gene sequences to create phylogenetic trees and analyse molecular evolution statistically (Kumar *et al*., 1994). The sequences were aligned using MUSCLE set at default parameters (Edgar, 2004). The evolutionary relationships between the aligned isolates were inferred using the Tamaru-Nei model based on the Maximum Likelihood method, which seeks the tree and related model parameters that maximise the likelihood of generating a specified collection of leaf genomes (Lin *et al.*, 2013; Tamaru *et al.,* 2021). Bootstrap replications of 1000 base substitution were used, which made

the tree more reliable, and *Xenorhabdus Beddingii* (NR_119153.1) was used as the outgroup and to root the tree.

The following taxa were selected for the construction of the phylogenetic tree of the unknown bacterial isolate:

Enterobacter cloacae strain DSM 30054 (NR 117679.1), *Enterobacter cloacae* strain 279-56 (NR 028912.1), *Enterobacter cloacae* subsp. *dissolvens* strain LMG 2683 (NR 044978.1), *Enterobacter ludwigii* strain EN-119 (NR 042349.1), *Enterobacter mori* strain YIM Hb-3 (NR 146667.2), *Enterobacter hormaechei* strain 0992-77 (NR 042154.1), *Enterobacter bugandensis* strain 247BMC (NR 148649.1), *Enterobacter asburiae* strain JCM6051 (NR 024640.1), *Enterobacter cancerogenus* strain LMG 2693 (NR 044977.1), *Enterobacter hormaechei* subsp. *xiangfangensis* strain 10-17 (NR 126208.1), *Enterobacter quasihormaechei* strain WCHEs120003 (NR 180451.1), *Enterobacter cloacae* strain ATCC 13047 (NR 118568.1), *Xenorhabdus beddingii* (NR 119153.1).

3.2.5. Bacterial pathogenicity assay

3.2.5.1. Bacterial serial dilution and cell enumeration

Bacterial colonies were streaked on fresh MacConkey agar and grown for 24 hours at 25 ℃. Bacterial broth cultures were prepared using the freshly streaked colonies by inoculating a single colony (~0.2 mm) into a 50 ml falcon tube containing 25 ml of fresh sterile nutrient broth. The bacteria were then grown in the dark at 25 °C on a shaking incubator at 150 rpm for 24 hours. A 10-fold serial dilution was prepared in the ranges 10^{0}-10^{-3} and was used for intra-haemocoelic administration into *T. molitor* larvae. A standard curve for optical density measurements at 600 nm (OD600) versus viable cell count was also generated to approximate the number of colonies within each diluted sample. This was done by growing the bacteria to an OD600 of ~1 in nutrient broth and preparing several dilutions with readings ranging between 0.001 and 1. Serial dilutions were performed, and samples were plated on nutrient agar and incubated for 24 hours at 25 °C before counting the number of colony forming units (CFU). The equation obtained from the line

of regression was then used to approximate the CFU/ml in the 10^0-10^{-3} dilutions. The CFU/ml of each dose was multiplied by 0.005 ml to obtain close approximations of the bacterial CFU administered to each larva for the different dilutions.

Table 3.1. The bacterial CFU approximations administered into the *T. molitor* at 25°C.

Sample	Bacterial dose in CFU
10^0	1.6×10^6
10^{-1}	4.6×10^5
10^{-2}	2.1×10^5
10^{-3}	1×10^5

3.2.5.2. Intra-haemocoelic injection assay

A total of 75 *T. molitor* larvae were surface sterilised using 70% ethanol (v/v) to remove insect diet, faeces, and any microorganisms on its surface. The larvae were divided into five groups (control, 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions), which consisted of 3 Petri dishes containing five insect larvae in each plate. The Petri dishes were placed on ice for approximately 5 minutes to reduce insect motility and allow injection. Using a 0.3 ml BD Micro-Fine+ insulin syringe and needle, aliquots of 0.005 ml of the stock and diluted samples were drawn and injected at an angle (less than 45°) into the right proleg of each larva. Precautions were taken to prevent gut puncture by injecting the suspensions underneath the epidermal layer. The loss of haemolymph (clear, green-blue liquid) is expected; however, the emergence of particulate matter and yellow liquid from the injection site indicates gut puncture. The control larvae were injected with 0.005 ml of sterile nutrient broth. The injected larvae were then incubated at 25 °C. Larval mortality was observed daily over 3 days, and a Two-way Analysis of Variance (ANOVA) with replication test was used to analyse the results.

3.2.6. Confirmation of dependency using the lipid agar test

A lipid agar test was used to confirm dependency and symbiosis between the isolated nematode and bacterial species, as it mimics the natural environment of EPNs. Bacterial colonies were isolated from MacConkey agar plates and inoculated into 10 ml of sterile Nutrient Broth within 50 ml Falcon tubes. The bacteria were grown in the dark at 25 °C on a shaking incubator at 150 rpm for 24 hours. Bacterial spread plates were prepared on 5 lipid agar plates (See Appendix I) by inoculating 0.5 ml of the bacterial culture into each plate. The plates were incubated in the dark at 25 °C for 24 hours until a bacterial lawn was established. IJs were retrieved from White traps, surface sterilised using 0.1% sodium hypochlorite for 3 hours, and rinsed three times using autoclaved distilled water. A serial dilution was performed on the sterilised IJs, and their numbers were enumerated in 100 µl droplets under a light microscope. Approximately 50 IJs were inoculated onto the bacterial lawn, and nematode growth and reproduction was observed daily for 7 days.

3.3. Results

3.3.1. Isolation of a potential bacterial symbiont from *Cruznema* sp. NTM-2021

Bacterial species associated with *Cruznema* sp. NTM-2021 were cultured on MacConkey and NBTA media (**Figure 3.1**). The unknown bacterial isolate produced pinkish-red colonies on MacConkey agar; however, on NBTA, the colonies were dark red. The bacterial colonies were smooth, raised, and circular with a 1-2.5 mm colony diameter.

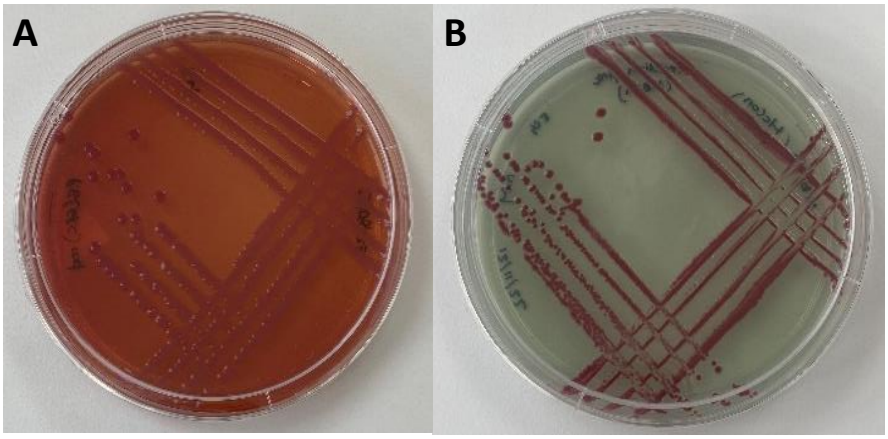

Figure 3.1. Bacterial species isolated from *Cruznema* sp. NTM-2021 and grown on MacConkey and NBTA media at 25 ^{o}C for 24 hours. A: Pinkish-red colonies observed on MacConkey agar. **B:** Dark red colonies observed on NBTA.

3.3.2. Morphological identification of potential bacterial symbiont

The unknown bacterial isolate is a gram-negative bacterial species, confirmed by the red/pink rod-shaped bacterial cells in **Figure 3.2**.

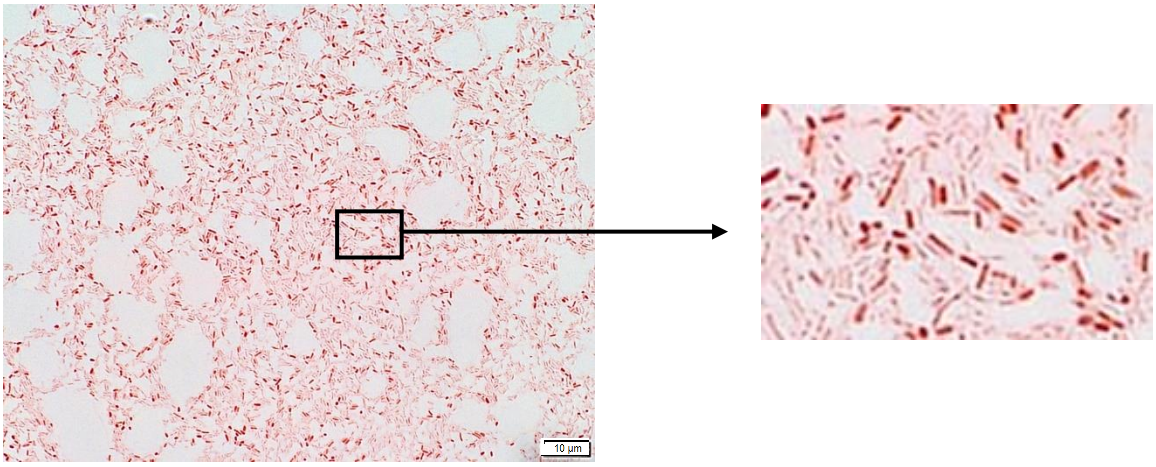

Figure 3.2. Gram stain of the unknown bacterial isolate at 1000X magnification. Pink and red rod-shaped colonies were observed.

3.3.3. Bioinformatics analysis

3.3.3.1. Sequence analysis

Chromatograms containing the sequence for the PCR amplicons of the 16S rDNA regions were obtained from Inqaba Biotechnical Industries (Pty) Ltd. These chromatograms were edited, error corrected using BioEdit version 5.0.9, and saved as FASTA formatted files. The 16S rDNA sequence of the unknown bacterial isolate is shown in **Figure 3.3**. The NCBI BLAST results revealed that the unknown bacterial isolate had the highest similarity to *Enterobacter ludwigii* strain EN-119 (NR 042349.1) with a percentage identity of 99.85%, query coverage of 100% and E value of 0 (see Appendix II). The sequence was deposited on NCBI GenBank, and the name *Enterobacter ludwigii* strain NTM-2021 was assigned with the accession number PP495524.

>CTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGAC
GATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA
GAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAA
TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG
GACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA

Figure 3.3. The 16S rDNA sequence of the unknown bacterial isolate. The NCBI BLAST results revealed a high similarity to *Enterobacter ludwigii* strain EN-119 (NR 042349.1).

3.3.3.2. Molecular phylogenetic analysis

Phylogenetic analysis of the isolated bacterial species was conducted on MEGA version 11.0.13, using the Maximum Likelihood Method to determine the phylogenetic relationships among the isolates. From the phylogenetic tree in **Figure 3.4**, the unknown bacterial isolate (which will be referred to as *Enterobacter ludwigii* strain NTM-2021) clustered on the same clade as *Enterobacter ludwigii* strain EN-119 (NR 042349.1) with a bootstrap percentage of 71%.

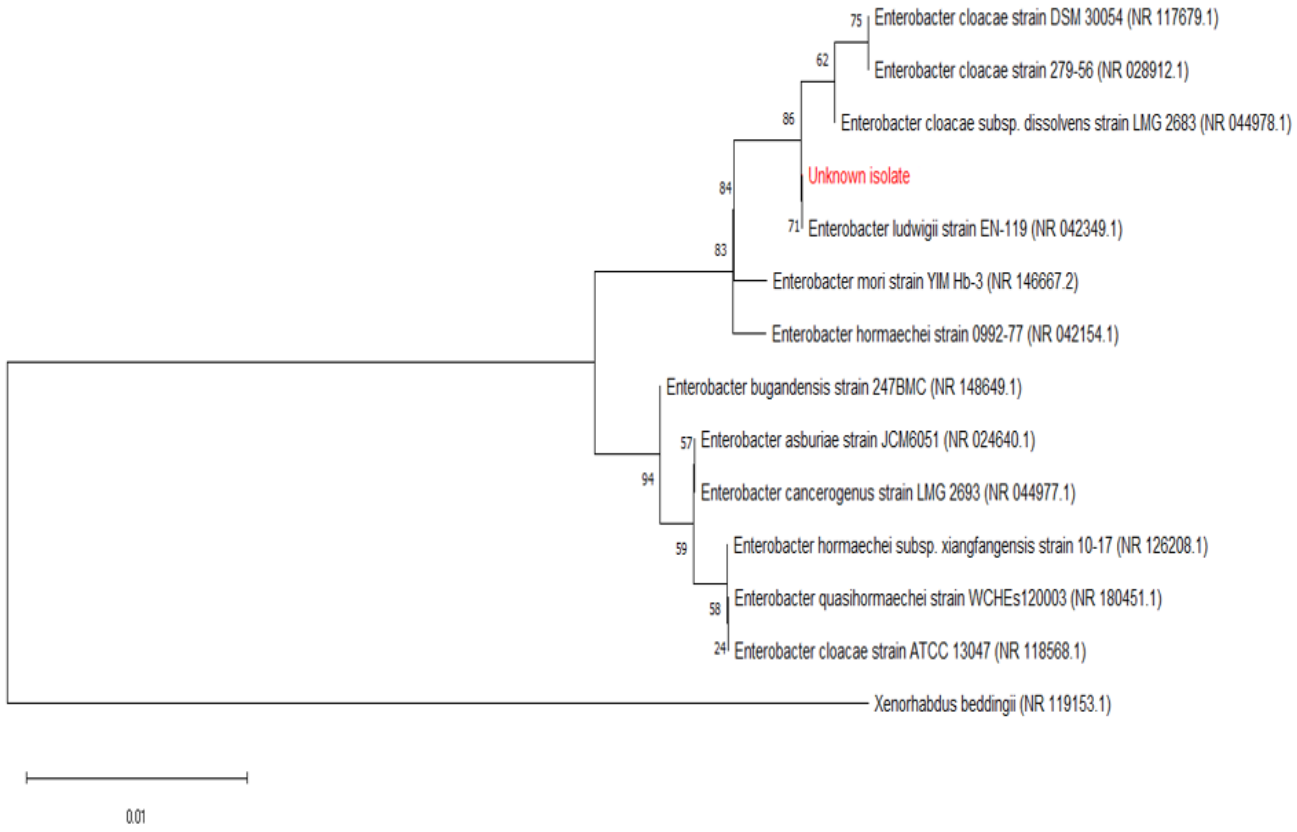

Figure 3.4. Molecular phylogenetic analysis of *Enterobacter* spp. based on the analysis of the 16S rDNA region. The unknown bacterial isolate is in red and *Xenorhabdus beddingii* (NR 119153.1) was used as the outgroup. The NCBI accession numbers are specified in brackets and the numbers adjacent to the tree branches are the bootstrap percentages. The tree was drawn to scale with the branch lengths measuring the number of substitutions per site.

3.3.4. Bacterial pathogenicity assay

The pathogenicity of *Enterobacter ludwigii* strain NTM-2021 to *T. molitor* larvae was determined by performing an intra-haemocoelic injection assay. Firstly, a bacterial standard curve for *Enterobacter ludwigii* strain NTM-2021 was performed, as shown in **Figure 3.5**. Using the obtained line of regression equation, the bacterial CFU for the doses of the 10^{0}-10^{-3} dilutions was determined (as shown in **Table 3.1**) and used for the assay. From **Figure 3.6**, *Enterobacter ludwigii* strain NTM-2021 is shown to be pathogenic to *T. molitor* larvae. Twenty-four hours post-injection, mortality was observed in all bacterial doses; however, insect mortality was <50%. After 48 hours, the insect mortality increased for all bacterial doses; however, a significant increase was observed in the bacterial doses of 1.6 x 10^6 CFU and 4.6 x 10^5 CFU. After 72 hours, the bacterial doses 1.6 x 10^6 CFU, 4.6 x 10^5 CFU, 2.1 x 10^5 CFU, and 1 x 10^5 CFU achieved insect mortality rates of 96.7%, 60%, 30% and 20% respectively. The control treatments in which larvae were injected with nutrient broth yielded 0% insect mortality after 72 hours.

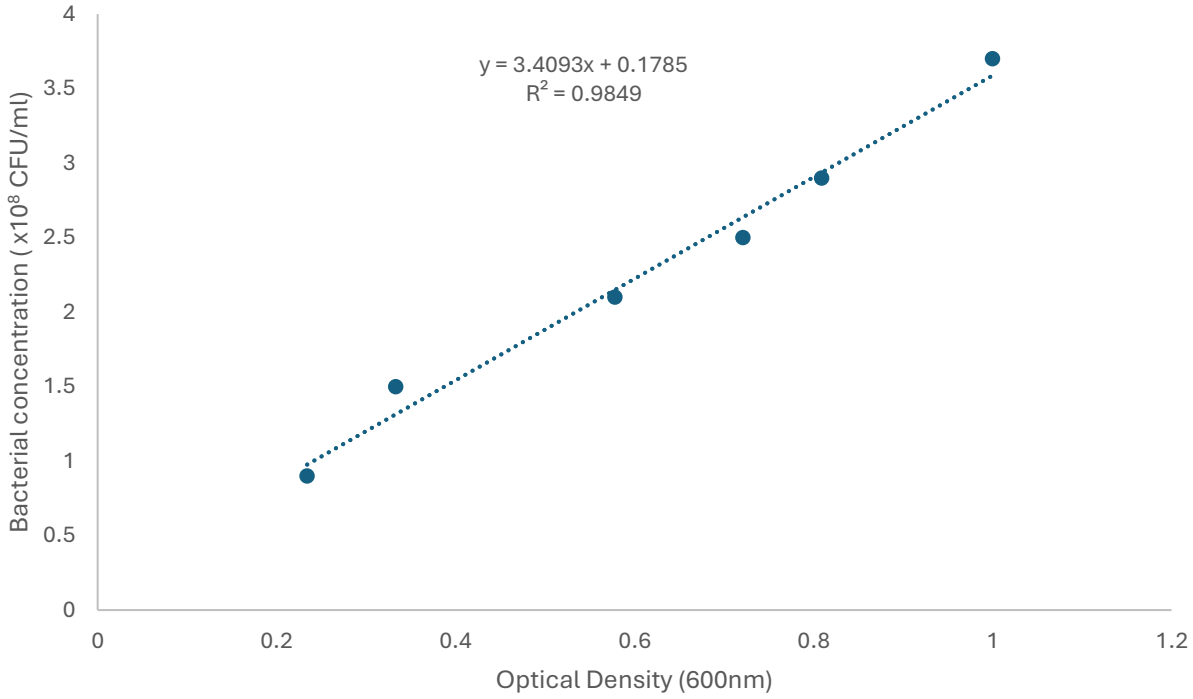

Figure 3.5. Bacterial standard curve. The slope of the linear standard curve is shown and used to approximate the bacterial cell densities shown in **Table 3.1.**

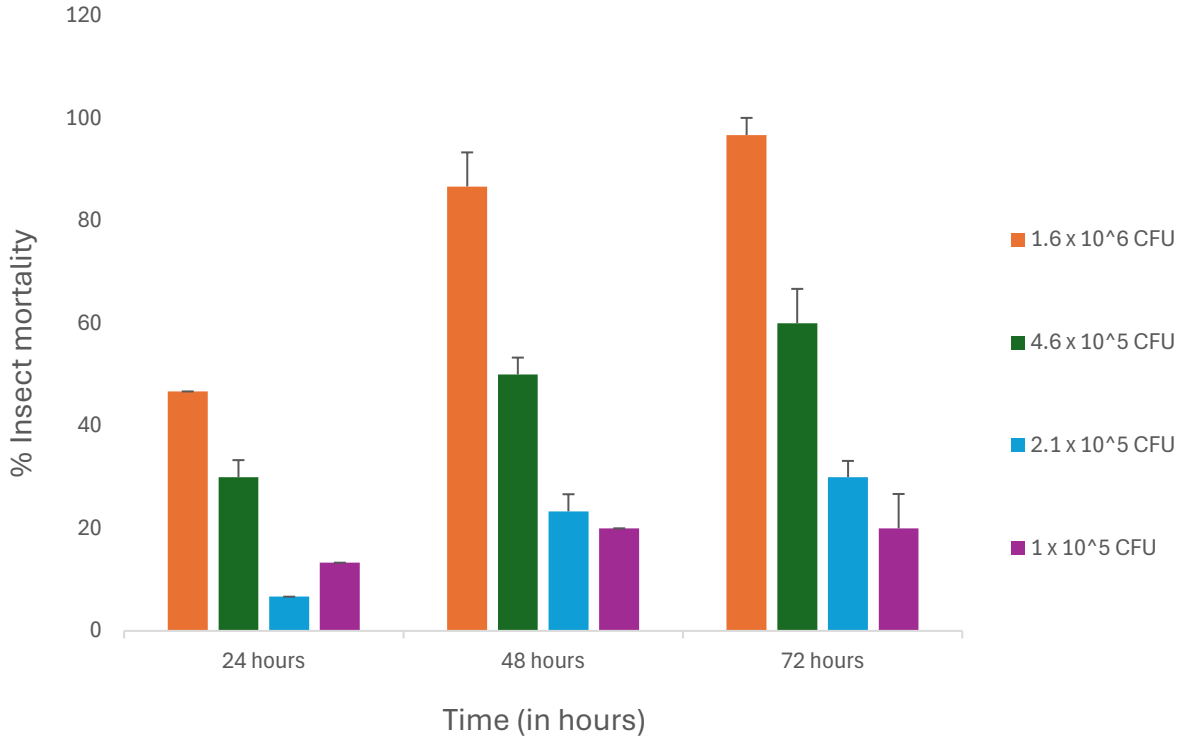

Figure 3.6. Intra-haemocoelic injection assay of *Enterobacter Ludwigii* strain NTM-2021 into *T. molitor* larvae assessed over 72 hours at 25 °C. The overall trend reveals that the administered bacterial doses significantly affect the insect mortality rate ($P<0.05$) based on a Two-way ANOVA (see Appendix IV). The control treatments, where larvae were injected with nutrient broth, resulted in 0% insect mortality after 72 hours. Three replicates were performed in the study, and error bars represent the standard error of the mean (SEM).

3.3.5. Confirmation of dependency using the lipid agar test

Lipid agar was used to determine if there was a symbiotic relationship between the isolated nematode and bacterial species. As shown in **Figure 3.7**, the nematodes reproduced into adults, indicating that they rely on the bacteria for reproduction and development.

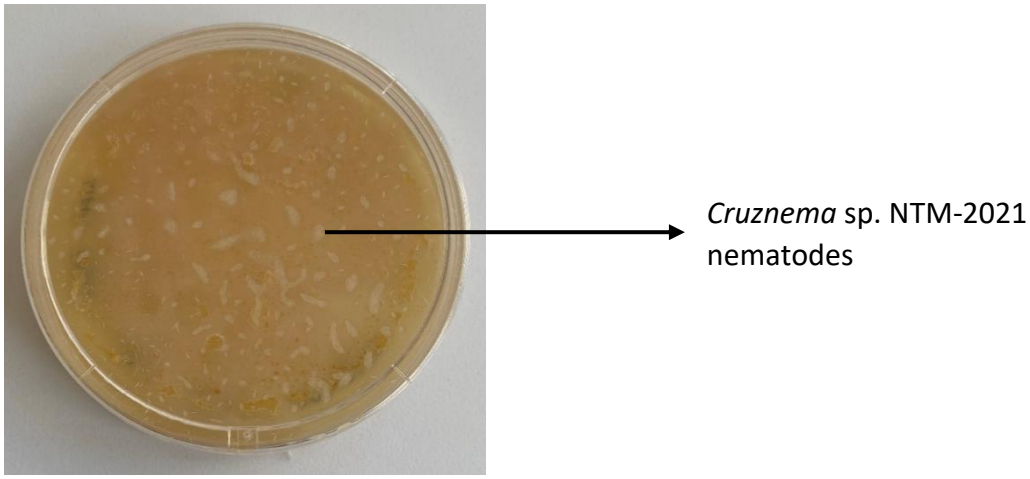

Figure 3.7 Confirmation of dependency using the lipid agar test. *Cruznema* sp. NTM-2021 nematodes developed from IJs and are visible as White clumps on the lid of the Petri dish.

3.4. Discussion

Bacterial species associated with EPNs are Gram-negative and belong to the family Enterobacteriaceae; therefore, this study used selective and differential media such as MacConkey and NBTA media to isolate bacterial species associated with *Cruznema* sp. NTM-2021. Selective culture media contains inhibitory substances that isolate specific bacterial species or genera, while differential culture media differentiates between closely related organisms based on their biochemical properties (Holmes and Jobling, 1996; Bonnet *et al.*, 2019). MacConkey agar was used to isolate gram-negative bacteria capable of fermenting lactose, whereas NBTA was used to isolate gram-negative bacteria that absorb bromothymol blue and reduce triphenyltetrazolium chloride (TTC) (Wanger *et al.*, 2017). On MacConkey agar, as shown in **Figure 3.1A**, *Enterobacter ludwigii* strain NTM-2021 produced pinkish-red colonies by dropping the pH of the media, suggesting that the bacteria can ferment lactose. Whereas, on NBTA media, as shown in **Figure 3.1B**, the colonies produced were dark red, indicating bromothymol blue absorption and TTC reduction. However, a limitation of using culture media such as MacConkey agar and NBTA is that they do not allow for the detection and identification of potential bacterial symbionts that may be viable but non-culturable (VBNC) (Fakruddin *et al*., 2013). Therefore, future studies should use next-generation sequencing (NGS) technologies such as shotgun metagenomics which allows for the sequencing of all genes in all organisms present within a

sample directly using a PCR-independent approach (Quince *et al.*, 2017; Boers *et al*., 2019). The gram stain procedure was performed to identify the isolated bacterial species further. The formation of pink/red colonies, as shown in **Figure 3.2**, suggested that *Enterobacter ludwigii* strain NTM-2021 is a gram-negative rod-shaped bacterium.

Due to its high conservation over time, the 16S rDNA gene was used as a molecular marker for taxonomical classification and differentiation of bacterial species. The NCBI BLAST results revealed that *Enterobacter ludwigii* strain NTM-2021 had the highest affinity to *Enterobacter ludwigii* strain EN-119 (NR 042349.1) with a percentage identity of 99.85%, query coverage of 100% and E value of 0. Additionally, based on the phylogenetic tree shown in **Figure 3.4**, *Enterobacter ludwigii* strain NTM-2021 clustered on the same clade as *Enterobacter ludwigii* strain EN-119 (NR 042349.1) with a bootstrap percentage of 71%. According to Dorris *et al*. (1999), a bootstrap percentage value higher than 65% is considered robust. However, 16S rDNA sequencing is not without its limitations. To begin with, the primers used to amplify the DNA are biased since they bind to regions that are not entirely conserved among all bacteria. Furthermore, the bacteria are mostly identified at the genus level since the 16S rDNA gene of closely related species or strains is highly similar (Gupta *et al.*, 2019). Therefore, whole-genome sequencing is crucial as it provides high resolution between closely related species or strains, by analysing the entire genome rather than just a single gene (Haworth *et al*., 2016).

The results of the intra-haemocoelic injection assay, as shown in **Figure 3.6**, revealed that *Enterobacter ludwigii* strain NTM-2021 is pathogenic to *T. molitor* larvae with different bacterial doses affecting the rate of insect mortality. After 72 hours, the highest bacterial dose (1.6 x 10^6 CFU) caused an insect mortality rate of 96.7%, while the lowest bacterial dose (1 x 10^5 CFU) caused an insect mortality rate of 20%. In a study, Loulou *et al*. (2023) investigated the entomopathogenic potential of bacterial species associated with rhabditid nematodes and revealed that *Enterobacter* sp. E_TC7 was highly entomopathogenic because, at doses of 10^6 CFU, it killed 100% of the *Galleria mellonella* larvae after 72 hours of infection. A study by Mohandkaci *et al*. (2015) also revealed that *Enterobacter ludwigii* strain EN-119 caused mortality (>70%) in

larvae of the migratory locust, *Locusta Migratoria* however, molecular drivers responsible for pathogenicity in this strain have not yet been studied. *Enterobacter ludwigii* strain EN-119 is the type strain of *Enterobacter ludwigii*, belonging to the *Enterobacter cloacae* complex (Ecc), which consists of five other species, namely *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, and *Enterobacter nimipressuralis*. According to several studies, species belonging to the genus *Enterobacter* produce endotoxins that serve as virulence factors of these bacteria. In a study, Liao *et al*. (2017) reported that *Enterobacter cloacae* produces toxin complexes (Tcs) and Makes caterpillars floppy (Mcf) proteins, which are insecticidal proteins. As such, these proteins may be the same virulence determinants found in *Enterobacter ludwigii* strain NTM-2021. Interestingly, similar protein complexes are produced by *Photorhabdus luminescens*, *Xenorhabdus nematophila* and *Serratia entomophila,* which are all bacterial symbionts of EPNs. *Enterobacter ludwigii* strain NTM-2021 has not been reported to be symbiotic with EPNs; however, the genus *Oscheius* is associated with bacterial strains from three classes: (1) beta-proteobacteria (*Alcaligenes* spp.), (2) gamma-proteobacteria (*Enterobacter*, *Proteus*, *Providera*, *Pseudomonas*, *Stenotrophomonas*), and (3) *Bacilli* (*Bacillus*, *Enterococcus*, *Lynsinibacillus).* Interestingly, a study by Liao *et al*. (2017) reported *Enterobacter gergoviae* and *Enterobacter cloacae* as a novel bacterial symbiont of the EPNs *Heterorhabditis* spp. PDSj1 and *Heterorhabditis* spp. PDSj2, respectively. This, therefore, suggests *Enterobacter* as another nematophilic genus. The pathogenicity of *Enterobacter ludwigii* strain NTM-2021 provides new biological control prospects for managing insect pests. Currently, the spore-forming bacteria, *Bacillus thuringiensis* (Bt) is the most used and commercialised biological control agent (Lacey et al., 2015). This is because Bt produces Cry proteins which are toxic to several insect species primarily of the order Lepidoptera (butterflies and moths), Coleoptera (beetles and weevils), and Diptera (flies and mosquitoes); however, there are several reports regarding their toxicity towards hymenopterans (wasps and bees) (Kumar *et al.*, 2021). Based on the application requirements, the Cry proteins produced by the bacteria may be formulated into sprays, suspension concentrates, and water-dispersible powders or granules (Bryant, 1994; Vimala Devi *et al.*, 2020). Therefore, future studies on *Enterobacter ludwigii* strain NTM-2021 should include

genomic and proteomic studies to identify the genes and proteins responsible for pathogenesis to assist in developing formulations that may be used for pest management.

In EPNs, Phase I bacterial symbionts facilitate nematode reproduction and growth. Thus, to determine the dependency of *Cruznema* sp. NTM-2021 on *Enterobacter ludwigii* strain NTM-2021, spread plates of Phase I colonies were prepared on lipid agar. From **Figure 3.7**, the nematodes developed from IJs to adults, confirming dependency and potential symbiosis between *Cruznema* sp. NTM-2021 and *Enterobacter ludwigii* strain NTM-2021. Bacterial symbionts play a crucial role in the fitness of their nematode hosts as they help establish nematode infection within insect hosts, defend the insect host from predators and competitors such as fungi, and promote normal nematode reproduction and development (Murfin *et al.*, 2012). However, based on the previous assays, IJs of *Cruznema* sp. NTM-2021 recovered from dead insects can reinfect insect hosts; however, their virulence decreases in *T. molitor* as a complex compared to when the bacteria is injected alone. This suggests that the nematode vector may be antagonistic to the bacteria or that the bacteria was released inefficiently (Dillman *et al.*, 2012). Therefore, similar to the *Caenorhabditis briggsae* strain and its bacterial symbiont *Serratia* sp. SCBI, *Cruznema* sp. NTM-2021 may range between necromenic and entomopathogenic, that it is a nascent entomopathogen and not yet efficient, or that *T. molitor* is a poor host.

3.5. Conclusion

MacConkey and NBTA media successfully isolated bacterial species associated with *Cruznema* sp. NTM-2021. Using the gram stain procedure, *Enterobacter ludwigii* strain NTM-2021 was identified as a gram-negative rod-shaped bacterium. Using DNA extraction, PCR amplification, Sanger sequencing and phylogenetic analysis of the 16S rDNA region, the unknown bacterial isolate was identified as belonging to the genus *Enterobacter.* The intra-haemocoelic injection assay revealed that *Enterobacter ludwigii* strain NTM-2021 is an insect parasitic strain and that its ability to support the nematode's reproduction and development may indicate a potential symbiotic relationship with *Cruznema* sp. NTM-2021. The identification of a new parasitic

bacterial strain warrants future genomic studies such as whole genome sequencing, and proteomics to determine genes and proteins responsible for pathogenicity. Additionally, formulation and application technology studies would be essential as they would provide storage formulations and application devices for the bacteria which would be appropriate for field trials.

3.6. References

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Chapter 4:

Investigating the foraging behaviour of a *Cruznema* sp. NTM-2021

Abstract

Foraging is an essential behavioural process that includes all the methods by which an organism acquires and utilises sources of energy and nutrients. The foraging behaviour of EPNs differs among species and affects their soil depth distribution and host range. EPNs are classified into a continuum between two endpoints namely cruise and ambush foragers, with many species (intermediate foragers) falling between these endpoints. The assignment of EPNs into foraging groups is primarily based on their response to abiotic and chemotactic cues. As such, the 2D sand substrate assay, vertical sand column assay and Pluronic F-127 (PF127) gel assay were used to determine the foraging behaviour of *Cruznema* sp. NTM-2021 by assessing its response to varying moisture levels as well as prenol as a chemotactic cue. Soil moisture was shown to affect insect mortality in both the 2D sand substrate assay and vertical sand column assay. The vertical sand column assay also revealed that *Cruznema* sp. NTM-2021 was able to move along the soil profile reaching depths of 32 cm. The Pluronic F-127 (PF127) gel assay revealed that *Cruznema* sp. NTM-2021 was strongly repelled by prenol. Based on the distance travelled along the column and its ability to respond to host-associated chemical cues such as prenol, *Cruznema* sp. NTM-2021 may be classified as a cruise forager.

4.1. Introduction

The selection of entomopathogenic nematodes (EPNs) as biological control agents (BCAs) mainly depends on their ability to locate, identify, and invade insect hosts within the soil. EPNs are classified into a continuum between two extremities known as cruise and ambush foragers, with many species (intermediate foragers) falling between these endpoints (Ruan *et al.*, 2018). Variations in the foraging behaviour of EPNs in soil occur naturally from differences observed in the allocation of foraging time to inert scanning and roaming (Campbell and Kaya, 2000). These differences are associated with a range of morphological, physiological, behavioural, ecological, and life-history characteristics, which allow the assignment of EPNs to appropriate foraging

groups. EPNs utilising an ambush foraging behaviour are usually located at or near the soil surface (Lortkipanidze *et al.*, 2016). This is because ambush foragers use an energy-conserving tactic known as the "sit-and-wait" method, where the nematode remains nearly sedentary while waiting to infect mobile hosts such as cutworms, codling moths, mole crickets, etc. Ambush foragers also exhibit action mechanisms, such as nictation (standing) and jumping behaviours, to increase their chances of encountering an insect host (Lewis *et al*., 1992). During nictation, nematodes reduce the surface tension forces holding the nematode to the surface through the elevation of the anterior portion of the body and waving it to enable attachment to a passing insect (Koppenhöfer *et al*., 2020). However, the jumping behaviour involves the nematodes standing on their tail and curling their bodies into a loop before propelling themselves towards an insect host with their entire bodies stretched (**Figure 4.1**). Typically, these jumps can reach a height of seven body lengths and function as a dispersal mechanism (Hallem *et al.*, 2011). Ambush foragers include *Steinernema* spp. such as *S. carpocapsae,* and *S. siamkayai* (Campbell *et al*., 2003; Campbell and Gaugler, 1997).

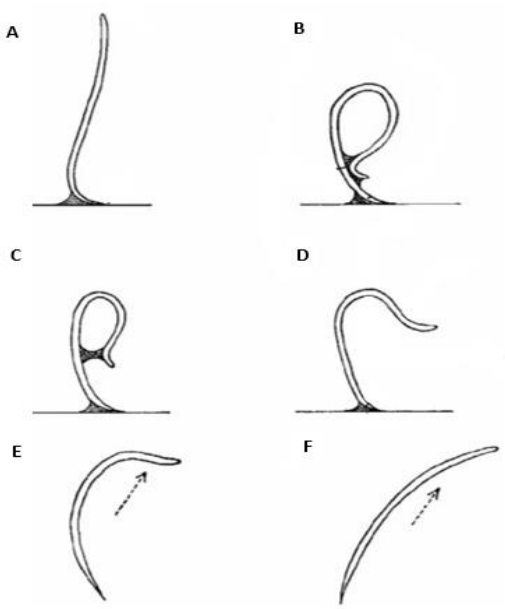

Figure 4.1. Jumping behaviour observed in ambush foraging nematodes (Image adapted from Frazer (2015)).

EPNs utilising a cruise foraging behaviour infect sedentary hosts such as scarab and lepidopterous pupae and hosts located deep below the ground or in cryptic habitats (Zhang *et al.*, 2021). Cruise foraging species rarely demonstrate standing behaviours and use most of their foraging time to crawl in soil or on substrates. Because host-seeking is more energetically expensive, cruise foragers store more lipids and tend to be larger and longer in size (>801 µl) (Campbell and Kaya, 2000). EPNs that exhibit this behaviour include all *Heterorhabditis* spp. and some *Steinernema* spp., such as *S. glaseri* and *S. kraussei* (Campbell and Gaugler, 1997). The intermediate foragers are species of EPNs that use behavioural characteristics common to both cruise and ambush foragers to locate insect hosts (Griffin, 2012). Intermediate foragers mainly parasitise insects occurring just beneath the soil, such as pupae of lepidopterous insects and weevil larvae and include species such as *S. riobravis* and *S. feltiae* (Kaspi *et al.*, 2010).

To locate susceptible insect hosts within the soil, cruise foragers respond to host volatile and contact cues, vibrations, water-soluble cues, and cues from insect-damaged plants (Lewis *et al.*, 2006). Short-range cues provide specific information regarding the host and include host contact cues such as the cuticle, carbon dioxide, and faeces. In contrast, long-range cues provide directional information for the host location and include volatile cues (Kaya and Campbell, 2002). Without host-associated cues, cruise foragers move using a linear movement pattern. Initially, it was thought that volatile cues did not affect ambush foragers. However, recent evidence has shown that ambush foragers respond to chemical cues, although their behavioural responses vary from those of cruise foragers and are context-specific (Lewis *et al.*, 2006). For example, when crawling, ambush foragers like *S. carpocapsae* are not attracted to host volatiles without prior contact with an insect host. However, when presented with host-associated cues during standing bouts, they wave backwards and forward and jump towards the source of the cues (volatile chemical cues and air movement (a mechanosensory cue)) (Bal and Grewal, 2015). Host volatiles are attractive to most intermediate foragers; however, since most species utilising an intermediate foraging behaviour also exhibit standing and jumping behaviours, they may show varying responses when host cues are introduced (Campbell *et al.*, 2003). In addition to host-associated cues, abiotic factors such as soil moisture affect the foraging behaviour of EPNs (le

Vieux and Malan, 2015). This is because, in soil, EPNs move through thin films of water coating the interstitial spaces; therefore, adequate soil moisture levels are essential for their survival and success as BCAs (Koppenhöfer *et al*., 2020). In dry conditions, the foraging behaviour of EPNs is limited; however, if moisture loss is gradual, EPNs may enter partial anhydrobiosis, which is a state of dormancy and persist. Additionally, soil texture is another factor affecting the movement and survival of EPNs (Rufai *et al*., 2020). In fine-textured soils, the movement of EPNs is more restricted than in sandy soils. However, sandy soils might dry up faster, reducing the activity of EPNs.

Therefore, this study aimed to investigate the foraging behaviour of *Cruznema* sp. NTM-2021 by exposing it to chemical cues and abiotic factors known to affect foraging. This is crucial because it enables a more accurate assignment of the nematode into a foraging group, which is essential for nematode-to-host matching. Additionally, information regarding the foraging behaviour of nematodes is also helpful in developing and improving formulation strategies and application technologies.

4.2. Materials and Methods

4.2.1. 2D Sand substrate assay

The 2D sand substrate assay was used to determine the foraging behaviour of *Cruznema* sp. NTM-2021 based on its response to varying soil moisture levels. This is because 2D substrates allow nictation and jumping; therefore, they may be used to determine if *Cruznema* sp. NTM-2021 is an ambush forager. A total of 15 Petri dishes containing 40 g of sterile sandy loam soil were prepared. The plates were divided into five groups (3 plates per group), with the control group adjusted to a moisture level of 10%, while the experimental groups consisted of 12.5%, 15%, 17.5% and 20% (see Appendix III). In each plate, 1000 IJs suspended in 250 µl of distilled water were inoculated in the centre before adding 10 *T. molitor* larvae. The plates were wrapped using parafilm and incubated at 25 °C. Insect mortality was recorded daily over 7 days, and insect cadavers were collected and transferred onto White traps to confirm nematodes as the cause of

infection. Three replicates were performed in the study, and a Two-way ANOVA with replication was used to analyse the results.

4.2.2. Vertical sand columns

The vertical sand columns were used to determine the foraging behaviour of *Cruznema* sp. NTM-2021 based on its response to varying soil moisture levels. This is because vertical sand columns allow the vertical movement of the IJs within the sand column and may be used to determine if *Cruznema* sp. NTM-2021 is an intermediate or cruise forager. A total of 15 vertical columns containing 650 g of sterile sandy loam soil were prepared. The columns were divided into five groups (3 columns per group), with the control group adjusted to a moisture level of 10%, while the experimental groups consisted of 12.5%, 15%, 17.5% and 20% (see Appendix III). Each column had 6 arenas at varying depths along the column, as shown in **Figure 4.2**. In each arena, a thin strip of filter paper was inserted and inoculated with water before a single *T. molitor* larvae was inserted. This was done to ensure that the nematodes had a film of water to enable movement toward the larvae. Each arena was then sealed at the ends using mounting putty (Prestik). In each column, 1000 IJs suspended in 250 µl of distilled water were inoculated onto the centre surface of the soil before loosely closing the columns with suitable lids to facilitate some level of aeration while preventing a significant loss of humidity and avoiding the suffocation of the IJs. The columns were stored at 25 °C, and mortality was recorded daily over 7 days. Insect cadavers were collected and placed on White traps to confirm nematodes as the cause of infection. Three replicates were performed in the study, and a Two-way ANOVA with replication was used to analyse the results.

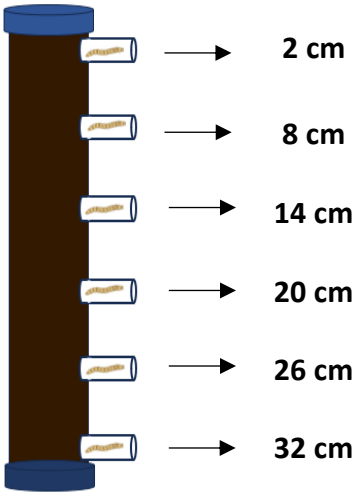

Figure 4.2. The column design for the vertical sand column assay.

4.2.3. Chemotaxis assay

To investigate the effects of host volatile cues on the foraging behaviour of *Cruznema* sp. NTM-2021, a chemotaxis assay was performed using Pluronic F-127 (PF127) gel. This is because PF127 gel is a non-toxic thermoreversible hydrogel which provides a 3D matrix in which the nematodes may experience similar tactile stimulation as observed while moving through the soil. Additionally, since the media is transparent, it allows real-time observation of the IJs response to the applied chemical stimuli. A 23% (w/v) PF127 gel was prepared and allowed to mix at 4 ℃ overnight. A total of 12 Petri dishes were set aside and 20 ml of the gel was poured into each plate and allowed to solidify at room temperature. The plates were divided into 4 groups (3 plates per group) for the prenol doses 2 M, 0.2 M, 0.02 M and 0.002 M (see Appendix III). In each plate, two opposing quadrants were left blank, and the remaining two quadrants were the control and test quadrants (as shown in **Figure 4.3**). The control quadrant was inoculated with 5 µl of 100% ethanol (diluent and control), while the test quadrant was inoculated with 5 µl of prenol (diluted in 100% ethanol to the different concentrations). In each plate, 100 IJs suspended in 25 µl of distilled water were inoculated beneath the gel's surface. The experiment was allowed to run for 24 hours, and the plates were scored using a dissecting microscope. IJs in the test and control quadrants were counted and used to determine the chemotaxis index (CI) (Baiocchi *et al*., 2020).

Three replicates were performed in the study, and a one-way ANOVA was used to analyse the results.

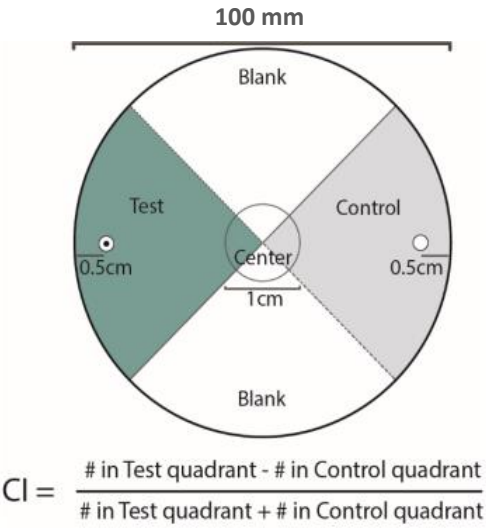

Figure 4.3. Assay arenas of the prenol chemotaxis assay on PF127 gel.

4.3. Results

The 2D sand substrate assays in **Figure 4.4** revealed that the infectivity of the IJs of *Cruznema* sp. NTM-2021 were affected by different soil moisture levels ($p<0.05$) based on a Two-way ANOVA (see Appendix IV). At 10% soil moisture levels, no insect mortality was observed over 7 days. However, soil moisture levels of 12.5%, 15%, 17.5%, and 20% showed insect mortality of 23.3%, 40%, 46.7%, and 66.7%, respectively. The vertical sand columns in **Figure 4.5** revealed that the IJs of *Cruznema* sp. NTM-2021 could travel to different depths (0-32 cm) along the column to cause insect mortality. However, from the results, it is evident that the soil moisture level significantly affects the movement and, therefore, the infectivity of the IJs ($p<0.05$) based on a Two-way ANOVA (see Appendix IV). At 10% soil moisture levels, the IJs travelled to a depth of 8 cm and caused <12% insect mortality at this depth. However, at soil moisture levels ranging between 15-17.5%, IJs of *Cruznema* sp. NTM-2021 reached depths of 32 cm, causing >85% insect mortality at all depths along the column. Interestingly, at 20% soil moisture level, IJs reached lengths of 26 cm; however, they could only parasitise <15% of the insect larvae. The dose-

response assay conducted using PF127 gel in **Figure 4.6** revealed that the IJs of *Cruznema* sp. NTM-2021 were repelled by high concentrations of prenol, as shown by the chemotaxis score of -0.80 at 2 M. However, as the concentration decreased, the IJs were attracted to the chemical stimuli such that at 0.002 M, the chemotaxis score was 0.18.

4.3.1. The effects of soil moisture on foraging behaviour using 2D Sand substrates.

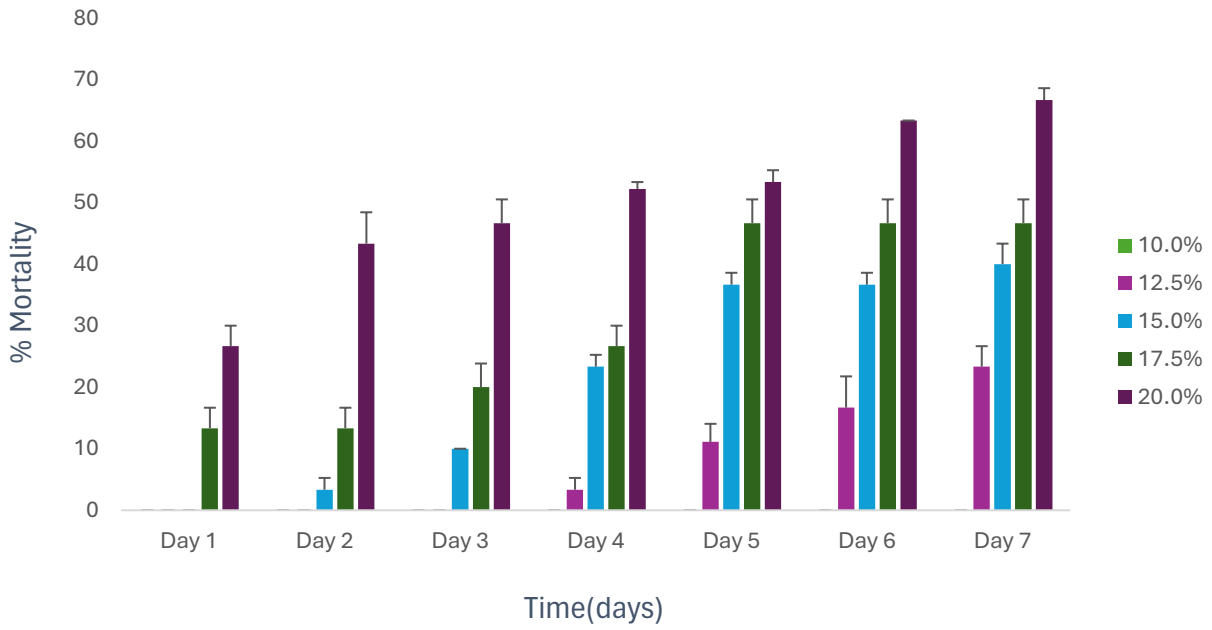

Figure 4.4. Average cumulative percentage mortality of *T. molitor* observed on 2D sand substrates in response to varying moisture levels (10%, 12,5%, 15%,17.5%, 20%). The overall trend reveals that an increase in soil moisture had a significant effect on the infectivity of *Cruznema* sp. NTM-2021 on 2D sand substates ($p<0.05$) based on a Two-way ANOVA (see Appendix IV). Insect mortality was recorded over 7 days at 25 °C. The error bars represent the standard error of the mean (SEM).

4.3.2. The effects of soil moisture on foraging behaviour using vertical sand columns

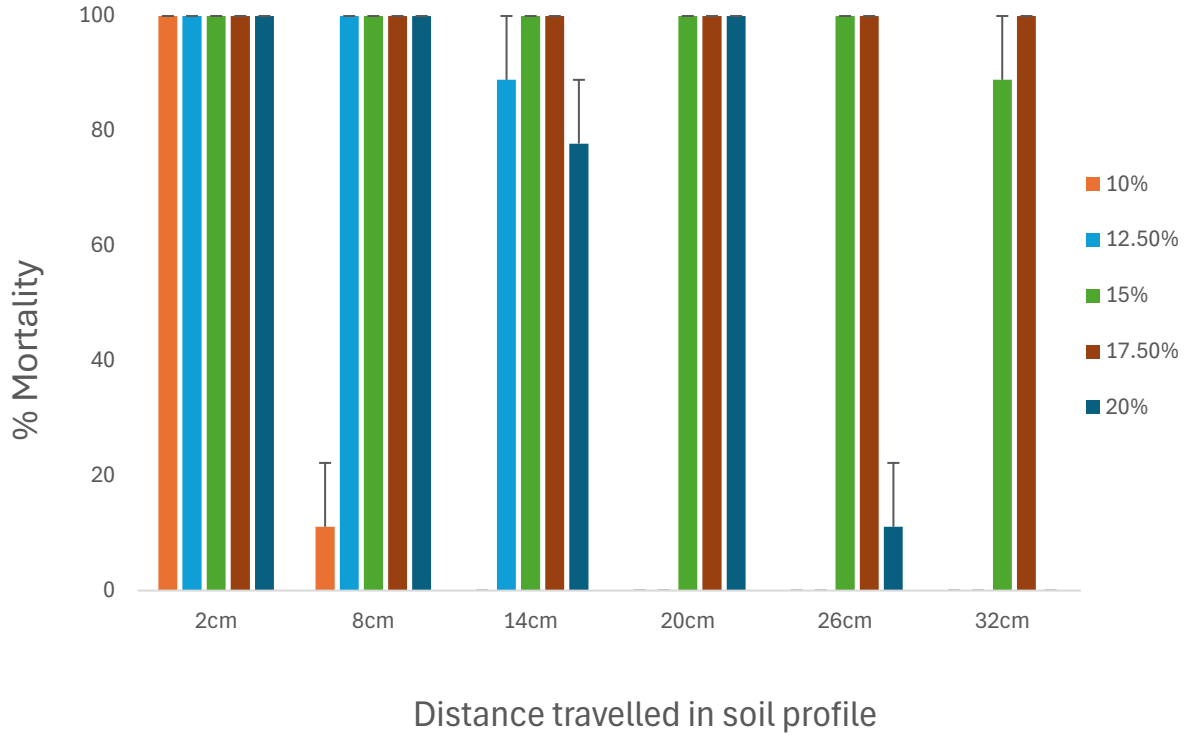

Figure 4.5. Average cumulative percentage mortality of *T. molitor* observed at different distances (2 cm, 8 cm, 14 cm, 20 cm, 26 cm, 32 cm) along vertical sand columns in response to different moisture levels (10%, 12,5%, 15%,17.5%, 20%). An increase in soil moisture had a significant effect on the movement and infectivity of *Cruznema* sp. NTM-2021 on vertical sand columns ($p<0.05$) based on a Two-way ANOVA (see Appendix IV). Insect mortality was observed over 7 days at 25 °C. The error bars represent the SEM.

4.3.3. Prenol chemotaxis assay using PF127 gel

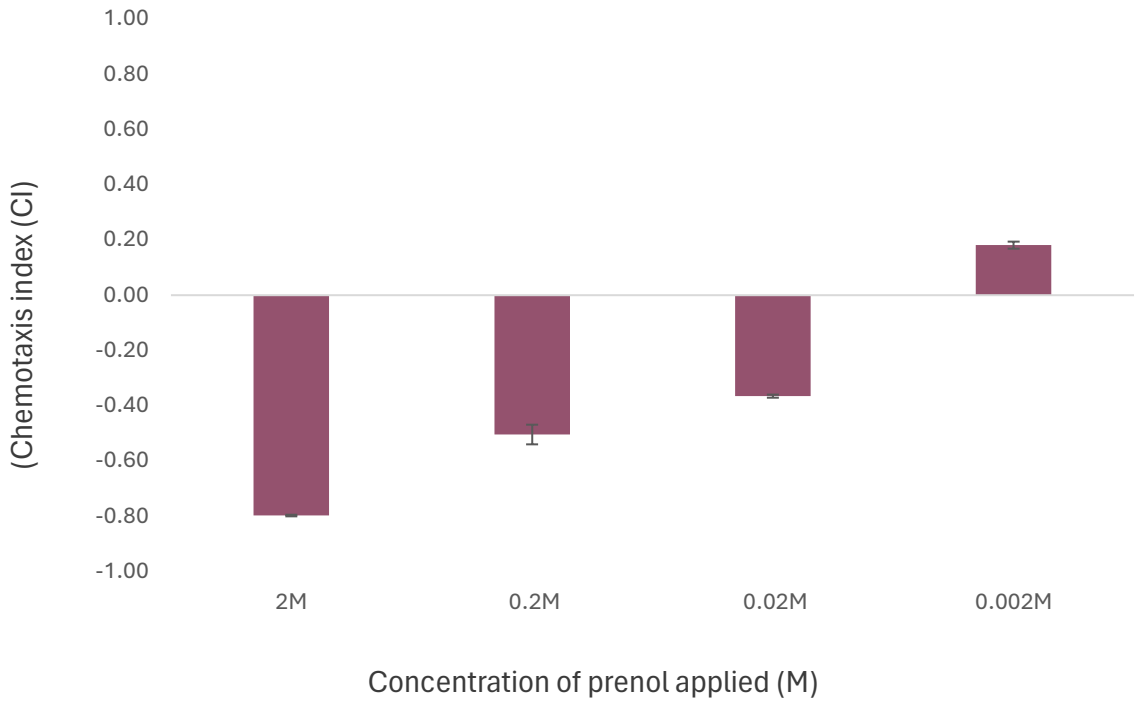

Figure 4.6. The response of *Cruznema* sp. NTM-2021 to different doses of prenol on PF127 media. A chemotaxis index score close to -1 suggests high repulsion, near 0 suggests neutrality, and +1 suggests strong attraction. The overall trend reveals that high doses of prenol strongly repelled the IJs whereas at low doses were attractive ($P<0.05$) based on a One-way ANOVA (see Appendix IV). Chemotaxis was recorded after 24 hours at 25 °C. The error bars represent the SEM.

4.4. Discussion

The foraging behaviour of nematodes exists along a continuum ranging between two endpoints, namely ambush and cruise foragers (Bal and Grewal, 2015). However, some nematode species exhibit a combination of these behaviours and are considered intermediate foragers. The foraging behaviour employed by a nematode species significantly influences their soil depth distribution and host preference. As such, foraging behaviour is crucial for selecting nematodes as BCAs (Yadav and Lalramliana, 2012). This study used 2D sand substrate assays to investigate whether the IJs of *Cruznema* sp. NTM-2021 utilise an ambush foraging behaviour. This is because 2D sand substrates provide a matrix that supports nictation and standing bouts, which are common behaviours observed in ambush foragers during host-seeking. From **Figure 4.4**, the IJs of *Cruznema* sp. NTM-2021 are shown to parasitise *T. molitor* larvae; however, the soil moisture level significantly influences the mortality rate. At a 10% soil moisture level (control), no insect mortality was observed over 7 days. In a study, Yadav and Lalramliana (2011) observed that *Heterorhabditis indica*, *Steinernema thermophilum* and *Steinernema glaseri* could induce insect mortality at moisture levels as low as 6%. However, nematodes differ in their ecological and behavioural traits regarding persistence and survival. Based on this, moisture levels of 10% and below may have caused nematode death, immobility, or the induction of dormancy in the IJs of *Cruznema* sp. NTM-2021, resulting in no infection of insect larvae. Contrastingly, after 7 days, the observed insect mortality in 12.5%, 15%, 17.5% and 20% soil moisture levels were 23.3%, 40% and 46.7% and 66.7%, respectively. These findings are similar to those of Grant and Villani (2003), who reported that nematode virulence increased with increases in soil moisture.

Although the IJs of *Cruznema* sp. NTM-2021 caused insect mortality on 2D sand substrates; this is not conclusive for the determination of the nematode's foraging behaviour. Therefore, vertical sand column assays were performed under the same conditions to eliminate biases and investigate whether the IJs of *Cruznema* sp. NTM-2021 utilise a cruise foraging behaviour. This is because vertical sand columns allow nematodes to move vertically within the soil in search of susceptible insect hosts. **Figure 4.5** shows that the distance travelled by the IJs of *Cruznema* sp. NTM-2021 within the sand column was dependent on the soil's level of saturation. At 10% soil

moisture levels, the IJs of *Cruznema* sp. NTM-2021 travelled to 2 cm and 8 cm soil depths, resulting in 100% and 11% insect mortality, respectively. These results are similar to those of Paunikar and Kulkarni (2020), who revealed that at a soil moisture level of 10%, IJs of *Steinernema dharanaii* could easily move to a distance of 2 cm, resulting in 100% insect mortality; however, as the distance travelled increased to 6 cm, insect mortality declined to 91% (Paunikar and Kulkarni, 2020). It is suggested that the observed decline occurs due to the restricted movement of the IJs due to insufficient moisture within the soil (Blatt and Barry, 2020). At 12.5% soil moisture levels, the IJs of *Cruznema* sp. NTM-2021 travelled to soil depths of 2 cm, 8 cm and 14 cm, resulting in 100%, 100% and 89% insect mortality, respectively. These results are similar to those of Blatt and Barry (2020), who revealed that the infectivity of *Heterorhabditis bacteriophora* was reduced (< 10%) when soil moisture levels were low (6 or 8%), and larvae were buried at depths of 5 or 7 cm however increases in soil moisture (15%) resulted in increased infectivity (> 80%) and migration in the column. At 15% soil moisture levels, IJs of *Cruznema* sp. NTM-2021 caused 100% insect mortality at all depths apart from 32 cm, where 89% insect mortality was observed. At 17.5% soil moisture levels, IJs of *Cruznema* sp. NTM-2021 caused 100% insect mortality at every distance. The high insect mortality observed at 15% and 17.5% may represent the optimum soil moisture of the IJs required for larval suppression (Shapiro-Ilan *et al.*, 2006). Interestingly, at 20% soil moisture levels, the IJs of *Cruznema* sp. NTM-2021 travelled to a soil depth of 26 cm; however, insect mortality in this region was ~11%. These results are similar to those of Koppenhofer *et al*. (1995), who observed that insect mortality resulting from nematode infection was very low or absent in high moisture (soil moisture levels > 19). This is because the reduced surface tension and anoxic environment in water-saturated soil may restrict movement and affect the survival of nematodes. Since IJs of *Cruznema* sp. NTM-2021 can reach soil depths of 32 cm; the nematode may be classified as a cruise forager. This classification is based on a study by Lortkipanidze *et al*. (2016), which revealed that *Heterorhabditis bacteriophora* could travel 35 cm vertically and, therefore, was classified as a cruise forager. Additionally, a study by Yamanaka *et al.* (1995) revealed that the cruise forager, *Steinernema glaseri,* reached depths of 30 cm in vertical sand columns while the ambush foragers, such as *Steinernema carpocapsae and Steinernema kushidai,* only reached depths ranging between 5-10

cm. Although vertical sand columns under varying moisture levels allow for the classification of *Cruznema* sp. NTM-2021 into a foraging group, other abiotic factors such as temperature could drastically affect their foraging behaviour during field applications and ultimately their use as biological control agents. Temperature is an environmental factor of great biological significance that influences the infectivity, virulence and reproductive ability of EPNs (Chen *et al.*, 2003). Different species of EPNs have been shown to possess different optimum temperatures for survival and infectivity which is mainly dependent on species type and the native habitat (Lacey and Georgis, 2012). Temperatures above 40˚C and below 0˚C are usually considered lethal to EPNs while temperatures ranging from 15-25˚C increase the rate of metabolism and shorten the life span of EPNs which reduces their efficacy (Susurluk and Ehlers, 2007). Heat-tolerant EPNs can maintain efficacy at temperatures of 29˚C and above and include *H. indica,* and *S. glaseri* whereas, cold tolerant EPNs such as *H. megidis* and *S. feltiae* are effective at temperatures of 15˚C and below (Shapiro-Ilan *et al.*, 2006). Therefore, future studies on how temperature affects *Cruznema* sp. NTM-2021 would allow the determination of the optimum temperature of the nematode while also providing information on the possible behaviour of the nematode during field applications. Additionally, in soils, EPNs are exposed to a variety of soil organisms, such as fungi, bacteria, and mites which may be beneficial, antagonistic or predacious (Shapiro-Ilan *et al.*, 2006). Therefore, investigating the interactions of *Cruznema* sp. NTM-2021 with other microorganisms is essential as it may reveal nematode fitness which is important for its success as biocontrol agents.

Another distinguishing characteristic between ambush, intermediate and cruise foragers is their ability to respond to chemical cues (Moore and Lepper, 1997). Therefore, to further confirm the cruise foraging behaviour, a chemotaxis assay using PF127 was used to investigate the use of prenol as a distal chemotactic cue. Prenol is a naturally occurring alcohol associated with resource-depleted insect cadavers infected by EPNs. As seen in **Figure 4.6**, the IJs of *Cruznema* sp. NTM-2021 are strongly repelled by prenol, which suggests that they can effectively detect odours released by nematode-infected cadavers using olfaction only. However, as the dose of prenol decreased, the IJs showed less sensitivity for the stimuli with chemotaxis indices of -0.80,

-0.50, -0.37 and 0.18 observed at 2 M, 0.2 M, 0.02 M, and 0.002 M, respectively. These results are similar to those by Kin *et al.* (2019), who reported that the sensitivity of *Heterorhabditis indica*, *Steinernema glaseri*, *Steinernema feltiae*, *Steinernema carpocapsae* and *Steinernema riobrave* to prenol reduced as the dose decreased. Therefore, the strong repulsion of IJs of *Cruznema* sp. NTM-2021 further supports the cruise foraging behaviour and the use of prenol as an information cue by informing the infection of a potential host by IJs and triggering the dispersal of IJs from a resource-depleted host. Interestingly, a study by Baiocchi *et al*. (2017) observed that the free-living nematode species *Caenorhabditis elegans* and *Levipalatum texanum* were highly attracted to prenol. Given that prenol is also associated with a range of plant materials, including various fruits, it is suggested that these species may find prenol attractive as a food cue, which reveals the potential ecological significance of prenol to other organisms. One limitation of the PF127 gel assay is that temperatures between 20-25°C cause the media to transition from liquid to semi-solid; therefore, the addition of the nematodes should be before the gel solidifies (at temperatures < 20°C) to allow their suspension into the matrix as well as 3D movement and host-seeking following solidification (Baiocchi *et al*., 2020). As such, temperature can affect the IJs behaviour, and result in behavioural changes in some cases (i.e., from attractive to repulsive or vice versa) (Lee *et al*., 2016). Therefore, several other host-related odorants such as butanoic acid, hexane, hexanol and isoprenol may be used to eliminate bias and further confirm foraging behaviour of the isolated nematode.

4.5. Conclusion

Cruznema sp. NTM-2021 may be classified as a cruise forager based on its ability to parasitise insects located at depths of 32 cm and its ability to respond to host-associated chemical cues. Additionally, in 2D sand substrate assays, soil moisture affects the foraging behaviour of the nematodes such that low soil moisture results in the absence or reduction of insect mortality, while high soil moisture results in increased insect mortality. In vertical sand columns, low soil moisture also results in the absence or reduction of insect mortality due to the inability of nematodes to disperse. However, increasing moisture increases insect mortality as nematodes

can move through water, coating the interstitial space. In water-saturated soils, however, nematodes may be unable to move freely due to oversaturation and oxygen depletion. The nematodes experienced strong repulsion to prenol, suggesting its use as a distal chemotactic cue. The exposure of *Cruznema* sp. NTM-2021 to varying biotic, abiotic, and chemotactic cues will further confirm the observed cruise foraging behaviour therefore suggesting that during field trials the nematode may strictly be used for the control of stationary hosts including those located deep within the soil or other cryptic habitats.

4.6. References

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Appendix I

0.1% JIK solution for surface sterilisation of IJs

1.4 ml 3.5% JIK

48.6 ml distilled water

1. Combine JIK and distilled water in 100 ml Schott bottles and autoclave for 15 minutes at 121 °C.

MacConkey agar (commercially available)

Composition (g/l)

20 g Peptone

10 g Lactose

1.5 g Bile Salts

5 g Sodium Chloride

0.03 g Neutral Red

0.0001 g Crystal Violet

13.5 g Agar

1. Suspend 52 g of MacConkey agar powder into 1000 ml of distilled water.
2. Autoclave the mixture for 15 minutes at 121 °C.
3. Cool the mixture and pour aseptically into clean Petri dishes, and set aside to solidify.

NBTA

1 L nutrient agar

0.04 g triphenyltetrazolium chloride (TTC)

0.025 g bromothymol blue (BTB)

1. Suspend 28 g of nutrient agar powder and BTB into 1000 ml of distilled.
2. Autoclave the mixture for 15 minutes at 121 °C.

3. Wait for it to cool down before adding TTC and mix by swirling.

4. Pour media aseptically into clean Petri dishes and set aside to solidify.

Nutrient broth (commercially available)

Composition (g/l)

1 g Meat extract

2 g Yeast extract

5 g Peptone

8 g Sodium chloride

1. Suspend 23 g of nutrient broth powder into 1000 ml of distilled water.

2. Autoclave the mixture at 121˚C for 15 minutes.

Lipid agar media (adapted from Kaya and Stock, 1997

10 g of honey was used instead of corn syrup

5 g yeast extract

25 g nutrient agar

2.5 ml cod liver oil

2g $MgCl_2.6H_2O$

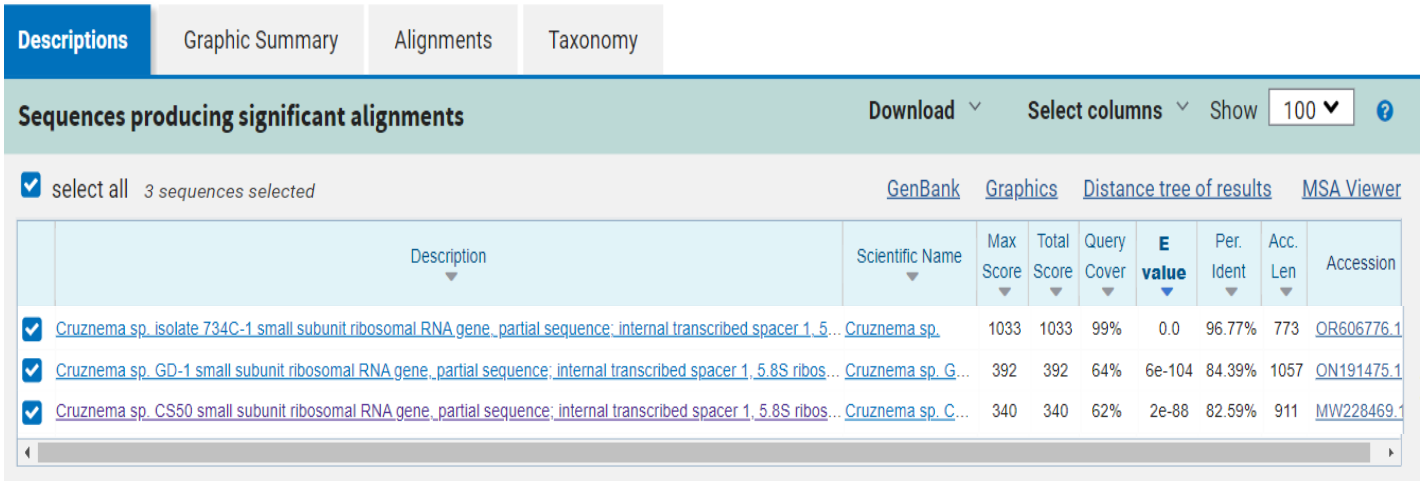
1 litre distilled H_2O

1. Prepare the mixture and autoclave at 121 °C for 20 minutes.

2. Cool the mixture and pour aseptically into clean Petri dishes, and set aside to solidify.

Appendix II

NCBI BLAST Analysis



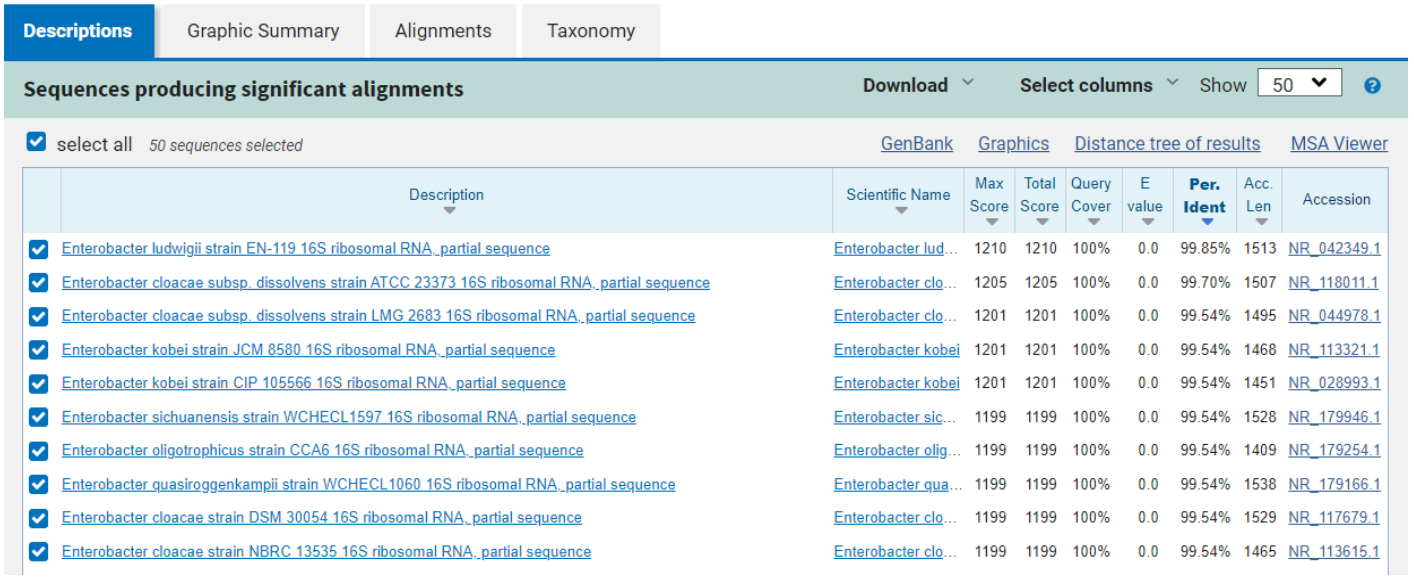
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 3 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cruznema sp. isolate 734C-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5...	Cruznema sp.	1033	1033	99%	0.0	96.77%	773	OR606776.1
Cruznema sp. GD-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos...	Cruznema sp. G...	392	392	64%	6e-104	84.39%	1057	ON191475.1
Cruznema sp. CS50 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribos...	Cruznema sp. C...	340	340	62%	2e-88	82.59%	911	MW228469.1

Figure A. NCBI BLAST analysis of the unknown nematode isolate.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 50

select all 50 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Enterobacter ludwigii strain EN-119 16S ribosomal RNA, partial sequence	Enterobacter lud...	1210	1210	100%	0.0	99.85%	1513	NR_042349.1
Enterobacter cloacae subsp. dissolvens strain ATCC 23373 16S ribosomal RNA, partial sequence	Enterobacter clo...	1205	1205	100%	0.0	99.70%	1507	NR_118011.1
Enterobacter cloacae subsp. dissolvens strain LMG 2683 16S ribosomal RNA, partial sequence	Enterobacter clo...	1201	1201	100%	0.0	99.54%	1495	NR_044978.1
Enterobacter kobei strain JCM 8580 16S ribosomal RNA, partial sequence	Enterobacter kobei	1201	1201	100%	0.0	99.54%	1468	NR_113321.1
Enterobacter kobei strain CIP 105566 16S ribosomal RNA, partial sequence	Enterobacter kobei	1201	1201	100%	0.0	99.54%	1451	NR_028993.1
Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA, partial sequence	Enterobacter sic...	1199	1199	100%	0.0	99.54%	1528	NR_179946.1
Enterobacter oligotrophicus strain CCA6 16S ribosomal RNA, partial sequence	Enterobacter olig...	1199	1199	100%	0.0	99.54%	1409	NR_179254.1
Enterobacter quasiroggenkampii strain WCHECL1060 16S ribosomal RNA, partial sequence	Enterobacter qua...	1199	1199	100%	0.0	99.54%	1538	NR_179166.1
Enterobacter cloacae strain DSM 30054 16S ribosomal RNA, partial sequence	Enterobacter clo...	1199	1199	100%	0.0	99.54%	1529	NR_117679.1
Enterobacter cloacae strain NBRC 13535 16S ribosomal RNA, partial sequence	Enterobacter clo...	1199	1199	100%	0.0	99.54%	1465	NR_113615.1

Figure B. NCBI BLAST analysis of the unknown bacterial isolate.

Appendix III

Foraging Behaviour experiments

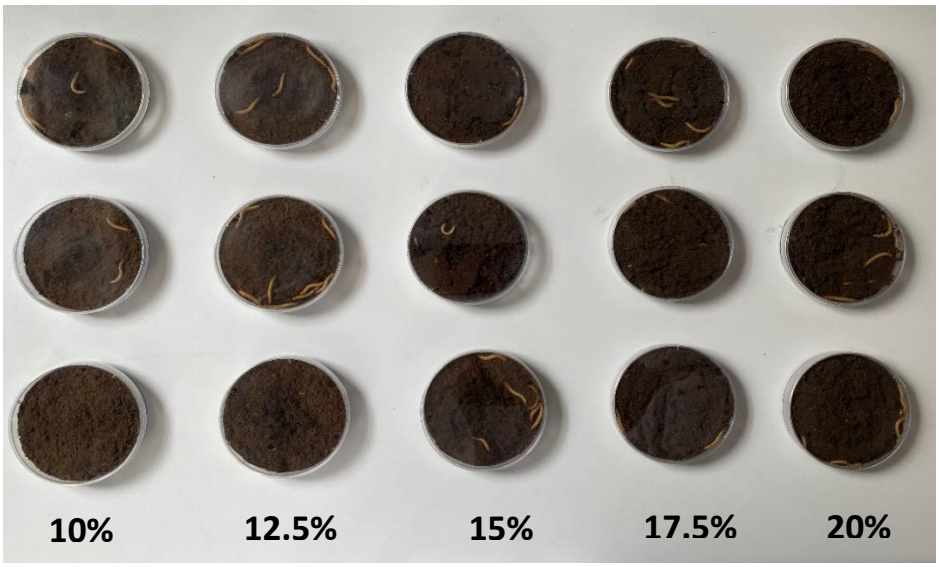


Figure C. 2D sand substrate experimental setup.

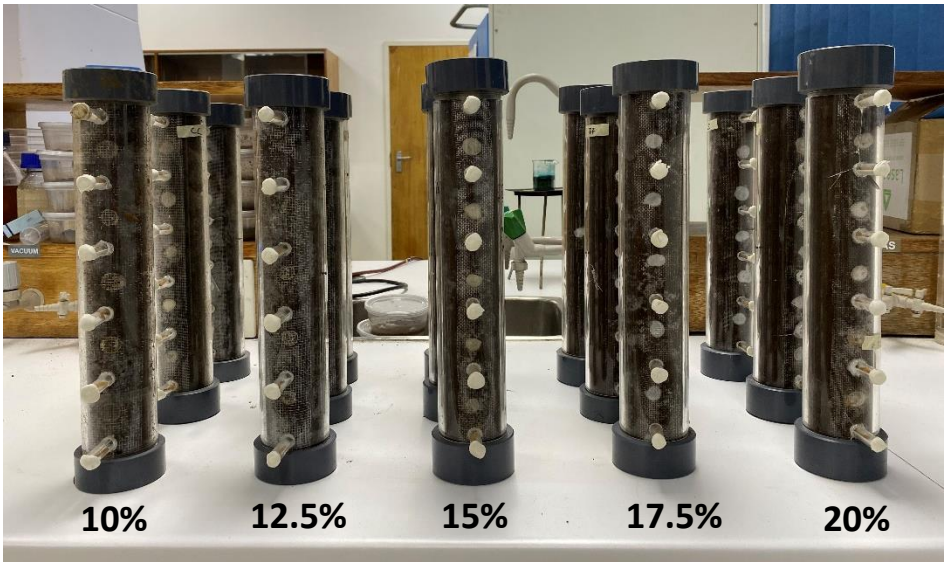


Figure D. Vertical sand column experimental setup.

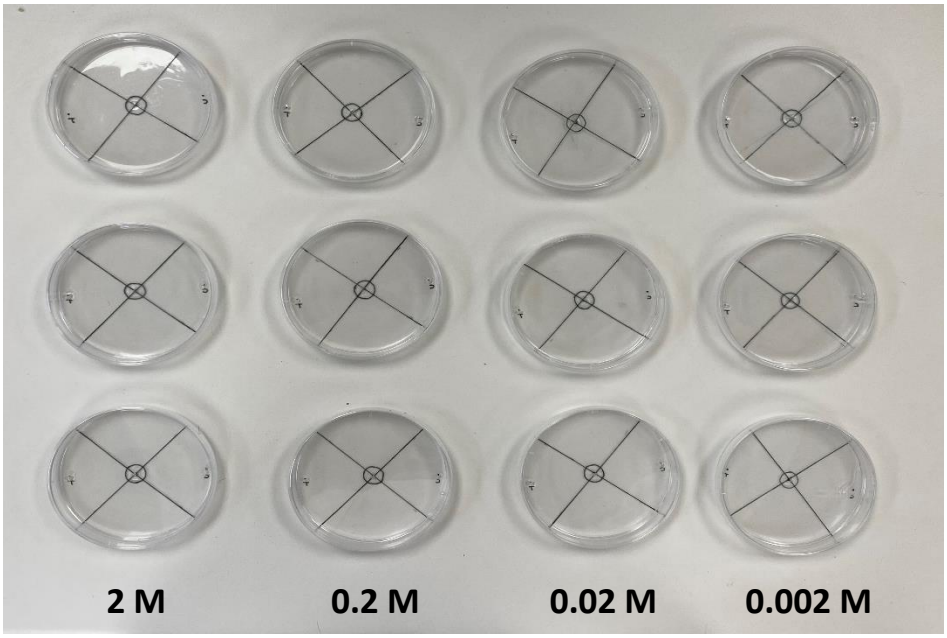


Figure E. Prenol chemotaxis assay using Pluronic F-127 gel.

Appendix IV

Statistical Analysis results

1. Statistical Analysis for the intra-haemocoelic injection assay

Two-way ANOVA results

Row Factor: Bacterial dose

Ho: No significant difference exists between the insect mortality means at each bacterial dose.

H1: There is a significant difference in the insect mortality means of at least one of the bacterial doses.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Column Factor: Time

Ho: No significant difference exists between the insect mortality means at each time interval.

H1: There is a significant difference in at least one of the insect mortality means at the different time intervals.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Interaction: Bacterial dose and time

Ho: No interaction exists between bacterial dose and time.

H1: An interaction exists between bacterial dose and time.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

The summary statistics of the Two-way ANOVA test with replication. A comparison was made between the insect mortality means at each bacterial dose and time interval. The bacterial dose was represented by the row factor and time by the column factor. An evaluation of the interaction between the two parameters was also part of the test. The study was conducted at 25° C.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (bacterial dose)	21488.30	4	5372.08	214.14	0.0000	3.06
Columns (time)	2626.29	2	1313.15	52.34	0.0000	3.68
Interaction	1731.16	8	216.40	8.63	0.0002	2.64
Within	376.30	15	25.09			
Total	26222.0547	29.0000				

2. Statistical Analysis for the 2D Sand substrate assay

Two-way ANOVA results

Row Factor: Soil moisture

Ho: No significant difference exists between the insect mortality means at each soil moisture.

H1: There is a significant difference in the insect mortality means of at least one of the soil moistures.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Column Factor: Time

Ho: No significant difference exists between the insect mortality means at each time interval.

H1: There is a significant difference in at least one of the insect mortality means at the different time intervals.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Interaction: Soil moisture and time

Ho: No interaction exists between soil moisture and time.

H1: An interaction exists between soil moisture and time.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

The summary statistics of the Two-way ANOVA test with replication. A comparison was made between the insect mortality means at each soil moisture and time interval. The soil moisture was represented by the row factor and time by the column factor. An evaluation of the interaction between the two parameters was also part of the test. The study was conducted at 25° C.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Soil moisture)	29129.89	4	7282.47	461.38	1.73E-49	2.50
Columns (Time)	14089.44	6	2348.24	148.77	8.26E-38	2.23
Interaction	5209.796	24	217.07	13.75	5.48E-18	1.67
Within	1104.89	70	15.78			
Total	49534.01	104				

3. Statistical Analysis for the vertical sand column assay

Two-way ANOVA results

Row Factor: Distance travelled

Ho: There are no differences between the mean insect mortalities obtained at each distance travelled.

H1: There is at least one mean mortality inequality present between the different distances travelled.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Column Factor: Soil moisture

Ho: No significant difference exists between the insect mortality means at each soil moisture.

H1: There is a significant difference in the insect mortality means of at least one of the soil moistures.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

Interaction: Distance travelled and soil moisture

Ho: There is no interaction between the distance travelled and soil moisture.

H1: There is an interaction between the distance travelled and soil moisture.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

The summary statistics of the Two-way ANOVA test with replication. A comparison was made between the insect mortality means at each distance travelled and soil moisture. The distance travelled was represented by the row factor, and soil moisture by the column factor. An evaluation of the interaction between the two parameters was also part of the test. The study was conducted at 25° C.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample (Distance travelled)	41246.91	5	8249.383	167.05	7.26E-34	2.36827
Columns (Soil moisture)	87950.62	4	21987.65	445.25	7.42E-44	2.525215
Interaction	57827.16	20	2891.358	58.55	5.94E-32	1.747984
Within	2962.963	60	49.38272			
Total	189987.7	89				

One-way ANOVA results

Ho: No significant difference exists between the chemotaxis indices mean obtained at each dose of prenol.

H1: There is a significant difference in the chemotaxis indices mean of at least one of the prenol doses.

H1: Reject H0 if F ≥ Fcrit

α = 0.05

The summary statistics of the One-way ANOVA test. The chemotaxis index obtained at different doses of prenol was compared. The study was performed at room temperature (20-25 °C).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.514404	3	0.504801	458.5243	2.74E-09	4.066181
Within Groups	0.008807	8	0.001101			
Total	1.523211	11				