Evaluating the Effects of Formulation Substrates on the Storage Duration of Entomopathogenic Nematodes

by

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Declaration

I, Onkgopotse Gomolemo Bantatetse (1593026), hereby declare that:

- The work on which this dissertation is based upon, is my original, unaided work (unless indicated otherwise), and neither the whole work nor any part thereof is being, has been, or is to be submitted for another degree in this or any other university.
- I am aware that plagiarism is wrong and that proper measures of referencing other people's ideas have been followed.
- I am aware that the university may take disciplinary measures if it appears that my work is aided, or I have failed to properly acknowledge the ideas of others.
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Research Summary

Although there has been satisfactory success attained by utilizing entomopathogenic nematodes (EPNs) as bio-predators, cultivation of formulations prolonging lifespan for at least a year, whilst warranting distribution of high-grade nematodes, have not been accomplished on the international commercial front. Hence, this study was aimed at evaluating the effects of various formulation substrates [Diatomaceous earth (DE), coarse silica sand, and the insect-cadaver-approach], on the storage duration of formulated nematodes, particularly *Oscheius* sp. OGB-2022 (OR879788) and *Cruznema* sp. NTM-2021 (OQ408141) which were isolated from the moderately dry climatic conditions of Johannesburg, South Africa, and the dry winter climate of Leribe, Lesotho using soil baiting and White trap setup technique. These isolates demonstrated high pathogenicity towards the last Instar larvae of *Tenebrio molitor* L. (*Coleoptera: Tenebrionidae*) at room temperatures ranging from 25-35℃ after 4-7 days, and were revealed by the NCBI BLASTn bioinformatics search parameters of the 18S rDNA nucleotide sequence data that they belonged to and are closely related to other nematode species in the *Oscheius* and *Cruznema* genera of the Nematoda phylum. Moreover, bacterial isolates successfully extracted from the gut of 0.1% Sodium hypochlorite (NaClO) surface sterilized infective juveniles (IJs) of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 were revealed by the NCBI BLASTn to have high affinity to species of genera *Alcaligenes*, *Pseudomonas*, and *Citrobacter*, respectively.

The study found no substantial statistical differences in the survival and virulence of formulated IJs across various substrates and incubation temperatures. However, *Oscheius* sp. OGB- 2022 IJs were more virulent after incubation at 30℃, while *Cruznema* sp. NTM-2021 appeared more virulent after incubation at 25℃. The insect cadaver formulation, DE, and coarse silica sand showed the highest survival and virulence (>85%), suggesting potential for agricultural field applications. Based on observations made from the results obtained in this study, the insect cadaver and DE formulation are the substrates best suited for field application with standard agronomic equipment. The *Oscheius* sp. OGB-2021 and *Cruznema* sp. NTM-2021 IJs showed high survivability in insect cadaver formulations (>85%) and DE (>85%) when stored at 25℃ and 30℃ incubation temperatures for 2, -7, -14, and 21-day incubation durations. This suggests that future commercialization of these nematode isolates will

have to be undertaken at these optimum temperatures in optimized formulation substrates.

Dedication

This work is dedicated to my loving parents Dorcas and Jeremiah Bantatetse-Tolman who always encouraged me to further my studies and continuously provided me with needed support. To my family, especially my brother Tshireletso Elvis Bantatetse and my mother for their love and immense support in difficult times. Thank you.

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Abbreviations

Biocontrol- Biological control

BLAST - Basic local alignment search tool.

bp – Base pair

COI - Cytochrome oxidase I

DNA - Deoxyribonucleic acid

EPN - Entomopathogenic nematode

G. mellonella - *Galleria mellonella*

IJ - Infective juvenile

IPM - Integrated pest management

ITS – Internal transcribed spacer.

MEGA – Molecular evolutionary genetics analysis.

MUSCLE – Multiple sequence comparison by log- expectation.

NaClO- Sodium hypochlorite

NCBI - National centre for biotechnological information

OTU - Operational taxonomic unit

rDNA - Ribosomal deoxyribonucleic acid

rRNA – Ribosomal ribonucleic acid.

T. molitor - *Tenebrio molitor*

tRNA – Transfer ribonucleic acid.

WDG – Water dispersible granule.

Research outputs

Manuscripts submitted for publication.

i. Isolation and Molecular Characterization of *Oscheius* sp. OGB-2022 (Nematoda: *Rhabditidae*), a potential entomopathogenic nematode (EPN) from semi dry climatic conditions of Johannesburg, South Africa.
ii. Isolation and Molecular Characterization of *Alcaligenes* sp. OGB-2022 and *Pseudomonas* sp. OGB-2022 from 0.1% Sodium hypochlorite (NaClO) surface sterilized *Oscheius* sp. OGB-2022 infective juveniles (IJs), potential associated gut bacterial isolates.
iii. Endo-parasitic dynamics of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 (Nematoda: Rhabditidae), insect parasitism inferred by nematodes.

Manuscripts under preparation for submission to journal

i. Formulation of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021, as endemic Southern African entomopathogenic nematode (EPN) species in Diatomaceous Earth (DE), Insect-cadaver, and Coarse Silica sand formulations.
ii. Insecticidal capacities of novel bacterial isolates extracted from gut of 0.1% NaClO surface sterilized *Oscheius* sp. OGB-2022 infective juveniles.

1 Chapter One: Literature Review

1.1 Nematode Taxonomy

Nematodes are invertebrate, non-segmented metazoans found free-living in almost every natural habitat (Flint *et al*, 1998; Baldwin, 2000; Hugot, 2001; Smythe *et al*., 2019). Anatomically, these microscopic roundworms possess a tubular shaped body covered with an elastic, complex, but flexible outer cuticular integument consisting of several layers of proteinaceous fibres (Flint *et al*, 1998). As multicellular, pseudo-coelomic, bilaterally symmetrical organisms, the Nematoda phylum falls under the kingdom Animalia, and have been incorporated in the super phylum Ecdysozoa along with the Arthropoda and multiple smaller phyla, based on the morphological and 18S rDNA molecular based evidence (Eernisse, 1992; Aguinaldo *et al*., 1997; Bert *et al*., 2011; Blaxter *et al*., 2015, 2016; Koutsovoulos, 2015).

The super phylum Ecdysozoa has two sub-divisions being the Panarthropoda and the Cycloneuralia as reported by Blaxter and Koustovolos (2015). The three phyla falling under Panarthropoda include: Onychophora, Arthropoda, and Tardigrada, whereas the other five phyla falling under Cycloneuralia, include: Nematomorpha, Nematoda, Loricifera, Priapulida, and Kinorhyncha, with Nematomorpha having been outlined as a parallel group regarding the Nematoda phylum (Eernisse, 1992; Aguinaldo *et al*., 1997; Dunn *et al*., 2008; Blaxter *et al*., 2016). Likewise, the phylum Nematoda initially had two divisions, being class Phasmida and class Aphasmida (Ivashkin, 1961), which were later renamed to class Secernentea and class Adenophorea, by Chitwood (1937, 1958). These two classes turned out to be the two foremost taxonomic groupings of the phylum Nematoda, in classical nematology (Chitwood, 1958, 1937). However, as reported by Blaxter *et al*., (1998) and De Ley (2002), the conventional grouping of the Nematoda phylum into the two classes, is no longer ratified by a phylogenetic system of classification based on the molecular evidence of the 18S rDNA sequence data (Smythe *et al*., 2019).

Most nematode species from class Secernentea were re-classified as Rhabditia, whereas the primary aquatic Enoplia and Chromadoria were elevated out of Adenophorea, and distinguished into three major phylogenetic lineages after subsequent morphological investigations were conducted by Andrássy (1976) and Malakhov (1994).

Table 1.1: A summary of all the phyla falling under both the respective sub-divisions of the super-phylum Ecdysozoa (Blaxter and Koustovolos, 2015; Dunn *et al*., 2008).

Ecdysozoa	
Panarthropoda	**Cycloneuralia**
Onychophora Arthropoda Tardigrada	Nematoda ✓ Adenophorea ✓ Secernentea Nematomorpha Loricifera Priapulida Kinorhyncha

As reported by Blaxter (2011), Holtermann *et al*., (2006) initially resolved the phylum Nematoda into 12 phylogenetic clades using the 18S rDNA sequence data, henceforth it is categorized into two classes, being class Enoplea and class Chromadorea as shown by recent literature. Moreover, this phylum was further resolved into five phylogenetic clades, namely: Dorylaimia (I), Enoplia (II), Spirurina (III), Tylenchina (IV), and Rhabditina (V). According to Blaxter (2011), invertebrate parasitic nematodes are classified into four evolutionary groups (Table 1.2) (Smythe *et al*., 2019).

Table 1.2: A simplification of all the major phylogenetic clades and lineages forming the mainstay of the phylogenetic cataloguing of the phylum Nematoda centred on the 18S rDNA sequences (Smythe *et al*., 2019).

	Nematoda			
	Enoplea		**Chromadorea**	
	Enoplia	**Dorylaimia**	**Chromodoria**	**Rhabditida**
Phylogenetic clades	**II**	**I**		**III** **IV** **V**
Major lineages	Enoplia Triplonchida	Trichinellida Dioctophymatida Mermithida Mononchida Dorylaimida	Rhabditida Plectida Araeolaimida Chromadorida	Tylenchina Rhabditina Spirurina

1.2 Entomopathogenic Nematodes (EPNs).

Entomopathogenic nematodes (EPNs) along with other nematode species are one of the most abundantly soil dwelling, endo-parasitic microorganisms found across the globe (Shapiro-Ilan 2014; Bhat *et al*., 2020). These microorganisms are usually the most studied among the minute nematode species due to their potential to serve as substitutes to the use of harmful, chemical pesticides in controlling problematic crop pests in forestry and agricultural industries (Alatorre-Rosas 1990; Malan *et al*., 2006; Jagodic, 2019). EPNs are obligate insect-parasites having a symbiotic association with the pathogenic bacteria found inside the frontal gut lumen and inner gut wall sections of their IJs (Poinar 1993; Hatting *et al*., 2009; Thakur *et al*., 2022b).

The three EPN genera known so far include: *Steinernema*, *Oscheius*, and *Heterorhabditis*, and they have symbiotic associations with the insect pathogenic bacteria *Xenorhabdus*, *Serratia*, and *Photorhabdus*, respectively (Adams *et al*., 2006; Zhang *et al*., 2008; Lulamba & Serepa-Dlamini, 2020). The EPNs' foraging strategies to ambush on soil surfaces and to cruise deep into the soil renders them suitable to act as biocontrol agents (Koppenhofer *et al*., 2006). However, EPNs normally found in diverse soil environments exhibit remarkable dissimilarities regarding infectivity, host range, conditions for survival, reproduction, and distribution (Campos-Herrera *et al*., 2012). Additionally, there has been successful applications of EPNs as biocontrol agents against substantial dipteran, coleopteran, and lepidopteran insects found on commercial produce (Athanassiou *et al*., 2010; Bhat *et al*., 2020).

As described by (Adams *et al*., 2006), EPNs normally infect susceptible insect hosts while still in the non-feeding, free-living infective juvenile, developmentally arrested phase, known as the dauer stage which is also the only life cycle stage with the ability to persevere in the soil apart from any host for a prolonged period (Dillman *et al*., 2012; Shapiro-IIan *et al*., 2019). The efficiency of EPNs as bio-control products is reliant on both the virulence of the EPNs' endosymbiotic bacteria to susceptible hosts and the capability of the EPNs to identify, detect and penetrate an insect host (Athanassiou *et al*., 2010). Moreover, the utilization of EPNs along with their endosymbiotic bacteria has been recognized as an essential approach in both the integrated pest management (IPM) and biocontrol industries (Stock 2015).

Furthermore, EPNs as eco-friendly, biodegradable pest control agents can be used as a suitable replacement for the usage of harmful insecticides for crop pest management, as they do not have any adverse environmental and human health consequences (Tomar *et al*, 2022). In addition, by stabilizing environmental changes, EPN-associated bacterial endosymbionts may have a remarkable potential to improve biocontrol approach of a wide variety of phyto-insect pests and diseases and minimize the usage of synthetic pesticides in crop pest control and may also have a significant potential for agricultural pest management due to the toxic bioactive complexes and proteins emitted as secondary metabolites (Thakur *et al*., 2020; Migunova and Sasanelli, 2021; Thakur *et al*., 2022a, 2022b).

1.3 Evolution of EPNs

The EPN genera *Heterorhabditis* and *Steinernema* respectively fall within the Rhabditina and Tylenchina phylogenetic clades under the Chromadoria lineage of the soil-dwelling nematodes (Blaxter, 2011). By adapting to new environments, these microscopic roundworms have gradually undergone evolution throughout time, and have evolved a wide range of distinctive morphological characteristics that allow them to locate and infect insect hosts (Tyson *et al*., 2012). Likewise, the general life cycle of nematodes is comprised of the egg, larval, and adult stage, henceforth, the advancement of the nematodes' parasitism requires various adaptations in terms of the efficient roles accomplished with deference to each progression phase (Maule and Curtis, 2011; Thakur *et al*., 2022b). This consists of both physical and chemical cues that prompt the change from a free-living to a parasitic state.

One of the most evident traits of evolution amongst EPNs is the interchangeable switch from non-feeding, developmentally arrested state to the bacterial-feeding and reproductive life cycle state (Tyson *et al*., 2012: Bhat *et al*., 2020). This has been observed in both *Heterorhabditids* and *Steinernematids.* Moreover, EPNs normally perseveres for prolonged time in the soil when in the dauer state, even under unfavourable environmental conditions such as desiccation (Bhat *et al*., 2020). During this developmentally arrested stage, the IJs harbour the endosymbiotic bacteria within their gut lumen until a suitable insect host has been located (Dillman *et al*., 2012; Thakur *et al*., 2022).

Conversely, in favourable conditions, when EPNs are in their normal reproductive and developmental stage, they tend to feed on their endosymbiotic bacteria (Feldhaar, 2011; Dillman *et al*., 2012; Bhat *et al*., 2020).

Additionally, EPNs have evolved a conjoined union with their endosymbiotic bacteria, involving a collaborative commensal phase in which EPNs act as a vector for their endosymbiotic bacteria and a trophic phase in which EPNs feed on their bacteria (feldhaar, 2011; Dillman *et al*., 2012; Thakur *et al*., 2022b). As previously described by (Dillman *et al*., 2012), there are four classifications describing a variety of relationships formed by EPNs and other organisms. These classifications include: necromenic which involves the use of the host cadaver as a nutrition source, and this can be observed when an intricate network of bioactive complexes is secreted by their endosymbiont as secondary metabolites for host insect tissue disintegration (Dillman *et al*., 2012; Thakur *et al*., 2022b).

Other classifications include facultative, which is when an EPN is not entirely dependent on their host to complete its life cycle; phoretic, which can be observed between EPNs and their symbiotic bacteria. The bacterial endosymbiont is heavily dependent on EPNs for mobility and dispersal from host to host (Dillman *et al*., 2012; Bhat *et al*., 2020). Contemporary scientific research has been focused mainly at studying how the bioactive complexes produced by the endosymbiotic bacteria of EPNs as secondary metabolites may be utilized to manage problematic insect pests in the agricultural industries as an alternative technique for the use of pesticides (Thakur *et al*., 2022b). Lastly, obligate parasitism involves complete dependence on the host for life cycle completion (Dillman *et al*., 2012; White *et al*., 2017). These EPNs have commonly been known since the seventeenth century, or probably earlier (Nguyen and Smart Jr, 2004), while substantial studies on EPNs were only undertaken in the nineteenth and twentieth centuries, and it has since been reported that EPNs are globally distributed.

1.4 Life cycle of Entomopathogenic Nematodes (EPNs)

In the agricultural industries globally, EPNs have essentially been utilized as ideal 'bio-predators' in the regulation of numerous problematic soil-dwelling crop pests (Nguyen & Smart, 1996; Bhat *et al*., 2020). EPNs as naturally occurring enemies of crop pests have played a significant role in the development of IPM systems since they are highly

specific, able to locate and infect their insect hosts and effectively repress the crop pest population in a brief window of time, and they are highly pathogenic (Bhat *et al*., 2020). Furthermore, EPNs offer a better and environmentally friendly substitute to the utilization of synthetic insecticides, due to their safety to humans and non-target organisms; whilst maintaining their ecological pleasantness and comparative effortlessness of field usage (Lulamba & Serepa-Dlamini, 2020).

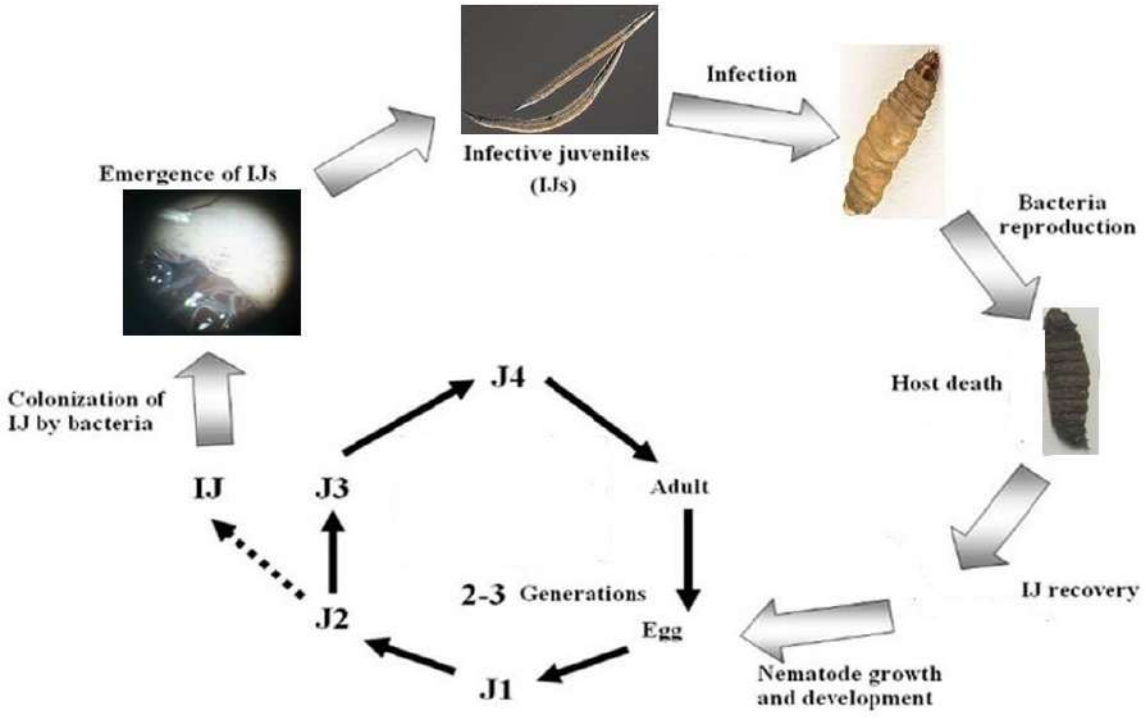

Figure 1.1: The general life cycle of EPNs (Lephoto and Gray, 2019).

EPNs have a life cycle that is comparable to that of other nematodes (Dunn *et al*., 2020). These soil-dwelling, free-living microscopic roundworms penetrate the susceptible insect hosts through any natural openings being either the respiratory spiracles, mouth, or anus, then discharge their pathogenic symbiotic bacteria into the host insect's hemocoel (Mráček *et al*., 2009; Thakur *et al*., 2022b). Once in the hemocoel, the bacterial cells proliferate exponentially and secrete toxic bioactive complexes, with high cytotoxic and insecticidal capacities, causing septicaemia and eventual death of the insect host within 48-72 hours (Adams and Nguyen 2002). These antimicrobial toxins and immune depressors regurgitated by the pathogenic symbiont into the prey's hemocoel perform a substantial task in facilitating the infection and eventual death of the infected host (Alia *et al*., 2014; Shapiro-Ilan *et al*., 2018, 2019). Additionally, IJs and the pathogenic bacteria utilize the invaded insect host as their

new nutrition source taking advantage of the nutrient-rich environment to reproduce for 2–3 generations (Stuart *et al*., 2006: Dillman *et al*., 2012; Tomar *et al*., 2022).

These endosymbionts maintain the nutritional physiology of EPNs within the insect cadaver (Feldhaar, 2011; Thakur *et al*., 2020; Migunova and Sasanelli, 2021; Thakur *et al*., 2022a, 2022b). Furthermore, after nutrition sources have been depleted, third stage IJs emerge from within the cadaver and persevere within the soil matrix while scouting for new host (Tarasco *et al*., 2008; Campos-Herrera *et al*., 2012; Shapiro-Ilan *et al*., 2018, 2019). The extent of the EPN life cycle is temperature-reliant and varies by species and location; nevertheless, EPNs normally completes their life cycle mostly within two weeks at temperature ranges of 18 to 28°C (Subramanian & Muthulakshmi, 2016).

1.5 Ecology of Entomopathogenic Nematodes (EPNs)

Dauer juveniles are the only EPN life cycle with the capacity to remain in the soil environments for extended period (Shapiro-Ilan *et al*., 2017, 2018). The ecology of these microscopic roundworms in various habitats can be impacted by a vast array of both biotic and abiotic factors (Stuart *et al*., 2015; Bhat *et al*., 2020). Some of the abiotic factors influencing IJ survival and distribution in soil habitats include, but are not limited to pH, temperature, soil porosity, and soil moisture content (Stuart *et al*., 2015). EPNs have been reported to be mostly active in moist environments, while being less active and having reduced lifespan in arid soil environments (Shapiro-Ilan *et al*., 2018).

Several biotic factors also influence the presence and perseverance of EPNs in the soil, however, the most crucial element is the availability of a suitable insect host and competing nematode species in the soil (Stuart *et al*., 2015). Soil-dwelling organisms are more susceptible to infection by EPNs under certain soil conditions as EPNs have developed various mechanisms of recognizing and infecting their hosts by simply employing specialized physical and chemical cues to locate and penetrate susceptible insect hosts. Moreover, EPNs are passively disseminated via phoresy in response to a wide range of volatile chemical cues and signals displayed by susceptible insect hosts within the soil matrix (Rasmann *et al*., 2005; Ali *et al*., 2010; Hiltpold *et al*., 2010; Turlings *et al*., 2012).

Additionally, their various foraging and ambushing techniques are extremely essential for their ecology and utilization as effective biocontrol agents in the agricultural sectors, as they allow them to seek for susceptible insect hosts (Griffin *et al*., 2005; Lewis and Clarke, 2012; Griffin, 2015; Shapiro-Ilan *et al*., 2017, 2018). Furthermore, EPNs are reported to be mostly active in moist environments, where they can effectively ambush on soil surfaces and employ their specialized feeding behaviours such as nictation which enhance their infectivity towards susceptible insect hosts with reduced mobility. Such species of EPNs include the previously identified *Steinernema scapterisci* and *S. carpocapsae* (Bal *et al*., 2014; Bhat *et al*., 2020). Conversely, *Heterorhabditis bacteriophora* and *S. glaseri* on the other spectrum, are EPN species that aggressively cruise and disperse deep into the soil (Wilson *et al*., 2012; Hiltpold and Hibbarb, 2018, Stelinski *et al*., 2019). When considering EPNs as a potential alternative to the use of insecticides in the agricultural industries, recent scientific contributions to the rapidly expanding research field have shed light and emphasized the significance of understanding the optimized EPN-insect interactions (Chiriboga *et al*., 2017; Helms *et al*., 2019; Stelinski *et al*., 2019).

1.6 EPNs as effective biocontrol agents

Biocontrol as an activity, encompasses the utilization of natural predators to regulate the prevalence of problematic crop pest population down to controllable levels in a distinctive environment without damaging beneficial organisms (Poinar, 1979; Flint *et al.*, 1998; Jagodic, 2017; Geisert *et al*., 2018). This eco-friendly approach can have a remarkably positive impact in both the forestry and agricultural industries if there is a well-established interaction between bio-predators and problematic crop pest populations (Van Lenteren, 2012). Moreover, the standard approach for selecting potential biocontrol agents is heavily dependent on the organism's abilities to locate and identify prey populations, to thrive in the event of low crop pest quantities, to have short and rapid reproduction duration, and to be vastly direct towards target preys (Bale *et al*., 2007; Shapiro-IIan *et al*., 2017, 2018).

As described earlier by (Adams *et al*. 2006), the toxic antimicrobials released by EPN's bacterial endosymbiont into the insect host's hemocoel, after penetration through natural openings, cause infection and eventual death of the infected host in a span 48-72 hours (Dillman *et al*. 2012; Banu *et al*., 2017).

EPNs are thereby deemed a suitable pest control measure and an even better alternative of pest management as they do not have adverse environmental and health implications (Adams *et al.*, 2009). Additionally, the non-specificity of most of synthetic insecticides gradually lead to regression in non-target pest populations and have been reported to instil damage to non-direct entities and lead to the incident of insecticide forbearance in direct entities (Georgis *et al*, 2006; Gill and Garg, 2014; Özkara *et al*., 2016). Hence, there is a dire need to incorporate IPM approach when controlling problematic crop pests in the agricultural industries (Mazid *et al*., 2011).

1.7 Ecological factors affecting EPN-insect host- associations.

Various EPN species have successfully been classified and are presently utilised commercially in several forestry, entomology, and agricultural industries (Adams, 2009). Suitable environmental conditions as well as innate EPN virulence must be taken into consideration when coordinating the potential EPN species to the prey of interest and environment as EPN persistence in the soil habitats is dependent on the endurance of individual IJs after emergence and the reproduction cycles within the insect host cadavers (Shapiro-IIan *et al*., 2017, 2018). EPNs are normally in frequent interaction with general microenvironments such as the soil matrix, macropore, and insect host hemocoel (Webster, 1973; Shapiro-IIan *et al*., 2017, 2018). Moreover, numerous interactions of EPNs with a variety of insect hosts in natural ecosystems render them highly suitable biocontrol agents (Shapiro- IIan *et al.*, 2018).

However, these interactions with various insect hosts may sometimes be limited by adverse climate conditions and soil types (Dritsoulas, 2020). Additionally, ecological barriers such as soil texture, soil moisture content, and even pH levels < 4 and > 8 may negatively interfere with the life cycle of EPNs (Susurluk, 2003; Dritsoulas, 2020; Shapiro-IIan *et al*., 2017, 2018). Soil-dwelling pathogens usually compete with EPNs for resources whilst numerous other invertebrate species such as parasitic nematodes tend to scavenge on the EPN-infected hosts (Shapiro-IIan *et al*., 2018).

Moreover, EPN population may not always be negatively affected by competition with other pathogens, however, may, in some instances, bring about interactive effects on host mortality without substantially having undesirable impacts on IJ reproduction in the hosts (Shapiro-IIan *et al*., 2018).

When insect host populations are extremely low or during adverse environmental conditions, the IJs gradually adapt by undergoing either desiccation or partial anhydrobiosis, to thrive in the soil for extended periods until the environmental conditions are favourable (Stuart *et al*, 2006). Furthermore, to better understand how EPNs interact with insect hosts in the natural habitat, factors that affect EPNs' virulence, such as motility must be taken into consideration to maintain the efficiency of EPNs as biocontrol agents (Shapiro-IIan *et al*., 2018, 2019). By taking these factors into consideration, efficient EPN biocontrol products could be developed, and suitable climatic conditions could be identified for field application (Flint *et al.*, 1998).

1.8 EPN desiccation tolerance and anhydrobiosis

Due to their vulnerability to the recurring cycles of soil desiccation, EPNs and countless other soil microorganisms essentially adapt to the continuously changing environmental conditions by undergoing evolution to survive desiccation (Askary and Ahmad, 2017). However, they have gradually evolved partial anhydrobiotic and desiccation tolerance abilities as their means of survival (Tyson *et al*., 2012). Additionally, the soil habitat has limited moisture retaining capabilities during winter and summer rainfall regions, when most soil habitats are prone to desiccation, considering its corresponding high surface area to volume ratios and microscopic size (O'leary *et al*., 2001). Hence, a saturated ecosystem with elevated relative humidity to decelerate moisture forfeiture is a major requirement for EPNs to successfully complete their life cycle as moisture content is very critical for their dispersal and host locating strategies in soil habitats (Perry *et al*., 2012).

Moreover, the sheath possessed by EPNs prevents rapid water loss by acting as a protective barrier between the IJs and the environment during soil desiccation, thus allowing IJs to thrive for prolonged periods in the soil until conditions are favourable (Perry *et al*., 2012). In addition, it has beforehand been recounted that desiccation tolerance capabilities among EPNs vary (Womersley *et al*., 1998; Askary and Ahmad, 2017). For instance, *Heterorhabditids* have been reported to have better water retaining capabilities due to their tight-fitting sheaths as compared to *Steinernematids* with loose-fitting sheaths.

Hence, *Heterorhabditids* are more likely to survive desiccation unlike *Steinernematids* (Womersley *et al*., 1998; Askary and Ahmad, 2017). Furthermore, during partial

anhydrobiosis, the free-living IJs in the soil micro-fauna have been reported to have acclimatized reaction rigorously expedited by the steady forfeiture of water from the ecosystem with elevated comparative humidity (Womersley *et al*., 1998; Askary and Ahmad, 2017). The EPNs' rate of metabolism is reduced by the induction of anhydrobiosis, thus making them extra forbearing to both tepid and arctic temperatures (Glazer and Salame, 2000; Grewal and Jagdale, 2002).

1.9 Temperature

Since EPNs can adapt to the fluctuating environmental conditions, multiple scientific studies have reported the correlation between EPNs and the environment (Zadji *et al*. 2014). Among other environmental conditions, temperature remains the most significant aspect in the growth and survival of EPNs as potential biocontrol agents (Chen *et al*., 2003; Sharmila *et al*., 2018). Likewise, the EPNs' infectivity, reproducibility, host seeking abilities, adhesion, and persistence, can directly be influenced by the heat stress to a much greater extent (Chen *et al*., 2003; Pervez *et al*., 2008; Susurluk, 2008; Ali *et al*., 2010). However, the severity of unfavourable temperature varies among the EPN isolates as they tend to adapt differently to various environmental conditions due to advanced temperature adaptability (Berry *et al*., 1997; Pervez *et al*., 2008).

Temperature and desiccation are some of the few stress factors that have great influence on the endurance, pathogenicity, and survival of EPNs in soil habitats (Sharmila *et al*., 2018). Ascertaining the idylic temperatures for the field usage of EPN products in the agricultural industries is vital in reducing the risks of EPNs' overexposure to extreme temperatures (Griffin, 1993; Sharmila *et al*., 2018). Moreover, identifying the desirable EPN strain with physiologically suitable adaptations for virulence and persistence in a distinctive agroecological habitat is vital in the establishment of the ideal temperature for field application of EPN biocontrol products (Sharmila *et al*., 2018).

Furthermore, the IJ lifespan is shortened by the rapid lipid reserve depletion rate which is amplified by high temperatures as EPN metabolism is temperature-dependent (Sharmila *et al*., 2018). Lipid saturation plays a key role in temperature forbearance and adaptability to adverse climatic siettings (Selvan *et al*., 1993). *Steinernematids* maintain a comparatively elevated quantity of replete fatty acids, which may justify why

they have a longer shelf life than *Heterorhabditids*. Furthermore, desiccating the IJs conserves lipid reserves and requires less oxygen (Womersley, 1990). Temperatures alternating from 25°C to 28°C have been observed to be optimal for field application of EPN biocontrol agents in agricultural sectors (Grewal *et al*., 1994).

However, the EPN species isolated from various climate regions tend to have different temperature tolerances with some being able to reproduce and remain virulent at both lower and higher temperature thresholds (Sharmila *et al*., 2018). EPNs isolated from high altitudes and cold climatic regions like the arctic and the sub-arctic climate regions are physiologically capable of enduring cold stress by preventing ice crystal formation (Stuart *et al*., 2015). Additionally, *S. feltiae* and *H. bacteriophora* are species of EPNs that can thrive at temperatures below -10℃ (Glazer, 2002; Koppenhöfer and Fuzy, 2003; Gungor *et al*., 2006; Stuart *et al*., 2015). Furthermore, *S. feltiae* has been reported to release cryo-protectants such as trehalose and glycerol, when exposed to extremely cold temperatures (Ali and Wharton, 2015). Conversely, the heat stress-related proteins like heat-shock proteins enables the *Heterorhabditids* to withstand extremely hot temperatures in semi-arid and desert regions (Glazer, 2002; Koppenhöfer and Fuzy, 2003; Stuart *et al*., 2015).

1.10 EPN Industrialization

EPNs as commercialized biocontrol products are an excellent substitute to the use of harmful pesticides in controlling problematic insect pests in the agricultural industries (Smart 1995; Gulcu *et al*., 2017). Over 100 EPN species have been identified and used as commercial biocontrol products in the agricultural industries, with the most common being: *S. carpocapsae, H. megidis, S. riobrave, S. feltiae, S. kushidai,* and *H. bacteriophora* (Askary *et al*., 2017; Labaude and Griffin, 2018). Moreover, the successful mass-production of EPNs as biocontrol products is dependent on an array of factors, involving survival during storage, ease of mass-production, formulation, and field application tactics, among others. Hence, refining these dynamics will heighten the usefulness of EPNs as stable profitable goods (Lacey *et al*., 2015; Abate *et al*., 2017; Labaude and Griffin, 2018).

Furthermore, EPNs are target-specific, environmentally friendly biopesticides, with a broader host range and relative ease of application when applied using standard agronomic spray equipment after mass production (Ehlers, 2002; Gulcu *et al*., 2017).

The most essential prerequisites for the effective industrialization of EPNs as biocontrol products is the engineering of appropriate commercial bioreactories for mass-industrialization of EPN IJs such as liquid and solid phase bioprocess machineries (Shapiro-Ilan *et al*., 2012; Ramakuwela *et al*., 2016). According to recent reports, airlift bioreactors are idylic IJ mass-creation bioprocess machineries and the vast significant aspect in ascertaining the economic viability of EPN production technology is the exact volumetric manufacturing ability of the specific biorepository system (Strauch and Ehlers, 2001; Shapiro-Ilan *et al*., 2012).

According to Shapiro-IIan *et al*., (2012), *In vitro* solid fermentation, *In vivo* production, and *in vitro* liquid fermentation, are the three primary approaches for yielding EPNs for commercial use (Shapiro-IIan *et al*., 2012). Since liquid fermentation has been reported to be more efficient as compared to *in vivo,* most EPN product are commercially produced using the liquid fermentation approach (Shapiro-IIan *et al*., 2012). However, highest amount of start-up capital and expertise are required for liquid culture fermentation, whereas labour and raw material costs are cheaper for solid culture fermentation even though the cost and economies of scale for solid fermentation fall between those of liquid culture and *in vivo* fermentation (Leite *et al*., 2017). Henceforth, solid culture fermentation is deemed to be the most profitable approach for commercial EPN production in certain countries (Gulcu *et al*., 2017).

Conversely, other numerous approaches such as optimization of media and fermentation characteristics, could be implemented in improving the *in vitro* fermentation of EPNs for commercial manufacturing (Shapiro-IIan *et al*., 2012; Leite *et al*., 2017). Moreover, the small-scale manufacturing of EPNs is normally using large quantities of model organisms such as *T. molitor* and *G. mellonella* last Instar larvae for the *in vivo* rearing of EPNs (Shapiro-IIan *et al*., 2012). However, this approach is usually a two-dimensional system relying on the White trap system and intensive labour and is reckoned to be of a curtailed economic scale (Shapiro-Ilan *et al*., 2012).

This *in vivo* culturing approach could however still be improved by improving the quality of diet used for rearing the insect host larvae as it is proportional to the quality of EPNs produced (Shapiro-IIan *et al*., 2012, 2016b). Alternatively, *in vivo* culturing approach can be greatly improved by mechanizing the whole production processes to minimize the labour expenses, and this can be achieved by mechanizing from the

insect host rearing stage to EPN inoculation of insect hosts, garnering and parcelling of IJs (Morales-Ramos *et al*., 2011; Shapiro-IIan *et al*., 2016b).

1.11 Formulation of EPNs.

Formulation of IJs in a substrate that ensures viability and infectivity for extended periods has been a major challenge experienced in developing EPNs as effective biocontrol agents (Poinar Jr and Grewal, 2012). Several techniques such as the incorporation of inactive carriers and the physical entrapment of IJs inside the inactive medium have been implemented in the formulation of EPNs. The simplicity and ease of production are some of the advantages of inert carriers. However, the downsides of carrier-based formulations include the prerequisite for frequent refrigeration throughout storage and transportation, which makes them expensive (Willett *et al*., 2018).

EPNs can be formulated in either gel, larval cadaver, liquid, or solid formulation substrates which normally consist of various components such as antimicrobials, absorbents, emulsifiers, UV-ray protectants, surfactants, and humectants, which serve various functions (Grewal, 2002; Kagimu, 2018; Willett *et al*., 2018). Moreover, to maintain the viability, pathogenicity, and lifespan of formulated EPNs, appropriate temperature and moisture conditions are required to ensure the formulated EPN product reaches the consumer in perfect usable conditions and that the EPNs are effective during field application (Askary, 2010; Willett *et al*., 2018). The induction of partial anhydrobiosis prior to EPN formulation has shown encouraging results, particularly in the context of diverse formulation strategies (Shapiro-IIan *et al*., 2012).

Moreover, the utilization of cadavers of infected insect hosts has been a promising advancement in formulation tactics (Hiltpold *et al*., 2012). Anhydrobiosis can be induced by powders, granules, and gel formulations (Grewal, 2002; Kagimu *et al*., 2018). EPNs have been reported to undergo partial anhydrobiosis in the case of unfavourable ecological circumstances such as extreme temperature, low moisture levels, or limited availability of insect hosts in soil habitats. Furthermore, when EPNs are in partial anhydrobiotic state, their energy consumption decreases (Heriberto *et al*., 2017). Environmental variations, notably water loss, lead EPNs to undergo physical and physiological changes (Willett *et al*., 2018).

1.12 Reasons for EPN formulation

The overarching goal of EPN formulation is to warrant the longstanding viability and infectivity of formulated IJs (Heriberto *et al*., 2017). Inducing partial anhydrobiosis during IJ formulation plays a commercially significant role in developing EPNs as effective biocontrol agents (Grewal, 2000; Heriberto *et al*., 2017). Furthermore, formulated EPN products ought to be consumer-friendly, have a prolonged durability, and require minimal groundwork. The formulation practice necessitates a thorough apprehension of numerous abiotic and biotic factors, such as desiccation and the impact of other ecological factors such as UV radiation, temperature, and interactions with other soil organisms. EPN products are commercially formulated to have relative ease of field application, prolonged shelf-life, and to reach the end-user in good useable conditions. According to Grewal (2002a), there have been no reports of EPNs in formulation that are cost and shelf life equivalent to synthetic pesticides, with EPNs being more costly to manufacture due to the elevated manufacturing costs required and having a very short shelf life (Jaffuel *et al*., 2017; Shapiro-IIan *et al*., 2018).

Furthermore, EPNs are typically preserved in water suspensions in culture flasks at temperature ranges of 4 °C to 15 °C, where they can survive for approximately a period of 3 to 12 months depending on the species utilized (Chen and Glazer 2005). Although mass-produced EPNs may be maintained in suspension in large refrigeration bubble storage tanks for weeks, this approach is fraught with contamination and places a significant demand on oxygen (Bečvář and Mráček, 2002). Considering the reasons stated above, it is reasonable to assume that the production and formulation of EPNs is done primarily for the sake of expediency and quality preservation.

1.13 Formulation for storage and transportation of EPNs.

Formulation of EPNs in a specific formulation substrate should be done when IJs are slightly desiccated to limit mobility and consumption of stored energy reserves, and to minimize the IJ deterioration rate (Grewal, 2000a; Lewis and Perez 2004). This technique allows formulated IJs to persevere for prolonged periods during storage and transportation (Grewal and Peters 2005). Moreover, the induction of partial anhydrobiosis reserves the stored lipid energy by reducing the EPNs' metabolic activity (Shapiro-IIan, 2000). Enhancing the techniques necessary for the improved effectiveness of formulated IJs during field application requires critical focus and

understanding on the timing of application and the type of formulation substrate used (Shapiro-IIan, 2000).

Furthermore, good formulation and storage measures must be implemented to ensure that formulated EPN products reach the end-user in acceptably good usable conditions, henceforth, the understanding of EPNs' ecology and the environmental conditions influencing their metabolic activity and pathogenicity is of utmost significance (Grewal, 2000; Lewis *et al*, 2006). Moreover, the inadequate storage can gradually impede the commercial usage of formulated EPN biocontrol products (Shapiro-IIan *et al*., 2006). EPN IJs can persevere at temperatures alternating from 4 to 10°C depending on the EPN strain and can therefore be stored under refrigeration conditions as aqueous suspensions for 6-12 months (Koppenhöfer and Fuzy, 2003; Gungor *et al*., 2006).

Nevertheless, countless scientific studies have been conducted to establish EPN formulation techniques that sustains the pathogenicity and survival of formulated IJs in aqueous and dry formulation substrates for prolonged storage durations (Grewal, 2002; Andalo *et al*., 2010; Leite *et al*., 2018). Moreover, the EPNs' temperature sensitivity and oxygen requirements must be taken into consideration during formulation and the sterility and toxicity of the formulation substrate against EPN IJs must also be thoroughly assessed (Kaya and Stock, 1997; Grewal, 2002, Bedding, 2006; Andalo *et al*., 2010; Leite *et al*., 2018).

1.14 Dynamics influencing the durability of formulated EPNs.

The implementation of an EPN formulated product with a relatively long lifespan of up to 3 months at 18-28℃ room temperature is significantly crucial for the profitable industrialization of EPNs as potential bio-control products (Shapiro-IIan *et al*., 2018). Additionally, understanding several fundamental factors influencing the quality of formulated EPN biocontrol products is critical for modifying and improving formulation techniques for better EPN yield, pathogenicity, and survival as well as relative ease of transport and environmental field applications (Shapiro-IIan *et al*., 2012). Environmental factors such as moisture content, aeration, and temperature play a substantial role in the successful commercialization of EPNs (Grewal *et al*., 1994; Dolinski *et al*., 2007; Shapiro-IIan *et al*., 2012).

Moreover, the optimum temperatures are normally found in the range of 18 °C and 28 °C in the EPN *in vivo* production programs (Hazir *et al*., 2001). The effects of temperature have been reported to influence the infectivity and survival of EPN IJs. For instance, it has been reported that a maximum number of IJs penetrated the last Instar larvae of the model organism *G. mellonella* after 96-hour exposure at 25 °C controlled temperature (Sunanda, 2009). The pathogenicity and survival of *S. carpocapsae* and *S. abbasi* has been reported to be higher at temperature ranges of 25 and 32 °C (Shapiro-IIan et al., 1996). However, the suitable temperature range for the development of *S. feltiae* is between 12 and 30 °C, with 25 °C being the optimum temperature for best maturation results (Shamseldean *et al*., 1996). Conversely, IJs of most EPN strains tend to lose viability at temperatures less than 12 °C and above 35 °C (Shamseldean *et al*., 1996; Willett *et al*., 2018).

As accounted for by previous scientific research, the emergence of higher numbers of *S. abbasi* IJs from *G. mellonella* insect cadaver was recorded at only 30 °C, whereas *H. indica* had excessive emergence of IJs from the *G. mellonella* insect cadaver at even higher temperatures (Shamseldean *et al*., 1996).

Moreover, *H. indica* has been reported to be more virulent than *S. abbasi* when exposed to last Instar larvae of model organism *G. mellonella* at temperature ranges of 20, 25, and 30 °C, thus making these temperatures vital for *in vivo* commercial rearing of EPNs (Shapiro-IIan *et al*., 1996). Lastly, the efficiency of commercial production of EPNs as effective biocontrol agents could be achieved through the sophistication and advanced mechanization of the production processes thereby improving the economic viability (Shapiro-Ilan *et al*., 1996).

1.15 EPN field application tactics and environmental consideration.

A single strain of a particular EPN species has the capacity to be utilized as a bio-control agent towards a wide variety of problematic crop pests in the agricultural industries, due to the broad host range of EPNs (Jafuel *et al*., 2017). The effectiveness of EPNs as potential bio-control products is heavily reliant on the adequate field application technology, which normally include a variety of irrigation system and standard agronomic spray equipment (Willett *et al*., 2018; Oliveira-Hofman *et al*., 2019; Shapiro-IIan., 2018, 2019). Moreover, the EPN field application can be through nearly all horticultural ground and/or standard agronomic equipment including electrostatic,

pressurized, and aerial sprayers, or mist blowers (Wright *et al*., 2005; Willett *et al*., 2018; Oliveira-Hofman *et al*., 2019; Shapiro-Ilan *et al*., 2018, 2019). However, the field application equipment is dependent on the type of cropping system, and in every instance, there are various handling considerations including nozzle type, volume, pressure and recycling time, agitation, spray dissemination configuration, and system ecological settings (Wright *et al*.,2005; Shapiro-Ilan et al., 2006a; 2018, 2019; Lara *et al*., 2008).

Additionally, field application of EPN biocontrol products to larger agricultural plots needs to be done using spraying devices such as boom sprayers, and there should be frequent agitation for proportional dispersal of EPNs across the field (Willet *et al*., 2018). Handheld tools such as water cans or back-pack sprayers may be useful for small plot applications (Willet *et al*., 2018).

Moreover, EPNs in aqueous suspension can be applied using a variety of formulations, including vermiculite, polyurethane sponge, activated charcoal, polyacrylamide gels, water dispersible granules (WDG), and alginate (Georgis *et al*., 1995; Willet *et al*., 2018; Shapiro-Ilan *et al*., 2018, 2019).

Since the efficacy of EPNs can be influenced by a vast array of both abiotic and biotic factors, analysing the environment of EPN field application must be a priority (Willett *et al*., 2018). The effectiveness of EPNs as biocontrol agents may be enhanced by means of agricultural tactics that augment both the biotic and abiotic factors to be more conducive for their virulence, survival, and dispersal (Campos-Herrera *et al*., 2015a; Shapiro-Ilan *et al*., 2015a, 2017). Moreover, several organic components such as manure could be added to the soil to increase the population of earthworms and - hereby improve the dispersal of EPNs via phoresy (Jaffuel *et al*., 2017; Shapiro-Ilan *et al*., 2017).

1.16 Research motivation

The main objective of this research project was to develop a cost-effective formulation substrate that will sustain the survival of formulated EPN biocontrol products at room temperature storage for relatively long periods. The formulated product had to have relative ease of application and transportation, thus allowing them to serve as effective biocontrol agents in the agricultural sector. This study could have positive implication towards knowledge generation and improved quality of life in both humans, the

environment, and animals, and this is in line with the national strategy that aims to eliminate or minimize the use of harmful synthetic chemical pesticides in farms by successfully commercializing the EPNs products. Moreover, through the commercialization of the research data and possible establishment of Spinoff Company, there is a potential to address the issues of poverty and unemployment among the youth in the communities.

1.17 Aims & Objectives

This study aimed to evaluate the survival and virulence of entomopathogenic nematodes formulated in different substrates being the coarse silica sand, diatomaceous earth (DE), insect cadaver approach (ICA), and coarse river sand, and the objectives were:

- ✓ To isolate and molecularly characterize the novel nematode species retrieved from the semi dry climatic conditions of Johannesburg, South Africa and dry winter climate of Leribe, Lesotho through soil baiting, White trapping, and 18S rDNA Sanger sequencing.
- ✓ To isolate and molecularly characterize the potential endosymbiotic gut-associated bacteria from the 0.1% sodium hypochlorite (NaClO) surface sterilized IJs of succesfully retrived nematodes using the 16S rDNA Sanger sequencing.
- ✓ To formulate the nematodes in different substrates and incubating at both 25℃ and 30℃ incubation temperatures for 2, 7, 14, and 21 days, respectively.
- ✓ To assss nematode survival on different formulation substrates after incubation.
- ✓ To evaluate virulence of nematodes against *T. molitor.*

1.18 Economic benefits and research impact

This study has significant ecological and health implications since it can help to provide an alternate method for controlling and reducing harmful agricultural pests that have no negative ramifications for human or domestic animal health. This research could indirectly contribute to social development since farmers can be trained and informed on the use and usage of these new and enhanced biocontrol techniques. Furthermore, employment will be created as there could be a demand for development programme facilitators. This study also intended to preserve a healthy environment free of hazardous insecticides that constantly impact the ecology. Moreover, EPNs are

generally cheap to isolate and maintain, therefore their employment as biocontrol agents can be cost-effective.

1.19 References

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2 Chapter Two: Isolation and 18S rDNA Molecular Characterization of Nematode species.

2.1 Introduction

Nematodes are microscopic, omnipresent, and varied roundworms found free-living in almost all environmental conditions such as aquatic and terrestrial habitats (Hugot, 2001; Smythe *et al*., 2019). The actual diversity of the phylum Nematoda has been reported to be closer to approximately one million species, with fewer than 30, 000 species having been described as parasitic towards most plants and animals (Baldwin, 2000; Hugot, 2001). Over the years, numerous intensive scientific studies have focused research mainly on parasitic nematodes of agricultural and medical significance (Smythe *et al*., 2019). Majority of free-living soil nematodes are commonly found in the Dorylaimia and Enoplia phylogenetic clades, along with virus- transmitting plant pests, plant and vertebrate parasites, model organism *Caenorhabditis elegans*, and free- living aquatic nematodes (Bik *et al*., 2010; Holterman *et al*., 2006).

Furthermore, nematodes serving as vectors to pathogenic bacterium and with active insect host-seeking capacities can be deemed entomopathogenic as they fulfil a certain criterion for entomopathogenicity. However, the life cycle of these nematodes can only be sustained if there is (i) reassociation of the pathogenic bacterium with the new dauer generation, (ii) nematode-bacterium reproduction, (iii) rapid mortality after the pathogenic bacterium is released into the insect host's hemocoel, and (iv) if there is emergence of a new cohort of dauer juveniles from the infected insect host's cadaver into the soil (Dillman *et al*., 2012; Shahina *et al*., 2017). Moreover, EPNs are obligate insect parasites with a potential to serve as an alternative to the use of harmful insecticides in IPM programmes (Shahina *et al*., 2017).

Globally, intensive scientific research has been undertaken to exploit these optimistic organisms as biocontrol agents of a broad range of problematic crop pests to convene the innovative values in IPM programmes (Shahina *et al*., 2017; Tabassum and Salma 2020). Additionally, benefits of using these EPNs as biocontrol agents include: the reduction in pesticide resistance by crop pests, reduced manufacturing expenses, and minimum health and environmental implications (Shahina *et al*., 2017). Beside EPN genera *Heterorhabditis* and *Steinernema* of the families *Heterorhabditidae* and *Steinernematidae* is the *Oscheius* genus of the family *Rhabditidae* first proposed in

Osche 1952, with *Oscheius Insectivorus* (=*Rhabditis insectivora* Körner) as type species (Andrássy, 1976). This *Oscheius* genus was further differentiated morphologically into two groups being the Insectivorus and the Dolichura group (Sudhaus 1976, 2011). Moreover, the morphological classification of these two groups indicated that the Insectivorus group included species with a bursa leptoderan and spicules with crochet needle-shaped tips, whereas species in the Dolichura group had peloderan bursa and spicules with thin tubular tips (Sudhaus, 2011).

Conversely, these two groups were further categorized into subgenera *Oscheius* and *Dolichorhabditis* based on the initially proposed division (Abolafia and Peña-Santiago, 2019). However, most species from these genera do not have adequate molecular support and are poorly described, henceforth their revision is vital in elucidating their taxonomic status (Rana *et al*., 2021). Furthermore, comparing the populations of already existing *Oscheius* species by molecular analysis of available genetic markers such as the ITS rDNA sequence data can substantially group systematics and heighten understanding of species diversity (Rana *et al*., 2021). Moreover, the nematodes' molecular taxonomy relies on the phylogenetic reconfigurations based on rDNA genetic markers such as the variable and conserved segments of the 18S and 28S subunits and the more variable ITS region (Adams, 2009).

However, the resolution essential to differentiate between the closely interrelated lineages is lacking in the 18S and 28S genes of some nematode groups (Landa *et al*., 2008). Additionally, individual variability or even intrapopulation may be displayed by the quickly evolving ITS region, which could in turn complicate its usage in group systematics, henceforth, mitochondrial genes thus however denote a practical alternative in nematode systematics (Hugall, 1999; Kiewnick *et al*., 2014; P° uža *et al*., 2015). Unfortunately, no mitochondrial sequence data has been published for *Oscheius* nematodes, except for *Oscheius onirici*, which has several published sequences of the cytochrome oxidase I (COI) gene available in the National Centre for Biotechnological Information (NCBI) GenBank database (Torrini *et al*., 2015; Foye, 2020). An overall of 42 valid nematode species are included in the genus *Oscheius* with 28 belonging to the Insectivora group, and a total of 14 belonging to the Dolichura group (Tabassum *et al*., 2016; Abolafia and Pena- Santiago, 2019).

However, *O. onirici* is the only species in the Dolichura group, along with seven other nematode species in the Insectivora group including *O. chongminensis*, *O. carolinensis*, *O. siddiqii*, *O. amsactae*, *O. rugaoensis*, *O. niazii* and *O. microvilli* that have been recognized as entomopathogenic. Moreover, it was revealed by the phylogenetic analysis of the ITS rDNA nucleotide sequence data that these two groups are monophyletic (Ye *et al*., 2010, Torrini *et al*., 2015). Furthermore, *Heterorhabditidoides* was initially recommended as a new genus (Zhang *et al*., 2008), although it was not accepted as a valid genus, and was however considered to be a junior synosym of *Oscheius* (Ye *et al*., 2010; Liu *et al*., 2012; Campos-Herrera *et al*., 2015; Torrini *et al*., 2015; Tabassum *et al*., 2016; Valizadeh *et al*., 2017). Additionally, the *Cruznema* genus which includes free-living bacterivorous as well as invertebrate parasitic nematodes belongs to the family *Rhabditidae*, along with the *Oscheius* genus (Tabassum *et al*., 2017). The genome of this genus consists of 14, 067 bp nucleotides and contains 22 tRNA, 2 protein-coding, and 2 rRNA genes (Valizadeh *et al*., 2017).

Furthermore, being the first sequenced representative of the genus *Cruznema*, the full mitochondrial genome of *Cruznema tripartite* was identified using high-throughput sequencing (Tabassum *et al*., 2016; Valizadeh *et al*., 2017). Based on amino acid data phylogenetic analysis, the *C. tripartitum* nematode has been supported as a sister clade to *O. chongmingensis* and *C. elegans* (Valizadeh *et al*., 2017). In addition to these, gene arrangement analysis of *C. tripartitum* revealed that this nematode belongs to the same order as *Caenorhabditis*, *O. chongmingensis*, *Diplocapter coronatus*, *Pristionchus pacificus*, and *Litoditis marina*; thus, the mitochondrial gene arrangement is highly conserved in the family *Rhabditidae* and some *Diplogasteridae* (Tabassum *et al*., 2016; Valizadeh *et al*., 2017). This study aimed to isolate and molecularly characterize the 18S rDNA region of the nematode species from semi dry climatic conditions of Johannesburg, South Africa, and the dry winter climate of Leribe, Lesotho with potential to serve as EPN biocontrol agents.

2.2 Materials and Methods

2.2.1 Collection of soil samples

A sum of thirty-two soil samples were amassed from different geographical locations of Johannesburg, around the Braamfontein area: Graaff street (-26.19379°S, 28.02763°E); University of the Witwatersrand, West campus (-26.18135°S, 28.02768°E); Wits university, East campus (-26.193171°S, 28.033198°E), and Leribe, Lesotho (28°59'17.4''S, 28°08'25.0''E), respectively. Soil samples were collected at a 16cm depth beneath the soil surface, using a metal hand trowel, and approximately 2meter spaces were left in between the sites of soil sample collection (Stuart *et al*., 2006). Approximately ±2kg of the collected soil samples were separately packaged in sterile 2L plastic vessels and kept at 25℃ room temperature during carriage to the laboratory (Cimen *et al*., 2016). Prior to soil baiting, sterilized distilled water was continuously added to the soil samples previously stored at room temperature in sterile 2L plastic containers. This was done to retain the soil moisture content at 8% (Kaya & Stock, 1997).

2.2.2 *Tenebrio molitor* (*T. molitor*) larval insect culture rearing

T. molitor (L.) (*Coleoptera*: *Tenebrionidae*) last Instar larvae were used as model organisms for *In vivo* rearing of nematodes. These insects, commonly known as Mealworms, were collected from Amazon pets, Lenasia, Gauteng (-26. 318586°S, 27.827079°E) and were reared in the laboratory on an artificial Oat bran diet supplemented with carrot and apple slices (Han & Ehlers, 2000; Tran *et al*., 2018). The insects were kept in 2L plastic containers and incubated in the insect room at 25 ℃ room temperature under natural light (Tran *et al*., 2018).

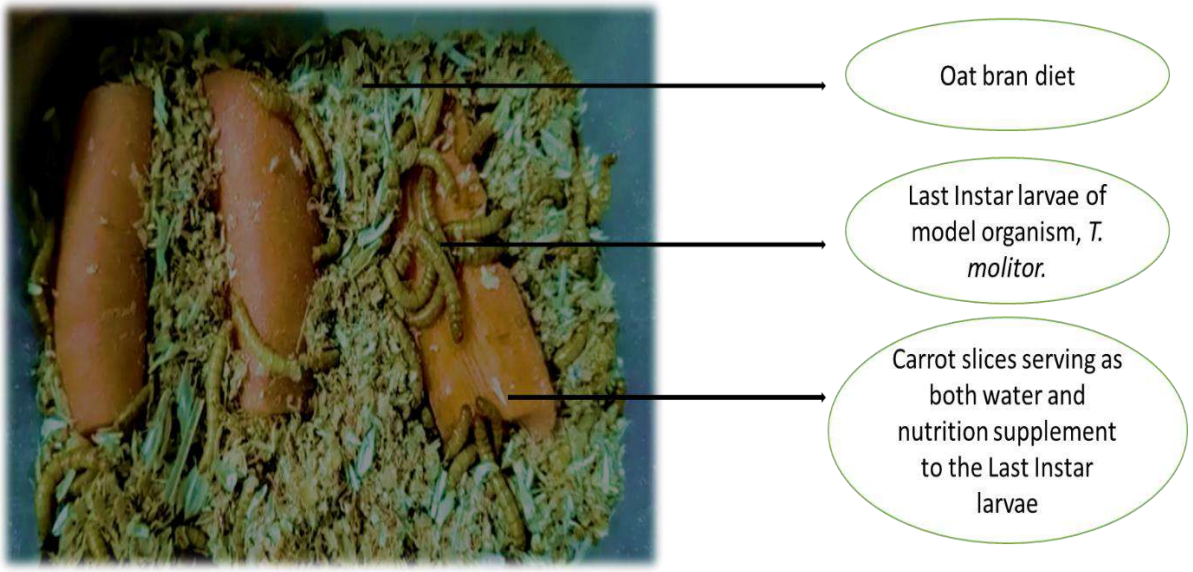

Figure 2.1: A diagrammatic representation of the Oat bran diet rearing of the last Instar larvae of the model organism *T. molitor* incubated in the insect room at 25℃ room temperature under natural light.

2.2.3 Soil baiting technique: Isolation of nematode IJs from the collected soil samples.

Last Instar larvae of *T. molitor* L. (*Coleoptera*: *Tenebrionidae*) were utilized as the model organisms in this study to recover IJs from the previously collected soil samples. A total of twelve *T. molitor* larvae were posited on the surface of the soil in each of the separate 2L containers. These containers were then concealed with lids and incubated at 25℃ (Kaya & Stock, 1997). During baiting, sterile distilled water was continuously applied to the soil samples to retain the moisture content. Observations on the soil samples were done at a 24-hour interval for a period of 4-7 days to facilitate infection, and cadavers of dead insect larvae were then collected. Throughout the extent of this study, fresh *T. molitor* larvae were used for re-baiting the soil samples and the symptoms observed on the cadavers after infection were recorded and used to diagnose infections initiated by nematodes. Cadavers showing signs of infection were collected and surface sterilized by spraying with 70% ethanol, and thoroughly rinsed with deionized water before being individually posited on the modified White trap setup to expedite the IJ emergence from within the insect cadaver (Kaya & Stock, 1997). Nematode IJs were successfully recovered from the two soil sample containers

collected from Johannesburg, around the Braamfontein area: Graaff street (-26.19379°S, 28.02763°E); and Leribe, respectively.

2.2.4 Isolation of infective juveniles (IJs)

The modified White trap setups assembled following protocols detailed previously by (Kaya & Stock, 1997) were used for isolating IJs from cadavers of insect larvae showing signs of infection. An inverted plastic watch glass of 45mm diameter, concealed with a damp 45mm diameter Whatman filter paper was placed inside a 90mm diameter petri dish filled with sterile autoclaved water to a 45mm mark, and the Individual dead larvae infected by nematodes were set on the damp Whatman filter paper before concealing the dish with the lid. The insect host cadavers were surface sterilized with 70% ethanol and deionized water before being placed on the White trap setups to prevent potential contamination (Stock & Kaya, 1997).

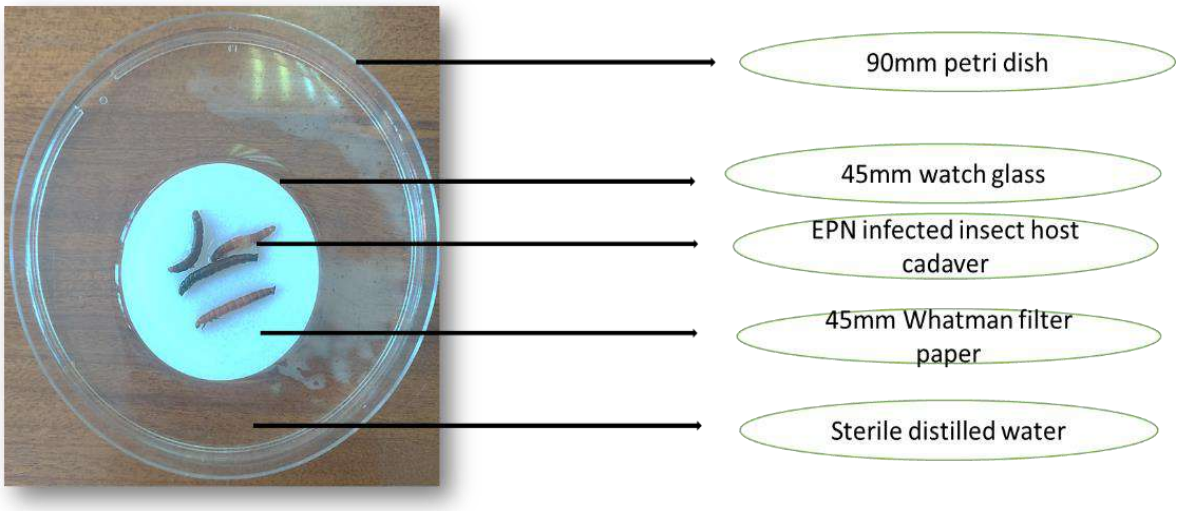

Figure 2.2: A diagrammatic representation of the White trap setup for nematode IJ isolation from the infected host cadavers.

2.2.5 Direct contact pathogenicity test assays: *In vivo* rearing and confirmation of pathogenicity of isolated nematodes.

The freshly emerged nematode IJs were collected into sterile 50mL falcon tubes and allowed to settle at the bottom over a 24-hour period. Excess water was then removed from the falcon tubes leaving only the nematode paste at the bottom. The IJs were then surface sterilized for 3 hours with a 0.1% solution of sodium hypochlorite (NaClO)

to prevent potential microbial contamination (see Appendix 2). Moreover, the excess NaClO solution was discarded, leaving only the IJs. The IJs were further rinsed thoroughly with 4mL of sterile distilled water. The pathogenicity of the recovered nematode isolates was tested on the last Instar larvae of the model organism *T. molitor.* Multiple 90mm diameter petri dishes with autoclaved coarse river sand (45g) were inoculated with 300IJs/μL before 6 *T. molitor* last Instar larvae were posited on the sand surface. The autoclaved coarse river sand bioassays were maintained at 8% moisture w/v. The plates were incubated at 25℃ room temperature for 72 hours, and mortality was recorded at 24-hour intervals from then on. Moreover, the pathogenicity test trials were performed three times, and plates with high larval mortality were selected for further experimental analyses. This was done to approve or disprove the Koch's postulates of infectivity and to also acquire IJs for classification and establishment of their cultures. Live insect host cadavers were continuously exposed to 300IJs/μL doses of freshly emerged IJs obtained from White trap setups prepared from coarse river sand bioassay infected insect host cadavers (Hominick, 1991).

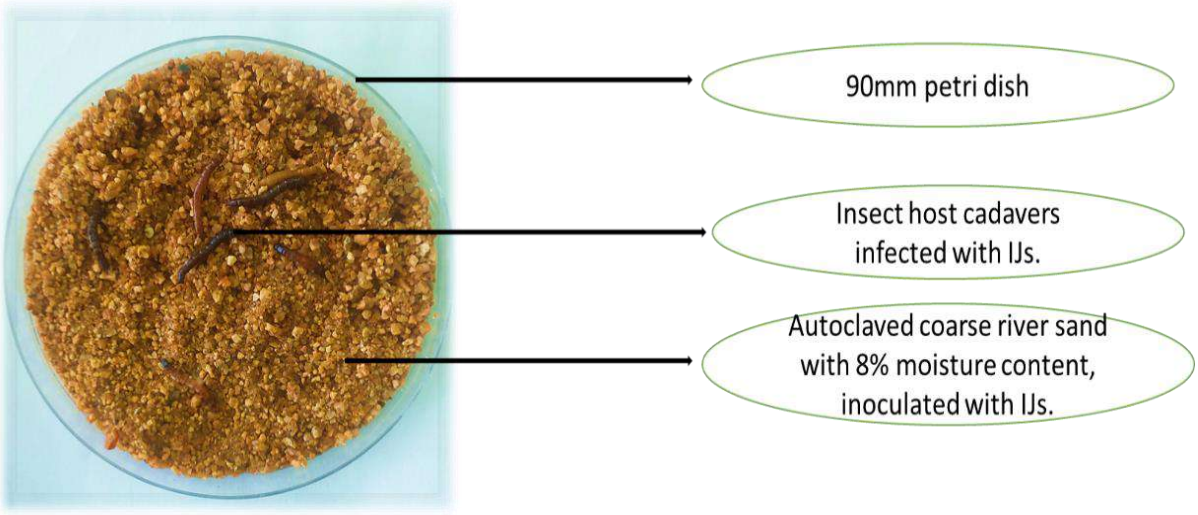

Figure 2.3: A diagrammatic representation of the autoclaved coarse river sand bioassays for confirming pathogenicity of recovered nematode isolates.

2.2.6 18S rDNA Molecular Characterization of nematodes.

2.2.6.1 Extraction of nematodes' genomic DNA

Nematodes' genomic DNA was extracted from the surface sterilized IJs enclosed in 1.5mL microcentrifuge tubes following the whole genomic DNA extraction protocol

specified on the E.Z.N.A® insect DNA extraction Kit (see Appendix 3). This procedure involved adding 350μL of CTL Buffer and 25μL of proteinase K solution to pulverized nematode paste in microcentrifuge tubes. The tubes were vortexed for 30 seconds, then incubated at 37℃. After incubation, chloroform: isoamyl alcohol was added, and the tubes were centrifuged at 10,000 x g for 2 minutes. The uppermost aqueous film was then moved to sterile tubes, whilst avoiding contaminants and inhibitors. HiBind® DNA Mini Columns were inserted into collection tubes, and 750μL cleared lysate was aspirated. The HiBind® DNA Mini Columns were then transferred to collection tubes, diluted with 500 μL of HBC Buffer and 700μL of DNA Wash Buffer. Moreover, the empty HiBind® DNA Mini Columns were centrifuged for 2 minutes, and the extracted DNA purity was checked on a Nano-Drop ND-1000® spectrophotometer before storage at -80℃.

2.2.7 Agarose gel electrophoresis

The NEB Fast Ladder was used on all gels (N3238) as size standard when visualizing the integrity of the PCR amplicons on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye.

2.2.8 Polymerase chain reaction (PCR) for amplification of ITS rDNA region

During this procedure, the amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was accomplished by PCR in a thermocycler using desired universal primers. DNA samples were prepared in PCR tubes before proceeding with the initial DNA denaturation step at 94℃ for 5 minutes. The initial denaturation step was followed by a 60 second denaturation stage entailing of 35 cycles run at 95℃. Moreover, a DNA strand annealing step followed at 64℃ for 60 seconds, followed by a 120 second primer elongation step at 72℃ and a final primer elongation step at 72℃ for 10 minutes. The TW81 Forward and AB28 Reverse primers (Powers *et al*. 1997) were utilised in the 18S rDNA sequencing of the amplified DNA using Sanger sequencing at Inqaba biotechnical industries Pty Ltd.

Table 2.1: Polymerase chain reaction (PCR) reagents and volumes for 18S rDNA region amplification.

PCR Reagents	Volume ($25\mu L$)
PCR Master mix	12.5
Nuclease-free water	9.5
TW81 Forward primer	1
AB28 Reverse primer	1
Template DNA	1

Table 2.2: PCR conditions for 18S rDNA region amplification.

Amplification stage	Temperature (℃)	Time
Initial denaturation	94 ℃	5 minutes
Denaturation	95 ℃	60 seconds
Annealing	64 ℃	60 seconds
Primer elongation	72 ℃	120 seconds
Final primer elongation	72 ℃	10 minutes

Table 2.3: Universal primer sequence information for amplification of 18S rDNA region.

Primer	Primer sequence	Tm (℃)	Ta (℃)
TW81 Forward Primer	5'-GCGGATCCGTTTCCGTAGGTGAACCTGC-3'	71.94	66.4
AB28 Reverse Primer	5'-GCGGATCCATATGCTTAAGTTCAGCGGGT-3'	68.87	63.87

2.2.9 Sanger sequencing of 18S rDNA region for molecular identification and sequence alignment.

The 18S rDNA amplification of the PCR products was accomplished by Sanger sequencing method using the desired TW81 Forward and AB28 Reverse universal primers at Inqaba Biotechnical Industries (Pty) Ltd, South Africa. FinchTV Version 1.4.0 trace data viewing software was used for trimming the Sanger sequenced DNA chromatograms generated for the nematodes' genomic DNA samples. The trimmed DNA sequence data for both the TW81 Forward and AB28 Reverse universal primers were uploaded to BioEdit Version 7.2 biological sequence alignment editor software for FASTA formatted consensus sequence generation. The FASTA formatted consensus sequence data were uploaded as queries to the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLASTn) for the identification of the unknown nematode sequence. The submitted query sequences were searched and aligned against the existing EPN genomic DNA sequence data already in the GenBank sequence database, by the NCBI BLASTn tool. The statistical similarities of the query sequence data were generated by the NCBI BLASTn alignment algorithmic tool against sequences in the GenBank database, ranging from highest to lowest percentage from which interim notions regarding phylogenetic affinities were roughly deduced in relation to the submitted query sequence.

2.2.10 Phylogenetic Analysis using MEGA11 software.

The FASTA formatted DNA nucleotide sequences generated from the 18S rDNA amplification of the PCR products were used in the phylogenetic analysis of the newly isolated nematode species. These FASTA formatted sequences of known EPN species were recovered from the National Institute of Health (NIH) GenBank® genetic sequence database using their designated accession numbers. The Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software was used for analysing the DNA nucleotide sequence data from known and unknown nematode species, using *Caenorhabditis elegans* (NR_000054.1) as an outgroup. The phylogenetic trees with the highest log likelihood were constructed using the Maximum Likelihood method and Tamura-Nei model after aligning the nucleotide sequences data with the Multiple Sequence Comparison by Log- Expectation (MUSCLE) tool (Tamura 1993). The

construction and the percentage of the phylogenetic trees was based on the clustering of the associated taxa on 1000 replicates in the bootstrap statistical test, with branch lengths measured in the number of substitutions per site (Tamura 2021).

The phylogenetic tree for unknown nematode isolate was constructed on the MEGA11 software using the selected taxa below: *Oscheius* sp. FVV-2 (MF196096.1), *Oscheius* sp. FDL-2014 (LN613267.1), *Oscheius* sp. FDL-2014 (LN613266.1)*, O. onirici* isolate GA3 (KX036751.1), *Oscheius* sp. 16 33834 (MF196100.1), *Oscheius* sp. MCB (KF684370.1), *O. tipulae* (KJ938579.1), *O. microvilli* strain NJAU-N19 (KT825914.1) , *Oscheius* sp. 67 P20 (KJ938578.1), *O. oniricii* isolate AKF Kurd (OP348877.1),

O, oniricii isolate GA3 (KX036758.1), *Oscheius* sp. FDL-2014 (LN613265.1), *C. elegans* (NR_000054.1).

The phylogenetic tree for *Cruznema* sp. NTM- 2021 was constructed on the MEGA11 software using the selected taxa below: *Cruznema* sp. NTM- 2021 (OQ408141), *Cruznema* sp. GD-1 (ON191475.1), *Cruznema* sp. CS50 (MW228469.1), *Oscheius* sp. MCB (KF684370.1), *O. chongmingensis* strain Tumian (EU273598.1), *Heterorhabditis safricana* (EF488006.1), *Steinernema feltiae* strain Ustinov (KT809344.1), *Steinernema abbasi* isolate CS35 (MG189974.1), *Steinernema yirgalemense* (AY748450.1), and *C. elegans* (NR_000054.1).

2.3 Results

2.3.1 Entomopathogenicity analysis, symptoms, and emergence of IJs from infected insect host cadavers.

Mortality of the last Instar larvae of the model organism *T. molitor* was observed after 4-7 days post exposure to the successfully isolated, surface sterilized nematodes of the unknown isolate and *Cruznema* sp. NTM-2021. The pigment of the insect host cadavers infected by these nematodes turned from yellowish- brown colour to dark brown, and the insect body had a mushy texture due to tissue deterioration. The observed pigmentation on the insect host cadavers preliminarily served as markers of nematode entomopathogenicity. These symptoms were preliminarily indicative of infection by some nematode species from the *Rhabditidae* family (Blinova and Ivanova, 1987; Park *et al*., 2011).

Figure 2.4: Nematode-infected insect host cadaver of the last Instar larvae of *T.molitor*. Depicted on (**b**) of both section **A** and **B** of **Figure 2.4** are the Nematode-infected insect host cadavers of the last Instar larvae of model organism *T. molitor,* 7 days after inoculation with surface sterilized pure nematode cultures of the unknown nematode isolate and *Cruznema* sp. NTM- 2021, respectively. Shown on section (a) on both image A and B is the healthy, uninfected last Instar larvae of model organism *T. molitor*, serving as a control. This experimental setup was designed to test the Koch's postulates of pathogenicity.

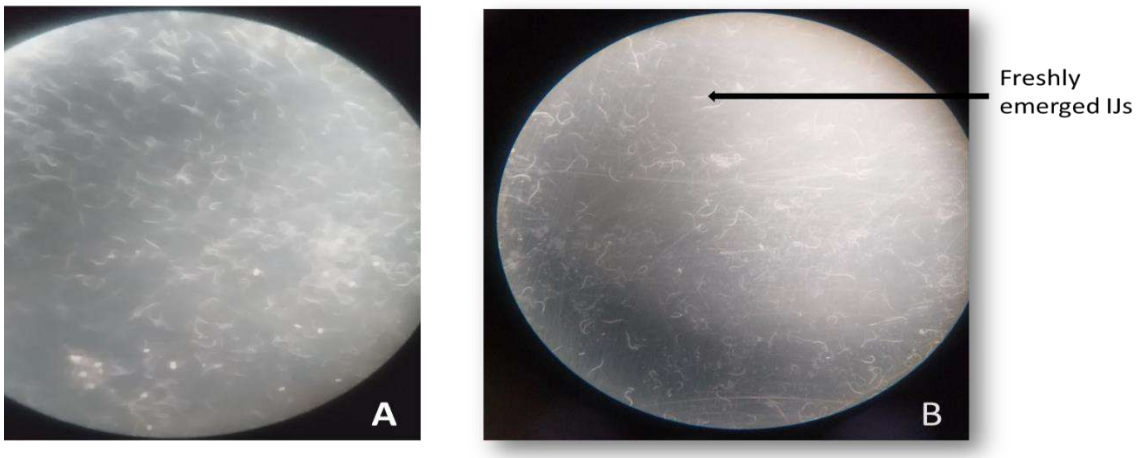

Figure 2.5: White trap setup for the isolation of IJs from infected insect host cadavers (Kaya and Stock, 1997). Depicted on A are the freshly emerged IJs of the unknown nematode isolate. Depicted on B are the freshly emerged IJs of *Cruznema* sp. NTM-2021.

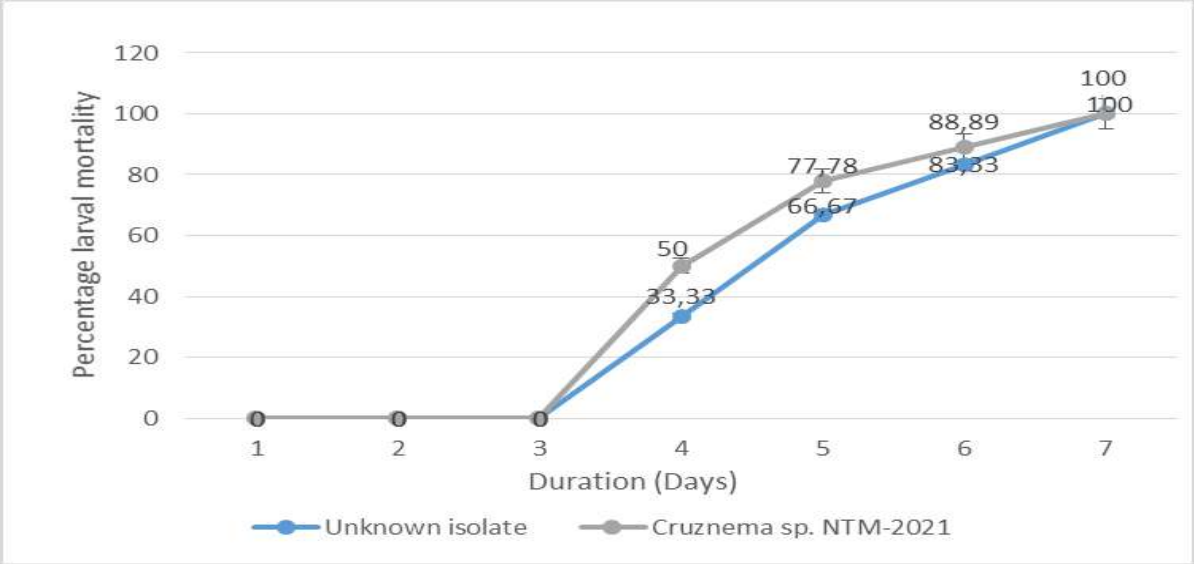

Figure 2.6: Percentage mortality of the last Instar larvae of the model organism *T. molitor* (n= 6) instigated by 0.1% NaClO surface sterilized IJs (300IJs/μL) of unknown isolate and *Cruznema* sp. NTM-2021 after 4-7 days post exposure. The error bars graphically represented the standard error of the means (Fcrit = 4.75; *P*- value > 0.05).

2.3.2 18S rDNA Molecular Characterization of the nematode isolates

2.3.2.1 Nematodes' Genomic DNA Extraction

FASTA formatted 18S rDNA nucleotide sequence data uploaded as queries to the NCBI BLASTn database for the identification of the unknown nematode isolates are illustrated in (Figure 2.6 and 2.7) below.

>TCTCTGCTTCAATTCAGCGGGTAATCACGACTGACATCAGGAAGCAATTCTGTGCACGAGAGCCAATAACAACGAT
GGAAACTACACCACAAGGGCAACCCAATAACATGATACCACTGTCAAGCCGTCATGGCACCGATCTGGGCAGTGCAG
TCTACGAGCTCTCGCCAGACCTACATGTTCCTACGAGGTCCGGTAGCCCCGTACGACGTGGCAACTATGATTCTAAGC
TGAAGACGCTGCATGGCACAAAGAGGCCCCGCCATAACCAGTCCGACAGACCAGTCATCTCAGGTACGTGGGTACTA
TTTAAGACTTCCCCGACATCAGACACCCCGGTACCAGAAGGGCCAGAGGTGCTAACTGCCTACAAGATTTTGCCACTC
AACGATCGATCGCAATTCGTCGAAAATAAGCGGAGCTAGCTCCGCTATTTCATCGATCTAAGGAAGCCCCCGATCAAT
CCCTAAAGGGAGATCAAGGATCGTTAAATGCAAAAAGAGAATATGAACCGGACAACGCAAAAATAAAACTTCAGAT
AGTTATATAGAACATACCGCCGCCAGGCAAAAAACGACCAGTCACTAACCACTCACTTTTAATTTCAAAAGCTGAAAT
AGGCACCAGAAAAAATACGCGATGCGAGGCAAACCGGTACTGCAGCTACGGTATTTCCCTACCGGTCCATCGACTAA
AGGTACGGAAGTTCTTTTGCTATTACGCTATTAAAGAAAACAGCGACCGAGTCAACCCAGCTGCCGCAAGCAAAACC
ATAGGGAAACCTCGAGGTCGGACAAGCTCGAGGACGATCAAAAAGCAGAACAAGCAACAGACAAGATCTACTTCGA
TTGCGGCAACGACTGATCAACCGCCTAAAGATAGGAAAACATTATAGAATCAGCGATGATCCACCTGCAGTTCACTG

Figure 2.7: FASTA formatted 18S rDNA nucleotide sequence of the Novel nematode isolate with high affinity to *Oscheius* species, *Oscheius microvilli* NJAU- N19, (KT825914.1) as revealed by the NCBI BLASTn search parameters.

>GAGAGGATGGCGATGAATCGATGCACGTTGCTTTTGCGACGATACTTAATCGCTCTGCCTGAGAGTGGCCTCGGATT
GGTTTGGTGGCTTGGAAGCCTTGTGCTTCTAGTCTTAATGAGATAGCTTGCTATCCGCCATCCAATCACTTCTTTCATAA
CTGAGTTGGTAATCGAAACGTGACAGGCTCACGCTAAAAAGCCTACTAACTAGCATTAGCGATGGATCGGTTGATTTG
GTCATCGATGAAAAACGCAGCCAGCTGCGTTATTTACCACGAATCGCAAATTTGTAGAGTGGTAAAATTTTGAATGCAT
AGCGCCGAAGGGTATCATCCCTTCGGCACGCCTGGTTCAGGGTTGTTTAATCGAATTCCGTCGACAGACAGTTGATGG
ATAGTCGAGGGGCTGCGCTTCGGCGTACCCTCTCTGGTTTCGAGAACTATGTTCCTAGTGAACTGTGAATTCGCGTTTG
CGTAGCCTCCATG

Figure 2.8: FASTA formatted 18S rDNA nucleotide sequence of the novel *Cruznema* sp.NTM-2021 isolate with high affinity to *Cruznema* species, Cruznema sp. GD-1 (191475.1) as revealed by the NCBI BLASTn search parameters.

2.3.3 Phylogenetic Analysis

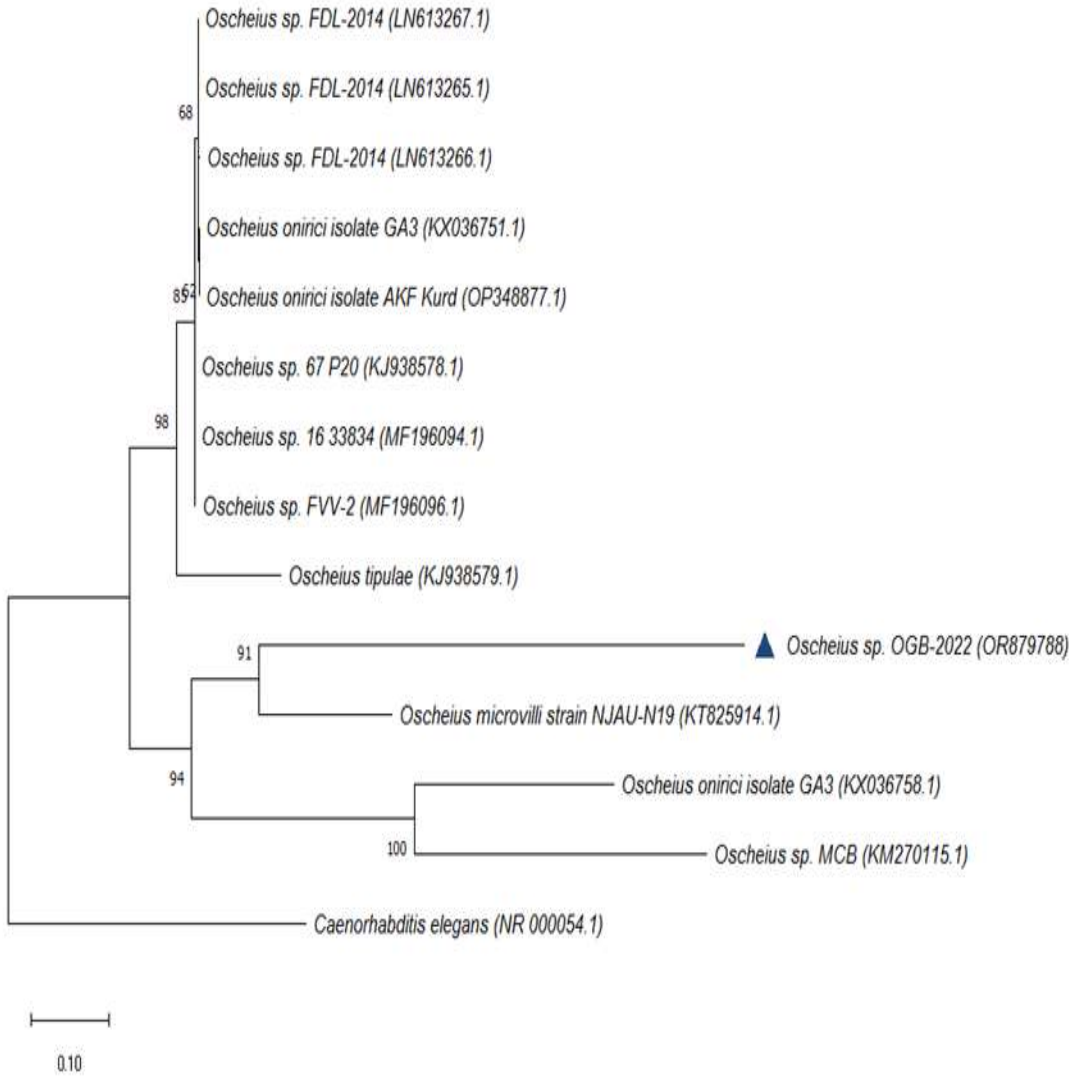

Figure 2.9: The evolutionary history of the initially unknown isolate (*Oscheius* sp. OGB-2022), inferred using the Maximum Likelihood method. Tamura-Nei model (Tamura, 1993) and the Maximum Likelihood approach were utilized to infer the evolutionary history of the initially unknown isolate, represented by a blue triangle, and numerous other EPN species. Shown on the figure is the tree with the highest log likelihood (-10131.93) with 14 nucleotide sequences. Displayed next to the branches is the percentage of the trees with the related taxa grouped together. The initial tree(s) for the heuristic search were automatically generated by incorporating the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances computed using the Tamura-Nei model, and then picking the topology with the highest log likelihood value. The tree

is drawn to scale, with branch lengths calculated using the number of substitutions per site (next to the branches). There were a total of 3099 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, 2021). The *Oscheius* sp. OGB-2022 isolate is shown on the first branch of the phylogenetic tree on top. *Caenorhabditis elegans* was used as outgroup.

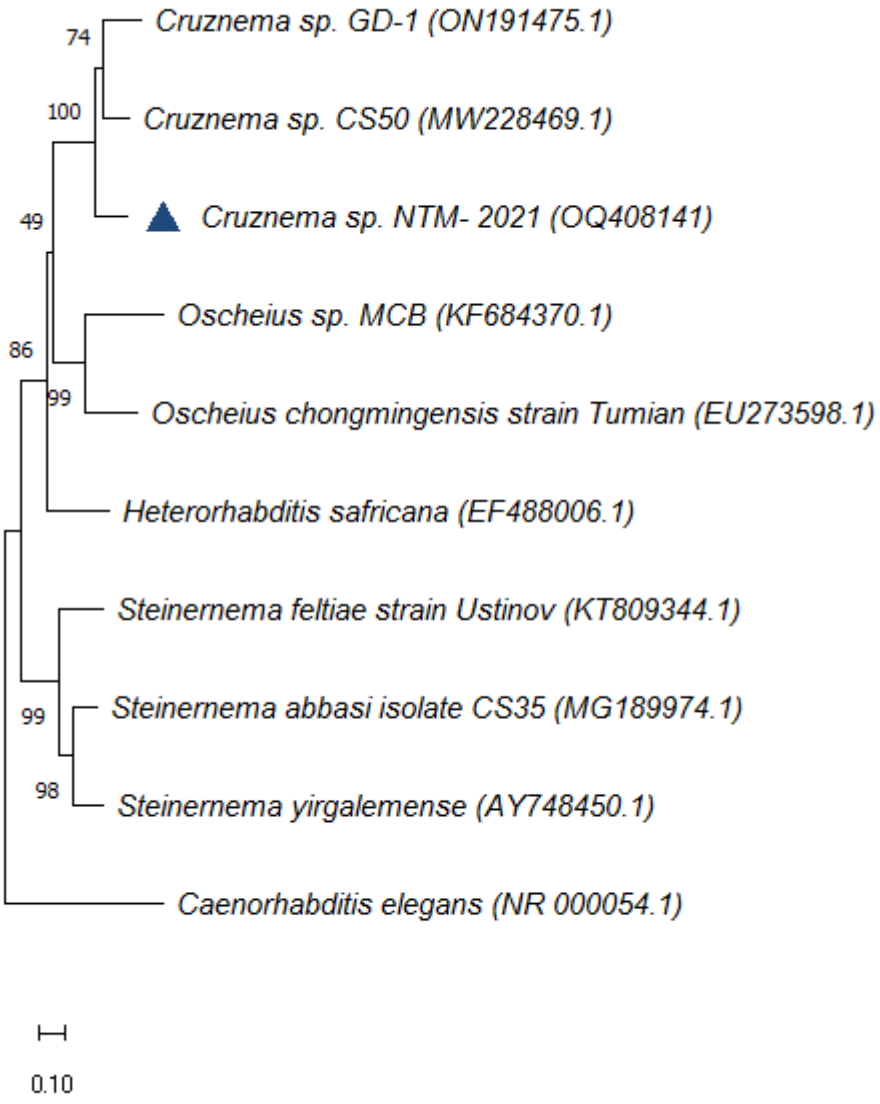

Figure 2.10: The evolutionary history of *Cruznema* sp. NTM-2021 nematode and several other EPN species inferred using Neighbor-Joining and Tamura 3-parameter method (Saitou and Nei, 1987). The tree with the highest log likelihood (-19336.15) is shown. The percentage of trees with the relevant taxa grouped together in the bootstrap test (1000 repetitions) is shown next to the branches (Felsenstein, 1985).

The tree is drawn to scale, with branch lengths matching the evolutionary distances used to estimate the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter technique (Tamura, 1992) and expressed as the number of base substitutions per site. The study includes 10 nucleotide sequences. All unclear sites in each sequence pair were removed (using the pairwise deletion method). There were a total of 2108 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, 2021). *Caenorhabditis elegans* was used as outgroup.

2.3.4 Evolutionary Divergence

The molecular evolutionary divergence between the various EPN species was computed in relation to the molecular evolutionary distances between the 18S rDNA nucleotide sequences using MEGA11 software pairwise deletion option.

Table 2.4: Estimates of evolutionary divergence between the nucleotide sequences of 13 *Oscheius* species with the initially unknown isolate (*Oscheius* sp. OGB- 2022). The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Tamura 3-parameter model (Tamura 1992). This analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence

pair (pairwise deletion option). There were a total of 3099 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Oscheius_sp._OGB-2022_(OR879786)		0,820	0,833	0,831	0,798	0,798	0,842	0,834	0,806	0,910	1,285	0,804	1,348	1,737
Oscheius_sp._67_P20_(KJ938578.1)	0,820		0,001	0,001	0,000	0,002	0,003	0,002	0,003	0,161	0,651	0,484	0,745	0,797
Oscheius_sp._FDL-2014_(LN613267.1)	0,833	0,001		0,000	0,005	0,007	0,002	0,001	0,001	0,159	0,651	0,490	0,755	0,806
Oscheius_sp._FDL-2014_(LN613265.1)	0,831	0,001	0,000		0,005	0,007	0,002	0,001	0,001	0,159	0,651	0,489	0,755	0,803
Oscheius_sp._16_33834_(MF196094.1)	0,798	0,000	0,005	0,005		0,001	0,007	0,002	0,003	0,165	0,647	0,433	0,785	0,586
Oscheius_sp._FVV-2_(MF196096.1)	0,798	0,002	0,007	0,007	0,000		0,009	0,005	0,004	0,168	0,646	0,434	0,786	0,587
Oscheius_sp._FDL-2014_(LN613266.1)	0,842	0,003	0,002	0,002	0,007	0,009		0,002	0,003	0,162	0,651	0,494	0,752	0,801
Oscheius_onirici_isolate_GA3_(KX036751.1)	0,834	0,002	0,001	0,001	0,002	0,005	0,002		0,000	0,166	0,637	0,519	0,758	0,814
Oscheius_onirici_isolate_AKF_Kurd_(OP348877.1)	0,806	0,003	0,001	0,001	0,003	0,004	0,003	0,000		0,157	0,645	0,529	0,738	0,825
Oscheius_tipulae_(KJ938579.1)	0,910	0,161	0,159	0,159	0,165	0,168	0,162	0,166	0,157			0,522	0,824	0,865
Oscheius_onirici_isolate_GA3_(KX036758.1)	1,285	0,651	0,651	0,651	0,647	0,646	0,651	0,637	0,645	0,761		0,721	0,594	1,464
Oscheius_microvilli_strain_NJAU-N19_(KT825914.1)	0,804	0,484	0,490	0,489	0,433	0,434	0,494	0,520	0,529	0,522	0,721		0,847	0,961
Oscheius_sp._MCB_(KM270115.1)	1,348	0,745	0,755	0,755	0,785	0,786	0,752	0,758	0,738	0,824	0,594	0,847		1,535
Caenorhabditis_elegans_(NR_000054.1)	1,737	0,796	0,806	0,803	0,586	0,587	0,801	0,814	0,825	0,865	1,464	0,961	1,535	

Table 2.5: Estimates of evolutionary divergence between nematode nucleotide sequences and *Cruznema* sp. NTM 2021. The number of base substitutions per site from different sequences is displayed. Tamura's 3-parameter model was used in the analyses. The analysis included 10 nucleotide sequences. Each sequence pair had all ambiguous places removed (the pairwise deletion option). The completed dataset

had a total of 2041 locations. Tamura *et al*. (2021) used MEGA11 to conduct evolutionary analysis.

	1	2	3	4	5	6	7	8	9	10
Cruznema_sp._NTM-_2021_(OQ408141)		0,296	0,292	1,256	1,123	0,832	0,754	0,751	0,621	0,628
Cruznema_sp._GD-1_(ON191475.1)	0,296		0,262	1,066	1,285	0,795	0,794	0,772	0,619	0,719
Cruznema_sp._CS50_(MW228469.1)	0,292	0,262		1,288	1,244	0,741	0,759	0,706	0,577	0,646
Oscheius_sp._MCB_(KM270115.1)	1,256	1,066	1,288		0,209	1,183	1,167	1,121	1,152	1,146
Caenorhabditis_elegans_(NR_000054.1)	1,123	1,285	1,244	0,209		1,107	1,151	1,164	1,202	1,086
Steinernema_feltiae_strain_Ustinov_(KT809344.1)	0,832	0,795	0,741	1,183	1,107		0,349	0,358	0,667	0,774
Steinernema_abbasi_isolate_CS35_(MG189974.1)	0,754	0,794	0,759	1,167	1,151	0,349		0,203	0,711	0,708
Steinernema_yirgalemense_(AY748450.1)	0,751	0,772	0,706	1,121	1,164	0,358	0,203		0,682	0,742
Heterorhabditis_safricana_(EF488006.1)	0,621	0,619	0,577	1,152	1,202	0,667	0,711	0,682		0,503
Oscheius_chongmingensis_strain_Tumian_(EU273598.1)	0,628	0,719	0,646	1,146	1,086	0,774	0,708	0,742	0,503	

2.4 Discussion

The two novel nematode species isolated from the moderately dry climatic conditions of Johannesburg, South Africa, and the dry winter climate of Leribe, Lesotho using the soil baiting and White trap setup techniques showed great pathogenicity towards the last Instar larvae of model organism *T. molitor* L. (*Coleoptera*: *Tenebrionidae*) under laboratory conditions. The two nematode isolates from this study have not been officially reported as EPNs. However, these isolates have shown to be effective in producing highest larval mortality at ambient room temperature ranging from 25- 35℃ after 4-7 days in the direct contact pathogenicity test assays. The overwhelming evidence observed from the experimental data indicates that both the newly isolated nematode species have promising potentials to be formulated and used in pest management systems as effective biocontrol agents.

Changes in the pigmentation of the nematode-infected insect host cadavers from tan to dark brown, over time, were preliminarily used to suggest entomopathogenicity and possible bacterial endosymbiosis of the nematode isolates (Park *et al*., 2011). Additionally, the infected insect host cadavers of the last Instar larvae of model organism *T. molitor* retained their original body shape and were moist and had mushy texture, of which was indicative of the presence of endosymbiotic bacteria released by

the nematodes (Blinova and Ivanova, 1987). The many symptom similarities observed on the insect host cadavers infected by these nematode isolates could be since they both belong to the same family of the phylum Nematoda. The more reliable measure of identifying these nematode isolates was done using sophisticated molecular identification techniques, which comprised of the extraction of nematode's genomic DNA, PCR amplification of the conserved ITS regions, and phylogenetic analysis (Půža *et al*., 2015).

Firstly, the FASTA formatted 18S rDNA nucleotide sequences obtained after Sanger sequencing at Inqaba Biotechnical Industries (Pty) Ltd were uploaded onto the NCBI BLAST bioinformatics tool for fair and rapid identification by aligning against the already existing nucleotide sequence data of other species on Genbank (Půža *et al*., 2015). The operational taxonomic unit (OTU) and the reliable metric of the highly conserved and hypervariable ITS regions of the 18S rDNA made it possible for the unknown nematode isolates to be successfully placed in their respective genera or families and phylogenetic affinities with other nematode species to be formed (Půža *et al*., 2015). The evidence from the 18S rDNA molecular characterization and the phylogenetic analysis of these two nematode isolates confirmed that they belong and are closely related to other nematode species from the *Oscheius* and *Cruznema* genera of the phylum Nematoda, under the family *Rhabditidae*. The bioinformatics analysis enabled the accurate identification of the new isolates using 18S rDNA.

The highest match was shown amongst the query sequences and the subject sequences by the NCBI BLAST algorithm parameters. The first query sequence obtained from the nematode isolates recovered from the soil samples collected in Johannesburg, South Africa around the Braamfontein area showed highest similarity to the subject nucleotide sequences of *Oscheius onirici* (OW015469.1) and *Oscheius* sp. FDL-2014 (LN613267.1), with the maximum similarity score of 749 and 739, respectively, implying that a significant proportion of similarity was obtained between the query sequences and the subject sequences. These sequences further showed the query coverage values of 98% and 99%, implying that about 98% and 99% of the query sequence lengths were incorporated in the aligned sequence segments of the subject sequences (Newell *et al*., 2013).

The number of hits with similar scores predicted by probability when searching a database of specific size was defined by the expected value (E-value), with low E-values implying relevance in terms of alignment and hit scores. The E-values of 0.0 indicated that the hits on the NCBI BLAST were of significance, for the subject sequences, with the highest identity parameter representing how closely the subject and query sequences were related over the length of coverage (Newell *et al*., 2013). The hit scored 82.38% and 82.05% similarities, respectively, indicating that the nucleotide sequences of the query and the subject diverged by 17.62% and 17.95%. The phylogenetic analysis was used to determine the taxonomic affiliations of the nematode isolate with other EPN species, and a sister taxon was formed with *Oscheius microvilli* strain NJAU-N19 (KT825914.1), henceforth a suggested name of *Oscheius* sp. OGB-2022 and an accession number OR879788 were assigned to this nematode isolate. Moreover, observations made from the NCBI BLAST search results of the query sequence of the nematode isolate recovered from the soil sample collected in Leribe, Lesotho showed that there was a maximum similarity score of 379 and a query coverage of 64%, with a percentage identity of 88.44% between the query nucleotide sequence and the subject sequence of *Cruznema* sp. GD-1 (ON191475.1). A suggested name of *Cruznema* sp. NTM-2021 and an accession number OQ408141 were assigned to this nematode isolate.

Only molecular based identification techniques were utilized for the accurate characterization and identification of these nematode isolates because identifying nematodes based on their morphological characteristics is often difficult due to their highly anatomical similarities which may be due to convergent evolution which resulted in the occurence of identical anatomical qualities and characteristics in evolved nematodes (Blaxter *et al*., 1998). Due to high similarities of morphological characteristics, the identification of nematode species within a genus is therefore very challenging (Tabassum *et al*., 2016). Furthermore, there is a difficulty in confirming the bacterial symbiosis of *Cruznema* and other nematode species from the family *Rhabditidae*, as most of them especially the bacterivorous nematodes possess morphological features such as the probolae, which projects from the lip region and is used for harvesting soil bacteria as the primary nutrition source (Ye *et al*., 2018). Other nematode species such as *Acrobeles complexus* and most members of the order Rhabditida ingest soil fluid with bacteria without any specific morphological

adaptations (Tabassum *et al*., 2016). Furthermore, *Cruznema tripartite* is one of the nematode species that has a tube-shaped mouth used take up soil fluid. The EPN-like characteristics and virulence exhibited by the nematode isolates from this study implies potential entomopathogenicity. The nematode isolates *Oscheius* sp. OGB-2022 (OR879788) and *Cruznema* sp. NTM-2021 (OQ408141) could potentially be used and commercialized as effective biocontrol agents due to their insect-parasitic abilities.

2.5 Conclusion

Under laboratory conditions, the two nematode species isolated from the moderately dry climatic conditions of Johannesburg, South Africa, and the dry winter climate of Leribe, Lesotho using soil baiting and White trap setup techniques demonstrated high pathogenicity towards the last Instar larvae of the model organism *T. molitor* L. (*Coleoptera: Tenebrionidae*). The 18S rDNA molecular characterisation and phylogenetic analysis of these two nematode isolates verified that they belong to and are closely related to other nematode species in the *Oscheius* and *Cruznema* genera of the Nematoda phylum. The bioinformatics analysis allowed for the correct identification of the isolates using 18S rDNA. The *Cruznema* sp. NTM-2021 and *Oscheius* sp. OGB-2022 nematodes from this investigation have not officially been classified as EPNs. In direct contact pathogenicity tests, these isolates produced the maximum larval mortality at room temperatures ranging from 25-35℃ after 4-7 days. The overwhelming evidence from the experimental data suggests that both the newly isolated *Cruznema* sp. NTM-2021 and *Oscheius* sp. OGB-2022 isolates have promising potential for usage in pest management systems as EPN biocontrol agents. Furthermore, the utilization of the cytochrome oxidase subunit I mitochondrial gene (COI) will be extremely useful in the molecular identification of newly isolated EPN species.

2.6 References

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3 Chapter Three: Isolation and 16S rDNA Molecular Characterization of bacteria associated with *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes.

3.1 Introduction

EPNs under the families *Steinernematidae*, *Heterorhabditidae*, and *Rhabditidae* of the phylum Nematoda are insect-parasitic roundworms exhibiting a holoparasitic mode of survival (Bhat *et al*., 2020). These microorganisms can survive in almost every natural habitat aside from the psychrophilic conditions of Antarctica (Hominick 2002). EPN species from the genera *Steinernema*, *Heterorhabditidae*, and *Oscheius* have been considered parasitic towards a wide variety of problematic crop pests in the agricultural industries (Liu *et al*. 2020). Approximately 100 species of the genus *Steinernema,* 17 species of genus *Heterorhabditis,* and 8 species of genus *Oscheius* have globally been reported as entomopathogenic towards a wide range of insect pests (Bhat *et al*. 2020).

These nematode species live in mutualism with their Gram-negative endosymbiotic bacteria which also play a critical role in their nutritional physiology (Feldhaar 2011). These endosymbionts include *Xenorhabdus*, *Photorhabdus*, and *Serratia*, respectively (Kaya and Gaugler 1993). The free-living dauer juveniles of EPNs residing in the soil with non-foraging behaviours act as vectors for the endosymbiotic bacteria responsible for the pathogenicity of the insect hosts (Adams and Nguyen 2002). This endosymbiotic bacterium is released into the insect host's hemocoel after the dauer juveniles invade the host through any inherent apertures being either the mouth, anus, or respiratory spiracles (Adams and Nguyen 2002; Bhat *et al*., 2020). After release into the bloodstream of the insect host, bacterial cells then proliferate and generate toxic antimicrobials with high insecticidal properties, thus causing infection and eventual death of the host within 48hours (Adams and Nguyen 2002).

Moreover, nourishment, growth, and reproduction of EPNs within the insect host cadaver is then promoted by the bacteria until the nutrition sources are depleted (Stock 2015). A new cohort of EPNs emerge from within the insect host cadaver into the soil matrix to scout for more susceptible insect hosts to infect, following the depletion of nutrition sources within the cadaver (Tomar *et al*., 2022). The field application of both EPNs and their bacterial endosymbionts to control problematic crop pests in the

agricultural sectors has become a common practice in the biocontrol sectors as well as in IPM systems (Stock 2015). Ecologically safer pest management products have been used in a sustainable agricultural perspective to diminish the over utilization of harmful chemical insecticides which have shown emerging dissatisfaction, unfavourable outcomes on human health and the ecology (Tomar *et al*. 2022).

Bacterial endosymbionts have recently been discovered to be a treasure trove of bioactive and insecticidal compounds, as they can reduce residues of chemical pesticides used in pest management and plant fortification by steadying ecological variations (Thakur *et al*., 2020, 2021; Migunova and Sasanelli 2021). Because of the high potential of the harmful bioactive complexes and proteins they produce as secondary metabolites, these bacterial endosymbionts can surely become useful alternatives in the biocontrol of several phyto-crop pests (Thakur *et al*. 2022a, 2022b). The purpose of this study was to isolate and molecularly characterize the 16S rDNA region of the related bacterial isolates extracted from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes.

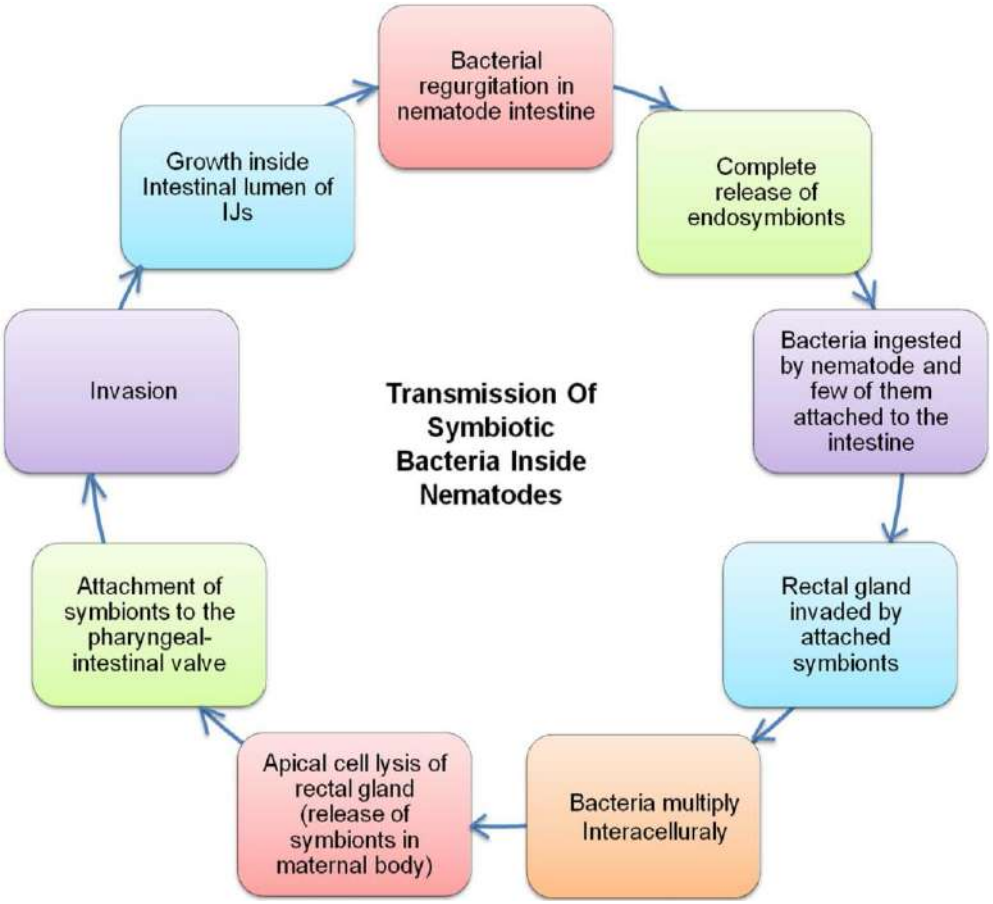

Figure 3.1: Cyclic events occurring during the transmission of the bacterial endosymbionts within the nematodes (Tomar *et al*., 2022).

3.2 Materials and Methods

3.2.1 Extraction of associated bacteria from 0.1% NaClO surface sterilized nematodes.

A pure suspension of freshly emerged nematode IJs was collected from the White trap setups into sterilized 50mL Falcon tubes and allowed to settle for approximately 2 hours. After settling, excess water was removed using sterile 5mL plastic Pasteur pipettes and the IJs were collected into a single tube. The nematode suspension in the 50mL Falcon tubes was surface sterilized by immersing in 5mL 0.1% (NaClO) for 2 hours (see Appendix 2). Moreover, the nematode suspension in the 50mL Falcon tubes were further rinsed once with 5mL of the Ringer's solution and left for approximately 2 hours to settle. The excess Ringer's solution was removed from the Falcon tubes using the sterile 5mL plastic Pasteur pipettes and the nematodes were then rinsed thrice with 10mL sterile autoclaved water. Furthermore, the water was

removed from the Falcon tubes and the nematodes were then aseptically transferred into sterile 1.5mL microcentrifuge tubes under the laminar flow hood before being pulverized into a thin paste using a sterile autoclaved plastic pestle. Approximately 1mL of nutrient broth bacterial growth medium was added into the 1.5mL microcentrifuge tubes (see Appendix 1). The tubes were then placed inside the 50mL Falcon tubes and incubated in the dark at 37 ℃ for 24 hours on an orbital shaker set at 150rpm. After the incubation period had elapsed, 500μL of the inoculum was aseptically streaked on the MacConkey and Nutrient Bromothymol Triphenyltetrazolium Agar (NBTA) selective and differential bacterial growth media using a sterile inoculation loop (see Appendix 1). The plates were incubated at 25℃ in the dark for 24 hours to allow for bacterial colony growth.

3.2.2 Evaluation of bacterial cell-free supernatant toxicity against *T. molitor* last Instar larvae.

The cell-free supernatant was acquired using the modified method described by Vicente-Díez *et al* (2021). Pure bacterial colonies of each bacterial isolate were inoculated into nutrient broth and incubated in the dark at 25℃ overnight, on an orbital shaker set at 150rpm. Minimal 1mL aliquots from each bacterial suspension were obtained and stored at -80℃ for 24 hours prior to inoculation of 100 μL aliquots in 50mL Luria-Bertani (LB) broth in sterile 50mL Falcon tubes. The 50mL Falcon tubes were further incubated for 72 hours at 25℃ in the dark, on an orbital shaker set at 150rpm. After the incubation period had elapsed, the bacterial suspensions were each centrifuged 9 times (3x in nuclease-free tubes) at full speed (10 000xg) and stored in the cold room (4℃) for 40 minutes to ensure the retrieval of a cell-free supernatant. Prior to utilization in toxicity tests and briefly after the final centrifugation step, the filtrates were defined as cell-free supernatant.

Moreover, surface sterilized last Instar larvae of *T. molitor* (n=10) were set in 90mm diameter petri dishes lined with two 90mm diameter Whatman filter paper. The filter papers were moistened with approximately 3mL of sterile distilled water before the foliar application of a 2-3mL concentration of the cell-free supernatant obtained after 15 minutes centrifugation of a pure bacterial colony at 10000xg. After application, the petri dishes were sealed with parafilm and incubated at 25℃ incubation temperature for a 7-day incubation period. A 3mL aliquot of a sucrose- based solution (2g/ 20mL)

was applied every 2 days to supplement the nutritional physiology (Vicente-Díez *et al*., 2021). Larval mortality induced by the bacterial cell-free supernatant was assessed every 24hours for a period of 7 days. These experiments were conducted for both *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode isolates. There were three trials of which each had three plates. Application of 2-3mL sterile distilled water served as a control (Kumar *et al*., 2011).

3.2.3 Morphological and biochemical analysis of bacteria isolated on different media.

3.2.3.1 Gram staining

A bacterial colony was obtained from the NBTA plate and mixed with water to create a smear on a sterilized glass slide. The smear was air-dried, heat-fixed, soaked with crystal violet stain for 60 seconds, washed with distilled water for 30 seconds, prior to the addition of Gram's iodine which was left for 60 seconds then washed off for 30 seconds. The smear was decolorized for 15 seconds and counterstained with Safranin for 60 seconds, then rinsed with distilled water, air-dried, and examined under an Olympus light microscope at x100 magnification (Kumar *et al*., 2011).

3.2.3.2 Catalase test

A sterilized inoculation loop was utilized to attain unalloyed bacterial colony from the NBTA plate onto a sterile glass microscope slide. A drop of freshly prepared solution of 3% Hydrogen peroxide was mixed into the inoculum. Interpretation of the test was made upon the catalase (fizzling) reaction. To avoid false-positive catalase results, a hardwood stick was utilized rather than a metal loop.

3.2.3.3 Endospore staining test.

A pure bacterial colony was obtained from the NBTA culture plate using a sterile inoculation loop and posited on a minimal drop of deionized water on a sterile microscope slide. The bacterial colony was thinly and thoroughly blended with a drop of water to create a smear. The smear was air-dried under a laminar hood flow, and heat fixed by passing over a Bunsen burner flame. The heat-fixed smear was then coated with a square blotting paper and the malachite green stain solution was used to contantly saturate the blotting paper (Kumar *et al*., 2011). The blotting paper covered glass slide was steamed for 5 minutes over a 25mL glass beaker of boiling

water, while continuously applying the dye. After the steaming period had elapsed, the slide was washed under running tap water for 30 seconds. Safranin was used to counterstain for 30 seconds. The slide was then washed under running tap water for 30 seconds, blot dried, and examined under the Olympus light microscope for presence of endospores.

3.2.4 16S rDNA Molecular Characterization of the nematode-associated bacteria.

3.2.4.1 Bacterial Genomic DNA Extraction

Bacterial genomic DNA was extracted from the pure bacterial colonies on the selective culture media following the bacterial genomic DNA extraction and purification protocol specified on the E.Z.N.A. ® Bacterial DNA Kit (omega BIO-TEK Revision No: 2.0) (see Appendix 4). Prior to this protocol, overnight bacterial cultures were prepared by inoculating bacterial colonies on 25mL Luria-Bertani (LB) broth in 50mL Falcon tubes and incubating in the dark at 30 ℃ for 24 hours on an orbital shaker at 150rpm. After the incubation period had elapsed, 1.5mL of the bacterial cultures was obtained from the 50mL Falcon tubes and dispensed into sterile 1.5mL using a 1000μL pipette. The 1.5mL microcentrifuge tubes containing 1.5mL bacterial samples were centrifuged at 4000 xg for 10 minutes at room temperature. This step was repeated.

100μL TE Buffer was added, and the tubes were vortexed to resuspend the pellet. Furthermore, 10μL Lysozyme resuspended with Elution Buffer was added and the tubes were incubated at 37℃ for 10 minutes. A 20μL Proteinase K solution and 100μL TL Buffer were added, and the tubes were vortexed to thoroughly mix the contents before incubating at 55℃ in a shaking water bath. A 5μL RNAse A solution was added, and the tubes were inverted several times to mix, and allowed to sit at room temperature for 5 minutes. This was followed by centrifugation at 10,000xg for 2 minutes to pellet any undigested material. The resulting supernatant was transferred to new 1.5mL microcentrifuge tubes without disturbing the pellet. Moreover, 220μL BL Buffer was added, and the tubes were vortexed to mix thoroughly prior to incubation at 65℃ for 10 minutes.

A 220μL volume of the 100% ethanol was further added to the tubes before briefly vortexing for 20 seconds at maximum speed to mix thoroughly. The precipitates were broken by pipetting up and down 10 times. Additionally, HiBind® DNA Mini columns

were inserted into 2mL Collection tubes, and the entire sample together with any precipitate that may have resulted was conveyed into the HiBind® DNA Mini Column. The tubes were briefly centrifuged at 10,000xg for 1 minute. The filtrates and the collection tubes were then discarded. Prior to the addition of 500μL HBC Buffer and centrifugation at 10,000xg for 1-minute, new HiBind® DNA Mini Columns were inserted to new 2mL collection tubes. Following centrifugation, filtrates were discarded, and the collection tubes were reused. Moreover, 700μL DNA Wash Buffer diluted with 100% ethanol was added to the tubes before further centrifugation at 10,000xg for 1 minute. Filtrates were discarded and the collection tubes were reused. This step was repeated for a second DNA Wash Buffer wash. For eliminating trace ethanol that may interfere with downstream application, the empty HiBind® DNA Mini Columns were centrifuged at maximum speed for 2 minutes to dry the column and were inserted in new nuclease-free 1.5mL microcentrifuge tubes. Elution buffer (50μL -100μL) heated to 65℃ was added to the tubes and let sit for 3-5 minutes at room temperature before centrifuging at 10,000xg for 1 minute to elute the DNA. This step was repeated for a second elution and the eluted DNA was stored at -20℃.

3.2.4.2 Polymerase chain reaction (PCR) for amplification of 16S rDNA region.

Table 3.1: PCR components for amplification of 16S rDNA region.

Reagents	Volume
NEB OneTaq 2X MasterMix	12.5µL
Genomic DNA	1µL
16S-27 Forward primer	1µL
16S-1492 Reverse primer	1µL
Nuclease free water (E476)	9.5µL

Table 3.2: PCR conditions for amplification of 16S rDNA region.

Amplification stage	Temperature (℃)	Time
Initial denaturation	94°C	5 minutes
Denaturation	94°C	30 seconds

Annealing	50°C	30 seconds
Primer elongation	68°C	1 minute
Final primer elongation	68°C	10 minutes

Table 3.3: Universal primer sequence information for amplification of 16S rDNA region.

Primer	Primer Target	Primer Sequence
16S-27 Forward Primer	16S rDNA Sequence	5'-AGAGTTTGATCTGGCTCAG-3'
16S-1492 Reverse Primer	16S rDNA Sequence	5'-CGGTTACCTTGTTACGACTT-3'

3.2.4.3 Agarose gel electrophoresis

The NEB Fast Ladder was used on all gels (N3238) as size standard when visualizing the integrity of the PCR amplicons on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye.

3.2.4.4 Sanger sequencing of nematode-associated bacteria's 16S rDNA region for molecular identification and sequence alignment.

The 16S rDNA amplification of the PCR products was performed by Sanger sequencing method using the desired 16S-27 Forward and 16S-1492 Reverse universal primers at Inqaba Biotechnical Industries (Pty) Ltd, South Africa. FinchTV Version 1.4.0 trace data viewing software was used for trimming the Sanger sequenced DNA chromatograms generated for the EPN genomic DNA samples. The trimmed DNA sequence data for both the 16S-27 Forward and 16S-1492 Reverse universal primers were uploaded to BioEdit Version 7.2 biological sequence alignment editor software for FASTA formatted consensus sequence generation. The FASTA formatted consensus sequence data were uploaded as queries to NCBI BLASTn for the identification of the unknown bacterial sequence. The submitted query sequences were searched and aligned against the bacterial genomic DNA sequence data already in the GenBank sequence database, by the NCBI BLASTn tool. The statistical similarities of the query sequence data were generated by the NCBI BLASTn alignment algorithmic tool against sequences in the GenBank database, ranging from

highest to lowest percentage from which interim notions regarding phylogenetic affinities were roughly deduced in relation to the submitted query sequence.

3.2.4.5 Phylogenetic Analysis using MEGA11 software.

The FASTA formatted DNA nucleotide sequences generated from the 16S rDNA amplification of the PCR products were used in the phylogenetic analysis of the newly isolated bacterial species. These FASTA formatted sequences of known bacterial species were recovered from the NIH GenBank® genetic sequence database using their designated accession numbers. The MEGA11 software was used for analysing the DNA nucleotide sequence data from known and unknown bacterial species, using *Escherichia coli* strain U 5/41 (NR_024570.1) as outgroup. The phylogenetic trees with the highest log likelihood were constructed using the Maximum Likelihood method and Tamura-Nei model after aligning the nucleotide sequences data with the MUSCLE tool (Tamura 1993). The construction and the percentage of the phylogenetic trees was based on the clustering of the associated taxa on 1000 replicates in the bootstrap statistical test, with branch lengths measured in the number of substitutions per site (Tamura 2021).

The phylogenetic tree for bacterial isolate S1 was constructed on the MEGA11 software using the selected taxa below: *Alcaligenes faecalis* strain ARD39 (KX023244.1), *A. faecalis* subsp. faecalis strain SK12 (KC790302.1), *A. faecalis* strain Y5 (KF925435.1), *A. parafaecalis* strain G (NR_025357.1), *A. faecalis* strain IAM 12369 (NR_043445.1), *A. aquatilis* strain LMG 22996 (NR_104977.1), *A. faecalis* strain NBRC 13111 (NR_113606.1), *A. aquatilis* strain LMG 22996 (NR_114959.1), *A. pakistanensis* strain NCCP-650 (NR_145932.1), *A. endophyticus* strain AER10 (NR_156855.1), *A. ammonioxydans* strain HO-1 (NR_180910.1).

The phylogenetic tree for bacterial S2 was constructed on the MEGA11 software using the selected taxa below: *Pseudomonas fluorescens* strain MG26 (MN295943.1), *Pseudomonas* sp. strain 4B3 (MH379786.1), *P. brenneri* strain PR05 (KX187328.1), *P. fluorescens* strain NU04 (KX187323.1), *P. marginalis* strain KP02 (KX187317.1), *P. proteolytica* strain BD08-00551 (KU647672.1), *P. yamanorum* strain LBUM636 (MG461475.1), *P. aeruginosa* strain ARM2 (PP101309.1), *P. syringae* strain K15 (OR725081.1), *P. viridiflava* strain ZHDD (PP032941.1), *P. savastanoi* strain 30CH (OR603169.1).

The phylogenetic tree for bacterial isolate O1 was constructed on the MEGA11 software using the selected taxa below: *Citrobacter freundii* ATCC 8090 (KM515969.1), *C. freundii* strain PYI-2 (MT039504.1), *C. portucalensis* strain YPI-2 (MN744335.1), *C. murliniae* strain HNJZ (OQ771952.1), *Citrobacter* sp. strain A316 (OP364004.1), *C. freundii* strain CPO 2.209 (OR426155.1), *Citrobacter braakii* strain CDC 80-58 (AF025368.1), *C. portucalensis* (PP095661.1).

The phylogenetic tree for bacterial isolate O2 was constructed on the MEGA11 software using the selected taxa below: *C. freundii* strain PYI-2 (MT039504.1), *Citrobacter* sp. strain A316 (OP364004.1), *C. portucalensis* strain YPI-2 (MN744335.1), *C. freundii* strain CPO 2.209, *C. braakii* strain CDC 80-58 (AF025368.1), *C. braakii* strain 167 (NR_028687.1).

The phylogenetic tree for bacterial isolate C1 was constructed on the MEGA11 software using the selected taxa below: *C. freundii* strain PYI-2 (MT039504.1), *C. portucalensis* strain YPI-2 (MN744335.1), *Citrobacter* sp. strain A316 (OP364004.1), *C. freundii* strain CPO 2.209, *C. braakii* strain CDC 80-58 (AF025368.1), *C. freundii* ATCC 8090 (KM515969.1), *C. freundii* (AF025365.1), *C. freundii* strain GZC171 (MW898667.1).

3.3 Results

3.3.1 Isolation of associated bacteria from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes.

Associated bacteria extracted from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes were grown on both NBTA and MacConkey agar selective and differential bacterial growth media.

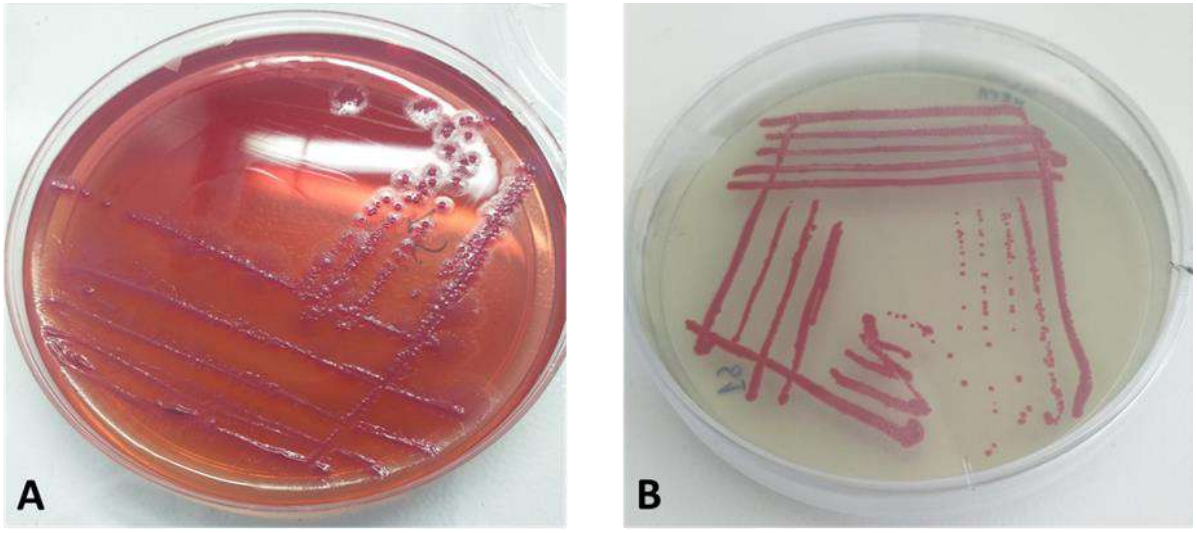

Figure 3.2: Bacterial colonies of associated bacteria isolated from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 nematode isolate. Brick-red bacterial colonies of bacterial isolate S1 grown on both selective and differential media MacConkey agar (A) and NBTA (B).

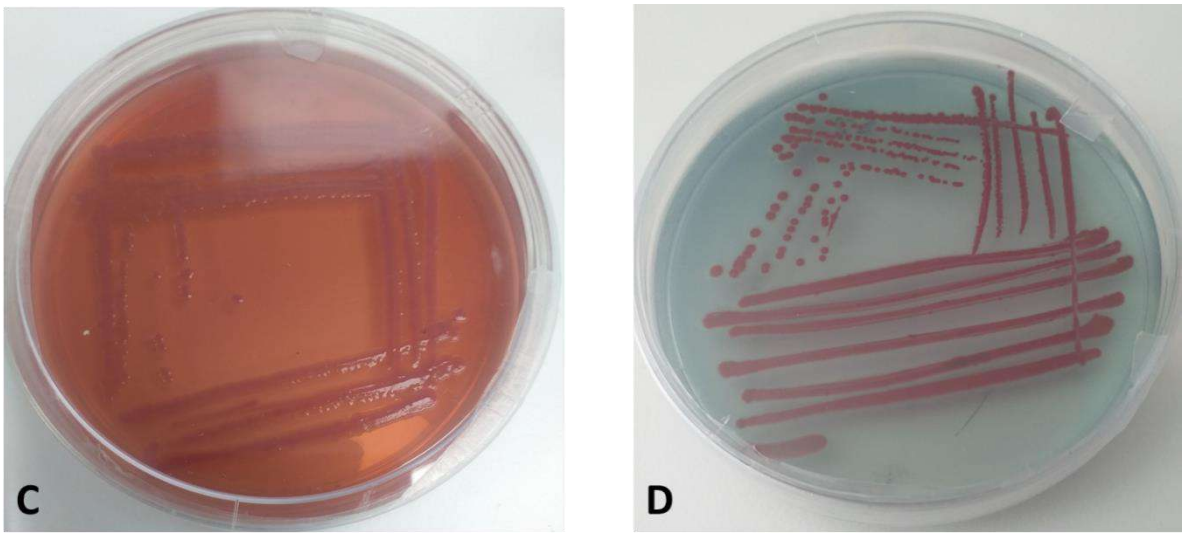

Figure 3.3: Bacterial colonies of associated bacteria isolated from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 nematode isolate. Brick-red bacterial colonies of isolate S2 grown on both selective and differential media MacConkey agar (C) and NBTA (D).

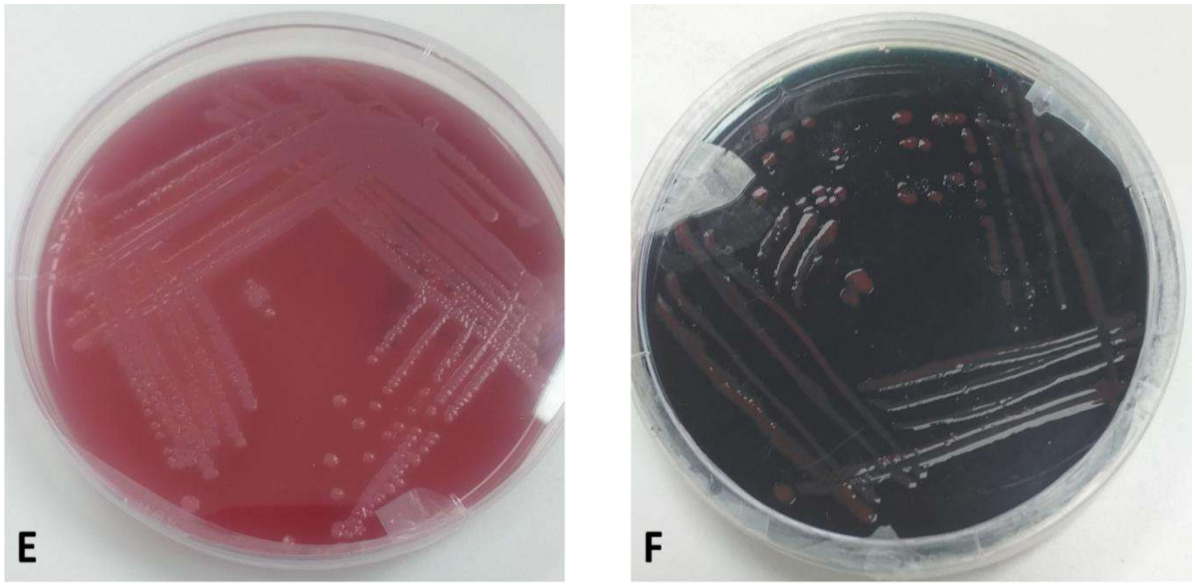

Figure 3.4: Bacterial colonies of associated bacteria isolated from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB- 2022 nematode isolate. Brick-red bacterial colonies of isolate O1 grown on both selective and differential media MacConkey agar (E) and NBTA (F).

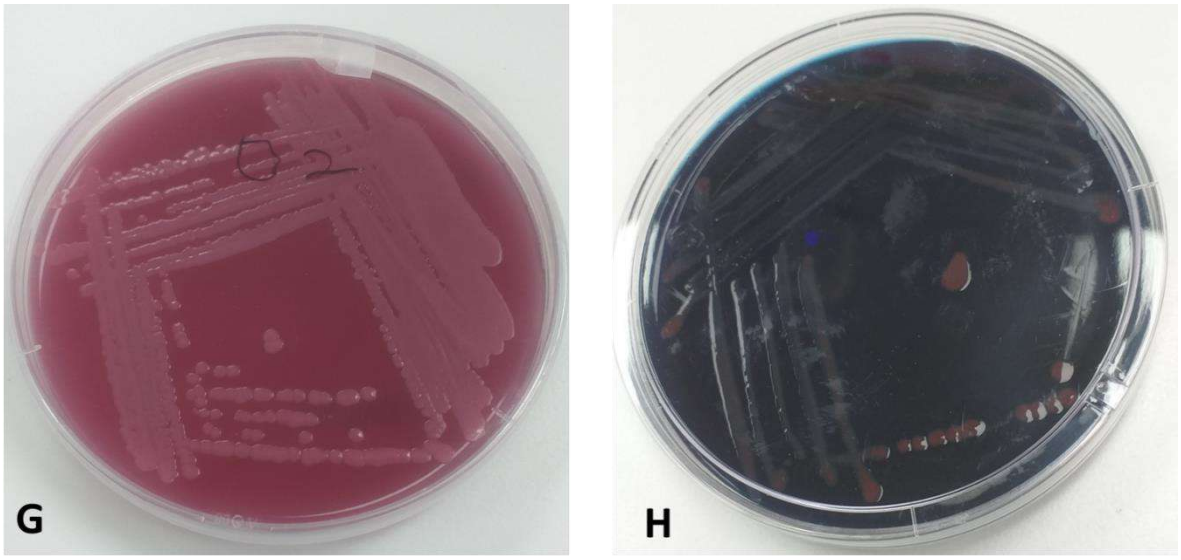

Figure 3.5: Bacterial colonies of associated bacteria isolated from 0.1% NaClO surface sterilized IJs of *Cruznema* sp. NTM-2021 nematode isolate. Brick-red bacterial colonies of isolate O2 grown on both selective and differential media MacConkey agar (G) and NBTA (H).

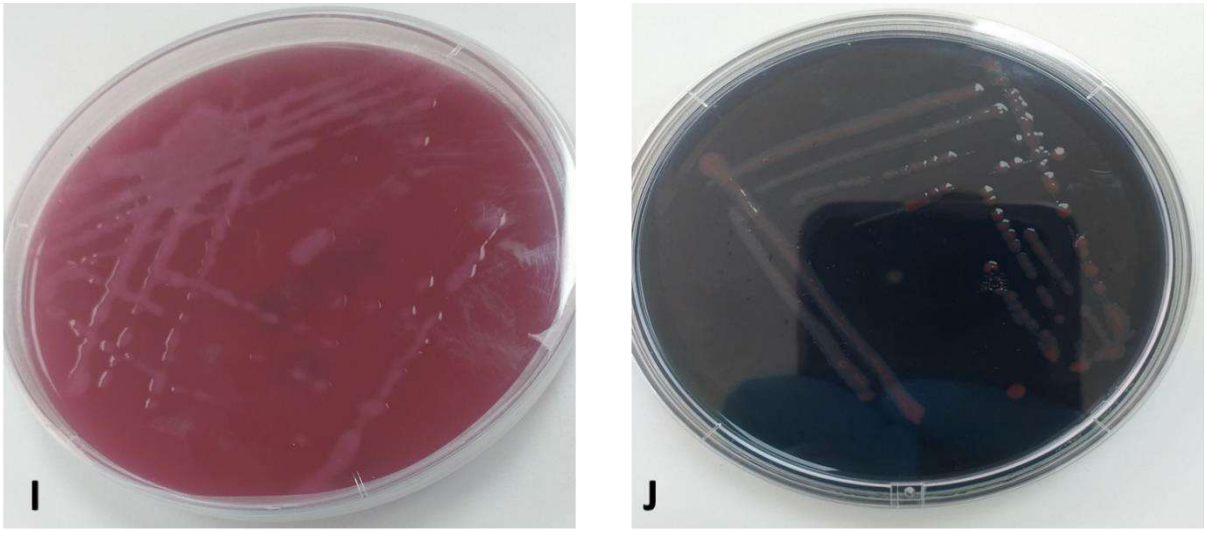

Figure 3.6: Bacterial colonies of associated bacteria isolated from 0.1% NaClO surface sterilized IJs of *Cruznema* sp. NTM-2021 nematode isolate. Brick-red bacterial colonies of isolate C1 grown on both selective and differential media MacConkey agar (I) and NBTA (J).

3.3.2 Evaluation of bacterial cell-free supernatant toxicity against *T. molitor* last Instar larvae.

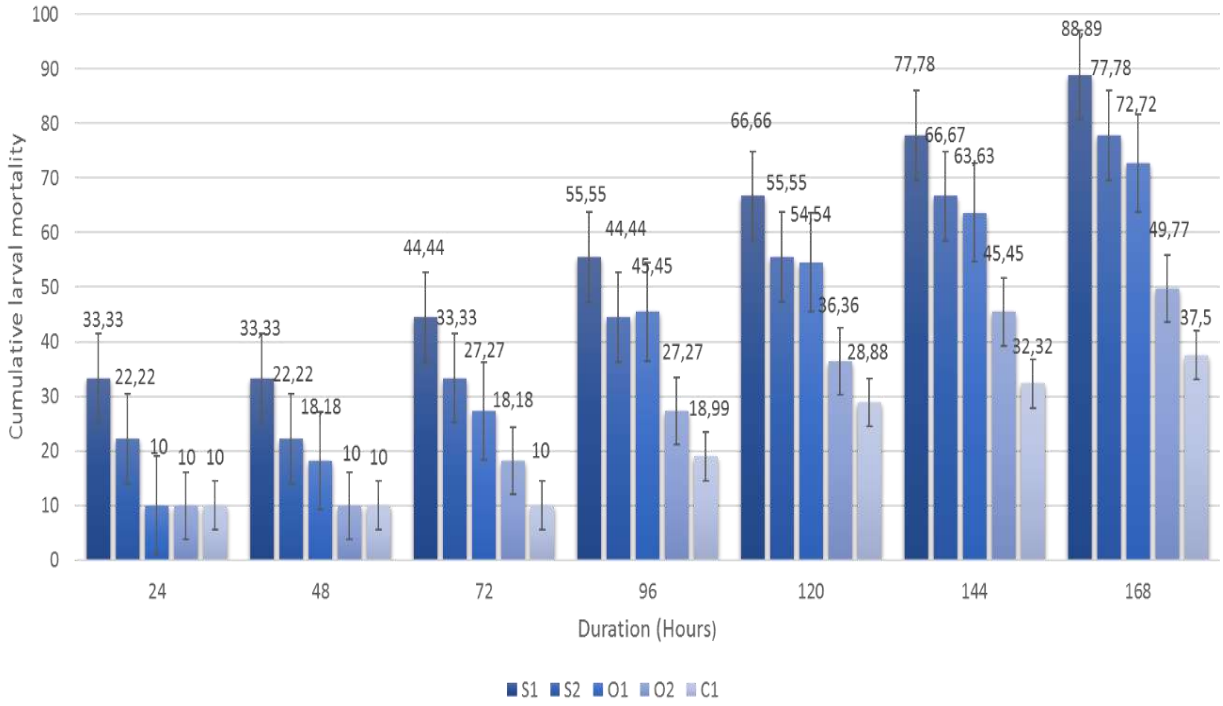

Figure 3.7: Cumulative larval mortality instigated by the toxicity of the cell-free supernatant from bacterial isolates extracted from 0.1%NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021. The error bars graphically represented the standard error of the means (Fcrit= 3.76; p< 0.05).

3.3.3 Morphological and biochemical analysis of bacterial isolates.

3.3.3.1 Gram-stain

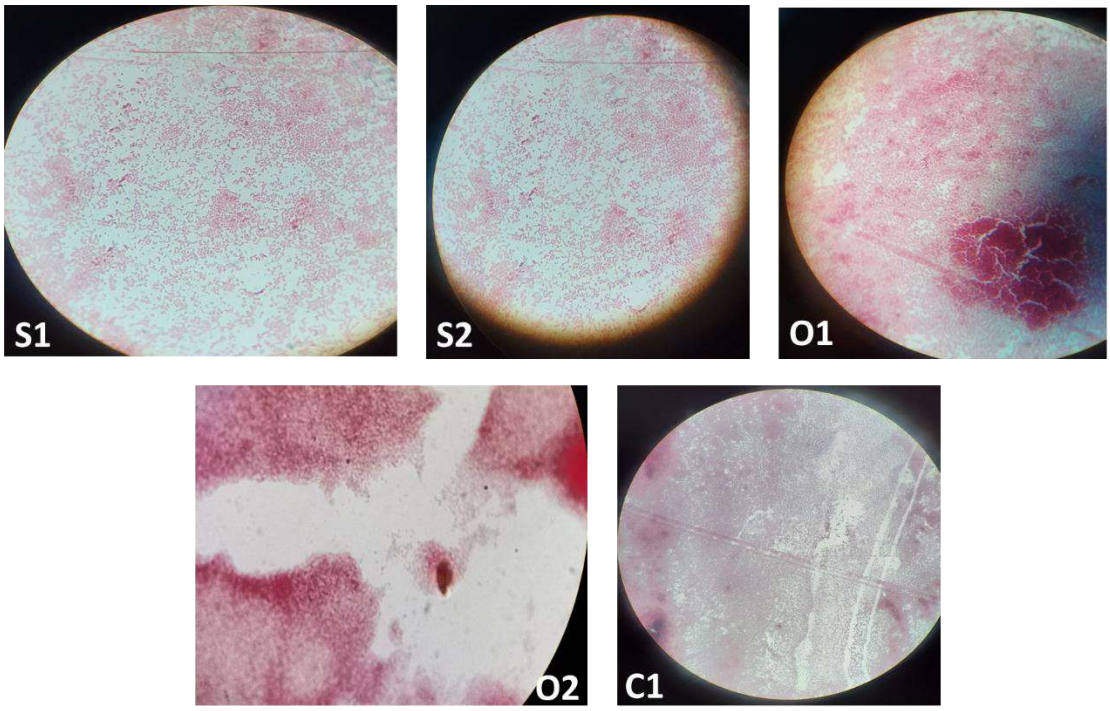

Figure 3.8: Gram stain of all the associated- bacterial isolates, all isolates appeared to have a Gram-negative (-) stain.

3.3.3.2 Catalase test

Figure 3.9: A diagrammatical representation of a catalase negative (-ve) bacterial isolate. A single bacterial isolate extracted from Oscheius sp. OGB-2022 nematodes serving a representative for catalase (-ve) isolates extracted from *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes.

3.3.3.3 Endospore staining test.

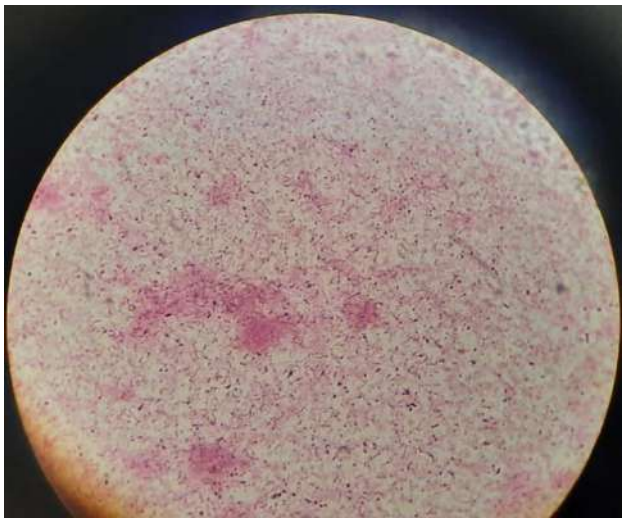

Figure 3.10: A diagrammatic representation of a negative endospore stain bacterial isolate. A single bacterial isolate serves as a representative for non-spore forming bacterial isolates extracted from *Oscheius* sp.OGB-2022 and *Cruznema* sp. NTM-2021 nematodes.

Table 3.4. Morphological characteristics of bacterial isolates on NBTA.

Bacterial isolates	Cell shape	Colony morphology					Gram stain	Spore formation
		Shape	Elevation	Size	Colour	Texture		
S1	Short rods	Regular margins (Circular)	Flat	Small	Brick-red	Smooth	-	-
S2	Short rods	Regular margins (Circular)	Slightly elevated	Small	Brick-red	Smooth	-	-
O1	Short rods	Regular margins (Circular)	Slightly elevated	Medium	Brick-red	Smooth	-	-
O2	Short rods	Regular margins (Circular)	Slightly elevated	Medium	Brick-red	Smooth	-	-
C1	Rod shaped	Regular margins (Circular)	Slightly elevated	Small	Brick-red	Smooth	-	-

Positive (+); Negative (-).

Table 3.5: Morphological characteristics of bacterial isolates on MacConkey agar.

Bacterial Isolates	Colony morphology				
	Shape	Elevation	Size	Colour	Texture
S1	Irregular margins	Flat	Small	Pinkish Red	Rough
S2	regular margins	Flat	Medium	Pink	Smooth

O1	Regular margins	Slightly elevated	Small	Pinkish red, Brick-red	Rough
O2	Regular margins	Slightly elevated	Medium	Brick- red	Smooth
C1	Regular margins (Circular)	Flat	Medium	Brick- red	Smooth

Table 3.6: Biochemical characteristics of bacterial isolates.

	Bacterial isolates				
	S1	S2	O1	O2	C1
Biochemical test					
Catalase test	+	+	+	+	+
Carbohydrate fermentation					
Lactose	+	-	+	+	+

+Positive, - Negative

3.3.4 16S rDNA Molecular Characterization of nematode-associated bacteria.

3.3.4.1 Bacterial Genomic DNA Extraction

FASTA formatted 16S rDNA nucleotide sequence data uploaded as queries to the NCBI BLASTn for the identification of the unknown bacterial isolates are illustrated in (Figure 3.8, 3.9, 3.10, 3.11, and 3.12) below.

>ATGCTTTACACATGCAGTCGAACGGCAGCGCGAGAGAGCTTGCTCTCTTGGCGGCGAGTGG
CGGACGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTG
GCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAG
CGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCT
GGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATTTTGGACAATGAGGGAAACCCTGATCCAGCCATCCCGCGTGTATGATGAA
GGCCTTCGGATAGTAAAGTACTTTAGGCAGAGAAGAAAAGGTATCCCCTAATACGGGATGCTG
CTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGA
TGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTCAGA
GGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCG
AAGGCAGCCCCTCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCGTTAGGCCTTA
GTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAA
AACCTTACCTACCCTTGACATGTCTGGAAAGCCGAAGAGATTTGGCCGTGCTCGCAAGAGAAC
AGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACAC
GTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCC
GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGC
GGATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGG
GAGTGGGTTTCACCAGAAGTAGGTAGCCTAACCGTAAGGAGGGCGCTACCACGTGAT

Figure 3.11: FASTA formatted 16S rDNA nucleotide sequence of the bacterial isolate S1 extracted from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 nematodes, showing high affinity to *Alcaligenes* species as revealed by the NCBI BLASTn search parameters.

>GGCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGC
GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAA
CGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTG
CGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAA
GGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC
CGTGATCCAGCCATGTCACACGTGTGTGAAGAACCGTCTTCAAGCCAGTGGGTAAA
GCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACC
GACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTAGTTAAGTTGG
ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTA
TGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG
AACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGC
GTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCC
GTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCA
ATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATG
GCTGTTCATAACCCAAACCCGTGTCGTAACACCATGTTGGGTTAAGTCCCGTAACGA
GCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG
GCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAG
GTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAG
CTAGTCTAACCTTCGGGAGGACGGTTACCACGAA

Figure 3.12: FASTA formatted 16S rDNA nucleotide sequence of the bacterial isolate S2 extracted from 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 nematodes, showing high affinity to *Pseudomonas* species as revealed by the NCBI BLASTn search parameters.

>TCTAGACCTGCCCCCTGGCTCAGATGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAA
CGGTAGCACAGAGGAGCTTCCTCCTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGG
AAACTGCCCGATGGAGGGGGATACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAG
ACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATCAGTCCCA
CTAGGTGGGGTAACGGCTCACCTAGGCCACCATCCCTAGCCGGTCTGAGAGGATGACCAGC
CACACTCGAACTGAGACACGGTCCAGACTCCTACGCGAGGCACCATGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGACCCAGCCATGCCGCGGGTATGAAGAAGGCCTTCGGGTCGTAAAG
TACTTTCACCGAGCAGGAAGGCCTTGCGGTTAATAACCGCAGCAATTGACGTTACTCGCAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTCCAAGCGTTAATCG
GAATTACTGGCCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCT
CAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGG
ACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAAC
GCGTTTAAGTCCACCGCCTCGGGACTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACT
CTTGACATCCAGCGAACTTCGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGCTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTAC
AATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAG
AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTGCTC

Figure 3.13: FASTA formatted 16S rDNA sequence of bacterial isolate O1 extracted from 0.1% NaClO surface sterilized IJs of Oscheius sp. OGB-2022 nematodes, showing high affinity to *Citrobacter* species, as revealed by NCBI BLASTn search parameters.

>AGTTCCCTCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTA
GCACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACT
GCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCA
AAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGACATATGACTAGTAC
CTGGGGTAACCGCTCACCTAGCGCGACGATCCACTAGCTGGTCTGACAGGAATGACCAGCCA
CACTGGAACTGCAGACACGGTCCAGACTCCTACGGGAGGCAGCAGCGGGGAATATTGCACAA
TGGGCCCAAGCCTGATGCAGCCATGCCGCGGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGCGAGGAGGAAGCCCTTGCGGTTAATAACCGCAGCCATTGACGTTACTTNCGCAGAA
GAAGCACCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCCCACCCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGC
TCAACCTGGGAACCCCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCCGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGG
ACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGGCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAAC
GCGTTAAGTCGACCCGCCCGGGGAAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACT
CTTGACATCCAGCGAACTTCGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TATCCTTTGTTGCCAGCGCTTCGGNCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGG
AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATG
GCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCG
GATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCC
ACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAA
AAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTACCACCTTTGCCTACAAG

Figure 3.14: FASTA formatted 16S rDNA nucleotide sequence of the bacterial isolate O2 extracted from 0.1% NaClO surface sterilized IJs of *Cruznema* sp. NTM-2021 nematodes, showing high affinity to Citrobacter species as revealed by the NCBI BLASTn search parameters.

>CTAGACTTTGATCCTGGCTCAGATCCACCCTGGCGGCAGCCTAACACATCCAAGTCGAACG
GTAGCACAGAGGAGCTCCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAA
ACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAG
ACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGT
AGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGCGCAAGCCCCCATGCACCCACCGCCGCGTCCACCAAGAAGGCCTCCGGGTCGTAAA
GTACTTTCAGCGAGGCAGCAAGCCCTCCCCGTTAACAACCGCAGCCATTGACGTTACTCGCA
GAAGAAGCACCGGCTAACTCCGTGCCAGCACCTCGCGGTAATACGGAGGGTGCAAGCGCTA
ATCGGAATTACTGGGCGTAAAGCGCACCCAGCCCGTCCCTCAAGTCGGATGTGAAATCCCC
GGGCTCAACCTGGGAACTCCATCCGAAACCGCCAGGCTAGAGTCTTGTAGAGGGGGGTAGA
ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCC
CCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGACCTACGACTTGCAGGTTGTGCCCTTGAGGCGTGGCTTC
CGGAGCTAACGCGTTAACTCCGACCCGCCCCCGGGCAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA
AGAACCTTACCTACTCTTGACATCCAGCGAACTTCGCAGAGATGCTTTGGTGCCTTCGGGAA
CTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCTTCGGCCGGGAACTCAAAGGAGACT
GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTCG
CGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAC
CTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
CTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTA
CCACCTTCCACTAG

Figure 3.15: FASTA formatted 16S rDNA nucleotide sequence of the bacterial isolate C1 extracted from 0.1% NaClO surface sterilized IJs of *Cruznema* sp. NTM-2021 nematodes, showing high affinity to *Citrobacter* species as revealed by the NCBI BLASTn search parameters.

3.3.5 Phylogenetic Analysis

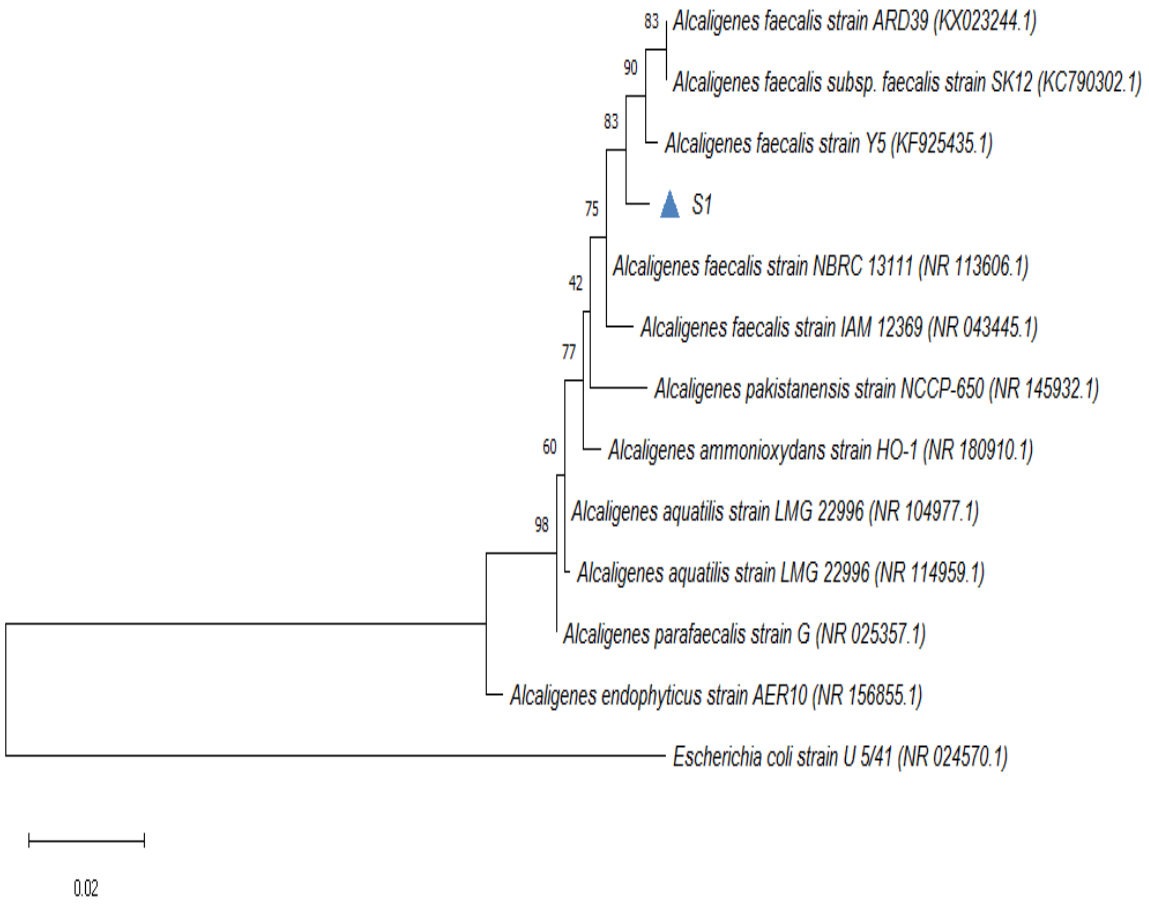

Figure 3.16: The evolutionary history of the bacterial isolate S1 with strong affinity for *Alcaligenes* species inferred using the Maximum Likelihood technique and the Tamura-Nei model on MEGA11. The tree with the highest log probability (-3534.70) is displayed (Tamura 1993; Tamura *et al*., 2021). The percentage of trees with the correlated taxa grouped together is presented next to the branches. The initial tree(s) for the heuristic search were automatically generated by incorporating the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances computed using the Tamura-Nei model, and then picking the topology with the highest log likelihood value. The tree is drawn to scale, with branch lengths calculated using the number of substitutions per site. This analysis included 13 nucleotide sequences. There were a total of 1555 positions in the final dataset. The nucleotide query sequence showed

high affinity to *Alcaligenes* species. *Escherichia coli* U 5/41 (NR024570.1) was used as outgroup.

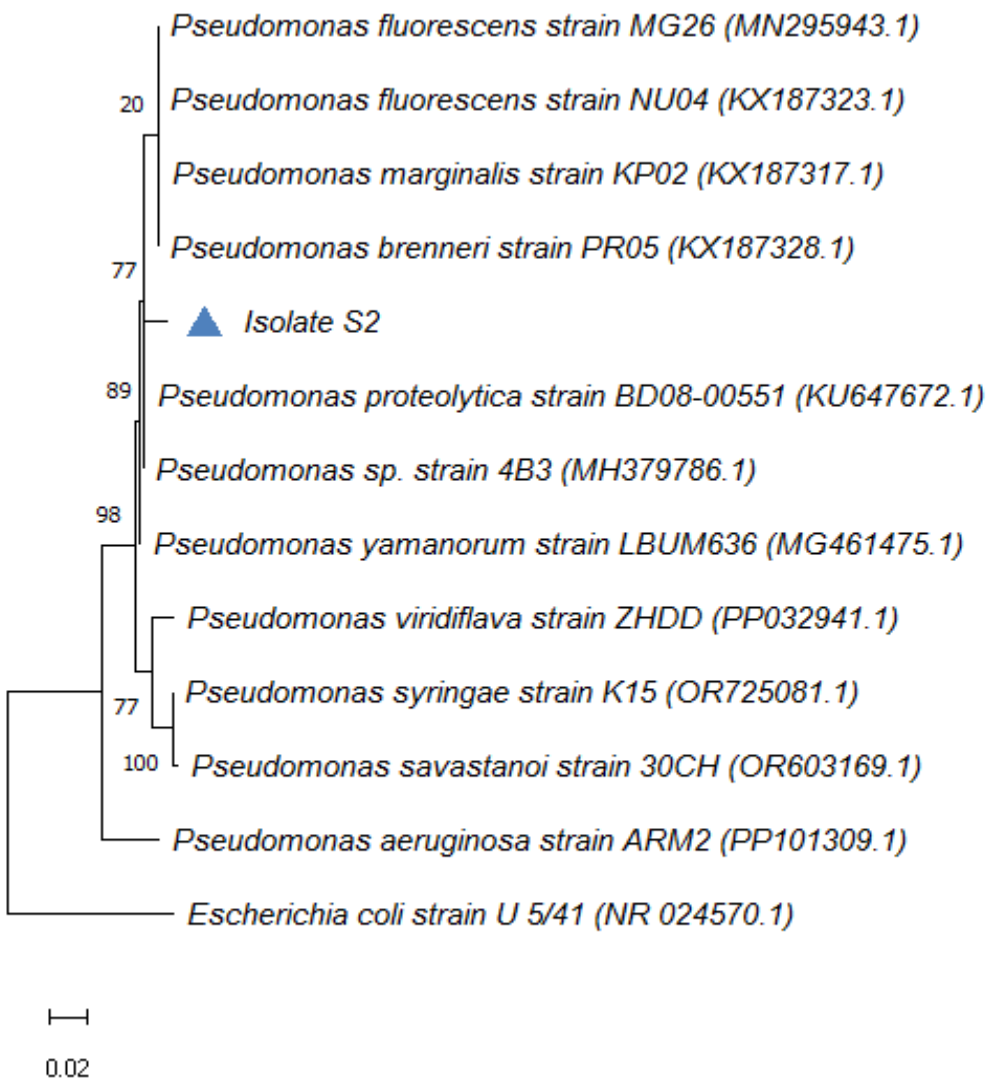

Figure 3.17: The evolutionary history of the bacterial isolate S2 with strong affinity for *Pseudomonas* species inferred using the Maximum Likelihood technique and the Tamura-Nei model on MEGA11. The tree with the highest log likelihood (-3760.42) is shown, with the percentage of trees in which the linked taxa grouped together presented next to the branches (Tamura 1993; Tamura *et al*., 2021). The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances computed using the Tamura-Nei model, and then picking the topology with the highest log likelihood value. The tree is drawn to scale, with branch lengths calculated using the number of substitutions per

site. This analysis included 13 nucleotide sequences. There were a total of 1562 positions in the final dataset. The nucleotide query sequence showed high affinity to *Pseudomonas* species. *E. coli* U 5/41 (NR024570.1) was used as an outgroup.

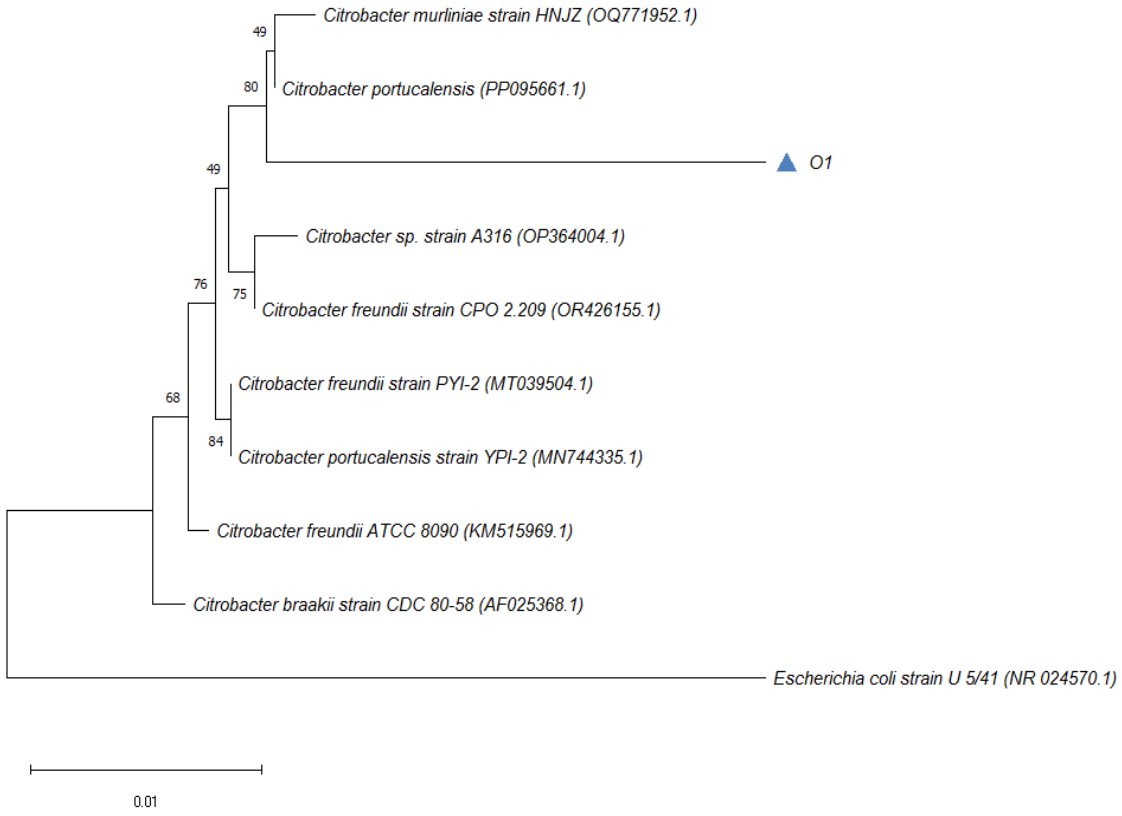

Figure 3.18: Evolutionary history of the bacterial isolate O1 with high affinity to *Citrobacter* species, inferred by utilizing the Maximum Likelihood approach and Tamura-Nei model on MEGA11. The tree with the highest log probability (-3371.56) is displayed (Tamura 1993; Tamura *et al*., 2021). The percentage of trees with the associated taxa grouped together is presented next to the branches. The initial tree(s) for the heuristic search were automatically generated by incorporating the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances computed using the Tamura-Nei model, and then picking the topology with the highest log likelihood value. The tree is drawn to scale, with branch lengths calculated using the number of substitutions per site. This investigation included ten nucleotide sequences. This analysis involved 10 nucleotide sequences. There were a total of 1867 positions in the final dataset. The query sequence showed high affinity to *Citrobacter* species. *E. coli* U 5/41 (NR024570.1) was used as outgroup.

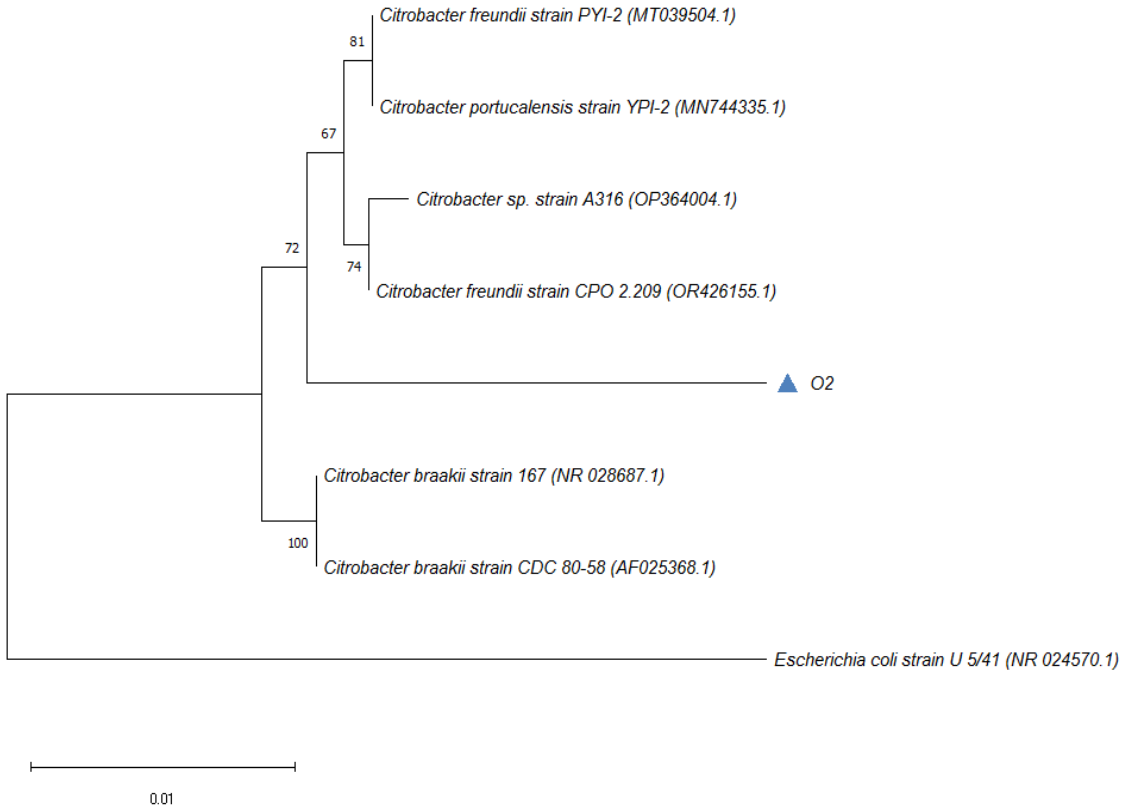

Figure 3.19: The evolutionary history of the bacterial isolate O2 showing strong affinity to *Citrobacter* species inferred using the Neighbor- Joining method on MEGA11. The correlated taxa which grouped together in the bootstrap test (1000 replicates) are shown next to the branches of the optimal tree showing high percentage of replicate trees (Felsenstein, 1985). The evolutionary analysis included 9 nucleotide sequences and the evolutionary distances were inferred by the Tamura 3- parameter approach (Tamura, 1992). All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1876 positions in the final dataset (Saitou and Nei, 1987; Tamura *et al*., 2021). The query sequence showed high affinity to *Citrobacter* species. *E. coli* U 5/41 (NR024570.1) was used as outgroup.

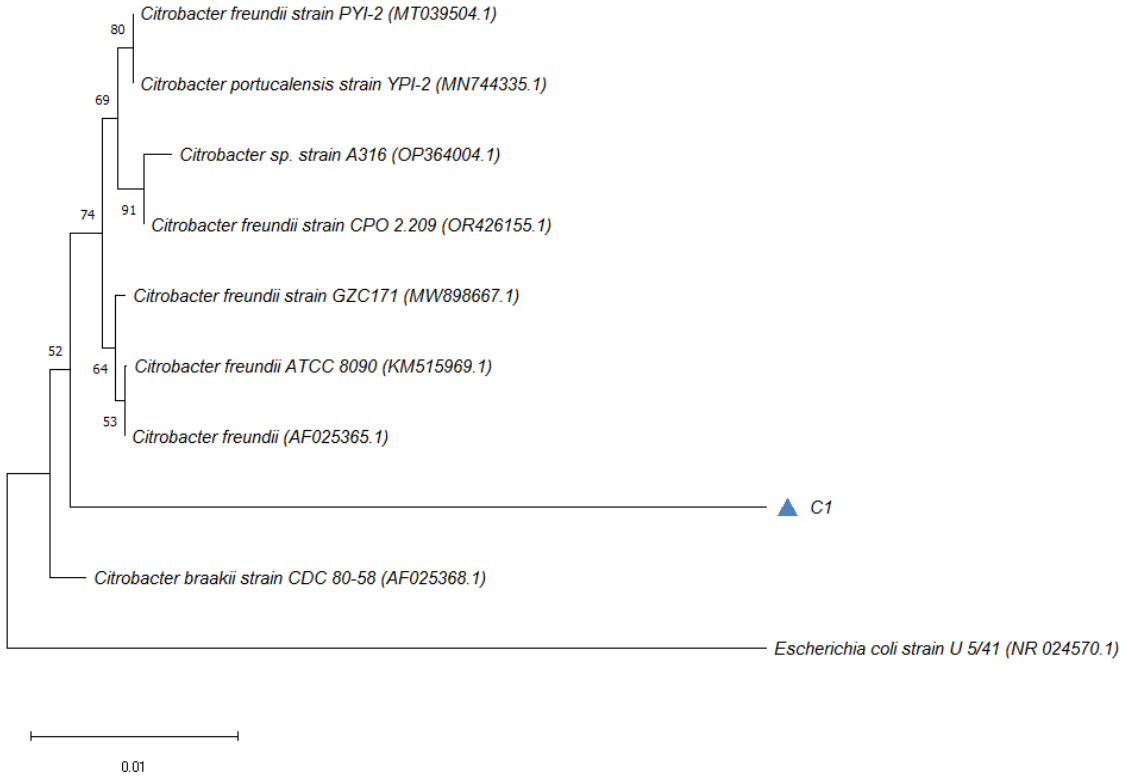

Figure 3.20: The evolutionary history of bacterial isolate C1 with strong affinity to *Citrobacter* species inferred using the Neighbor-Joining method on MEGA11 software. The proportion of replicate trees whereby the connected taxa grouped together in the bootstrap test (1000 replications), along with the optimal tree drawn to scale is depicted (Feisenstein, 1985). The phylogenetic tree was inferred by the branch lengths corresponding to the evolutionary distances which were calculated using the Tamura 3- parameter model (Tamura 1992) and expressed as number of base substitutions per site. The evolutionary analysis included 10 nucleotide sequences, with all ambiguous positions removed for each sequence pair (pairwise deletion) (Saitou and Nei, 1987; Tamura *et al*., 2021). There were a total of 1873 positions in the final dataset. The query sequence showed high affinity to *Citrobacter* species. *E. coli* U 5/41 (NR024570.1) was used as outgroup.

3.3.6 Evolutionary Divergence

The molecular evolutionary divergence between the various bacterial isolate species was computed in relation to the molecular evolutionary distances between the 16S rDNA nucleotide sequences using MEGA11 software pairwise deletion option.

Table 3.7: The number of base substitutions per site between sequences along with the estimates of evolutionary divergence between Alcaligenes species' nucleotide data inferred by the Tamura 3-parameter model (Tamura, 1992) analyses are shown. The evolutionary analysis including 13 nucleotide sequences was conducted on MEGA11 (Tamura *et al*., 2021). Each sequence pair had all ambiguous places eliminated (the pairwise deletion option). The completed dataset had a total of 1555 locations.

	1	2	3	4	5	6	7	8	9	10	11	12	13
S1		0,006	0,006	0,006	0,014	0,011	0,011	0,006	0,017	0,019	0,026	0,008	0,212
Alcaligenes_faecalis_strain_ARD39_(KX023244.1)	0,006		0,000	0,001	0,009	0,008	0,008	0,004	0,014	0,014	0,025	0,007	0,211
Alcaligenes_faecalis_subsp._faecalis_strain_SK12_(KC790302.1)	0,006	0,000		0,005	0,009	0,013	0,013	0,008	0,016	0,014	0,029	0,011	0,213
Alcaligenes_faecalis_strain_Y5_(KF925435.1)	0,006	0,001	0,005		0,009	0,013	0,013	0,008	0,019	0,014	0,030	0,012	0,215
Alcaligenes_parafaecalis_strain_G_(NR_025357.1)	0,014	0,009	0,009	0,009		0,012	0,001	0,006	0,002	0,012	0,016	0,009	0,208
Alcaligenes_faecalis_strain_IAM_12369_(NR_043445.1)	0,011	0,008	0,013	0,013	0,012		0,010	0,005	0,014	0,018	0,026	0,011	0,210
Alcaligenes_aquatilis_strain_LMG_22996_(NR_104977.1)	0,011	0,008	0,013	0,013	0,001	0,010		0,005	0,001	0,012	0,016	0,007	0,204
Alcaligenes_faecalis_strain_NBRC_13111_(NR_113606.1)	0,006	0,004	0,008	0,008	0,006	0,005	0,005		0,009	0,011	0,019	0,006	0,206
Alcaligenes_aquatilis_strain_LMG_22996_(NR_114959.1)	0,017	0,014	0,016	0,019	0,002	0,014	0,001	0,009		0,019	0,021	0,009	0,210
Alcaligenes_pakistanensis_strain_NCCP-650_(NR_145932.1)	0,019	0,014	0,014	0,014	0,012	0,018	0,012	0,011	0,019		0,029	0,014	0,210
Alcaligenes_endophyticus_strain_AER10_(NR_156855.1)	0,026	0,025	0,029	0,030	0,016	0,026	0,016	0,019	0,021	0,029		0,021	0,199
Alcaligenes_ammonioxydans_strain_HO-1_(NR_180910.1)	0,008	0,007	0,011	0,012	0,009	0,011	0,007	0,006	0,009	0,014	0,021		0,204
Escherichia_coli_strain_U_5/41_(NR_024570.1)	0,212	0,211	0,213	0,215	0,208	0,210	0,204	0,206	0,210	0,210	0,199	0,204	

Table 3.8: The number of base substitutions per site between sequences along with the estimates of evolutionary divergence between *Pseudomonas* species' nucleotide sequence data inferred by the Tamura's 3-parameter model (Tamura, 1992) analyses are shown. The evolutionary analysis including 13 nucleotide sequences was conducted on MEGA11 (Tamura *et al*., 2021). Each sequence pair had all ambiguous

places eliminated (the pairwise deletion option). The final dataset had a total of 1562 locations.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Isolate_S2		0,013	0,013	0,013	0,013	0,014	0,013	0,016	0,067	0,037	0,038	0,044	0,171
Pseudomonas_fluorescens_strain_MG26_(MN295943.1)	0,013		0,007	0,000	0,000	0,001	0,000	0,003	0,054	0,030	0,024	0,031	0,154
Pseudomonas_sp._strain_4B3_(MH379786.1)	0,013	0,007		0,000	0,001	0,001	0,000	0,003	0,054	0,022	0,024	0,031	0,156
Pseudomonas_brenneri_strain_PR05_(KX187328.1)	0,013	0,000	0,000		0,000	0,001	0,000	0,003	0,054	0,022	0,024	0,031	0,154
Pseudomonas_fluorescens_strain_NU04_(KX187323.1)	0,013	0,000	0,001	0,000		0,001	0,000	0,003	0,054	0,023	0,024	0,031	0,154
Pseudomonas_marginalis_strain_KP02_(KX187317.1)	0,014	0,001	0,001	0,001	0,001		0,001	0,004	0,054	0,022	0,023	0,031	0,155
Pseudomonas_proteolytica_strain_BD08-00551_(KU647672.1)	0,013	0,000	0,000	0,000	0,000	0,001		0,003	0,053	0,022	0,023	0,031	0,156
Pseudomonas_yamanorum_strain_LBUM636_(MG461475.1)	0,016	0,003	0,003	0,003	0,003	0,004	0,003		0,050	0,022	0,023	0,029	0,154
Pseudomonas_aeruginosa_strain_ARM2_(PP101309.1)	0,067	0,054	0,054	0,054	0,054	0,054	0,053	0,050		0,056	0,067	0,064	0,171
Pseudomonas_syringae_strain_K15_(OR725081.1)	0,037	0,030	0,022	0,022	0,023	0,022	0,022	0,022	0,056		0,023	0,003	0,159
Pseudomonas_viridiflava_strain_ZHDD_(PP032941.1)	0,038	0,024	0,024	0,024	0,024	0,023	0,023	0,023	0,067	0,023		0,033	0,158
Pseudomonas_savastanoi_strain_30CH_(OR603169.1)	0,044	0,031	0,031	0,031	0,031	0,031	0,031	0,029	0,064	0,003	0,033		0,161
Escherichia_coli_strain_U_5/41_(NR_024570.1)	0,171	0,154	0,156	0,154	0,154	0,155	0,156	0,154	0,171	0,159	0,158	0,161	

Table 3.9: The number of base substitutions per site across sequences, including the estimates of evolutionary divergence between *Citrobacter* species' nucleotide sequence data inferred by the Tamura's 3-parameter model (Tamura, 1992) analyses are shown. The evolutionary analysis included 10 nucleotide sequences and were conducted on MEGA11 (Tamura *et al*., 2021). Each sequence pair had all ambiguous places eliminated (the pairwise deletion option). The completed dataset included a total of 1867 locations.

	1	2	3	4	5	6	7	8	9	10
O1		0,026	0,026	0,026	0,024	0,027	0,021	0,027	0,021	0,064
Citrobacter_freundii_ATCC_8090_(KM515969.1)	0,026		0,003	0,003	0,007	0,007	0,001	0,003	0,004	0,043
Citrobacter_freundii_strain_PYI-2_(MT039504.1)	0,026	0,003		0,000	0,005	0,003	0,001	0,004	0,001	0,043
Citrobacter_portucalensis_strain_YPI-2_(MN744335.1)	0,026	0,003	0,000		0,005	0,003	0,001	0,004	0,001	0,043
Citrobacter_murliniae_strain_HNJZ_(OQ771952.1)	0,024	0,007	0,005	0,005		0,006	0,005	0,008	0,001	0,045
Citrobacter_sp._strain_A316_(OP364004.1)	0,027	0,007	0,003	0,003	0,006		0,000	0,013	0,002	0,042
Citrobacter_freundii_strain_CPO_2.209_(OR426155.1)	0,021	0,001	0,001	0,001	0,005	0,000		0,003	0,002	0,042
Citrobacter_braakii_strain_CDC_80-58_(AF025368.1)	0,027	0,003	0,004	0,004	0,008	0,013	0,003		0,005	0,040
Citrobacter_portucalensis_(PP095661.1)	0,021	0,004	0,001	0,001	0,001	0,002	0,002	0,005		0,046
Escherichia_coli_strain_U_5/41_(NR_024570.1)	0,064	0,043	0,043	0,043	0,045	0,042	0,042	0,040	0,046	

Table 3.10: The number of base substitutions per site across sequences including the estimates of evolutionary divergence between *Citrobacter* species' nucleotide sequence data inferred by the Tamura's 3-parameter model (Tamura, 1992) analyses are shown. The evolutionary analysis included 8 nucleotide sequences and were conducted on MEGA11 (Tamura *et al*., 2021). Each sequence pair had all ambiguous places eliminated (the pairwise deletion option). The completed dataset included a total of 1876 locations.

	1	2	3	4	5	6	7	8
O2		0,022	0,057	0,021	0,021	0,020	0,018	0,022
Citrobacter_braakii_strain_167_(NR_028687.1)	0,022		0,040	0,004	0,004	0,013	0,003	0,000
Escherichia_coli_strain_U_5/41_(NR_024570.1)	0,057	0,040		0,043	0,043	0,042	0,042	0,040
Citrobacter_freundii_strain_PYI-2_(MT039504.1)	0,021	0,004	0,043		0,000	0,003	0,001	0,004
Citrobacter_portucalensis_strain_YPI-2_(MN744335.1)	0,021	0,004	0,043	0,000		0,003	0,001	0,004
Citrobacter_sp._strain_A316_(OP364004.1)	0,020	0,013	0,042	0,003	0,003		0,000	0,013
Citrobacter_freundii_strain_CPO_2.209_(OR426155.1)	0,018	0,003	0,042	0,001	0,001	0,000		0,003
Citrobacter_braakii_strain_CDC_80-58_(AF025368.1)	0,022	0,000	0,040	0,004	0,004	0,013	0,003	

Table 3.11: The number of base substitutions per site across sequences along with the estimates of evolutionary divergence between *Citrobacter* species' nucleotide sequence data inferred by the Tamura 3-parameter model (Tamura, 1992) analyses are shown. The evolutionary analysis included 10 nucleotide sequences and were conducted on MEGA11 (Tamura *et al*., 2021). Each sequence pair had all ambiguous

places eliminated (the pairwise deletion option). The completed dataset included a total of 1873 locations.

	1	2	3	4	5	6	7	8	9	10
C1		0,036	0,036	0,037	0,037	0,036	0,038	0,072	0,036	0,038
Citrobacter_freundii_ATCC_8090_(KM515969.1)	0,036		0,000	0,003	0,003	0,001	0,007	0,043	0,001	0,003
Citrobacter_freundii_(AF025365.1)	0,036	0,000		0,003	0,003	0,001	0,003	0,043	0,001	0,009
Citrobacter_freundii_strain_PYI-2_(MT039504.1)	0,037	0,003	0,003		0,000	0,003	0,003	0,043	0,001	0,004
Citrobacter_portucalensis_strain_YPI-2_(MN744335.1)	0,037	0,003	0,003	0,000		0,003	0,003	0,043	0,001	0,004
Citrobacter_freundii_strain_GZC171_(MW898667.1)	0,036	0,001	0,001	0,003	0,003		0,003	0,044	0,003	0,004
Citrobacter_sp._strain_A316_(OP364004.1)	0,038	0,007	0,003	0,003	0,003	0,003		0,042	0,000	0,013
Escherichia_coli_strain_U_5/41_(NR_024570.1)	0,072	0,043	0,043	0,043	0,043	0,044	0,042		0,042	0,040
Citrobacter_freundii_strain_CPO_2.209_(OR426155.1)	0,036	0,001	0,001	0,001	0,001	0,003	0,000	0,042		0,003
Citrobacter_braakii_strain_CDC_80-58_(AF025368.1)	0,038	0,003	0,009	0,004	0,004	0,004	0,013	0,040	0,003	

3.4 Discussion

The isolation, morphological characterization, 16S rDNA molecular identification, and the phylogenetics of the nematode-associated gut bacteria with the potential to be the endosymbiotic bacteria of the two nematode isolates used in this study were conducted. A sum of five bacterial isolates were successfully extracted from the 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes. Both the differential and selective bacterial culture media, MacConkey agar and NBTA were used to selectively grow desired organisms and take advantage of their biochemical properties, whilst preventing the growth of non-target organisms (Kumar *et al*., 2011; Kampfer *et al*., 2017). Of the five bacterial isolates, three (S1, S2, and O1) were isolated from *Oscheius* sp. OGB- 2022 and two (O2, and C1) were isolated from *Cruznema* sp. NTM-2021.

The bacterial colonies of the isolates all exhibited varying phenotypical properties on the bacterial culture media used. Phenotypically, bacterial isolates mostly appeared to have Brick-red, pinkish colonies on both MacConkey agar and NBTA. Bacterial isolate S1 phenotypically exhibited irregular margins, flat elevation, small rough textured colonies on MacConkey agar, as opposed to the regular (circular) margins, and small smooth textured flat colonies observed on NBTA. Moreover, isolate S2 and C1 had

regular margins (circular), flat elevation, medium size, and smooth texture on MacConkey agar, whilst they had flat elevation on NBTA. Bacterial isolate O1 and O2 exhibited slightly elevated, medium sized, smooth colonies with regular margins on NBTA and MacConkey agar. However, O1 exhibited small sized rough colonies on MacConkey agar. All the bacterial isolates had a negative Gram's stain reaction with pink coloration of the short-rod shaped cells, suggesting the possession of thin peptidoglycan cell wall and the inability to retain the crystal violet primary stain (Kumar *et al*., 2011; Kampfer *et al*., 2017). The Gram-negative reaction of the bacterial isolates was further confirmed by the growth of brick-red colonies on the MacConkey agar as only Gram-negative bacteria have been reported to utilize and vigorously grow in the bile salt component of the MacConkey growth medium, thus absorbing the neutral red dye and hence the brick-red colony appearance (Kumar *et al*., 2011; Kampfer *et al*., 2017). Except for bacterial isolate S2, all the other bacterial isolates were lactose fermenters.

The positive catalase test results suggested the ability of the bacterial isolates to decompose the hydrogen peroxide (H_2O_2) and release water and oxygen, further suggesting the presence of the catalase enzyme (Kumar *et al*., 2011; Kampfer *et al*., 2017). Rapid bubble formation was observed on the catalase reaction of bacterial isolate S1 and S2. However, slowed bubble formation was observed on the catalase reaction of the bacterial isolates O1, O2, and C1. Moreover, no endospore formation was observed for all the five bacterial isolates. Bacterial isolate S1 and S2 were pathogenic and showed higher virulence against last Instar larvae of model organism *T. molitor* when applied as a cell-free supernatant. Isolate O1, O2, and C1 also exhibited satisfactory mortality towards the *T. molitor* larvae, suggesting the capacity of the bacterial isolates to suppress the insect host's immune system after ingestion even during foliar application (Mohan *et al*., 2003), further suggesting the penetration of the bacteria into the insect host's hemocoel possibly through the natural openings being the mouth, anus, or respiratory spiracles (Shan *et al*., 2019; Vicente-Díez *et al*., 2021).

Moreover, the NCBI BLASTn bioinformatics search parameters of the query sequences obtained from the bacterial genomic DNA samples sent to Inqaba Biotechnical Industries (Pty, Ltd) for 16S rDNA Sanger sequencing, indicated high affinity to the Gram-negative bacteria species of the *Alcaligenes*, *Pseudomonas*, and

Citrobacter genera, respectively. Bacterial isolate S1 was revealed to have high affinity to *Alcaligenes* species, hence, the suggested name *Alcaligenes* sp. OGB-2022 (PP163383). The NCBI BLASTn bioinformatics search parameters revealed the high affinity of bacterial isolate S2 to *Pseudomonas* species and the name *Pseudomonas* sp. OGB-2022 (PP165496) was thereby suggested. Moreover, bacterial isolate O1, O2, and C1 were revealed to have high affinity to the *Citrobacter* species.

3.5 Conclusion

Recently, the application of the bacterial endosymbionts along with their nematode vectors have become a popular tactic in the IPM systems. Numerous bioactive compounds secreted by the bacterial endosymbionts as secondary metabolites possess great insecticidal and antimicrobial properties that can counteract the growth of various organisms such as fungi and plant parasitic nematodes, henceforth, suggesting the suitability of the endosymbionts to be applied as bioformulations in the agricultural sectors. Bacterial isolate S1, *Alcaligenes* sp. OGB-2022 (PP163383) and S2, *Pseudomonas* sp. OGB-2022 (PP165496) exhibited great potentials of being incorporated in the IPM systems as bioformulations which could be applied directly using standard spray equipment. The bioactive complexes released as secondary metabolites by the bacterial endosymbionts could also be optimized and preserved in the form of wettable powders, cell pellet, and cell-free supernatant. The bacterial isolates successfully extracted from the 0.1% NaClO surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes may be classified as nematode-gut associated bacteria with possible endosymbiosis. Bacterial endosymbiosis tests are required to better understand the association of the successfully isolated bacteria with *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode species. Moreover, isolation and characterization of the secondary metabolites released as toxic antimicrobials by nematodes from this study is crucial in understanding their toxicity towards crop pests and in improving their field application as bio-formulated pest control agents.

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4 Chapter Four: Evaluating the Effects of Different Formulation Substrates on the Storage Duration of Nematode Isolates.

4.1 Introduction

Beneficial insect-parasitic roundworms from the EPN genera *Steinernema*, *Heterorhabditis*, and a few from the *Rhabditis* genus are currently used as effective biocontrol agents in the IPM systems due to their safety to both humans and the environment, and due to the restriction of the utilization of harmful synthetic chemical insecticides in the agricultural sector (Askary & Ahmad, 2017). However, various contributing factors such as high production costs, poor field application practices, and a comparatively short lifespan of about 4-6 weeks at low temperature storage greatly compromise the efficient commercialization of EPN biocontrol products (Askary & Ahmad, 2017). These EPN genera have a mutualistic relationship with the symbiotic bacteria of *Xenorhabdus*, *Photorhabdus*, and *Serratia* found within the gut of the nematode (Poinar, 1990).

The symbiotic bacteria are regurgitated inside the insect host's hemocoel after penetration through any natural openings (mouth, anus, or respiratory spiracles) by EPN IJs (Campbell and Gaugler, 1991; Hazir *et al*., 2003). The bacteria thus in turn overcome the insect host's defence system. This eventually cause infection and death within 24-48 hours while supressing the microbial expansion of potential competitors (Dowds and Peters, 2002; Hazir *et al*., 2003). EPNs have been deemed efficient biocontrol agents of numerous problematic crop pests due to their wide host range, high reproductive rate, rapid infective rate, and their suitability for mass rearing in artificial media (Kaya and Gaugler, 1993; Gaugler *et al*., 1997; Ehlers, 2001). These microorganisms can potentially be incorporated with other pest control practices as they can easily be dispersed using prevailing agronomic spray machinery and due to their compatibility with most agrochemicals (Koppenhöfer and Grewal, 2005).

Formulating IJs in a suitable substrate that ensures viability and infectivity for extended periods has been a major challenge experienced in developing EPNs as effective biocontrol agents (Poinar Jr and Grewal, 2012). Several techniques such as the incorporation of inactive carriers and the physical entrapment of IJs within the inactive medium have been implemented in the formulation of EPNs. The simplicity and ease of production are some of the advantages of inert carriers. However, the downsides of

carrier-based formulations include the need for frequent refrigeration during storage and transportation which makes them expensive (Willett *et al*., 2018).

EPNs can be formulated in either gel, larval cadaver, liquid, or solid formulation substrates which normally consist of various components such as antimicrobials, absorbents, emulsifiers, UV-ray protectants, surfactants, and humectants, which serve various functions (Grewal, 2002; Kagimu, 2018; Willett *et al*., 2018). Moreover, to maintain the viability, pathogenicity, and lifespan of formulated EPNs, appropriate temperature and moisture conditions are required to ensure the formulated EPN product reaches the farmer in perfect usable conditions and that the EPNs are effective during field application (Askary, 2010; Willett *et al*., 2018). Furthermore, the induction of partial anhydrobiosis prior to EPN formulation has shown encouraging results, particularly in the context of diverse formulation strategies (Shapiro-IIan *et al*., 2012). The utilization of cadavers of infected insect hosts has been a promising advancement in formulation tactics (Hiltpold *et al*., 2012).

It has been stated that EPNs undergo partial anhydrobiosis in the case of unfavourable environmental conditions such as extreme temperature, low moisture levels, or limited availability of insect hosts in soil habitats. When EPNs are in partial anhydrobiotic state, their energy consumption decreases. It has been argued that EPNs can only attain partial anhydrobiosis since they still need a limited quantity of water to survive and are hence classified as drought forbearing rather than real anhydrobiotes (Heriberto *et al*., 2017). Moreover, environmental changes, notably water loss, cause EPNs to undergo physical and physiological changes (Willett *et al*., 2018). Additionally, the EPNs' morphological adaptations such as clomping and curling minimize the amount of exposed surface area to the environment, resulting in less water forfeiture (Perry *et al*., 2012). These functional alterations occurring in response to dehydration stress help slow down metabolic activities, thus retaining lipid reserves (Perry *et al*., 2012; Willett *et al*., 2018).

Successfully commercializing EPNs after mass-production necessitates the implementation of suitable transportation, formulation, and storage tactics that restrict the loss of nematode viability, and nematode mortality. However, their short durability is a disadvantage, particularly in large-scale industrial applications (Grewal 2000a, b). Maintaining high-quality EPNs at all stages, from the bioreactor to formulation and

storage until the product reaches the end user in good usable conditions is of critical importance (Grewal and Peters, 2005; Kagimu *et al*., 2017). Such maintenance is of significance since EPNs are affected by both biotic and abiotic factors. During formulation moisture content, temperature, water loss, and aeration as well as microbial contamination and toxicity of anti-microbial proxies in terms of IJs are particularly critical (Grewal and Peters 2005; Kagimu *et al*. 2017). Moreover, the recommended storage temperature of formulated endemic South African EPN species has been reported to range from 14 to 25 °C, due to the comparatively elevated survival and virulence rates observed at such temperatures (Kagimu and Malan, 2019).

The major significant difficulties encountered when using EPNs as biocontrol agents is their poor post-application survivability influenced mainly by the abiotic factors such as pH, soil type, temperature, moisture content, UV radiation, and relative humidity (Koppenöffer and Fuzy, 2007; Shapiro-IIan et al., 2006, 2012). Unfavourable environmental conditions usually lead to desiccation, and depletion of energy sources, making the mentioned parameters major determinants of EPN survival and mobility (Smits, 1996). Moreover, EPN efficacy and persistence after field application is usually improved by pre- and post- irrigation practices since EPNs are sensitive to low soil moisture content (Wright *et al*., 2005; Georgis *et al*., 2006).

Great progress has been achieved by many European countries in the commercialization and formulation of mass produced EPNs (Tridan *et al*., 2020). However, similar EPN research for the South African markets is still in its early development with no effective EPN products readily available on the local markets other than Kenyan and European EPN product imports. Nonetheless, budding success in the mass-culturing and formulation of endemic South African EPN species such as *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel & Gaugler and Adams, and *Steinernema jeffreyense* Malan, Knoetze & Tiedt, has been showcased by current tentative studies conducted by Kagimu and Malan (2019) and Dunn *et al*., (2020). Additionally, a South African endemic EPN species, *Steinernema innovation* was successfully produced and formulated in a sponge carrier by Ramakuwela *et al*. (2015).

Under laboratory settings, EPN species of *Steinernematids*, and *Heterorhabditids* tend to have survival span of 6-9 months and 3-4 months, respectively, when stored in aqueous suspensions in aerated culture flasks at low temperature storage (Koppenhöfer, 2007; Stock and Goodrich- Blair, 2014). However, the preservation of EPNs maintained in aqueous suspensions for industrial usage is costly due to the necessity for a suitable cooling system as well as bulk transportation (Chen & Glazer, 2005), making the storage approach unsustainable for commercialization (Grewal *et al*., 2002; Grzywacz *et al*., 2014). The undertaken research was aimed at developing an improved EPN formulation substrate with suitable storage stability at room temperature for relatively long periods of time, as well as increased ease of transportation and field- application by end users. Formulations which lowered the metabolic activity of IJs and had moderate desiccation effects were reported to have been successful in maintaining EPN viability (Bedding, 1988; Grewal, 2000a, b; Silver *et al*., 1995).

Variations in formulation type and application tactics are important elements in the efficient usage of EPNs. EPNs are typically manufactured in aqueous solutions and delivered through agricultural irrigation systems or sprinklers (Shapiro-IIan *et al*., 2006). Various formulation substrates such as Diatomaceous earth (DE), infected insect cadaver, coarse silica sand, and coarse river sand, were used in this study. DE has been employed in several forms, including pelleted, granulated, and wettable powders (Cortés- Martines *et al*., 2016, 2017; Kagimu and Malan, 2019). DE's desiccating characteristics and capacity to soak up moisture make it suitable for use in microbial insecticide formulations as it also has minimal direct toxicity towards EPN IJs (Behle and Birthisel, 2014; Bohinc *et al.,* 2018; Kagimu and Malan, 2019).

EPN- infected insect host cadavers prepared in laboratory settings may also be used as formulations. As a result, EPN-infected insect host cadavers are released into the environment, causing IJs to disperse from within the cadaver into the soil matrix to scout for fresh insect hosts to infect, and suppress target pest populations (Dolinski, 2006). Moreover, during field- application, EPNs formulated in the cadaver method have demonstrated greater dispersive capacities, as well as elevated survival and pathogenicity levels in the soil, contributing to successful arthropod control (Shapiro and Lewis, 1999; Del Valle *et al*., 2008). Ground or aerial agronomic spraying

equipment are normally used for the field application of EPNs, depending on the cropping system in the targeted area (Shapiro-IIan *et al*., 2006, 2012).

This study aimed to evaluate the effects of different formulation substrates on the storage duration of formulated nematode species (*Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021), with objectives including to:

- ✓ Evaluate the survival and assess virulence of formulated nematode species after different incubation periods at different temperatures had elapsed.
- ✓ Assess survival mainly on different substrates.

4.2 Materials and Methods

4.2.1 Nematode culture preparation

Freshly emerged IJs were collected from the White trap setups into sterilized 50mL Falcon tubes using sterile 3mL plastic Pasteur pipettes. The nematodes were allowed to settle before excess water was removed from the tubes. Subsequently, the nematodes of the same species were collected into the same Falcon tube and were surface sterilized with sterile distilled water for 3hours. This step was performed thrice as a form of quality control. Nematode counts were performed from the surface sterilized nematode suspension in 50mL Falcon tubes. A 200IJ/μL concentration was obtained from the nematode suspension onto a sterile petri dish using a micropipette, for the nematode count which was done under the Olympus stereomicroscope (see Appendix 5).

4.2.2 Formulation of nematode IJs in different formulation substrates.

The IJs of nematode isolates (*Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021) were formulated in Diatomaceous earth (DE), Coarse silica sand, and the Insect-cadaver formulation (Insect-cadaver-approach) for 2-, 7-, 14-, and 21-day duration at 25°C and 30°C incubation temperatures, with the coarse river sand serving as a control (see Appendix 6). The experiment had three replicates for each nematode species, incubation temperature, and incubation period, with each replicate having three petri dishes (see Appendix 7). The solid formulation substrates (DE, Coarse silica sand, and Coarse River sand) were autoclaved and the last Instar larvae of

model organism *T. molitor* were surface sterilized with 70% ethanol as a quality control measure (see Appendix 5).

4.2.2.1 Formulation of nematode IJs in Diatomaceous earth (DE).

Eco-Earth: Food Grade Diatomaceous Earth (DE) (Newtown Fertilizers, Newtown, JHB, South Africa) was transferred into 250mL Erlenmeyer flasks and autoclaved to kill off potential contaminants. Sterilized DE (20g) was transferred into 90mm diameter petri dishes under the laminar hood flow using a sterile metal spatula as a form of quality control. A 1600 IJs/μL concentration of freshly emerged surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode species were formulated in 20g of DE and 8% w/v moisture content was retained. The inoculated nematodes were thoroughly and gently mixed with the DE component of the formulation (Kagimu and Malan 2019). Subsequently, plates were sealed with a layer of parafilm and incubated at 25 °C and 30 °C incubation temperature for 2-, 7-, 14-, and 21-day duration. The experiment was performed thrice with each replicate having three plates.

4.2.2.2 Coarse silica sand formulation.

For the coarse silica sand formulation, silica sand, particle size 0.05-0.8mm was transferred into 250mL Erlenmeyer flasks and autoclaved at 121°C. 20g of sterilized coarse silica sand was subsequently dispensed into sterile 90mm diameter petri dishes under the laminar hood flow. A 1600 IJs/μL concentration of freshly emerged surface sterilized IJs of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode species were inoculated to the plates while retaining 8% moisture content w/w (Caroli *et al*., 1996). Plates were then sealed with a layer of parafilm and subsequently incubated at both 25 °C and 30 °C for 2, 7, 14, and 21 days, respectively. This experiment was conducted thrice under same laboratory conditions with each replicate having three plates.

4.2.2.3 Nematode-infected insect host cadaver preparation for insect cadaver formulation (Insect cadaver approach).

Ten surface sterilized last Instar larvae of model organism *T. molitor* were set in 90mm diameter petri dishes lined with two sheets of 90mm Whatman filter paper. Desired concentrations of each nematode species were added to the damp filter paper in

separate petri dishes by transferring 1600IJ/μL of the stored aqueous nematode suspension in 50mL Falcon tubes (Del Valle *et al*., 2008). Petri dishes were then sealed with a layer of parafilm and incubated at room temperature storage 25℃ for a duration of 3-4 days. The dead insect host cadavers with signs of infection by nematodes (Dark brown pigmentation with mushy texture) were transferred to sterile 90mm diameter petri dishes lined with a single sheet of dry 90mm Whatman filter paper. The plates were subsequently incubated at both 25 °C and 30 °C for 2, 7, 14, and 21 days, respectively. This experiment was conducted thrice under same laboratory conditions with each replicate having three plates.

4.2.2.4 Evaluating survival and virulence of formulated nematodes.

The survival of IJs formulated in different substrates was evaluated using two approaches being the direct nematode count and the direct-contact pathogenicity test assays after the incubation period had elapsed. A single petri dish from the three of each trial was used for direct nematode counts by dissolving 5g from the 20g solid substrate into sterile 10mL distilled water. Serial dilutions of the suspensions were conducted and the viable IJs were counted under the stereomicroscope. However, the counts were complicated for the solid substrates, hence, the remainder of the plates were used for the direct-contact pathogenicity test assays. Survival was evaluated by placing ten last Instar larvae of model organism *T. molitor* (n=10) on the surface of the formulation substrate with 8% moisture content w/w. The insect host larvae were added at various durations (2, 7, 14, and 21 days) post-inoculation and larval mortality was recorded daily for a period of 7 days. Larval insect host cadavers displaying symptoms of nematode infection were collected and placed on the White trap setups for evaluation and recovery of IJs (Lephoto and Gray, 2019).

4.2.2.5 Direct-contact pathogenicity test assays (Nematode entomopathogenicity).

Entomopathogenicity of nematodes was evaluated by placing last Instar larvae of model organism *T. molitor* (n=10) in 90mm diameter petri dishes partially filled with sterile 35g of sandy loam soil autoclaved at 121 ºC. The moisture content of the bioassays was maintained at 8% w/w after inoculation with 175IJs suspended in 1mL of sterile distilled water (Lephoto and Gray, 2019). Larval mortality of the insect hosts post-inoculation was monitored daily for a period of 7 days, and cadavers displaying symptoms of nematode infection were collected and placed on modified White-trap

setups for evaluation and recovery of freshly emerging cohort of IJs (Lephoto and Gray, 2019).

4.3 Results

Both the *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode isolates formulated in different substrates (DE, Coarse silica sand, Insect-cadaver, coarse river sand) at different incubation temperatures of 25 and 30° C showed significant survival and virulence, post resuscitation, respectively. A significantly ($p<0.05$) higher survival rate was attained by formulated IJs of both the used nematode isolates in DE, Insect cadaver and coarse silica sand. The Two-way ANOVA statistical analysis on Statistical Computing and Graphics software R Script Version 4.2.1 (R-4.2.1) and graphs drawn on Microsoft Excel 2021 revealed that the experimental data was significantly different with $p<0.05$ for all the nematode isolates (Refer to Index 8).

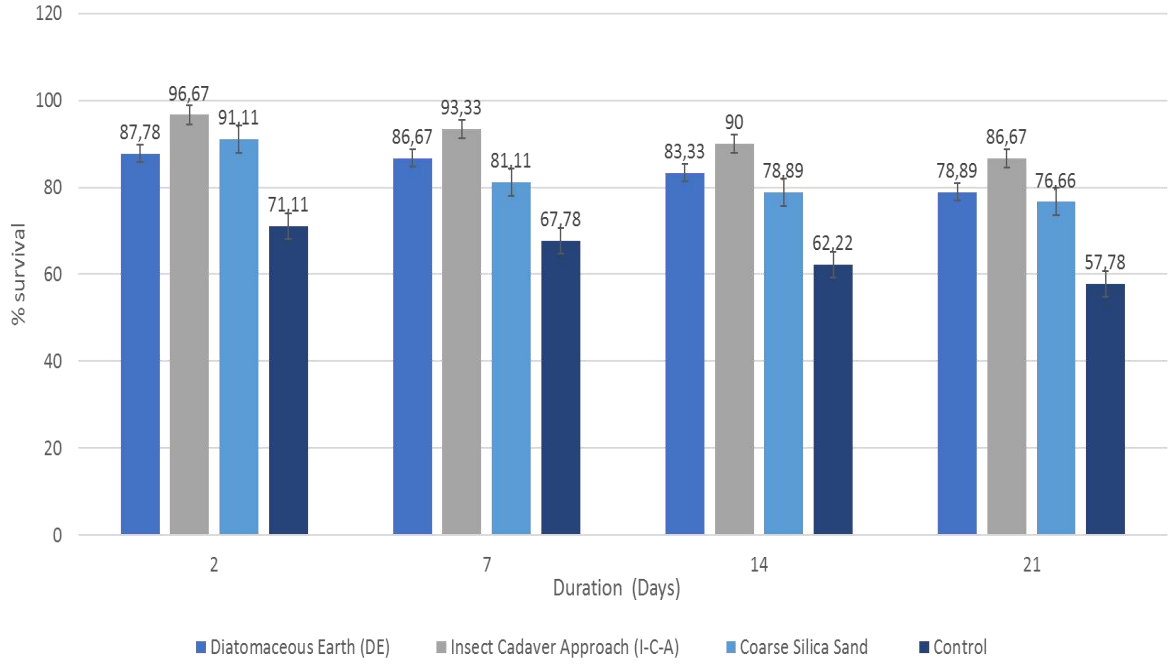

Figure 4.1: Percentage survival of *Oscheius* sp. OGB-2022 IJs formulated in different formulation substrates (95% Confidence Interval) for 2-, 7-, 14-, and 21-day incubation period at 25 º C incubation temperature.

The mean percentage viability (survival and virulence) of formulated IJs on the last Instar larvae of model organism *T. molitor* (n=10) was evaluated daily for a 7-day period, post inoculation with 1600IJs/μL. Highest rate of nematode survival was observed on the Insect-cadaver formulation, DE, and coarse silica sand with gradual

decreasing viability over time. The survival percentage of formulated IJs was maintained above 55% in the control, and above 75% in other substrates [DE (78.89%), Insect cadaver (86.67%), and coarse silica sand (76.66%)] after a 21-day incubation duration in a substrate (F=159.02; Fcrit=3.49; p<0.05) (Refer to Index 8). The error bars graphically represented the standard error of the means.

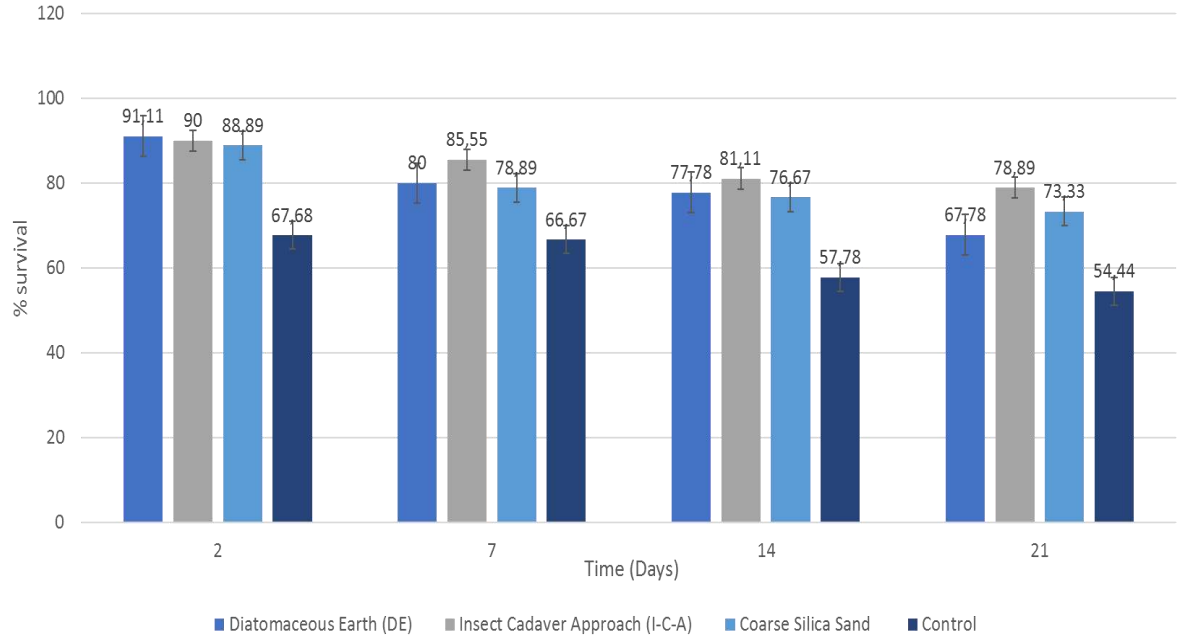

Figure 4.2: Percentage survival of *Oscheius* sp. OGB-2022 IJs formulated in different formulation substrates (95% Confidence Interval) for 2-, 7-, 14-, and 21-day incubation period at 30 º C incubation temperature.

The mean percentage viability (survival and virulence) of formulated IJs on the last Instar larvae of model organism *T. molitor* (n=10) was evaluated daily for a 7-day period, post exposure. Highest rate of nematode survival was observed on the Insect-cadaver formulation, DE, and coarse silica sand with gradual decreasing viability over time. The percentage survival of formulated IJs was maintained above 50% in the control, and above 66% in other substrates [DE (67.78%), Insect cadaver (78.89%), and coarse silica sand (73.33%)] after a 21-day incubation duration in a substrate (F= 50.92; Fcrit= 3.49; p< 0.05) (Refer to Index 8). The error bars graphically represented the standard error of the means.

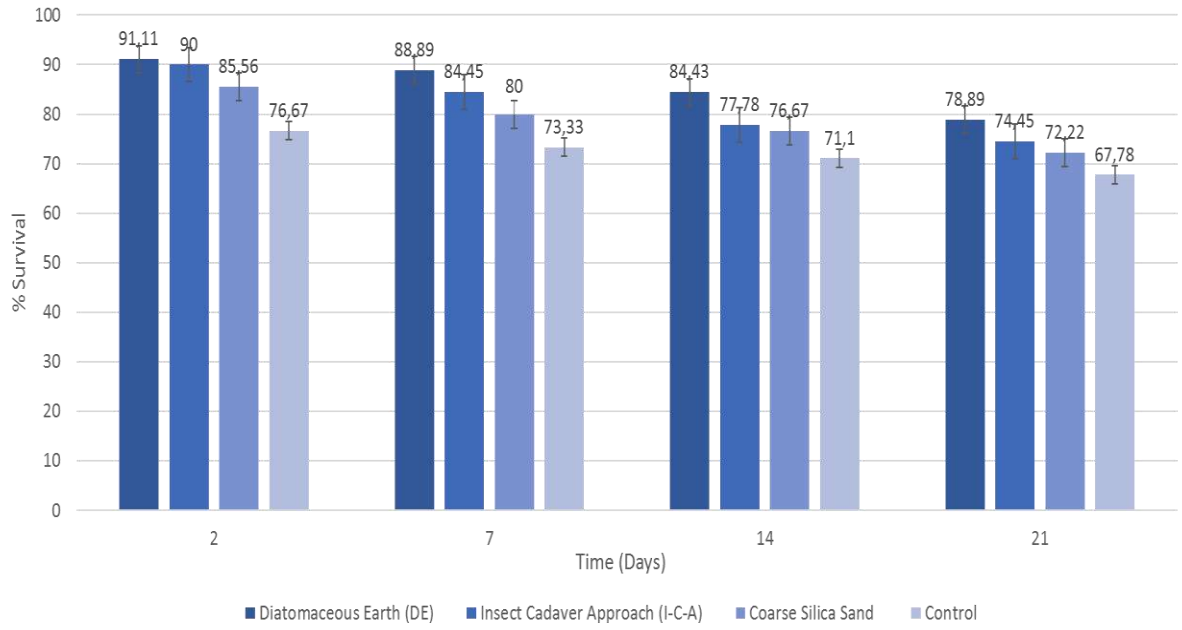

Figure 4.3: Percentage survival of *Cruznema* sp. NTM-2021 IJs formulated in different formulation substrates (95% Confidence Interval) for 2-, 7-, 14-, and 21-day incubation period at 25 º C incubation temperature.

The mean percentage viability (survival and virulence) of formulated IJs on the last Instar larvae of model organism *T. molitor* (n=10) was evaluated daily for a 7-day period, post exposure. Highest rate of nematode survival was observed on mainly DE, Insect cadaver, and coarse silica sand with gradual decreasing viability over time. DE had the highest survival rate of IJs observed. The survival percentage of formulated IJs was maintained above 65% in the control, and above 70% in other substrates [DE (78.89%), Insect cadaver (74.45%), and coarse silica sand (72.22%)] after a 21-day incubation duration in a substrate ($F=62.05$; $Fcrit= 3.49$; $p< 0.05$) (Refer to Index 8). The error bars graphically represented the standard error of the means.

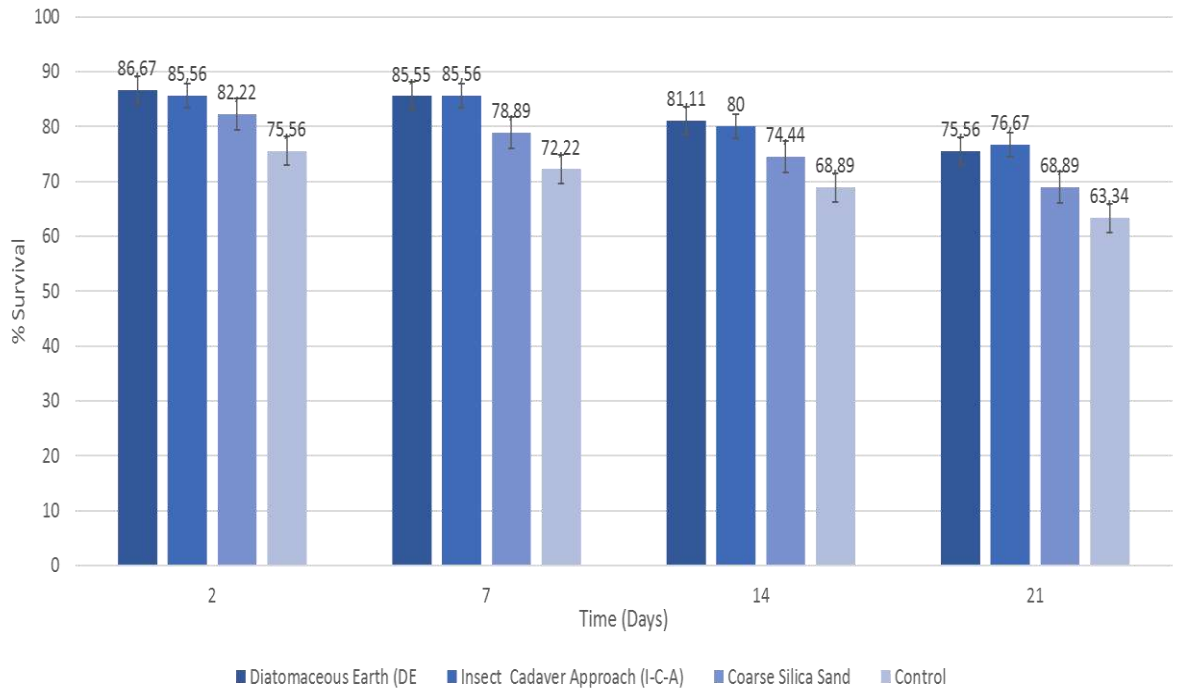

Figure 4.4: Percentage survival of *Cruznema* sp. NTM-2021 IJs formulated in different formulation substrates (95% Confidence Interval) for 2-, 7-, 14-, and 21-day incubation period at 30 º C incubation temperature.

The mean percentage viability (survival and virulence) of formulated IJs on the last Instar larvae of model organism *T. molitor* (n=10) was evaluated daily for a 7-day period, post exposure. Highest rate of nematode survival was observed on DE, Insect cadaver, and coarse silica sand with gradual decreasing viability over time. Similar survival rate of IJs observed was observed between DE and the insect cadaver formulation. The survival percentage of formulated IJs was maintained above 60% in the control, and above 65% in other substrates [DE (75.56%), Insect cadaver (76.67%), and coarse silica sand (68.89%)] after a 21-day incubation duration in a substrate ($F=134.12$; $F_{crit}= 3.49$; $p< 0.05$) (Refer to Index 8). The error bars graphically represented the standard error of the means.

4.4 Discussion

Numerous EPN species have emerged as effective biocontrol agents for use in several agricultural sectors against various problematic crop pests and are often considered suitable alternative for the use of harmful, synthetic chemical pesticides and for incorporation in the IPM programs (Kary *et al*., 2017). The ability for mass production of EPNs in liquid cultures in huge bioreactors (>90000 L), combined with the possible implementation of novel formulations that maintain IJ survival and pathogenicity for extended periods of time, has resulted in a wider availability of economical EPN-based products. Nonetheless, as compared to other biocontrol products, such as those based on *Bacillus thuringiensis* (Bt), EPN-based solutions remain rather expensive, necessitating the development of novel production and formulation technologies that can further reduce costs.

Microbial contamination and the metabolic rate of formulated IJs have been reported as the two major factors influencing survival of EPNs during the storage duration at a particular incubation temperature (Chen and Glazer, 2005; Kary *et al*., 2017). During the storage duration, longevity is severely affected by the higher rate of EPN movement within the formulation substrates as it leads to the rapid lipid reservoir depletion, especially in EPNs with cruising host seeking strategies (Grewal, 1998). Hence, it is of utmost significance to select a suitable formulation substrate that enhances survival and retains viability of EPNs, whilst physically trapping the IJs and reducing the rate of metabolisms. The experimental results of this study clearly illustrated how the suitable formulation substrates used significantly enhanced longevity and viability of formulated IJs.

It was evaluated that the ability of DE and the Insect cadaver formulation to retain virulence and viability of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 IJs for relatively extended periods and the relatively low cost and solubility in water renders them suitable substrates for the formulation of mass-produced IJs for commercialization (Kary *et al*., 2017, 2018). All the formulation substrates (DE, insect cadaver, coarse silica sand) that were used in this study significantly played critical roles in the survival of formulated IJs. DE, and coarse silica sand efficiently trapped the IJs in place by gradually immobilizing them throughout the storage duration of 2, 7, 14, and 21 days, whilst gradually inducing the partial state of anhydrobiosis (Kary

et al., 2017; 2018). The physical entrapment of IJs within the formulation prevented the overuse of stored lipid energy reserves by IJs and the migration from the substrate. The overly active formulated IJs usually have extremely reduced pathogenicity and viability and tend to develop increased sensitivity to extreme temperatures (Griffin, 1993; Hinchliffe *et al*., 2010).

The coarse silica sand retained the initial 8% moisture content (w/w) throughout the duration of the entire experiment and was able to maintain IJ viability at above 70% for both *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes at 25℃ and 30℃ incubation temperatures. However, slightly above 65% of *Cruznema* sp. NTM-2021 IJ viability was observed at 30℃ incubation temperature after a 21-day incubation period. The infected insect cadaver formulation on the other hand supported the nutritional physiology of the formulated IJs, thus extending their shelf-life up until a 21-day incubation duration whilst maintaining a percentage viability above 75% for both *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 IJs at both 25℃ and 30℃ incubation temperatures.

The lower moisture content (w/w) in these formulation substrates significantly played a role in preventing the possible microbial contamination during storage and maintaining viability and virulence of formulated IJs at respective incubation temperatures and periods. Formulations having a higher moisture content are more susceptible to microbial contamination, particularly by fungi, which can lower the quantity of accessible oxygen and, by contending with nematodes, cause their death. Contamination may as well occur during field application since dead particles and other contaminants may block spray nozzles. Moreover, the water content of the formulated product has a significant impact on the effective development of microbial agents, as demonstrated by studies of water activity in connection to the lifespan and integrity of formulated EPNs (Grewal, 2002a; Kagimu, 2018).

There was no significant variation observed on the survival and virulence of formulated IJs in different formulation substrates at both 25℃ and 30℃ incubation temperatures. However, *Oscheius* sp. OGB- 2022 IJs appeared to be more virulent after incubation at 30℃ and not at 25℃, unlike *Cruznema* sp. NTM-2021 IJs which indicated a slight gradual decrease from 25℃ to 30℃. *Cruznema* sp. NTM-2021 appeared more virulent at a lower incubation temperature of 25℃, and this could be well explained when the

ecology of these nematodes has been thoroughly investigated. The varied abilities possessed by the different formulation substrates with varying physical properties, sufficiently allowed for oxygen exchange (aeration) and IJ energy conservation without any major differential effect posed by the incubation temperature (Grewal, 2002).

Highest percentage survival and virulence of formulated IJs after incubation duration of 2, 7, 14, and 21 days at respective incubation temperatures was observed in the insect cadaver formulation, DE, and coarse silica sand, suggesting the potential usage of this media for field application of EPN products in agricultural sectors. There was a slight variation observed between the survival percentages of the two nematode isolates observed on the different formulation substrates when the last Instar larvae of model organism *T. molitor* was used as a proxy for survival. Throughout the study, there were clear indications that highest survival and viability rates were observed in the insect cadaver and DE formulation, followed by the coarse silica sand, thus suggesting that the storage conditions and chemical or physical properties of the storage media did not severely impact survival. This is likely because the total desiccation and lipid reservoir depletion of IJs was counteracted by the physical properties of these media, henceforth, maintaining infectivity and viability (Shapiro-IIan *et al*., 2010).

Based on observations made from the results obtained in this study, the substrates suitable for field application with relative ease of application using standard agronomic equipment are the insect cadaver and DE formulation. Numerous studies have reported that EPNs formulated in the insect cadaver have enhanced dispersive potentials and higher infectivity as compared to ones stored in aqueous suspensions (Shapiro-IIan and Glazer, 1996; Shapiro and Lewis, 1999). Perez *et al*., (2003) also reported that longer survival periods of three EPN species, *S. riobrave*, *S. carpocapsae*, and *H. bacteriophora*, with increased pest management stability was observed in the cadaver formulation than in the aqueous suspensions (Shapiro-IIan *et al*., 2003; Dolinski *et al*., 2015). These reports are also true to the nematode isolates (*Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021) used in this study. This method has also shown to support the nutritional physiology and provide nematodes with protection from adverse abiotic conditions.

Nematodes formulated in infected insect host cadavers have been reported to have delayed emergence from within the cadavers during unfavourable environmental conditions (Brown and Gaugler, 1996; Koppenhöfer *et al*., 1997). These nematodes thus tend to have enhanced protective mechanisms during both storage and after field application in agricultural sectors (Lewis and Shapiro-IIan, 2002). The infectivity, dispersive rate, and the biocontrol potentials of insect cadaver formulated nematodes are enhanced by the chemical cues or rather pheromones released within the infected host cadaver (Kaplan *et al*., 2012; Oliveira-Hofman *et al*., 2019; Shapiro-IIan *et al*., 2019). Moreover, various substances emitted by the infected insect host cadavers provide buffering capacity against abiotic stresses and assist in the activation of physiological processes guarding against several stress factors (Shapiro-IIan *et al*., 2000; Grewal and Jagdale, 2002). These reports from previous scientific studies could support the high virulence and viability observed on *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes formulated in the last Instar larvae of model organism *T. molitor* and potentially implicate the usage of insect cadaver technique in the agricultural sector. Furthermore, the insect cadaver formulation may broaden the wide range of abiotic factors (desiccation or higher temperatures) in which EPNs can be applied during field application and is also anticipated to improve EPN survival and persistence under adverse environmental conditions (Shapiro-IIan *et al*., 2011). EPNs applied as infected insect cadavers provide stability and economic benefits for commercial *in vivo* producers and can easily be distributed using automated agronomic equipment (Shapiro-IIan *et al*., 2001; Deol *et al*., 2011; Dolinski *et al*., 2015). However, extensive research must be undertaken to investigate potential modifications of this method.

Other more promising formulations for mass- produced EPNs which exhibited excellent percentage viability and virulence of *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematodes used in this study include powder formulations which can easily dissolve in water. Such kind of formulations include DE which has greatly emerged as the most prominent and popular powder for EPN formulation (Kagimu *et al*., 2017). The benefits of utilizing DE in EPN formulations, including ease of field application were recently reported by Kagimu *et al*., (2017). The optimization capacities of this kind of formulation gives it more potential to be incorporated in the EPN formulation technology (Kagimu and Malan, 2019). These formulations can be

applied during irrigation, utilizing the already existing farm irrigation systems without any requirement for specialized equipment. The coarse silica sand formulation showed satisfactory IJ maintenance capacities. However, the field application of IJs formulated in this substrate will be problematic as they pose the risks of blocking the spray nozzles of standard agronomic equipment, unless they are only applied manually by hand for small scale farms. The *Oscheius* sp. OGB-2021 and *Cruznema* sp. NTM-2021 IJs exhibited excellent survivability in the infected insect cadaver formulation and in DE, when store at both 25℃ and 30℃ incubation temperatures for varying incubation durations of 2, -7, -14, and 21- days, thus implying that future commercialisation of these nematode isolates will have to be undertaken at these optimum temperatures in optimized formulation substrates.

4.5 Conclusion

Interests on the use of EPNs as suitable effective alternatives for the use of harmful, synthetic insecticides in agricultural industries has exponentially grown in recent years. However, higher production costs involved in the storage, formulation, transportation, and overall maintenance of EPNs as biocontrol agents is a major hinderance in the large-scale commercialization at market value, hence, there is a dire necessity for developing cost effecient EPN formulation tactics. Although there has been significant progress in mass producing EPNs, the development of a formulation with the ability to maintain high- quality EPNs for relatively extended periods at room temperature storage without negatively impacting virulence and survival, and with minimal microbial contamination, has been a limiting factor for most EPNs due to their distinct ecology. Formulation substrates modified to induce EPNs into a reversible state of partial anhydrobiosis, whilst suppressing the metabolic rate and maintaining their survival at room temperature storage for relatively long periods are ideal for the successful commercialization of EPNs. *Oscheius* sp. OGB-2022 and *Cruznema* sp. NTM-2021 nematode IJs showed impressively high viability and survival when formulated in the insect cadaver and DE formulation, thus implying the potential for these substrates to be used as promising formulations for commercialization. However, there needs to be optimization for these substrates to effectively be used and applied in agricultural sectors. The insect cadaver formulation could for instance be coated with eco-friendly biodegradable powders to prevent the infected insect host cadavers from bursting open, whilst not interfering with EPN efficacy and survival. The DE formulation could

also be supplemented with antimicrobial agents to prevent any potential microbial contamination over time.

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Appendices

Appendix 1

Selective and differential bacterial growth media for endosymbiont isolation

Nutrient Bromothymol blue Triphenyltetrazolium Chloride agar (NBTA)

1 litre nutrient agar

Triphenyltetrazolium chloride (TTC) (0.04g)

Bromothymol blue (0.025g)

- ✓ Suspend nutrient agar and bromothymol blue in sterile distilled water and autoclave the mixture at 121°C for 15 minutes.
- ✓ Let the media cool down to 50°C. Add TTC and gently swirl to mix, before aseptically dispensing into sterile 90mm diameter Petri dishes.
- ✓ Leave the media plates to solidify under the laminar flow hood.

McConkey agar.

Composition (g/l)

Peptone (20.0g)

Lactose (10.0g)

Bile Salts (1.5g)

Sodium Chloride (NaCl) (5.0g)

Neutral Red (0.03g)

Crystal Violet (0.0001g)

Agar (13.5g)

- ✓ Weigh out McConkey agar powder on an electronic weighing balance and suspend in 1L sterile distilled water.
- ✓ Swirl to dissolve completely and autoclave at 121ºC for 15 min.
- ✓ Let the media cool down to 50ºC, then aseptically dispense into sterile 90mm diameter petri dishes under the laminar flow hood.
- ✓ Leave to solidify.

Nutrient broth

Composition (g/l)

Meat extract (1g)

Yeast extract (2g)

Peptone (5g)

NaCl (8g)

- ✓ Weigh out nutrient broth powder and suspend in 1L sterile distilled water.
- ✓ Swirl to completely mix and autoclave at 121˚C for 15 min.

Appendix 2

0.1% Sodium hypochlorite (NaClO) solution for surface sterilization of nematode IJs

34ml sterile distilled water

1ml 3.5% jik

- ✓ Mix 34mL of sterile autoclaved water and 3.5% jik in sterile autoclaved 1L bottle.
- ✓ Autoclave at 121˚C for 15 min.

Appendix 3

Nematode Genomic DNA Extraction protocol (E.Z.N.A® insect DNA extraction Kit).

- ✓ Surface sterilized IJs enclosed in 1.5mL microcentrifuge tubes with 0.1% NaClO solution and pulverize into a thin paste using a sterile plastic pestle.
- ✓ Add 350 μL CTL Buffer and 25 μL proteinase K solution to the pulverized nematode paste in the 1.5mL.
- ✓ Briefly vortex for 30 seconds, prior to an overnight incubation at 37℃ on the water bath.
- ✓ After the incubation, add 350 μL chloroform: isoamyl alcohol (24:1) and vortex to mix thoroughly.
- ✓ Centrifuge at 10,000 x g for 2 minutes at room temperature.
- ✓ Remove the upper aqueous phase that resulted after centrifugation and carefully transfer to sterile 1.5mL microcentrifuge tubes, whilst avoiding the milky interface containing contaminants and inhibitors.

- ✓ Add 350 μL BL Buffer and 2 μL RNAse A solution into the 1.5mL the tubes before vortexing at maximum speed for 15 seconds.
- ✓ Incubated the tubes at 70℃ on a Labotec EcoBlock dry heating block for 10 minutes.
- ✓ Add 350 μL solution of 100% ethanol and vortex at maximum speed for 15 seconds.
- ✓ Insert HiBind® DNA Mini Columns into 2mL collection tubes and aspirate 750 μL cleared lysate into the HiBind® DNA Mini Columns.
- ✓ Centrifuge the collection tubes at maximum speed for 1 minute and discard the filtrates.
- ✓ Reuse the collection tubes for step repetition until all the remaining samples have been transferred to the HiBind® DNA Mini Columns.
- ✓ Transfer the HiBind® DNA Mini Columns into the 2mL collection tubes and add 500 μL HBC Buffer.
- ✓ Centrifuge at maximum speed for 30 seconds. Discard the filtrates and the reuse the collection tubes.
- ✓ Add 700 μL DNA Wash Buffer and centrifuge at maximum speed for 1 minute. Discard the filtrates and reuse the collection tubes.
- ✓ Centrifuge empty HiBind® DNA Mini Columns for 2 minutes at maximum speed to dry the column matrix and transfer to sterile microcentrifuge tubes.
- ✓ Add 50 μL Elution Buffer solution preheated to 70℃ directly to the centre of the column membranes and allow to sit for 2 minutes at room temperature.
- ✓ Centrifuge the tubes at maximum speed for 1 minute and assess purity of the extracted DNA on the Nano-Drop ND-1000® spectrophotometer (Bio-Rad), before storage at - 80℃.

Appendix 4

Bacterial Genomic DNA Extraction Kit (E.Z.N.A. ® Bacterial DNA Kit; omega BIO-TEK Revision No: 2.0).

- ✓ Overnight bacterial cultures were prepared by inoculating bacterial colonies on 25mL Luria-Bertani (LB) broth in 50mL Falcon tubes and incubating in the dark at 30 ℃ for 24 hours on an orbital shaker at 150rpm.

- ✓ After the incubation, 1.5mL of the bacterial cultures was obtained from the 50mL Falcon tubes and dispensed into sterile 1.5mL microcentrifuge tubes using a 1000μL pipette.
- ✓ The 1.5mL microcentrifuge tubes containing 1.5mL bacterial samples were centrifuged at 4000 xg for 10 minutes at room temperature. This step was repeated.
- ✓ 100μL TE Buffer was added. Vortex to completely resuspend the pellet.
- ✓ Add 10μL Lysozyme resuspended with Elution Buffer. Incubate at 37℃ for 10 minutes.
- ✓ Add 100μL TL Buffer and 20μL Proteinase K solution. Vortex to mix thoroughly. Incubate at 55℃ in a shaking water bath. Usually no more than 1 hour is required for bacterial lysis. If a shaking water bath is not available, incubate the samples and shake or briefly vortex every 20-30 minutes.
- ✓ Add 5μL RNAse A. Invert the tube several times to mix. Let sit at room temperature for 5 minutes.
- ✓ Centrifuge at 10,000xg for 2 minutes to pellet any undigested material. Transfer the supernatant to a new 1.5mL microcentrifuge tube. Do not disturb the pellet.
- ✓ Add 220μL BL Buffer. Vortex to mix thoroughly. Incubate at 65℃ for 10 minutes.
- ✓ Add 220μL 100% ethanol. Vortex for 20 seconds at maximum speed to mix thoroughly. Break any precipitate by pipetting up and down 10 times.
- ✓ Insert a HiBind® DNA Mini column into a 2mL Collection tube. Transfer the entire sample to the HiBind® DNA Mini Column, including any precipitate that may have formed.
- ✓ Centrifuge at 10,000xg for 1 minute. Discard the filtrate and the collection tube.
- ✓ Insert the HiBind® DNA Mini Column to a new 2mL collection tube.
- ✓ Add 500μL HBC Buffer diluted with 100% Isopropanol. Centrifuge at 10,000xg for 1 minute. Discard the filtrate and reuse the collection tube.
- ✓ Add 700μL DNA Wash Buffer diluted with 100% ethanol. Centrifuge at 10,000xg for 1 minute. Discard the filtrate and reuse the collection tube.
- ✓ Repeat step the above step for a second DNA Wash Buffer Wash step.
- ✓ Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column. This step is critical for removal of trace ethanol that may interfere with downstream application.

- ✓ Insert the HiBind® DNA Mini Column a new nuclease-free 1.5 mL microcentrifuge tube.
- ✓ Add 50μL -100μL Elution Buffer heated to 65℃. Let sit for 3-5 minutes at room temperature. Yields may be increased by incubating the column at 65℃. Centrifuge at 10,000xg for 1 minute to elute the DNA.
- ✓ Repeat the above step for a second elution step.
- ✓ Store eluted DNA at -20℃.

Appendix 5

Methodological outline of the nematode formulation in different formulation substrates.

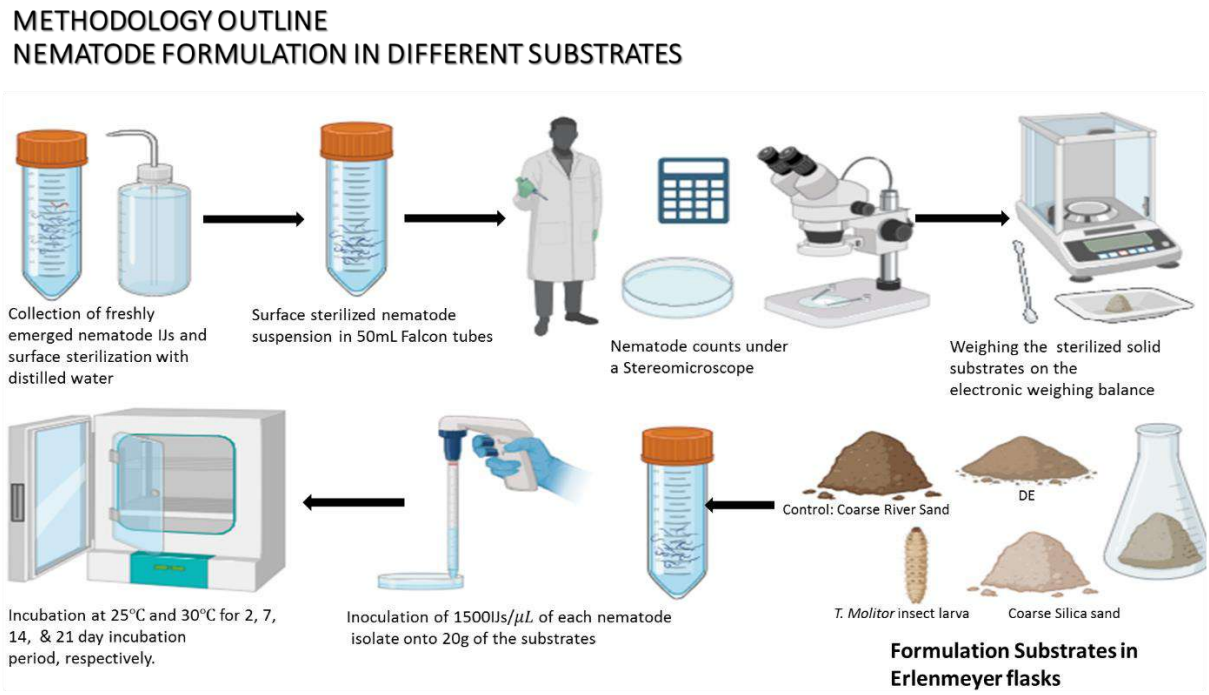

Figure 4.5: Methodological outline of the nematode formulation in different formulation substrates.

Appendix 6

Different formulation substrates used in this study.

Figure 4.6: Different formulation substrates used in this study.

Appendix 7

Overview of the formulation experimental setup for a single incubation period for both nematodes.

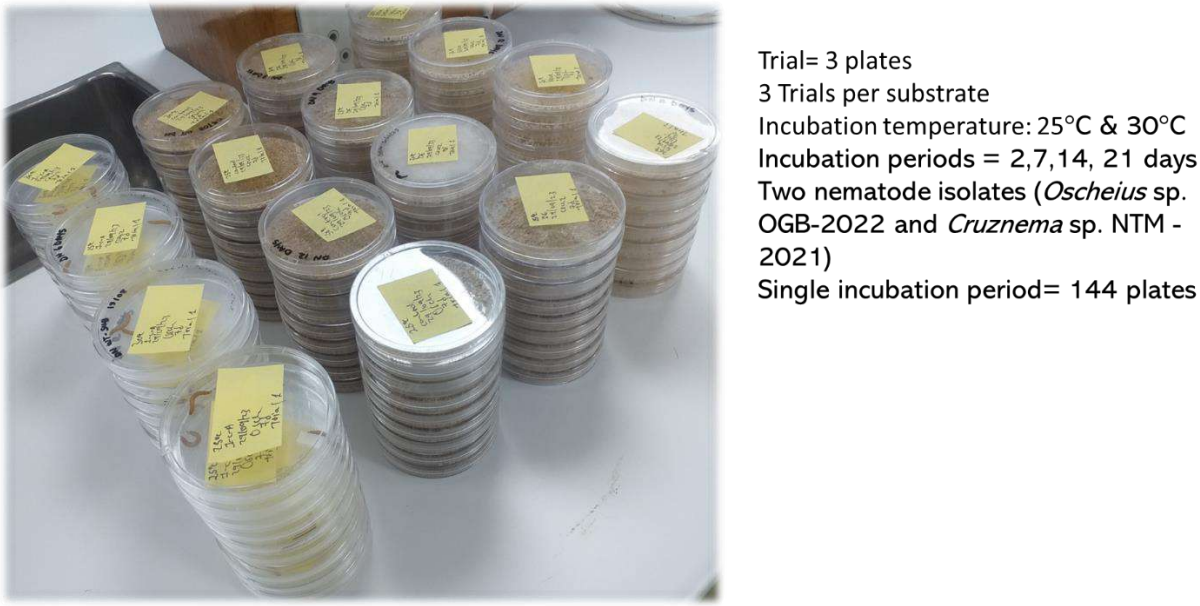

Figure 4.7: Overview of the formulation experimental setup.

Appendix 8

Two-Way Statistical Analysis of Variance (ANOVA)

Table 12: Analysis of Variance (ANOVA) Data for *Oscheius* sp. OGB-2022 at both 25℃ and 30℃ in different substrates for varying incubation durations.

	A	B	C	D	E	F	G
1	***Oscheius* sp.OGB-2022 Analysis of Variance (ANOVA/AOV) Data (25℃)**						
2		**Degrees of Freedom (Df)**	**Sum.Sq**	**Mean.Sq**	**F.value**	**Pr..F.**	
3	substrate	3	1556,4457	518,815233	159,017206	2,4405E-09	
4	Duration	1	294,089506	294,089506	90,1386247	1,2388E-06	
5	Residuals	11	35,8889941	3,26263582			
6							
7	***Oscheius* sp. OGB-2022 Analysis of Variance (ANOVA/AOV) Data (30℃)**						
8		**Degrees of Freedom (Df)**	**Sum.Sq**	**Mean.Sq**	**F.value**	**Pr..F.**	
9	substrate	3	1160,98587	386,99529	50,9199939	9,7135E-07	
10	Duration	1	527,264004	527,264004	69,3762447	4,4426E-06	
11	Residuals	11	83,6007206	7,60006551			
12							

The F and P-values indicate significant effects on the substrates and duration on the survival of IJs. The residuals had a relatively small Mean.sq value, indicating that the type of substrate, duration, and temperature all have an impact on the survival of formulated nematode IJs.

Table 13: Tukey (HSD) Post-Hoc Test Data for *Oscheius* sp. OGB-2022 at both 25°C and 30°C in different substrates for varying incubation durations.

	A	B	C	D	E
1	***Oscheius* sp. OGB-2022 Tukey (HSD) Test Data (25℃)**				
2	**Differences between Substrates**	**Differences of Means**	**95% Confidence Interval/ 95% CI**	**Adjusted P-value (p adj)**	
3	Insect Cadaver Approach (I-C-A)-Diatomaceous Earth (DE	7,5	(3.66, 11.34)	0,000528770	
4	Coarse Silica Sand-Diatomaceous Earth (DE	-2,225	(-6.07, 1.62)	0,349211680	
5	Control-Diatomaceous Earth (DE	-19,445	(-23.29, -15.60)	0,000000051	
6	Coarse Silica Sand-Insect Cadaver Approach (I-C-A)	-9,725	(-13.57, -5.88)	0,000052632	
7	Control-Insect Cadaver Approach (I-C-A)	-26,945	(-30.79, -23.10)	0,000000002	
8	Control-Coarse Silica Sand	-17,22	(-21.06, -13.38)	0,000000179	
9					
10	***Oscheius* sp. OGB-2022 Tukey (HSD) Test Data (30℃)**				
11	**Differences between Substrates**	**Differences of Means**	**95% Confidence Interval/ 95% CI**	**Adjusted P-value (p adj)**	
12	Insect Cadaver Approach (I-C-A)-Diatomaceous Earth (DE	4,72	(-1.15, 10.59)	0,130119205	
13	Coarse Silica Sand-Diatomaceous Earth (DE	0,2775	(-5.59, 6.14)	0,99890566	
14	Control-Diatomaceous Earth (DE	-17,525	(-23.39,-11.66)	1,08205E-05	
15	Coarse Silica Sand-Insect Cadaver Approach (I-C-A)	-4,4425	(-10.31,1.42)	0,162302308	
16	Control-Insect Cadaver Approach (I-C-A)	-22,245	(-28.11,-16.38)	1,0038E-06	
17	Control-Coarse Silica Sand	-17,8025	(-23.67,-11.94)	9,28469E-06	
18					

The Tukey (HSD) Post-Hoc test for *Oscheius* sp. OGB-2022 indicates statistically signicant differences between the specific pairs of variables, such as substrates and duration at specific temperatures.

Table 14: Analysis of Variance (ANOVA) Data for Cruznema sp.NTM-2021 at both 25℃ and 30℃ in different substrates for varying incubation durations.

	A	B	C	D	E	F	G
1	***Cruznema* sp.NTM-2021 (ANOVA Data) 25℃)**						
2		**Degrees of Freedom (Df)**	**Sum.Sq**	**Mean.Sq**	**F.value**	**Pr..F.**	
3	substrate	3	394,144869	131,381623	62,0512218	3,518E-07	
4	Duration	1	345,285874	345,285874	163,077681	6,1222E-08	
5	Residuals	11	23,2904012	2,1173092			
6							
7	**Cruznema sp.NTM-2021 (ANOVA Data) 30℃**						
8		**Degrees of Freedom (Df)**	**Sum.Sq**	**Mean.Sq**	**F.value**	**Pr..F.**	
9	substrate	3	400,827669	133,609223	134,12185	6,0736E-09	
10	Duration	1	304,673668	304,673668	305,842629	2,2433E-09	
11	Residuals	11	10,9579569	0,9961779			
12							

The ANOVA analysis indicate that both substrates and duration have significant impacy on the survival of *Cruznema* sp. NTM-2021 at both 25℃ and 30℃. The effect of the substrates is significant at $p<0.001$, and the effect of the duration is significant at $p<0.001$. The residuals have a relatively small Mean.sq value.

Table 15: Tukey (HSD) Post-Hoc Test Data for Cruznema sp. NTM-2021 at both 25℃ and 30℃ in different substrates for varying incubation durations.

	A	B	C	D	E
1	***Cruznema* sp. NTM-2021 Tukey (HSD) Post-Hoc Test Data (25℃)**				
2	**Differences between Substrates**	**Differences of Means**	**95% Confidence Interval (CI)/ 95% CI**	**Adjusted p-value (p adj)**	
3	Insect Cadaver Approach (I-C-A)-Diatomaceous Earth (DE	-4,16	(-7.26, -1.06)	0,0089	
4	Coarse Silica Sand-Diatomaceous Earth (DE	-7,2175	(-10.31, -4.12)	0,0001	
5	Control-Diatomaceous Earth (DE	-13,61	(-16.72, -10.51)	0,0000	
6	Coarse Silica Sand-Insect Cadaver Approach (I-C-A)	-3,0575	(-6.15, 0.04)	0,0533	
7	Control-Insect Cadaver Approach (I-C-A)	-9,45	(-12.55, -6.35)	0,0000	
8	Control-Coarse Silica Sand	-6,3925	(-9.50, -3.30)	0,0003	
9					
10	***Cruznema* sp.NTM-2021 Tukey (HSD) Post-Hoc Test Data (30℃)**				
11	**Differences between Substrates**	**Differences of Means**	**95% Confidence Interval (CI)/ 95% CI**	**Adjusted P-value (p adj)**	
12	Insect Cadaver Approach (I-C-A)-Diatomaceous Earth (DE	-0,275	(-2.40, 1.85)	0,97890207	
13	Coarse Silica Sand-Diatomaceous Earth (DE	-6,1125	(-8.24,-3.99)	0,00001553	
14	Control-Diatomaceous Earth (DE	-12,22	(-14.34,-10.10)	0,00000001	
15	Coarse Silica Sand-Insect Cadaver Approach (I-C-A)	-5,8375	(-7.96,-3.71)	0,00002414	
16	Control-Insect Cadaver Approach (I-C-A)	-11,945	(-14.07,-9.82)	0,00000002	
17	Control-Coarse Silica Sand	-6,1075	(-8.23,-3.98)	0,00001565	
18					

The Tukey (HSD) Post-Hoc test for *Cruznema* sp. NTM-2021 indicates statistically signicant differences between the specific pairs of variables, such as substrates and duration at specific temperatures.