DEVELOPMENT OF A NEW ENTOMOPATHOGENIC NEMATODE SPECIES, *STEINERNEMA INNOVATIONI*: BIOLOGICAL CHARACTERIZATION AND MASS PRODUCTION

By

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THESIS SUMMARY

Entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis* are employed as environmental friendly biopesticides to control key pests in high value agricultural crops. Their successful application in developed countries has proved their effectiveness. The production of EPN for large scale commercial application has been restricted to developed countries because high capital cost of setting up liquid fermentation, and the high running costs of such units. If EPNs are to be commercialized in developing countries, then this will probably be achieved using *in vivo* culturing in insect larvae, or by using *in vitro* solid culture on a small to medium scale. The Republic of South Africa bans the importation of exotic EPN products by Amendment 18 of Act 36 of 1983. Therefore EPN products in South Africa will need to be developed from indigenous species. This study focused on optimizing the solid culture production and biological characterization of an indigenous nematode, *Steinernema innovationi*, as a biocontrol agent. A good production system must achieve high yield, maintain high virulence and the product must have a reasonable shelf-life.

Optimum production temperature was determined using *in vivo* culturing of *S. innovationi* inside *Galleria mellonella* larvae. Performance was measured by quantifying the first day of emergence of infective juveniles (IJs) from cadavers, yield of IJs and IJ length, at five temperatures ranging from 18°C to 25°C. Optimum temperature was selected as 22°C based on high yield and slow emergence. There was a yield of 92,756 ± 28,089 IJs/larva, and 84,056 ± 27,832 IJs/larva, at 22°C and 25°C, respectively, which were not significant different. However, IJ emergence was significantly slower at 22°C, which provided more time for nutrient uptake and therefore these IJs had a greater nutrient reserve. There was no correlation observed between IJ length vs. temperature and IJ length vs. yield.

A medium containing a puree of the larvae of the common house fly (*Musca domestica)* and 3% canola oil produced the highest yield of IJs (781,678 ± 221 IJs/5g), the highest level of live IJs (>84%) and the lowest level of adults (<10%)

remaining in the medium at the time of harvest, compared to five other media formulations. All media were subjected to *in vitro* mass production at 22°C. A liquid inoculum of the EPN gave higher yields than a solid culture inoculum, irrespective of concentration. The length of IJ did not affect virulence against last instar larvae of *G. mellonella,* which was >90% in all experiments.

A study characterizing optimum storage temperature of *S. innovationi* was carried out at five temperatures ranging from 5°C to 25°C in aqueous suspension over a period of 84 days. Survival was highest and most stable at 15°C, ranging from 84% to 88% after 84 days storage. Storage of the EPN in a sponge at a concentration of 2.5 million IJs in 15ml 0.1% formalin solution was successful, with an improvement of 6% compared to aqueous storage at 15°C. Furthermore, storage of the EPN in a sponge at 25°C, after a period of low temperature (15°C) storage for 84 days, did not have a detrimental impact on IJ survival and infectivity (87% and 95%, respectively).

The new EPN (*S. innovationi*) was characterized by studying traits related to its infectivity (infectivity under a range of temperatures [10°C to 35°C], foraging behaviour & persistence under field conditions). Time till death was shortest at 25 and 30°C (average 1 day). The highest number of established IJs was recovered at 25°C (mean = 27°C). The nematode infected *Galleria mellonella* larvae at all depths and was capable of covering a distance of up to 15cm in 24 hours. There was no statistical difference between *in vitro* (40%) and *in vivo* (27%) cultured IJ persistence 4 weeks post application. Furthermore, there was no statistical difference in nematode recovery after 1 and 4 weeks post application of *in vitro* (33% & 40%, respectively) and *in vivo* (60% & 27%, respectively) produced IJs. Thermal activity was optimal at 25°C, the new species was classified as a cruiser and proved to survive under field conditions for at least 4 weeks.

Susceptibility of larvae and/or pupae of *Eldana saccharina* (Walker)*, Sesamia calamistis* (Hampson), *Chilo partellus* (Swinhoe)*, Tenebrio molitor* (Linnaeus), *Galleria mellonella* (Linnaeus), *Cydia pomonella* (Linnaeus), *Plutella xylostella* (Linnaeus), and *Gryllidae acheta* (Linnaeus) representing three orders (Coleoptera, Lepidoptera & Orthoptera) was tested at a low and high concentration of 50 and 500 IJs, respectively. The hosts *G. acheta, C. partellus* and *P. xylostella* showed least

susceptibility with maximum mortalities at the 500 IJs concentration of 28%, 45% and 92%, respectively. All other hosts suffered 100% mortality. Pupal mortality ranged from 47% to 68%. An LC_{50} and LC_{70} of 3 IJs/larva and 31 IJs/larva, respectively, was calculated for the black cutworm, *Agrotis ipsilon* (Hufnagel). These results provided a broad guideline on the relative pathogenicity of this new species against different hosts.

A cost analysis was calculated for *in vitro* solid culture of the EPN, including the cost of rearing an insect-based nutrient component. An estimated retail price was then compared to the costs of market products around the globe. An estimated retail price (R90.61) for *S. innovationi* was considerably lower than the market price for other *Steinernema* species, which ranged from R271.50 to R458.55, in South African rands. The production system developed in this study for *S. innovationi* offers a highly competitive small to medium scale production method to produce EPN products without having to invest in large scale liquid fermentation equipment, by using relatively cheap production media ingredients, and simple solid culture growing conditions.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCES DECLARATION

I, **Tshimangadzo Ramakuwela** Student Number: **211560084** declare that:

1. The research contained in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other University.

3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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    a. Their words have been re-written but the general information attributed to them has been referenced.

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STUDENT DECLARATION

I, **Tshimangadzo Ramakuwela** Student Number: **211560084** declare that:

The research reported in this thesis, except where otherwise indicated is the result of my own activities at the Agricultural Research Council-Small Grain Institute (ARC-SGI, Bethlehem), under the supervision of Dr Justin L. Hatting and Prof. Mark D. Laing (UKZN).

Signed at……..on the……. day of ……..... 2014

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SIGNATURE

CONFERENCE CONTRIBUTIONS FROM THIS RESEARCH

2013 Ramakuwela, T., Hatting, J. & Laing, M. D. (2013). Solid-state *in vitro* mass production of an entomopathogenic nematode (Steinernematidae). XVIII Congress of the Entomological Society of Southern Africa (ESSA), North-West University, Potchefstroom, South Africa. 30 June – 3 July 2013.

2014 Ramakuwela, T., Hatting, J. & Laing, M. D. (2014). "Solid-state in vitro mass production of an entomopathogenic nematode (Steinernematidae). The 6th International Congress of Nematology (ICN), The Southern Sun Cape Sun, Cape Town, South Africa. 4 – 9 May 2014

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DEDICATION

I dedicate this thesis to my beloved mom,

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Her hard work and achievements remain the source of my inspiration to work hard for the things to which I aspire.

TABLE OF CONTENTS

THESIS INTRODUCTION

Background and motivation

Entomopathogenic nematodes (EPNs) are naturally occurring round worms in the soil, which live in a symbiotic relationship with highly specialized bacteria (Family: Enterobacteriaceae) and are capable of causing mortality in an insect host within 48 hours (Kaya & Gaugler, 1993). Recent classification of insect parasitic nematodes add two *Oscheius* species (*Ocheius chongmingensis* and *Ocheius carolinensis*) to the EPNs group (Dillman *et al*., 2012). However, for biocontrol purposes, the families Steinernematidae and Heterorhabditidae are the most explored and by far the most successful.

EPNs are used as environmentally friendly bio-insecticides to control insect pests. The third-stage, infective juveniles (IJs) are used as bioinsecticides because they are free-living and infectious, and actively hunt for insect hosts in the soil environment. During this stage the nematodes do not feed or develop and can remain viable for many months (Hazir, Kaya, Stock & Kestin, 2003).

Nematode parasites of insects have been known since the 17th century (Nickel, 1984), but it was only in the 1930's that serious consideration was given to using nematodes to control an insect pest. In 1929, Glaser and Fox (1930) found a nematode infecting grubs of the Japanese beetle, *Popillia japonica* (Newman) at the Tavistock Golf Course near Haddonfield, New Jersey. Glaser and his colleagues isolated the nematode, multiplied it for field trials and used it for the control of Japanese beetles in 1931. Since then, there has been much research on the isolation ofEPNs from different localities, in order to develop them for biological control of insect pests. EPN-based bioinsecticides are already available internationally. However, in South Africa the importation of exotic EPN products has been blocked by Agricultural Pest Amendment Act 18 of Act 36 of 1983.

An important step in the development of bio-insecticidal nematodes is the mass production of the IJs. Mass production can be achieved by *in vivo*, *in vitro* solid

culture or by *in vitro* liquid culture methods. The choice of production method is determined by the cost of infrastructure, the cost of production and the quality of the product. Generally, EPN products are more expensive than chemical pesticides, or fungal and bacterial biocontrol agents because of their high costs of production.

EPN species have highly specific requirements for production and maintenance. Therefore, local protocols for production maintenance are needed if indigenous EPNs are to be commercialized. This particular study focused on developing a novel, low cost mass production medium for a new indigenous *Steinernema innovationi* (Çimen, Lee, Hatting & Stock 2014) isolated in South Africa (Hatting, Stock & Hazir, 2009) and curated by the Agricultural Research Council – Small Grain Institute (ARC-SGI).

EPN research in South Africa

In South Africa, the history of EPNs date back to 1953 when a *Steinernema* species was isolated from the maize beetle, *Heteronychus arator* (Fabricius), by Harrington (1953) in the Eastern Cape Province. Following this, many surveys were conducted and isolates of *Heterorhabditis* and *Steinernema* were collected in different provinces, *viz*., KwaZulu-Natal (Hatting *et al*., 2009; Pillay, Martin, Rutherford and Berry, 2009; Spaull, 1988, 1990, 1991), Western Cape (Hatting *et al*., 2009; Malan, Knoetze & Moore, 2011; Malan, Nguyen & Addison, 2006; Nguyen Malan and Gozel, 2006), Eastern Cape (Malan *et al*., 2006; Malan *et al.,* 2011), Mpumalanga and Free State (Hatting *et al*., 2009; Malan *et al.,* 2011). The most diverse and systematic survey was that reported by Hatting *et al*. (2009) covering seven geographic regions of South Africa (Figure 1), which yielded 76 positive samples including *Steinernema khoisanae* (Nguyen, Malan & Gozel, 2006) (Nguyen *et al.*, 2006), *Heterorhabditis bacteriophora* (Poinar, 1976) and three new species of *Steinernema,* from a total of 1506 soil samples. Two other indigenous species from other surveys were described as *Heterorhabditis safricana* (Malan, 2008) (Malan *et al.,* 2008) and *Steinernema citrae* (Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011) (Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011).

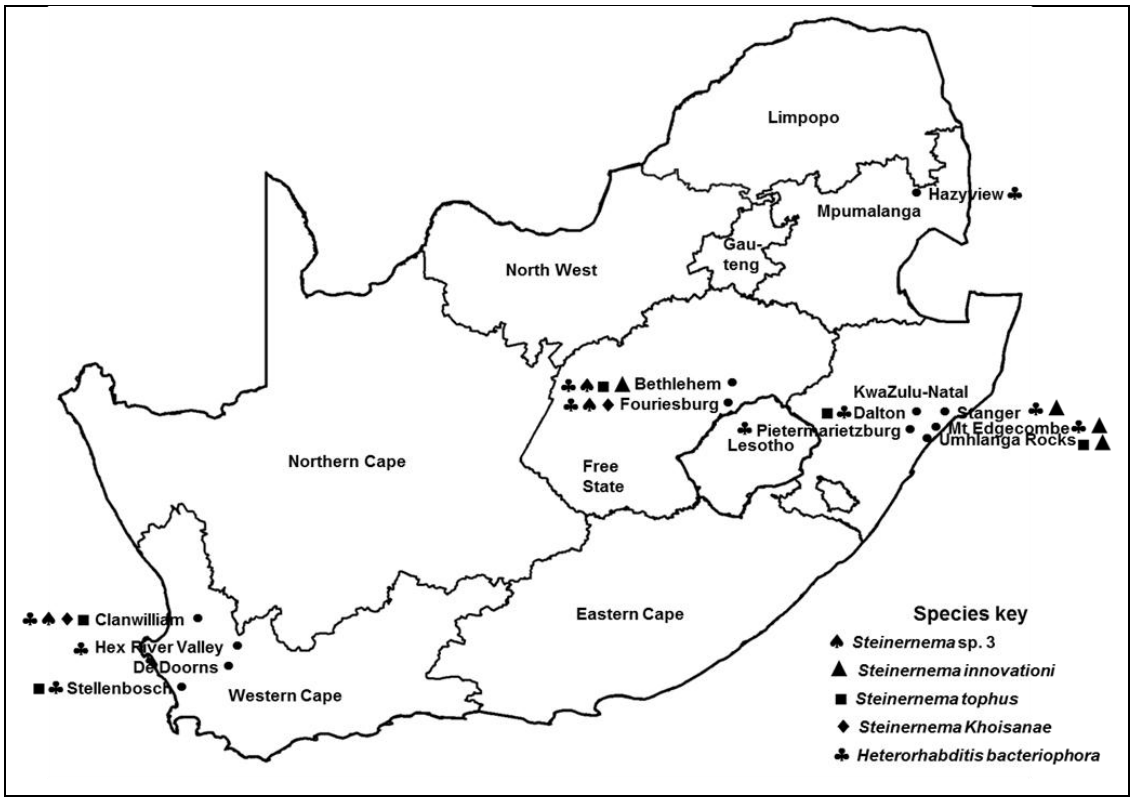

Figure 1. Diversity and distribution ofEPNs in South Africa Hatting *et al*. (2009).

Extensive research on EPNs has been conducted in South Africa, focusing on the virulence of EPNs against different insect pests of agricultural importance: the sugarcane stalk borer, *Eldana saccharina* (Walker) (Pillay *et al*., 2009); the codling moth*, Cydia pomonella* Linn (De Waal, Malan and Addison, 2011) (De Waal, Malan, Levings & Addison, 2010; Malan *et al.,* 2011; Malan & Manrakhan, 2009) the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and the Natal fruit fly, *Ceratitis rosa* (Karsch) (Malan & Manrakhan, 2009); and the citrus mealy bug, *Planococcus citri* (Risso) (Van Niekerk & Malan, 2012). In all these studies, EPNs proved their full potential to control or reduce infestations of insect pests in both laboratory and field trials. However, no indigenous EPN product has been developed on a commercial scale in South Africa which would allow EPNs to be used to their potential.

Problem statement

The purpose of this study was to develop a new indigenous *S. innovationi* isolated in South Africa for commercial production using in vitro solid culture. Factors taken into consideration included temperature, media formulation and biological characterization of the species.

The capital costs of setting up liquid mass production of nematodes are extremely high (Wright *et al.,* 2005). This method is currently employed by developed countries such as USA and several European countries (Kaya *et al.,* 2006). In addition, there is a risk of poor quality EPN IJs associated with liquid culture if the process is not monitored well (Ehlers & Shapiro-Ilan, 2005). On the other hand, *in vivo* mass production using insects can yield high quality nematode IJs. However, it is not feasible at a commercial level because of the high costs of labour associated with insect rearing.

Hypothesis

A low-cost solid substrate medium can be developed that will yield high quality IJs of an indigenous EPN, for use as a bio-insecticide.

Objectives

A. Determine the effect of temperature on nematode yield (i.e. optimum temperature for production)
B. Effect of different media on IJ yield
C. Determine optimum storage temperature
D. Shelf life of EPNs post production in sponge formulation
E. Ecological characterization of the new species
F. Effect of media on virulence of the EPN IJs against a range of insect hosts
G. Cost analysis of best *in vitro* medium

Thesis referencing and format style

The referencing system used in the chapters of this thesis is based on the Harvard system of referencing (De Montfort University), and follows the specific style used in "Journal of Biocontrol Science and Technology" (Appendix I).

The thesis is in the form of discrete research chapters, each following the format of a stand-alone research paper (whether or not the chapter has already been published). This is the dominant thesis format adopted by the University of KwaZulu-Natal because it facilities the publishing of research out of theses far more than the older monograph form of thesis. As such, there is some unavoidable repetition of references and some introductory information between chapters.

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CHAPTER ONE

Literature review

1.1 EPNs occurrence and distribution

Entomopathogenic nematodes (EPNs) occur naturally in the soil. EPNs are widespread; the only continent that has not recorded the presence of EPNs is Antarctica (Uribe-Lorío, Mora & Stock, 2005). Kaya *et al.* (2006) highlighted the diversity of EPNs in North America, Europe, Asia, Central America, South America and Africa. Scientists have isolated nematodes from different types of soil ranging from sandy soil to clay soil, and from dead insects (Hatting, Stock & Hazir, 2009). Natural, undisturbed environments are more likely to contain indigenous species compared to agricultural areas that may be contaminated by introduced nematodes (Stock, Buckner & Coyne, 2003). Factors such as geographic location, climate, habitat and soil type may have an effect on the occurrence and distribution of EPNs (Kanga, Waeyenberge, Hauser & Moens, 2012). Soil texture determines the pore size which affects air and moisture availability. Many surveys showed that clay content negatively affect EPN occurrence and distribution (Campos-Herera *et al.,* 2008; Koppenhöfer & Fuzzy, 2006; Kung, Gaugler & Kaya, 1991). However, *Steinernema riobrave* (Cabanillas, Poinar and Raulston, 1994) and *Heterorhabditis bacteriophora* (Poinar, 1976) performed better in Marl soil compared to sandy soils Shapiro-Ilan, McCoy, Fares, Obreza and Dou, 2000). *Heterorhabditis* often prefer sandy soils close to the coast whilst *Steinernema* is now known to be highly rich in species and widely distributed in tropical soils (Hunt, 2007) apart from *Heterorhabditis sonorensis* (Stock, Rivera-Orduno & Flores-Lara, 2009) which was isolated from Sonoran desert in Mexico (Stock, Rivera-Orduño and Flores-Lara, 2009). Few species have been isolated from desert or semi-desert areas. For example, only one *Steinernema* and two *Heterorhabditis* were isolated from the Negev desert (Glazer, Liran, Poinar Jr & Smits, 1993), and only one *S. riobrave* from a semi-arid habitat in the USA (Cabanillas, Poinar Jr, & Raulston, 1994).

1.2 Characteristics of EPNs

1.2.1 Nematode-bacterial symbiotic relationship

EPNs actually comprise a nematode-bacterium complex, which operates in a symbiotic relationship. Nematodes carry bacteria in the gut of its free living stage, the infective juvenile (IJ). The IJ carries between 200 and 2000 cells of its symbiont bacterium in the anterior part of its intestine (Endo & Nickel, 1994). In steinernematids, the bacterial cells are contained in a sack like structure called 'bacterial receptacle (Stock and Blair, 2008). The nematode may appear like a biological syringe for its bacterial partner, which is unable to infect a host on its own. Yet the relationship between these organisms is symbiotic. Nematode growth and reproduction depends upon suitable conditions established in the host cadaver by the bacterium. The bacterium further contributes anti-immune proteins to assist the nematode in overcoming host defenses and anti-microbials that suppress colonization of the cadaver by competing secondary invaders (Koppenhöfer & Gaugler, 2009). Conversely, the bacterium lacks invasive powers and is dependent upon the nematode vector to locate and penetrate suitable hosts.

Steinernematids are associated with *Xenorhabdus* spp. and *Heterorhabditis* with *Photorhabdus* spp., which are gram negative Enterobacteriaceae (Forst, Dowds, Boemare & Stackebrandt, 1997). These bacteria are not free-living and therefore can only be isolated from their associated nematode or infected insect. Both these bacteria exist in two major forms; Phase I or primary phase, which produce antibiotics and gives optimum conditions for in vitro production; and Phase II or secondary phase, which does not produce antibiotics (Akhurst, 1980). Phase II occurs as a result of *in vivo* or *in vitro* culturing under stressful environmental conditions, e.g., media with low osmotic strength. Krasomil-Osterfeld (1995)

demonstrated the reversal of Phase II bacteria to Phase I when IJs were cultured under suitable conditions.

1.2.2 Life cycle of EPNs

The life cycle (Figure 1.1) starts when the IJ, which is the only free-living stage, enters the insect host through the mouth, anus, spiracles, or by direct penetration through the cuticle (Koppenhöfer, Grewal and Fuzy, 2007; Nguyen & Hunt, 2007). When an IJ reaches the hemocoel of a host, it releases the symbiotic bacteria, which then multiply rapidly in the hemocoel. Usually the insect dies within 48 - 72 hours (Shairra, 2009). The nematodes feed upon the bacteria and liquefied host, subsequently maturing into adults. Steinernematid IJs become males or females, whereas heterorhabditids develop into self-fertilizing hermaphrodites in the first generation. Heterorhabditids produce males, females and hermaphrodites in the second generation. If there is plenty of food, the IJs develop into second generation adults and continue the life cycle (Gaugler *et al*., http://oardc.osu.edu/nematodes/video_and_poster.htm). They can progress to the third generation, depending on the availability of food. Once food is depleted in the cadaver, the life cycle is completed as many as hundreds of thousands of new IJs emerge from the cadaver to infect new hosts. The IJs do not have to feed but can live for weeks on stored reserves and for months by entering a near-anhydrobiotic state (Womersley & Higa, 1998). Persistence of EPNs in the soil depends on the availability of an insect host and nematode reproduction in that host.

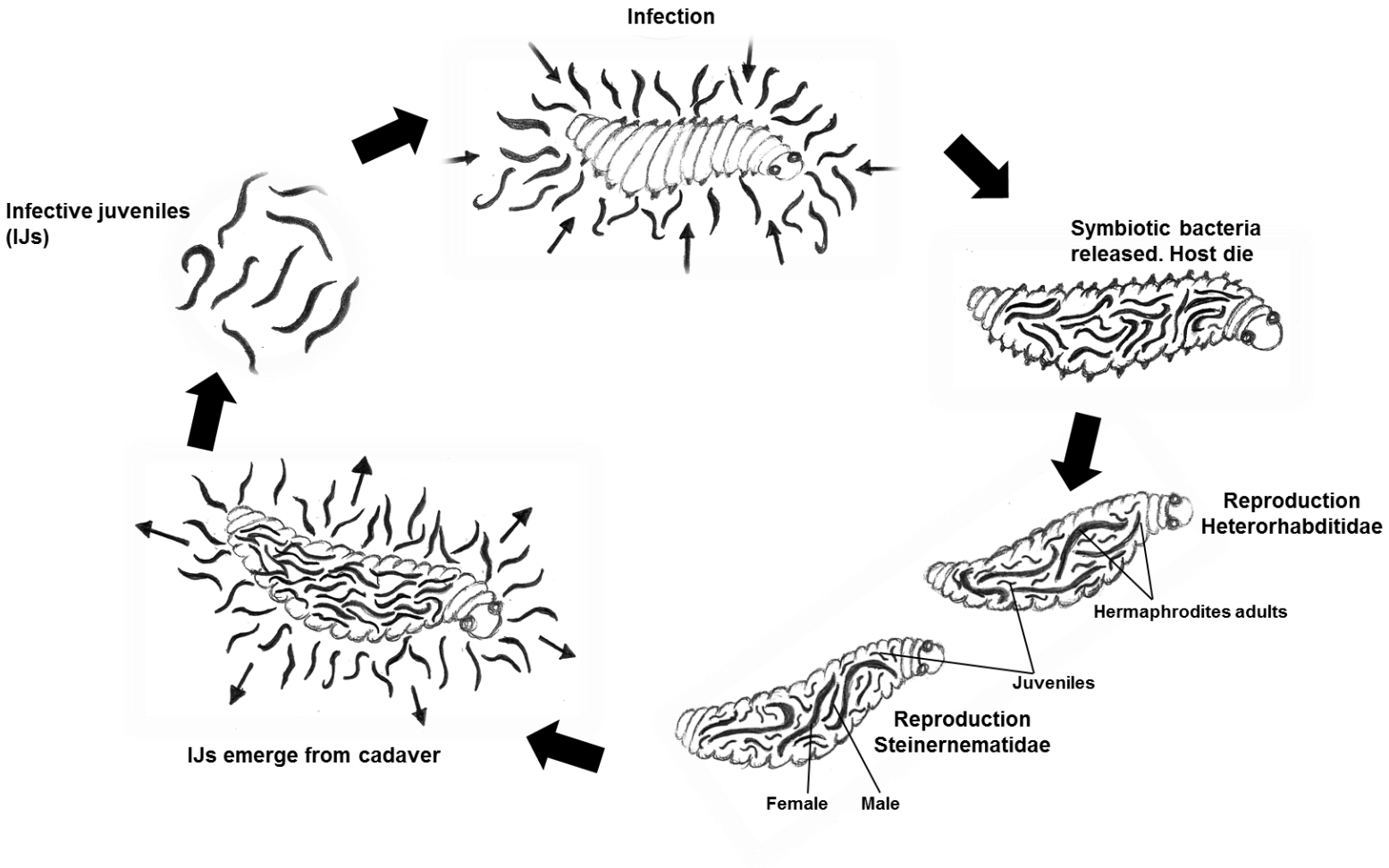

Figure 1.1 Life cycles of EPNs

1.2.3 Target insect pests

EPNs are pathogenic on more than 200 insect pests (Shapiro-Ilan, Gouge & Koppenhöfer, 2002). Their ability to kill the insect host varies from poor to excellent depending on the nematode species and the insect pest (Table 1.1). The success of nematode applications depends on many factors. The most important is that the biology and ecology of both the nematode and the pest should be understood (Hazir *et al*., 2003). Some insect pests cannot be killed by nematodes because entry portals are not accessible (Eidt & Thurston, 1995). For example, oral filters in the mouth, anus may be constricted by a muscle, the spiracle may be covered with a septum or it may be too narrow for passage, etc. Nematode infection may also be hindered by aggressive grooming or evasion behaviour (Gaugler, Wang & Campbell, 1994) such as the formation of impenetrable cocoons. The immune response of insects to the nematode also affects nematode success (Wang *et al.,* 1995).

EPNs are more effective in habitats that protect them from extreme environmental conditions such as UV, high temperature, drought, etc. Their application is more suitable against soil-borne insects, given that soil is the natural habitat of EPNs. Furthermore, many insect species spend at least part of their life cycle in the soil. EPNs have also been applied against insect pests in cryptic environments, in manure and on foliage (Wright, Schroer, Peters & Fife, 2005). To date, most EPN applications have been limited to terrestrial insects because nematodes are not adapted to motility in water (Hazir *et al.,* 2003).

Dillon, Foster, Williams and Griffin (2012) raised concern about the effect of EPNs on non-target organisms. In their study, they concluded that nematodes may not have a significant effect on non-target organisms in the year of application but possibly in subsequent years as EPN populations may remain high for years after application. However, both Bathon (2012) and Georgis, Kaya and Gaugler (1991) in two separate studies concluded that EPNs have negligible effect on non-target organisms.

Table 1.1. Examples of biological control by EPNs against various insect pests: adapted from P. M. Sharma, A. N. Sharma & Hussaini (2011).

Crop	Pest	EPN sp.	Dosage(IJs)	Efficacy (%)
Apple	*Cydia pomonella,* Lepidoptera: Tortricidae	Sc	5 billion/ha	83
	Hoplocampa testudinea, Hymenoptera:Tenthredinidae	Sc	1 x 10^5/500cm branch	100
Citrus	*Diaprepes abbreviates,* Coleoptera: Curculionidae	Native Sr	Soil baiting technique	65–80
Strawberry	*Otiorhynchus sulcatus,* Coleoptera: Curculionidae	Sc	7.6 x 10^9/ha	50–65
Litchi	*Comoritis albicapill,* Hemiptera: Yponomeadidae	Hb, Sc, Sg, Sf	-	14–92
Turf field	*Popillia japonica,* Coleoptera:Scarabaeidae	Sg	5 x 10^9/ha	44–66
	Popillia japonica, Coleoptera:Scarabaeidae	Hb	2.5 x 10^9/ha	34–98
	Cyclocephala borealis, Coleoptera:Scarabaeidae	Hb	2.5 x 10^9/ha	47–83
	Coleoptera: Scarabaeidae *Popillia japonica,* Coleoptera:Scarabaeidae	Hb, Sg	-	75

Golf course	*Scapteriscus spp.,* Orthoptera: Gryllotalpidae	Sc	2 x 10^5/m^2	100
	Phylloperta horticola, Coleoptera Rutelidae	Hb	0.5-1.5 x 10^6/	40–83
Carrot	*Listronotus oregonensis,* Coleoptera: Curculionidae	Hb		Significant reduction
Peanut	*Maladera matrida,* Coleoptera:Scarabaeidae	Hb	0.25 x 10^6/m^2	50–90
Field and plantation crops	*Maladera insanabilis*, Coleoptera:Scarabaeidae	Hb, Sf, Sg		Significant reduction
Cabbage	*Plutella xylostella,* Lepidoptera: Plutellidae	Sc	75 IJ/cm^2	80
Lettuce	*Liriomyza huidobrensis,* Diptera : Agromyzidae	Sf	1-2 x 10^{11}/ha	82
Bean	*L. trifolii*, Diptera : Agromyzidae	Sc	9 x 10^9/ha	Above 65
Peach	*Synanthedon exitiosa,* Lepidoptera: Sesiidae	Sc	1.5 x 10^6/tree	<20% infestation
Cauliflower	*Tropinota squalida,* Coleoptera: Scarabaeidae	Hi, Hb	2.4 x 104/m^2	Above 54
Turf	*Herpetogramma phaeopteralis*, Lepidoptera: Crambidae	Sc	2.5 x 10^8/ha	83-93

Peach	*Grapholita molesta,* Lepidoptera: Tortricidae	Hb	70 IJ/cm^2	94-97
Maize	*Spodoptera littoralis,* Lepidoptera: Noctuidae	Sc	9 x 10^4/m^2	89-100
Soil	*Ceratitis capitata*, Diptera :Tephritidae	Sf	150–5000/cm2	76–95
Mushroom	*Lycoriella auripila,* Diptera*:* Sciaridae	Sf	3 x 10^6/tray	8 and 11 increased
	L. solani , Diptera: Sciaridae	Sf	1-3 x 10^6/m^2	83

Sc, Steinernema carpocapsae; Sf, S. feltiae; Sr, S. riobrave; Sg, S. glaseri; Hb, Heterorhabditis bacteriophora; Hm, H. megidis.

1.3 Development of EPNs as biocontrol agents

1.3.1 Rationale

Development of a particular strain of an EPN involves several crucial steps, the first of which is strain selection. The choice of strain impacts on the final outcome of production more than any other feature of production. High virulence against the target pest and ease of production are prioritized over high yield (Gaugler & Han, 2002). A high yielding strain is useless if it possesses inferior virulence and the converse is equally true, where a highly virulent strain with low yield will result in high production costs. Genetic improvement (i.e., modification of genetics of an organism so that it expresses desirable traits) has also received some attention in EPN research. The second aspect is mass production: this can be achieved *in vivo* using insects, or *in vitro* using solid media or liquid media. *In vivo* production is appropriate for small scale applications, whereas *in vitro* production is typically practiced for large scale international markets (Ehlers & Shapiro-Ilan, 2005). The third aspect is formulation. There are various formulations of EPNs, but a fairly simple formulation is sponge formulation. A paste containing the EPNs is absorbed into a sponge and stored at low temperature (Strauch *et al*., 2000). The final step is commercialisation (discussed under Section 1.3.6 below).

1.3.2 Advantages and disadvantages of EPNs as biocontrol agents

Advantages of EPNs as biocontrol agents

EPNs have many advantages and few disadvantages. The advantages are presented in Table 1.2.

Table 1.2. Advantages of using EPNs as biocontrol agents

Advantageous Trait of EPNs	Comments
Environmental friendly and highly host specific (Lacey & Georgis, 2012)	They are safe to environment and non-target organisms. Humans, animals or beneficial insects are completely unaffected by their use.
EPNs as a group have a broad host range. They are efficacious against many soil insects and insects in cryptic habitats (Lacey & Georgis, 2012).	Over 200 species (Shapiro-Ilan *et al*., 2002).
There is no evidence of natural or acquired resistance to their symbiotic bacteria (Divya & Sankar, 2009).	This makes the use of EPNs for insect pest control a viable long-term alternative.
Nematodes can kill their insect host within 48 hours due to the pathological effect of their symbiotic bacteria (Shairra, 2009)	This ability is valuable when a rapid speed of kill of the host is required.
They have a resistant stage (infective or dauer juvenile) with several advantages	This stage is resistant to shear forces and can be applied through a conventional sprayer. IJs are non-feeding and they can live for some time without nourishment as they search for a host. They also enter a dormant stage through partial desiccation to aid survival during adverse environmental conditions (Womersley & Higa,

	1998).
They can actively seek their hosts within the soil environment (i.e., 'cruiser-type' EPNs) (Campbell, Lewis, Stock, Nadler & Kaya, 2003)	EPNs do not have to be applied accurately.
EPNs are also compatible with many agrochemicals, including fertilizers and some pesticides. (Sharma *et al.,* 2011)	They can be included into integrated pest management (IPM) programmes.
In most countries (including South Africa), EPNs are exempted from registration requirements (Lacey & Georgis, 2012).	Only a few countries have proposed some requirements for registration, which are usually not comparable with the data requirements needed for the registration of chemical compounds or other microbial agents.

Disadvantages of EPNs as biocontrol agents

Use of EPNs does have some disadvantages. The disadvantages are listed in Table 1.3 below:

Table 1.3. Disadvantages of using EPNs as biocontrol agents

Disadvantages of EPNs	Comments
Biological control using EPNs require more intensive management and planning (Lacey & Georgis, 2012).	It can take more time, require more record keeping, and demand more patience and education or training.
To be successful, the biology of both the pest and the nematode species must be understood (Hazir *et al*., 2003).	Usually you need to target a certain susceptible stage, and if the insect outgrows that stage it may be difficult to control its population.
Often, the results when using biological control are not as dramatic or immediate as the results obtained when using chemical pesticides (Sharma *et al*., 2011).	One should have long-term objectives.
Short shelf life relative to chemical pesticides. Short persistence	Largely depends on availability of insect host in the soil.
Also, nematode products are generally more expensive than their chemical counterparts (Wright *et al.,* 2005).	Affordability and profit concerns.

1.3.3 Mass production

Mass production at low cost is an important prerequisite in the successful development of EPNs as bio-insecticides. Nematodes have been cultured for more

than 70 years utilizing *in vivo* and *in vitro* techniques, with solid and liquid media being used for large-scale commercialization / research purposes (Gaugler & Han, 2002). *In vitro* mass production requires large capital investments (bioreactors, fermentation technology, etc.) and is therefore more suitable for large scale commercialization (Lacey & Georgis, 2012). *In vivo* production has been adopted by small scale farmers, small businesses focused on niche markets, and for laboratory use for its advantage of easy setup and low production capital. Although the capital requirement is low, this method entails high inputs of manual labour.

1.3.3.1 *In vivo* mass production

In vivo production is suitable for a cottage industry characterised by low volume production, and the use of White traps or modified White traps (Sharma *et al.,* 2011). This method takes advantage of the IJs natural migration away from the insect host. The latest development in *in vivo* mass production was the development of the LOTEK system by Gaugler *et al.* (2002), which does not rely on nematode migration to a reservoir. The system consists of perforated trays to secure insects, harvesters with misting nozzles that rinse IJs through the holding trays into a central bulk storage tank and use of a continuous deflection separator for washing and concentrating IJs.

In vivo production relies on the constant supply of susceptible hosts. The most common insect host used is the final instar larvae of the greater wax moth (*Galleria mellonella*, Linnaeus*)* which is susceptible to most EPNs, are easy to rear and offer high yields (Woodring & Kaya, 1988). The use of mealworms (*Tenebrio molitor,* Linnaeus) for mass production has so far received little research (Shapiro-Ilan & Gaugler, 2002). Mwaniki, Nderitu, Olubayo and Limenju (2013) achieved yields not significantly different from that of *G. mellonella* using silkworm (*Bombix mori,* Linnaeus) larvae for mass production of *H. indica* and *S. karii*. In general nematode yield is proportional to insect host size (Ehlers & Shapiro-Ilan, 2005). Other factors affecting yield are inoculum and temperature. Best yields are achieved with intermediate inoculum dosage because higher doses lower yield due to EPN competition for nutrients (Grewal, Selvan & Gaugler, 1994; Shapiro-Ilan *et al.,* 2002).

Optimum production temperatures lie between 18°C and 28°C for different species (Grewal *et al.,* 1994; Hazir, Stock, Kaya, Koppenhöfer & Kestin*,* 2001; Karagoz, Gulcu, Hazir & Kaya, 2009; Morton & Gracía-del-Pino, 2009). It is also crucial to maintain adequate aeration and humidity throughout the production process (Shapiro-Ilan & Gaugler, 2002).

1.3.3.2 *In vitro* mass production

Glaser (1931) is considered the pioneer of *in vitro* mass production. Continuous production, however, failed because the critical role of symbiotic bacteria was not known. Efforts to grow *Steinernema* sp. in axenic culture was successful, but with low yields (Stoll, 1952). A notable breakthrough was the description of the symbiotic bacterium *Xenorhabdus nematophilus* (Akhurst) for *in vitro* production of *Steinernema carpocapsae* (Weiser, 1955) (Poinar & Thomas, 1966). This study formed the foundation for modern-day *in vitro* mass production. For both solid and liquid *in vitro* production, the growth medium is first inoculated with symbiotic bacteria, allowing colonization of the medium before nematodes are added (Shapiro-Ilan *et al.,* 2012).

Monoxenic culture is also recognised as a key step for *in vitro* mass production (Gaugler &Han, 2002). The use of surface sterilised IJs may not be appropriate since it is more likely that surface sterilised IJs still carry non-symbiotic microorganisms in their gut and the bacteria under the extra cuticle of the IJ can survive the surface sterilization process (Lunau, Stoessel, Schmidt-Peisker & Ehlers, 1993). The growth of non-symbiotic bacteria may totally inhibit nematode growth or have a negative effect on production (Lunau *et al*., 1993). Contaminants are usually detected at a later stage of culturing because Phase I bacteria produce antibiotic compounds that inhibit the growth of some contaminants (Hu & Webster, 2000). The antibiotic effect is lost when Phase I cells shift to Phase II during prolonged *in vitro* sub-culturing, subsequently favouring the growth of contaminants. In addition, monoxenic culture is required to avoid loss of nematode lines during genetic selection to improve strains (Lunau *et al*., 1993). Essentially, only the presence of symbiotic bacteria in

monoxenic cultures ensures suitable conditions for nematode reproduction with high numbers of offspring (Ehlers, 2001).

1.3.3.2.1. *In vitro* solid culture

An important development in solid-state mass production was the innovation of a three-dimensional medium in flasks, using polyether-polyurethane sponge as a liquid medium carrier (Bedding, 1981). The media is first inoculated with the symbiotic bacteria and incubated at the optimum temperature for bacterial growth (2 - 3 days). Once bacteria have been established, flasks are inoculated with nematodes and incubated at the optimum temperature for nematode growth and development. The presence of nutrients stimulates nematode development and reproduction until food is depleted. Nematodes can, however, be added at the same time as bacteria, provided that a high concentration of bacteria is used (Sharma *et al*., 2011). Other innovations include: the use of autoclavable plastic bags with a gas permeable strip for ventilation; automated mixing and autoclaving; and harvesting using centrifugal sifters (Gaugler & Han, 2002). These developments took place in the process of scaling up of EPN production at Biotec Australia and Ecogen-Sylvan, USA.

Production can be carried out using a range of agar media (Dumphy & Webster, 1989; House, Welch & Cleugh, 1965; Wouts, 1981) in Petri dishes. Media based on animal waste (e.g., chicken offal) have been developed and later improved for cost and consistency. These media may include various ingredients such as peptone, yeast extract, eggs, soy flour and lard (Sharma *et al.,* 2011)

In vitro solid culture has several advantages over liquid culture:

1. The effect of bacteria phase variation on yields is less than in liquid culture (Han & Ehlers, 2001).

2. Less capital investment is required.

3. The risk of process failure is partitioned given several smaller production units.

Although the solid media process has been successful, the high cost of labour, vulnerability to contaminants during up- and down-stream processing and inability to monitor the process online limits its economic application (Sharma *et al.,* 2011). Also,

the uneven distribution of nematodes in the medium prevents systematic sampling (Ehlers & Shapiro-Ilan, 2005). Bedding (1990) recommended the use of this technology in countries where labour costs are low.

1.3.3.2.2. In vitro liquid culture

This method is commercially viable for developed countries where capital and expertise is available. EPNs were first produced in liquid media by Stoll (1952). The first attempt to use a bioreactor was by Pace, Grote, Pitt and Pitt (1986). Their study suggested that shear pressure from a flat blade disrupts adult females. Currently, bioreactors with a paddle stirrer are used instead of a flat blade impeller, to minimize disruption of adults. Liquid culture of EPNs has been scaled-up to production in 80.000 litre bioreactors with paddle stirrers (Sharma *et al.,* 2011).

A basic production medium should contain a carbon source, a variety of proteins (animal or plant), yeast extract and lipids (animal or plant) (Ehlers & Shapiro-Ilan, 2005). Published formulations contain varying concentrations of peptone, yeast extract, beef extract, lipids, carbohydrates, cholesterol and salts (Floyd, Singh & Holmes, 2012). Lipids should always be part of the production media to increase IJ fat content (Shapiro-Ilan *et al.,* 2012). Low IJ fat content can reduce efficacy and survival since IJs are non-feeding and rely on stored reserves (Patel, Stolinski & Wright, 1997).

Liquid culture presents more challenges than *in vivo* and *in vitro* solid culture. Such cultures are particularly vulnerable to contamination (Ehlers & Shapiro-Ilan, 2005). This is due to the even distribution of fluids and organisms obtained through the mixing of liquids in bioreactors and the long process time. Nematode recovery from non-developing IJ to adult is far less in liquid culture than *in vivo*, 18% - 90% vs. 100% (Gaugler & Han, 2002; Shapiro-Ilan *et al*., 2012). Poor recovery leads to low IJ concentrations and extended culture time. A symbiotic bacterium has to be maintained in the primary phase during fermentation, bearing in mind that the secondary phase is non-virulent. Other challenges include securing high yields, obtaining IJs with long shelf-life, maintaining high virulence against their host,

resistance against adverse environmental conditions and designing a bioreactor that will satisfy the oxygen requirements without damaging the nematodes (De la Torre, 2003).

1.3.4 Formulation

The success of EPN infectivity depends on the stability of the formulated product. Nematodes have been developed in a variety of formulations including sponges, charcoal, vermiculite, clay, dispersible granules, infected cadavers (not commercially available) and capsules (not commercially available) (Deol, Jagdale, Canas & Grewal, 2011; Hiltpold *et al*., 2012). Although it is difficult to obtain an optimal formulation for all nematode species (given their specific requirements for moisture and water), the most common type of formulation is the absorption of a nematode paste into sponges. These types of formulation require refrigeration in order to retain virulence (Hazir *et al*., 2003). To improve shelf-life, formulations which reduce IJ metabolism by partial desiccation or immobilization are also used. These include alginate gel, vermiculite, clay, polyacrylamide, activated charcoal and water dispersible granules as the carrier matrix (Georgis, Dunlop & Grewal, 1995; Georgis & Kaya, 1998; Grewal, 2002; Grewal & Peter, 2005). One example of such formulations is water dispersible granules, which combine long-term nematode storage without refrigeration, with ease of handling (Koppenhöfer, 2007). Droplets of thick suspension of IJs are encapsulated in a 10 - 20mm diameter granule consisting of mixtures of silica, clays, cellulose, lignin and starches (Georgis *et al*., 1995). The partial desiccation of these IJs is reversed upon exposure to a moist environment. However, to achieve optimal infectivity IJs require up to three days of rehydration (Baur, Kaya & Tabashnik, 1997). Water dispersible granules offer several advantages over other formulations (Grewal, 2002), including:

1. Extended nematode storage at room temperature.

2. Enhanced tolerance to extreme temperature

3. Improved ease of use by limiting the number of preparation steps

4. Decreased container size and ratio.

5. Decreased volume of disposal material.

Another promising formulation for small scale application entails the use of infected cadavers. Cadavers can be coated with a protective formulation (e.g. masking tape, starch and clay mixture) to prevent the cadaver from rupturing (Shapiro-Ilan, Lewis, Behle and Mcguire, 2001). Deol *et al.* (2011) demonstrated that the shelf life of *S. carpocapsae* can be extended to 180 days by the use of an infected cadaver formulation in commercial growing medium as compared to 60 days when stored in aqueous suspension.

1.3.5 Quality control

Nematode viability and virulence can be influenced by many factors during mass production, formulation and storage. These include: (1) Quality of media or host; (2) Exposure to adverse environmental conditions (temperature, aeration & sheer); (3), Contamination by non-symbiont bacteria; (4) Toxicity of antifoaming and antimicrobial agents (Grewal & Peters, 2005). These factors may cause nematode quality to differ from batch to batch. The quality of nematodes after production and formulation is determined by measuring virulence potential. Grewal *et al.* (1998) promoted the use of one-on-one assays (one IJ to one *G. mellonella* larva) as a standard quality control measure. This method measures the proportion of infective nematodes in a batch and is sensitive to “impaired” nematodes. The one-on-one procedure works well for steinernematids, whereas five-on-one (five IJs to one *G. mellonella* larva) is recommended for heterorhabditids (Gaugler, Grewal, Kaya, & Smith-Fiola, 2000). Another vital tool in quality assessment is the assessment of microbial contamination. Physical characteristics such as product colour and weight, granule size, formulation dispersibility, product temperature and packaging are also monitored to reduce batch-to-batch variability and maintain consistency (Grewal, 2002).

1.3.6 Commercialization

Nematode products are most popular in USA and Europe. Most of the nematode-based products recently available include formulations of various strains of *S. carpocapsae, Steinernema feltiae* (Filipjev, 1934), *Steinernema glaseri* (Steiner, 1929)*, Steinernema kraussei* (Steiner, 1923)*, S. riobrave, H. bacteriophora, Heterorhabditis indica* (Poinar, Karunaka & David, 1992) *Heterorhabditis megidis* (Poinar, Jackson & Klein, 1987) *(*Kaya *et al.*, 2006). Nematode products come in pack quantities at concentrations ranging from 5 million to 500 million (Floyd *et al.*, 2012). See Table 1.4 below for commercial producers of EPNs and their target market/s. Critical properties of a commercial strain are: high virulence against the target pest, ease of culturing, superior shelf–life and effectiveness against multiple insect pests (Hazir *et al.,* 2003). Nematode products are generally more expensive than their chemical counterparts (Wright *et al.,* 2005) due to high cost of production, formulation and transportation. Controlled environmental conditions (temperature & aeration) during production and transportation add to the high cost of these products.

Regulation on the use ofEPNs differ between countries. So far, EPNs have been exempted from registration in many countries (Sharma *et al.,* 2011). Some countries require some data for registration, but this still does not compare to the volume thereof required for registration of chemical pesticides (Lacey & Georgis, 2012). The only exception is Japan, where SDS Biotec had to provide data files similar to those required to register chemical pesticides, in order to register two EPN species, *S. carpocapsae* and *S. glaseri* (Ehlers, http://www.cost850.ch/legal_safety/safety%2Bregulation.pdf)

Table 1.4 Nematode producers and their target market/s (Kaya *et al.,* 2006)

Company & location	Nematode species	Web Site	Formulations
Andermatt Biocontrol, Grossdietwil, Switzerland	*Sc, Sf, Hm*	www.biocontrol.ch	Clay
Asa Jung Laboratory, Oakland, California, USA	*Sc, Sf*	www.asajunglab.com	Clay
BioLogic Willow Hill, Pennsylvania, USA	*Sc, Sf*	www.biologicco.com	Bulk, dispersable gra sponge, granular
Bionema, Umea, Sweden	*Sc, Sf*	www.bionema.se/	Polymer
BASF Corporation	*Sc, Sf, Sg, Sk, Ss, Hm*	www.basf.com	Sprayable, dispersab granule
e-nema GmbH, Raisdorf, Germany	*Sc, Sf, Hb*	www.e-nema.de	Clay, polymer
Hydrogardens, Colorado Springs, Colorado, USA	*Sc, H sp.*	www.hydro-gardens.com	Sponge
Koppert, Berkel en Rodenrijs, the Netherlands	*Sf, Hb*	www.koppert.nl/	Clay
M & R Durango, BayWeld, Colorado, USA	*Sc, Sf, Hb*	www.goodbug.com/	Sponge
Nematec, San Ramon, California, USA	*Hb*	N/A	Nematode wool
Owiplant, Owijska K/Poznania, Poland	*Sf*	www.owiplant.com.pl/	Clay
SDS Biotech, Japan	*Sc, Sg*	www.sdsbio.co.jp/english/ir/	Alginate gel
Novartis, Vienna, Australia	*Sc*	www.novartis.at	Alginate gel
ARBICO Organics, Oro Valley, AZ, USA	*Sc, Sf, Hb*	http://www.arbico-organics.com	
Natural insect control, Canada	*Sc, Sf, Hb*	www.naturalinsectcontrol.com/	

*Sc, Steinernema carpocapsae; Sf, S. feltiae; Sg, S. glaseri; Sk, S. kraussei; Sr, S. riobrave; Ss, S. scapterisci; Hb, Heterorhabditis bacteriophora; Hm, H. megidis; H sp., Heterorhabditis species.***1.4**

Application

A universal application method entails spraying a homogenous suspension of EPNs in water directly onto the soil surface. EPNs have been applied using spray equipment for chemicals and through standard irrigation systems without major modification (Georgis, 1990). This method is simple and quick, and it provides good coverage. Filters and sieves should be large enough to allow nematodes to pass through, at least 300µm wide, with nozzle aperture >500µm (Wright *et al.,* 2005). It might be necessary to remove screens when using some formulations to avoid clogging. Nematodes should never be exposed to high temperatures (above 35°C), be it in the tank, delivery hose or nozzle. Post application irrigation is recommended

to facilitate EPN movement into the soil before desiccation of spray droplets and/or exposure to harmful UV irradiation. Although moisture is required for EPN survival and movement, too much water can cause oxygen deprivation and restrict movement (Koppenhöfer, Kaya & Taormio, 1995). In addition, applying nematodes in the morning or late afternoons is advised (Shapiro-Ilan & Gardner, 2012). Other application methods include: injecting IJ suspensions into the insect-bored holes or blocking holes with sponges soaked in nematode suspensions (Yang, Zhang, Zhang & Jian, 1993); insect baits containing IJs for mobile insects (e.g. crickets) (Koppenhöfer, 2007); and application of infected cadavers. The latter hold much promise because IJs that emerge directly from infected-hosts can provide superior insecticidal performance compared to IJs in aqueous suspension (Shapiro-Ilan, Lewis, Tedders, & Son, 2003). Although nematodes are generally applied as curative treatments, prophylactic applications to the soil surrounding seedlings or seeds have been promoted by Grewal (2002).

The success of nematode applications can be influenced by their relationship with other organisms. Other organisms can have a positive, negative or neutral effect on EPN applications. For example, nematode pathogens and predators such as phages, bacteria, protozoa, nematophagous fungi, and predatory mites have a negative effect on the performance of EPNs (Karagoz, Gulcu, Cakman, Kaya & Hazir, 2007; Kaya, 2002). Phoretic relationships have been reported with other organisms, for example isopods (Eng, Preisser & Strong, 2005). The relationship between EPNs and other entomopathogens can be synergetic, additive and antagonistic depending on the nematode species and relative timing or application rate (Shapiro-Ilan *et al.*, 2012). Anbesse, Adge and Gebru (2008); Ansari, Shah and Moens (2006) and Ansari, Tirry and Moens (2004) showed synergy with the entomopathogenic fungus *Metarhizium anisopliae* (Sorok). On the other hand, antagonism with the popular entomopathogenic fungus, *Beauveria bassiana* (Balsamo) has also been reported (Brinkman & Gardner, 2000). Tarasco, Alvarez, Triggiani and Moraga (2011) concluded that different responses during interactions between pathogens are based on competition for development and survival.

1.5 Future prospects

Significant progress has been made regarding nematode mass production, formulation, application technologies and testing efficacy against various pests. However, confidence in the performance of EPNs has not yet reached a level or price at which they compete directly with chemical pesticides. Additional research focusing on lowering production cost, improving efficacy and enhancing ease-of-use will promote their use as biocontrol agent. Since EPNs are compatible with chemical pesticides and other entomopathogens (Sharma *et al*., 2011), nematodes show great potential for inclusion in integrated pest management programmes. Additionally, the advantages of using EPNs are underscored by operator and end-user safety.

Future research could also focus on the use of EPNs to suppress plant parasitic nematodes (PPNs). There are a number of reports which prove the suppression of PPNs (including root-knot nematodes) by EPNs (Grewal Martin, Miller & Lewis, 1997; Hussaini, Kiran Kumar, Adholeya & Shakeela, 2009; Perez & Lewis, 2002, 2004). However, there is very little information regarding the mechanism and effectiveness of the suppression. Grewal, Lewis and Venkatachari (1999) demonstrated allelopathic interaction between plant-parasitic nematodes, EPNs and their symbiotic bacteria as a mechanism in suppression of plant parasitic nematodes. In another study, Jagdale, Kamoun and Grewal (2009) concluded that EPNs and their symbiotic bacteria induce defence in plant which may contribute to reduced reproduction of plant parasitic nematodes.

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CHAPTER TWO

Effect of temperature on reproduction and development of an entomopathogenic nematode indigenous to South Africa, *Steinernema innovationi*

Abstract

Entomopathogenic nematodes (EPNs) have been observed to have diverse temperature optima for development and reproduction at the species or even strain level. The effect of temperature on an indigenous *Steinernema innovationi* was determined *in vivo* using *Galleria mellonella* (Linnaeus) larvae by quantifying the first day of infective juvenile (IJ) emergence from cadaver, yield and IJ length at five different temperatures (range 18°C to 27°C). *S. innovationi* showed the fastest emergence at 25°C and 27°C (mean of 5 days) and the slowest emergence at 18°C (mean of 8 days). On average, emergence at the two mid-range temperatures tested (20°C & 22°C) occurred on Day 6. Highest empirical yields were observed at 22°C and 25°C (393,403 ± 129,080 and 383,270 ± 139,957 IJs/gram, respectively), although regression analysis showed a peak at 23.08°C with an estimated maximum yield of 425,012 IJs/gram. No correlation was found between temperature vs. IJ length (R^2 = 0.2481) and/or yield vs. IJ length (R^2 = 0.0066). Based on these results, 22°C was selected as optimum production temperature, ensuring a high IJ yield while allowing enough time for lipid acquisition.

Keywords: Nematode reproduction; *Steinernema*; infective juvenile; temperature, yield; progeny emergence

2.1 Introduction

EPNs are soil inhabiting lethal insect parasites that occur in natural and agricultural soil globally (Koppenhöfer & Fuzy, 2003). Traditionally, interest lies with the isolation and use of EPNs collected from a specific locality against insects found in that particular region (Hazir, Kaya, Stock & Kestin, 2003). These nematodes were previously understood to be adapted to the soil ecological conditions as well as to the

insect pests found in that region (Kung, Gaugler & Kaya, 1991; Molyneux, 1985). Using indigenous nematodes isolated from a given soil environment should therefore negate the need to introduce foreign strains (Gungor, Kestin & Hazir, 2006). This perception, however, was challenged by the isolation of *Steinernema anatoliense* (Hazir, Stock & Keskin, 2003) showing an optimum temperature much higher (25°C) than that typically encountered in the region it was isolated from (Gungor *et al*., 2006). Ecological and biological studies are thus needed to define the performance of a particular EPN strain under a range of climatic conditions (Koppenhöfer & Kaya, 1999).

Temperature is an environmental factor of great biological significance (Chen, Li, Han & Moens, 2003). It influences EPN infectivity, virulence and reproductive ability. EPN species or even strains have been observed to have diverse temperature optima for development and reproduction (Grewal, Selvan & Gaugler, 1994; Grewal, Gaugler & Shupe, 1996; Mason & Hominick, 1995). EPN development is slow at low temperatures, increases to a peak and then decreases again close to the maximum tolerated temperature (Hirao & Ehlers, 2009). Most EPN species have their optimum temperature between 20°C and 30°C (Grewal *et al*., 1994; Gungor *et al*., 2006; Kaya, 1990; Shapiro-Ilan *et al*., 2006) Culture time is inversely related to temperature and it should be optimised to achieve maximum yield (Shapiro-Ilan, Han & Dolinski, 2012). Longer production times can provide higher yields but IJ mortality may also increase with time (Han, Cao & Liu, 1992; 1993). Long incubation times carry an opportunity cost in terms of space occupied by single batch production. The quality of the non-feeding IJ stage is also indirectly affected by temperature. Development of IJs is slowed by low temperatures, allowing the EPNs more time to feed and they accumulate more lipid reserves, which are essential for survival of the IJ stage (Hazir *et al*., 2001). The compromise to be resolved is that higher temperatures can offer a short production cycle, but nematode quality may be jeopardized if it is too high (Jagdale & Gordon, 1998).

This study aimed to determine the optimum production temperature for *Steinernema innovationi* (Çimen, Lee, Hatting & Stock, 2014) through quantification of yield, first day of emergence from the cadaver, IJ length; and to better understand the relationship between temperature, yield and IJ length for this particular species and strain.

2.2 Materials and Methods

2.2.1 IJs production for inoculum

IJs of *S. innovationi*, originally isolated from soils collected at Fouriesburg, Free State Province, South Africa, were used in this study. Nematodes were produced *in vivo* by passaging them through final instar wax moth larvae, *Galleria mellonella* (Lepidoptera: Pyralidae), according to the method described by Kaya and Stock (1997). IJ were stored in sterile water in 250ml flat angled tissue culture flasks (Lasec SA, Corning®) at 10°C and were used within two weeks of harvesting.

2.2.2 Temperature effect on reproduction

The effect of temperature was assessed using a Sandwell bioassay system by filling wells of a 24-well tissue culture (TC) plate with 0.5g sterile loam sand in which 100 IJs were pipetted in 100µl of distilled water per well (Fallon *et al*., 2004). The control treatment was inoculated with 100µl of sterile distilled water only. One final instar *G. mellonella* larva, of approximately 200 to 300mg (Koppenhöfer & Kaya, 1999), was added per well. A total of 30 larvae, as single replicates, were used per treatment and the control. Each culture plate was placed inside a 330×215mm Zip Seal bag (GLAD®, www.glad.co.za) to conserve moisture, and were incubated at 25°C for 48h in the dark. Plates were monitored for mortality every 24h. Infected cadavers were individually transferred into modified White traps (Kaya & Stock, 1997) and incubated at temperatures of 18, 20, 22, 25 and 27°C and monitored for EPN development and reproduction. The following parameters were recorded: first day of emergence from

infected cadaver; the total number of IJs produced. First day of emergence was recorded as the day on which IJs were visible on the cadaver, filter paper or in the water of the White trap. For yield, emerging IJs were periodically harvested by emptying the White traps and filling them with fresh water until the number of emerging IJs was less than 50 in a given White trap (Hazir, Stock, Kaya, Koppenhöfer & Keskin, 2001). Harvested IJs in water suspension were stored in a 250ml flat angled TC flask (Lasec SA, www.lasecsa.co.za) for each White trap. Three sub-samples were counted per flask to calculate the number of IJs produced per *G. mellonella* cadaver using the dilution method described by Kaya and Stock (1997). Yield was then converted to yield per gram of wax moth larvae using weight of the particular insects.

2.2.3 Relationship between temperature, yield and IJ length

The bottom of a 24 well TC plate was covered with 0.5g sterile loam sand per well. A total of 100 IJs was pipetted in 100µl of sterile distilled water into each well (Fallon *et al.*, 2004) and one final instar *G. mellonella* larva, of approximately 200 to 300mg (Koppenhöfer & Kaya, 1999), was introduced per well. Five *G. mellonella* larvae were used as single replicates per temperature. Plates were maintained individually inside Zip Seal bags (GLAD®, www.glad.co.za) and incubated at 20, 22, 23.5 and 25°C for 72h. Cadavers were then transferred individually into White traps and incubated at the same temperatures. Yield was quantified for each plate using the dilution method as above (see Kaya & Stock, 1997). A sample of 40 to 50 IJs were heat killed at 60°C and stored in triethanolamine formalin (TAF) solution (Kaya & Stock, 1997) in Eppendoff tubes to measure the lengths of the IJs. The length of 25 randomly selected IJs (5 from each tube) was measured from anterior to posterior end using an Olympus BX52 microscope fitted with a DP72 camera (Wirsam Scientific, www.wirsam.info). Measurements were performed using Cellsense imaging software (Celsence, www.celsense.com).

2.2.4 Statistical analysis

Day of nematode emergence and the total number of new generation IJs were analysed using one way ANOVA (Shapiro & Wilk, 1965). Separation of means was carried out with Duncan's test (SPSS, 1999) at the 5% level of significance. Regression analysis of yield data was performed with TableCurve (SYSTAT Software Inc., 2002). The relationship between yield, temperature and IJ length was determined using regression analysis (SAS Institute, 1999).

2.3 Results

2.3.1 First day of progeny emergence

Temperature had a significant effect on both time of emergence and total number of new generation IJs. *S. innovationi* showed the fastest emergence at 25°C and 27°C (mean of 5 days) and the slowest at 18°C (mean of 8 days) ($F= 24.757$; $df= 4, 116$; $P<0.05$) (Figure 2.1). Notably, there was no significant difference between 25°C and 27°C (5 days) or between 20°C and 22°C (6 days), but there was a significant difference between 22°C and 25°C (6 & 5 days, respectively).

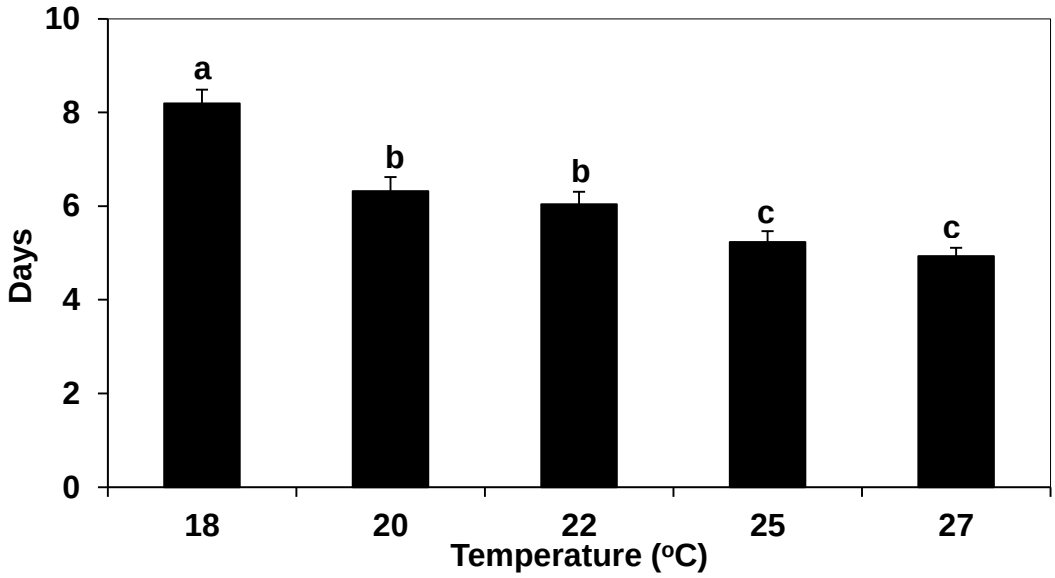

Figure 2.1 Time of first progeny emergence from host cadaver at different temperatures. Bars with the same lowercase letter indicate no significant difference (*P* > 0.05; Duncan's test).

2.3.2 Yield

Compared to the other temperatures, the number of emerged IJs was significantly higher at 22°C (393,403 ± 129,080 IJs/gram) and 25°C (383,270 ± 139,957 IJs/gram), whereas the lowest number of IJs was observed at 18°C (148,629 ± 104,235 IJs/gram) ($F= 13.865$; $df= 4, 116$; $P<0.05$). There was no significant difference between 22°C and 25°C or between 20°C and 27°C (Figure 2.2). Regression analysis estimated a maximum yield of 425,012 IJs/gram at 23.08°C (Figure 2.3).

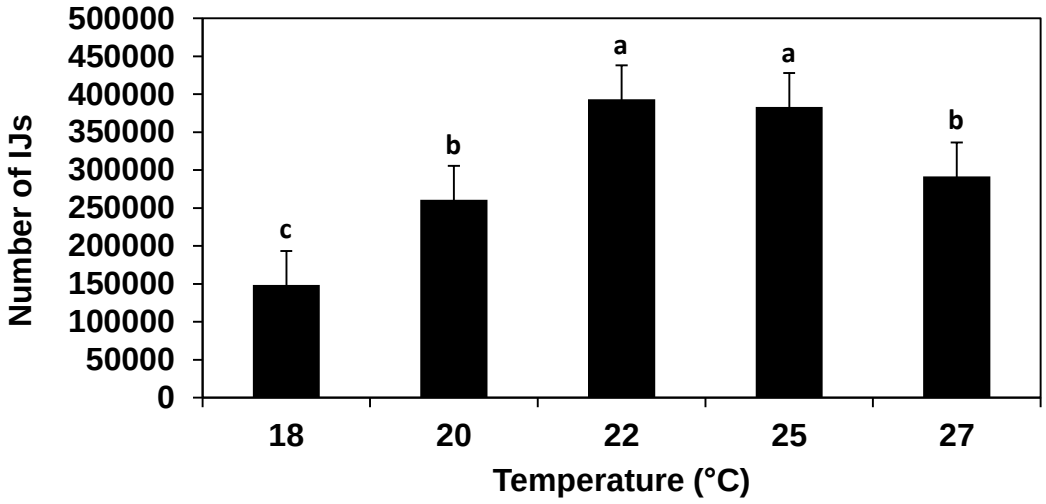

Figure 2.2 Yield (number of IJs) per gram of wax moth larvae at different temperatures. Bars with the same lowercase letter indicate no significant difference ($P > 0.05$; Duncan's test).

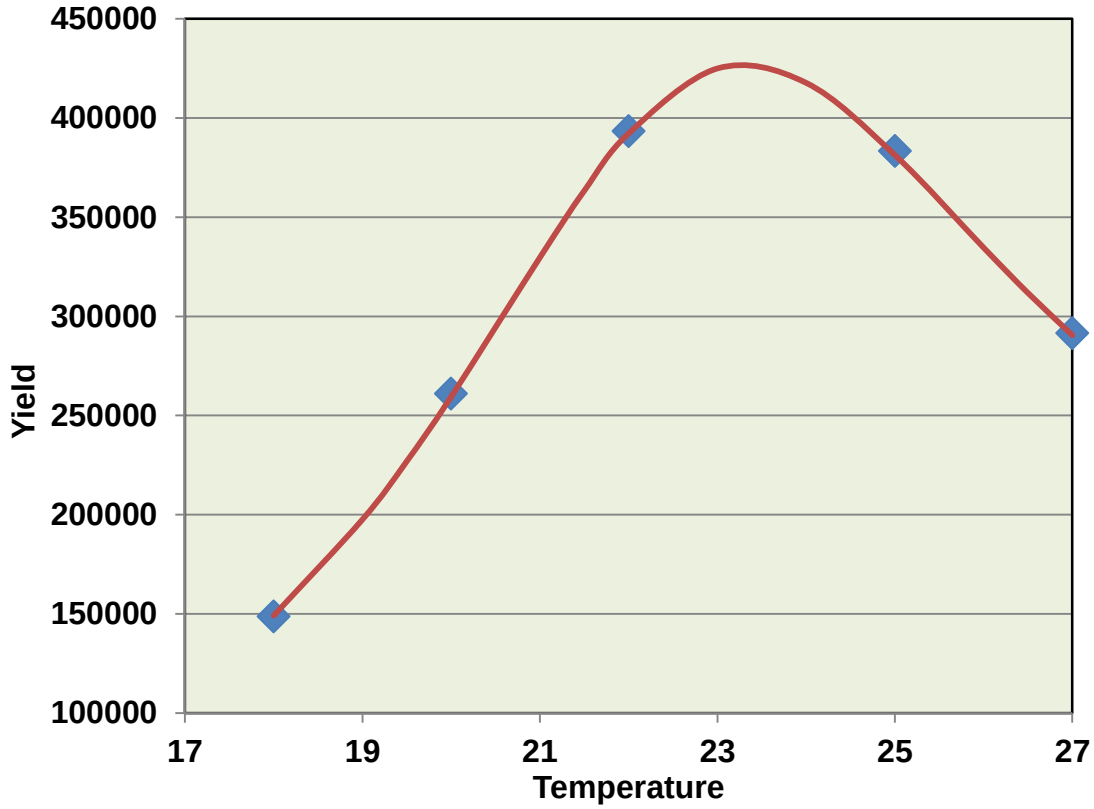

Figure 2.3 Regression analysis of yield of *S. innovationi* performed with TableCurve (SYSTAT Software Inc., 2002) showing a peak at 23.08°C; maximum yield = 425 012 IJs/gram.

2.3.3 Relationship between temperature, yield and IJ length

There was no correlation between yield and IJ length, R^2 = 0.0066 and 0.1208 for linear and polynomial regression, respectively (Figure 2.4). Although there was a tendency of IJs to be shorter when the production temperature increased, there was a weak correlation between temperature and IJ length; R^2 = 0.2481 and 0.5344 for the linear and polynomial regressions, respectively (Figure 2.5).

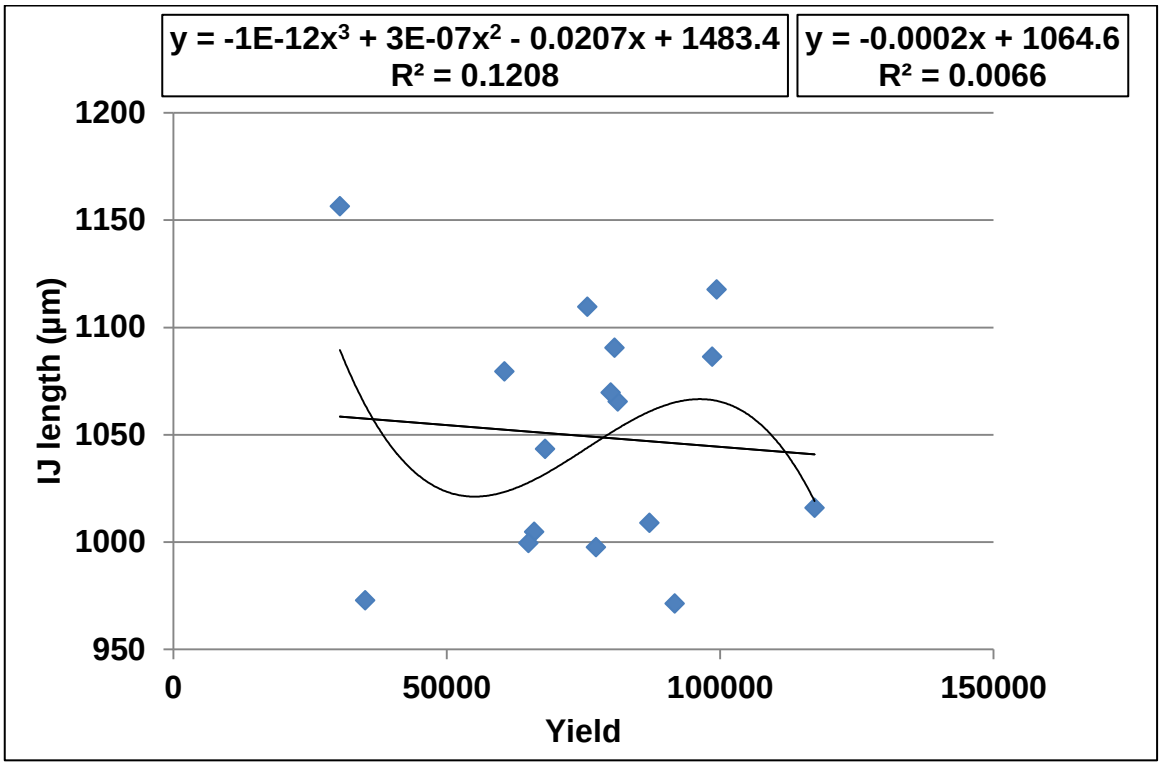

Figure 2.4 Regression of yield of *S. innovationi* vs. IJ length.

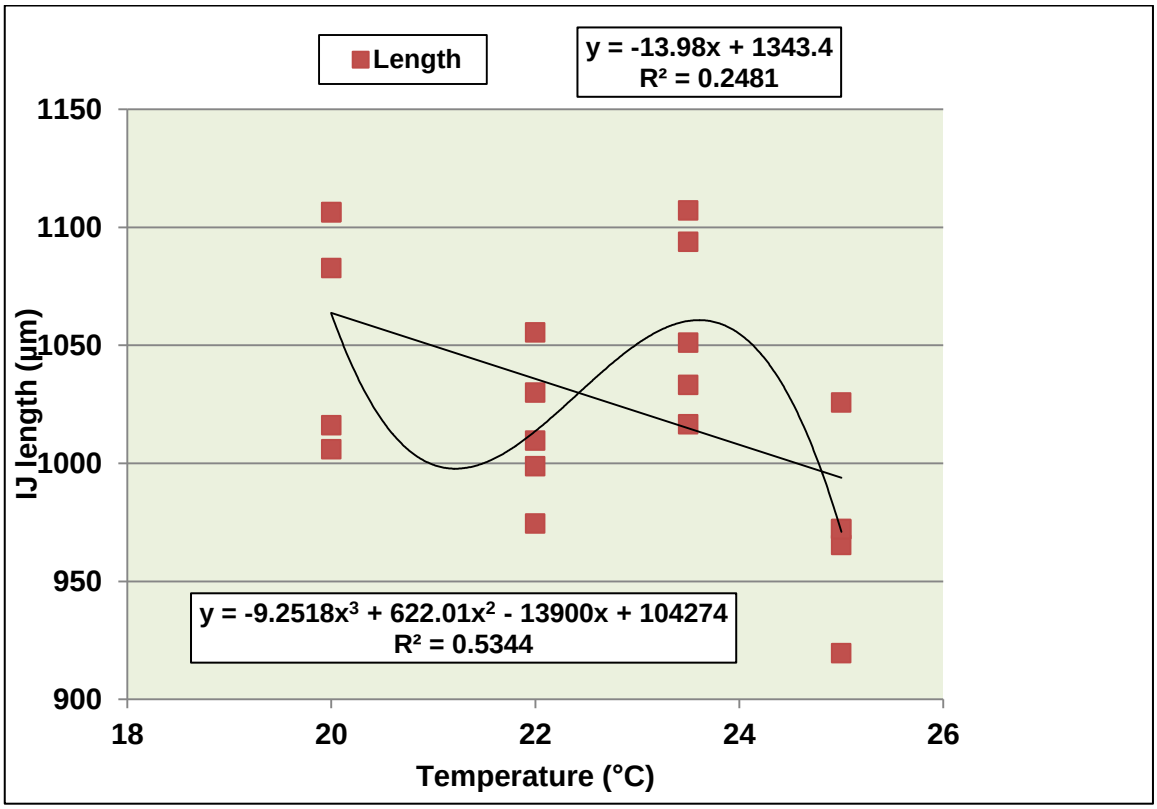

Figure 2.5 Regression of IJ length of *S. innovationi* vs. temperature

2.4 Discussion

Temperature is a crucial environmental parameter for commercial production of EPNs. This study presents the effects of temperature on reproduction and development of *S. innovationi* under laboratory conditions. Temperature had a significant effect on time of progeny emergence. Emergence was faster at the higher temperatures tested (25°C and 27°C) and slower at the lowest temperature (18°C). Jagdale and Gordon (1998) also observed the reproduction of more generations at higher temperatures than lower temperatures (15 to 18 generations at 10°C, 21 to 25 at 15°C, 30 to 35 at 20°C and 48 to 60 at 25°C) when different strains of *S. feltiae* (Flipjev), *S. carpocapsae* and *S. riobravis* were cultured at different temperatures over a period of two years. The faster emergence at 25°C and 27°C could be an

advantage because a shorter cycle during mass production would allow for a greater cumulative yield in the same period of time. On the other hand, Hazir *et al*. (2001) cautioned that a lower temperature, and hence a slower rate of emergence, gives the nematode juveniles more time to feed and accumulate more lipid reserves, which are necessary for the survival of the non-feeding IJ stage. The greater the lipid reserves that a juvenile acquires, the longer it can survive and retain virulence (Glazer, 2002). Schirockie and Hague (1997) observed that the primary effect of a longer development period at lower temperatures was increased nutrient uptake and therefore larger IJs when they compared the size of *S. feltiae* reared at 10°C versus 22°C. Similarly, Hazir *et al*. (2001) found that *S. feltiae* IJs reared at 8°C were significantly larger than IJs reared at 23°C, both *in vivo* and *in vitro*. However, there was no significant difference in IJ length in relation to rearing temperature in this study. It could be that the 5° range used here that was tested was too narrow a range to expose any significant differences.

Regression of the yield data showed a peak at 23.08°C, while the yields were not significantly different at 22°C and 25°C, suggesting that this strain may be adapted to warmer conditions. However, this does not correspond to the typical climate of the region (Fouriesburg, Free State Province, South Africa) from where it was originally isolated. This climate is defined as humid subtropical with summer rainfall, and is relatively cool (warmest month < 22°C) (Hatting, Stock & Hazir, 2009). Although many studies have shown that EPNs are adapted to the environment of their origin (Kung *et al*., 1991; Molyneux, 1985), the notion has been challenged by the discovery of a strain of *S. anatoliense* that has an optimum production temperature of 25°C, which is higher than the temperatures typically encountered in its geographical area of isolation in the interior of Turkey (Gungor *et al*. 2006). *S. innovationi* may be yet another example of such a phenomenon.

Molyneux *et al.* (1985) suggested that at low temperatures some nematode species remained in the host's hemocoel as IJs, and that they could remain there without killing the host until temperatures rose, allowing for bacterial growth and nematode

reproduction. The presumed adaptability of *S. innovationi* to a wider range of temperatures (18°C to 27°C) was supported by its subsequent isolation from the warmer Kwazulu-Natal province, which is characterised by a humid subtropical climate with annual rainfall and its warmest month >22°C (Hatting *et al.,* 2009).

Other studies have demonstrated differences in different isolates of the same species in response to various factors. Gaugler, Campbell and MacGuire (1989) found differences in response to host-finding ability and ultraviolet light tolerance of 22 isolates of *S. carpocapsae* isolated from four different continents. Hazir *et al*. (2001) also concluded that there were differences in five geographic isolates of *S. feltiae*. Alternatively, the higher optimum temperature of *S. innovationi* may have been caused by physiological changes while the isolate was maintained for several years through short term preservation by passaging through an insect host in an insectary maintained at 25°C (Morton & Garcia-del-Pino, 2009; Wang & Grewal, 2002). Or it could be resulting from acclimation to warmer temperatures because the test population was reared and re-cycled for many generations (> 5 years) at 25°C in the laboratory. Jagdale and Gordon (1998) demonstrated that the repeated use of a fixed rearing temperature led to an adaptation of the strain to that temperature and improved its performance at temperatures close to the rearing temperature. These authors also showed that infectivity at low temperatures was enhanced by propagating nematodes at such temperatures, whereas infectivity at warm temperatures was enhanced by rearing under warm conditions.

EPNs have been reported to reproduce at temperatures as low as 8°C (Grewal *et al*., 1994) and as high as 38°C, depending on the species (Salma & Shahina, 2012). Several studies have reported that the majority of EPNs are not virulent at low temperatures (5°C to 10°C), or at high temperatures (>35°C), with an optimum temperature range of 20°C to 25°C (Campos-Herrera *et al*., 2008; Gungor *et al*., 2006; Koppenhöfer, 2003; Kung *et al*., 1991; Molyneux *et al*., 1985). The optimum temperature of *S. innovationi* falls within the range 18°C to 28°C. Grewal *et al*. (1994) found this to be the optimal range for 12 species and strains of EPNs from diverse

climatic regions. This species has an optimum temperature closer to that of *S. anatoliense* (Gungor *et al.*, 2006), *S. rarum* (De Doucet) (Koppenhöfer & Kaya, 1999) as well as *S. feltiae* and *S. carpocapsae* (Hirao & Ehlers, 2009). Although *S. innovationi* reproduced at all temperatures tested, there were significant differences in yields at different temperatures (18°C = 148,629 ± 104,235 IJs/gram, 22°C = 393,403 ± 129,080 IJs/gram and 25°C = 383,270 ± 139,957 IJs/gram), emphasizing that production outside the optimum temperature will have a negative effect on yield. Gungor *et al*. (2006) observed a similar pattern in that IJ yield of *S. anatoliense* dropped significantly at production temperatures below 20°C and above 25°C.

While there was no significant difference in yield at the two highest yielding temperatures (22°C & 25°C), 22°C was selected as an optimum temperature for two reasons: 1. There was significantly slower emergence at 22°C, which would allow the EPNs to spend more time feeding and therefore accumulating more nutrients; 2. The tendency of sub-surface soil temperatures to be lower than ambient temperature would favour propagation at lower temperatures, in order to avoid acclimation of the nematodes to higher temperature, which could impair infectivity under field conditions.

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CHAPTER THREE

Effects of *in vitro* solid media on *Steinernema innovationi* reproduction and virulence

Abstract

Low-cost mass production of entomopathogenic nematodes (EPNs) is an important prerequisite towards the commercially successful development of EPN-based bio-insecticides. This study evaluated six low-cost solid substrate media for *in vitro* mass production of *Steinernema innovationi* at optimum temperature. The media comprised of a larval puree of house fly, *Musca domestica* (Linnaeus), amended with (1) 0.15g canola oil + 0.5g soy flour + 0.13g yeast, (2) 0.15g canola oil + 0.5g soy flour + 0.37g yeast, (3) 0.15g canola oil + 1.67g soy flour + 0.13g yeast, (4) 0.15g canola oil + 1.67g soy flour + 0.37g yeast, compared to (5) *M. domestica* larval puree only, and (6) *M. domestica* larval puree + 0.15g canola oil. The highest yield of infective juveniles (IJs) was obtained from Medium #6, a *M. domestica* larval puree + 0.15g canola oil, (781,678 ± 221 IJs/5g). Inoculum size did not affect yield but rather inoculum type (liquid versus solid). A liquid culture inoculum used at concentrations of 225 ± 7, 418 ± 79 and 918 ± 76 EPNs/flask, yielded 781,678 ± 221,420, 733,240 ± 74,761 and 657,080 ± 118,727 IJs/5g, respectively. However, with a solid culture inoculum (lipid agar) at a concentration of 733 ± 160 EPNs/5g, the yield dropped to only 361,439 ± 63,266 IJs/5g. Medium #6 also had the lowest number of adults remaining in the medium and dead IJs (<10%) at the time of harvest (Day 28). Although IJ length varied between experiments and being significantly shorter for Medium #6, this trait did not affect virulence. Pathogenicity against larvae of the greater wax moth, *Galleria mellonella*, remained >90% for all media tested.

Keywords: Entomopathogenic nematodes; mass production; nematode yield; substrate; infective juvenile; *in vitro*; infectivity; monoxenic culture

3.1 Introduction

Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae, coupled with their endosymbiotic bacteria, are utilized for the biological control of a wide variety of agricultural pests (Shapiro-Ilan, Gouge & Koppenhöfer, 2002). These nematodes pose a number of advantages when considered for inclusion in an integrated pest management strategy, including: safety towards mammals, non-target organisms and the environment, sustained control due to a durable IJ (non-feeding) stage, host seeking abilities, persistence in soil, rapid rate of mortality (48 hours), compatibility with chemical pesticides and exemption from registration procedures in many countries (Shapiro-Ilan & Gaugler, 2002). Control using EPNs has been more successful against soil inhabiting insects and in cryptic environments due to their susceptibility to adverse environmental conditions such as UV, moisture and extreme temperature (Wright, Schroer, Peters & Fife, 2005). In addition, the biology of the nematode species and the target pest should be matched accordingly (Hazir, Kaya, Stock & Kestin, 2003).

A key factor in the success of EPNs as bioinsecticides is their amenability towards mass production. Regardless of the importance of the production requirement to the commercial success of EPNs, only 52 papers were published on this topic from the year 2000 to 2010 (San-Blas, 2013). Generally, EPNs are produced either *in vivo*, *in vitro* solid or *in vitro* liquid culture. The choice of production method depends on the amount of product required, time, resources as well as the knowledge available (El-Sadawy, 2011). *In vivo* and *in vitro* solid cultures are typically employed by cottage industries. These methods use low technology but are labour intensive. Remarkable progress in solid-state mass production was achieved with the invention of a three-dimensional medium in flasks, using polyethylene-polyurethane sponges as liquid medium carriers (Bedding, 1981). *In vitro* culture is based on introducing nematodes to a pure culture of their symbiotic bacteria grown in an artificial medium followed by incubation at an optimum temperature until the culture nutrients are depleted (Shapiro-Ilan & Gaugler, 2002). Monoxenic culture is also recognised as being key to

in vitro mass production of EPNs. The use of surface sterilised IJs may not be appropriate since it is more likely that surface sterilised IJs still carry non-symbiotic microorganisms in their gut and under the extra cuticle (Hara, Lindergen & Kaya, 1981; Lunau, Stoessel, Schmidt-Peisker & Ehlers, 1993). The growth of non-symbiotic bacteria may totally inhibit nematode growth or have a negative effect on production (Ehlers, 2001; Hu & Webster, 2000; Lunau *et al.,* 1993). *In vitro* liquid culture is employed for large scale commercialization, mostly in developed countries (Kaya *et al.*, 2006). Culture time is inversely proportional to temperature and should be adjusted so that nematode quality is not jeopardized due to limited time for nematodes to accumulate sufficient lipid reserves (Hazir, Stock, Kaya, Koppenhöfer, & Keskin, 2001). According to Han, Cao and Liu (1992), an increase in lipid quantity and quality leads to increasing yield.

Despite the fact that quality control is essential in any industry to ensure success, there has been very little information published on quality control of EPN-based products (10 articles in the last three decades) (San-Blas, 2013). The quality of IJs depends on the method of production and media composition. Reportedly, the quality of nematodes produced *in vitro* solid culture is similar to that produced *in vivo* (Abu Hatab & Gaugler, 1999; Abu Hatab, Gaugler & Ehlers, 1998; Gaugler & Georgis, 1991). However, a number of reports indicate reduced quality of the nematodes produced *in vitro* using liquid culture. Contrary, high quality nematodes can be produced using liquid culture, but media composition and environmental conditions in the bioreactor and downstream processes may reduce quality (Shapiro-Ilan, Han & Dolinski, 2012).

The high cost of mass producing EPNs has been a limiting factor in their application as biocontrol agents. The use of offal from poultry, beef and pork industries is no longer a cheap alternative, given their value as high protein sources (San-Blas, 2013). Whey derived from the cheese industry (Islas-Lopez, Sanjuan-Galindo, Rodriguez-Hernandez & Chavarria-Hernandez, 2005) and protein from agave juice discarded during tequila production in Brazil (Chavarría-Hernández, Espino-Garciá,

Sanjuan-Galindo & Rodríguez-Hernández, 2005) have been exploited as economical alternatives to reduce the cost of production. The objective of the current study was to compare several low cost *in vitro* solid culture media for mass production of indigenous *Steinernema innovationi* (Çimen, Lee, Hatting & Stock, 2014) at optimum production temperature. Yield (number of viable IJs) was used as the main criteria to evaluate the media. Other parameters quantified included infectivity, IJ length, number of adults and number of dead IJs.

3.2 Materials and methods

3.2.1 Isolation of symbiotic bacteria

To isolate symbiotic bacteria, three *G. mellonella* larvae were inoculated with 100 IJs of *S. innovationi* in 100µl distilled water placed in a Petri dish lined with double filter paper. Three days after inoculation, cadavers were surface sterilized by dipping them in absolute ethanol, and allowing the ethanol to evaporate. The cadaver was held upside down with a forceps and the head was removed with sterile scissors. The first drop of haemolymph which oozed out was discarded, but the second drop was deposited onto a plate of NBTA agar medium (nutrient agar, bromothymol blue, 2-3-5 triphenyltetrazoliumchloride), and was streaked with an inoculation loop, to make primary, secondary and tertiary streaks to give isolated colonies. Plates were incubated at 27°C for 72h. After testing colony morphology, colony colour and catalase tests according to Boemare (2002), a single colony was then selected and used to inoculate 30 ml Nutrient Broth (Biolab) and incubated at 27°C with shaking at 100rpm for 24 to 48h.

3.2.2 Establishment of monoxenic culture

A monoxenic stock culture was established using nematode eggs isolated by hydrolysis of gravid females (Johnigk, Ecke, Poehling & Ehlers, 2004; Strauch, Stoessel & Ehlers, 1994). Ten final instar wax moth larvae were placed in a 90mm

Petri dish lined with double filter paper and inoculated with 1ml of sterile water containing 3000 IJs and incubated for three days at 25°C. Cadavers were then dissected in Ringer's solution by cutting off the head of a larva and squeezing out the haemocoel. The cuticle was removed and the haemocoel was separated using forceps. Gravid females were isolated by fishing them out and placing them in a watch glass filled with Ringer's solution. At least 100 gravid females were used. Gravid females were dissected using sharp forceps under a dissection microscope to release eggs. Pieces of adults were removed by sieving through a 53µm sieve and the filtrate with the eggs was collected and placed in 2ml micro centrifuge tubes (Eppendorf tubes). Eggs were then washed with Ringer's solution three times by centrifuging at 2000rpm for 1 min, removing the supernatant and replacing the Ringer's solution. In the laminar flow, the last wash supernatant was removed and the eggs were transferred into a sterile tube which was filled with an egg sterilization solution [0.5ml sodium hypochlorite (NaOCl 10% to14%), 1.5ml 4mol NaOH (160g in 1L distilled water) and 10ml distilled water]. The tube was shaken gently for 4min. followed by centrifugation at 2000rpm for 2 min. The sterilization solution was immediately removed and eggs were washed with nutrient broth three times by centrifuging at 2000rpm for 2 min in each wash. Nutrient lipid agar (16g Bacto nutrient broth, 12g Bacto agar, 5g sunflower oil and 1L distilled water) plates were inoculated with two drops of egg suspension and incubated at 25°C. Plates were checked daily for egg hatching and contamination. After 3 days, clean plates with axenic nematodes were inoculated with 2 to 3 drops of the primary form of the symbiotic bacteria and then incubated at 25°C. Once the surface of the agar plate was covered with nematodes, pieces of agar were used to inoculate 100ml Bovine Serum Albumin (BSA) medium in a 500ml flask for liquid stock culture. Flasks were incubated at 25°C with shaking at 100rpm until used (1 to 4 weeks).

3.2.3 Mass production

To determine yield, percentage live and dead IJs, and infectivity, 5g of different production media (as summarized in Table 3.1 and Table 3.2), was absorbed in 0.5g

of sponge cubes (measuring 0.5cm^2 each) in 100ml Erlenmeyer flask and autoclaved at 121°C for 15 min. The media was inoculated with 0.5ml of bacterial suspension and incubated at 27°C for 72h. The flasks were then inoculated with 0.5ml of monoxenic nematode liquid culture or agar pieces containing monoxenic nematode culture. Experiment 1: four weeks old liquid culture inoculum [918 ± 76]; Experiment 2: two weeks old solid culture inoculum [733 ± 160]; Experiment 3: two weeks old liquid culture inoculum [418 ± 79] and modified media (see Table 3.2); Experiment 4: 5five days liquid culture inoculum [225 ± 7]) and incubated at 22°C for 28 days. Five flasks were prepared for each production medium. Nematodes were harvest by washing sponges with 50ml distilled water six times. Sponges were squeezed five times during each wash. Dilution counts were made to determine yield and percentage survival. IJs were distinguished from other stages by their rapid movement and high concentration of fat reserves, resulting in a dark appearance of body content (Ehlers, Lunau, Krasomil-Osterfeld & Osterfeld, 1998). The experiment was repeated four times. Nematode infectivity was assessed using a Sandwell bioassay by filling wells of a 24 well Tissue Culture (TC) plate with 0.5g sterile loam sand in which 50 IJs were pipetted in 60µl of distilled water per well (Gungor, Kestin & Hazir, 2006). One final instar wax moth larva was added per well. Control larvae were inoculated with 60µl distilled water only. A total of 30 larvae (as single replicates) were used per treatment and control. Plates were incubated at 25°C for 72 hours. The experiment was repeated four times. A sample of 40 to 50 IJs from three best yielding media were heat killed at 60°C and stored in triethanolamine formalin (TAF) solution (Kaya & Stock, 1997) in Eppendoff tubes to measure the lengths of IJs.The length of 25 randomly selected IJs was measured from anterior to posterior end using an Olympus BX52 microscope fitted with DP72 camera. Measurements were performed via polyline using Cellsense imaging software.

Table 3.1 Production medium formulation.

Number	Culture Media
1	5g *M. domestica* larval puree [£] + 0.15g canola oil + 0.5g soy flour + 0.13g yeast*
2	5g *M. domestica* larval puree[£] + 0.15g canola oil + 0.5g soy flour + 0.37g yeast*
3	5g *M. domestica* larval puree[£] + 0.15g canola oil + 1.67g soy flour + 0.13g yeast*
4	5g *M. domestica* larval puree[£] + 0.15g canola oil + 1.67g soy flour + 0.37g yeast*
5	5g *M. domestica* larval puree[£]
6	5g *M. domestica* larval puree[£] + 0.15g canola oil

[£] Prepared by crushing frozen larvae in a food blender.
* Marmite® yeast extract, manufactured by Bokomo Foods. Typical value per 100g: protein 37.3g, total fat 0.5g, carbohydrates 18.1g, sodium 3480mg and fibre 2g.

Table 3.2 Media formulation for modified media experiment.

Number	Culture Media
1	5g soy flour
2	5g canola oil
3	5g *M. domestica* larval puree[£]
4	5G soy flour + 0.15g canola oil
5	2.5g soy flour + 2.5g *M. domestica* larval puree[£]
6	5g *M. domestica* larval puree[£] + 0.15g canola oil
7	2.5g soy flour + 2.5g *M. domestica* larval puree [£] + 0.15g canola oil

[£] Prepared by crushing frozen larvae in a food blender

3.2.4 *Musca domestica* rearing

Pupae of *M. domestica* in moist bran were supplied by Bio-Insectaries South Africa (BISA) (Pty) Ltd (www.bioinsectsa.com). A container with pupae in wet bran was placed without a lid in a cage with a mesh small enough to retain adults while

allowing adequate ventilation. Upon adult emergence, they were fed Nespray® milk powder (Nestlé®, South Africa). In a separate container, adults were provided with water absorbed in cotton wool. From Day 6, about 100g of wet bran was placed in the cage for adult females to lay eggs. Eggs were collected by removing the bran container and mixing bran containing eggs with larval diet (2000g bran, 300g Nespray® milk powder, 6g sodium benzoate, 20g brewer's yeast, 3000ml lukewarm water). Eggs hatched in less than 24h and instar larvae were collected and frozen until used or left to pupate and progress to the next production cycle. This culture was reared at temperatures between 20°C to 28°C (Chang, 2010).

3.2.5 Statistical analysis

Yield results were analysed by one way analysis of variance (ANOVA) (Shapiro and Wilk, 1965). IJ length was analysed using one way ANOVA and the treatment means were separated with Fisher's unprotected LSD test at the 5% level (Fisher, 1970).

3.3 Results

3.3.1 Yield: Experiment 1, 2 and 3

Media formulation had a significant effect on yield for all three experiments. Media number 6 (*M. domestica* larval puree + 0.15g canola oil) gave the highest yield (max = 733.240 ± 74.761 IJs/flask) in all three experiments, with significantly higher yields in Experiments 1 ($P < 0.05$; $f = 68.762$; $df = 4.737$) and 3 ($P < 0.05$; $f = 42.127$; $df = 4.116$). Notably, the addition of 3% canola oil improved production in all three experiments. Media number 2, which had the highest concentration of yeast extract and the lowest concentration of soy flour had the lowest yield (max = 54.139 ± 52.468 IJs/flask). Yield improved in all production media when two weeks old liquid culture inoculum (Experiment 3) was used (Table 3.3).

Table 3.3 Mean yield comparison of Experiments 1, 2 and 3, and ANOVA table. Means in a given column followed by a different letter(s) differed significantly at the 5% test level.

Media	Experiment 1 Yield	Experiment 2 Yield	Experiment 3 Yield
1. *M. domestica* larval puree + 0.15g canola oil + 5g soy flour + 0.13g yeast*	89,132 ± 20,260 c	145,553 ± 85,207 b	485,429 ± 203,879 b
2. *M. domestica* larval puree + 0.15g canola oil + 0.5g soy flour + 0.37g yeast*	2,819 ± 2,046 c	30,994 ± 12,462 c	54,189 ± 52,468 d
3. *M. domestica* larval puree + 0.15g canola oil + 1.67g soy flour + 0.13g yeast*	23,256 ± 5,127 c	199,925 ± 76,782 b	265,829 ± 136,795 c
4. *M. domestica* larval puree + 0.15g canola oil + 1.67g soy flour + 0.37g yeast*	3,211 ± 3,522 c	116,500 ± 78,421 bc	155,460 ± 12,638 cd
5. *M. domestica* larval puree	392,758 ±139,370 b	**345,223 ± 75,285 a**	460,200 ± 28,530 b
6. *M. domestica* larval puree + 0.15g canola oil	**657,083 ± 118,727a**	**361,439 ± 63,267 a**	**733,240 ± 74,752 a**
F	51.86	17.64	21.73
P	<0.05	<0.05	<0.05
LSD	111 786	90 954	155 736
CV%	38.65	34.85	33.23

* = Marmite® yeast extract, manufactured by Bokomo Foods

3.3.2 Percentage live, dead and adult: Experiment 1, 2 and 3

Percentage live IJs was highest (89%, 84% and 93% for Experiments 1, 2 and 3, respectively) for Medium # 6 (Figure 3.1). This medium contained less than 10% of dead IJs and adults at the time of harvest. Second to this was Medium # 5 with 79%, 85% and 85% for Experiment 1, 2 and 3, respectively, but % dead IJs was 20%, 9 %

and 14%, respectively. The lowest yielding medium was Medium # 2 which had the highest number of dead IJs (above 50%) for all three experiments. Percentage of adults was also the lowest compared to percentage live and percentage dead IJs in all production media across the three experiments.

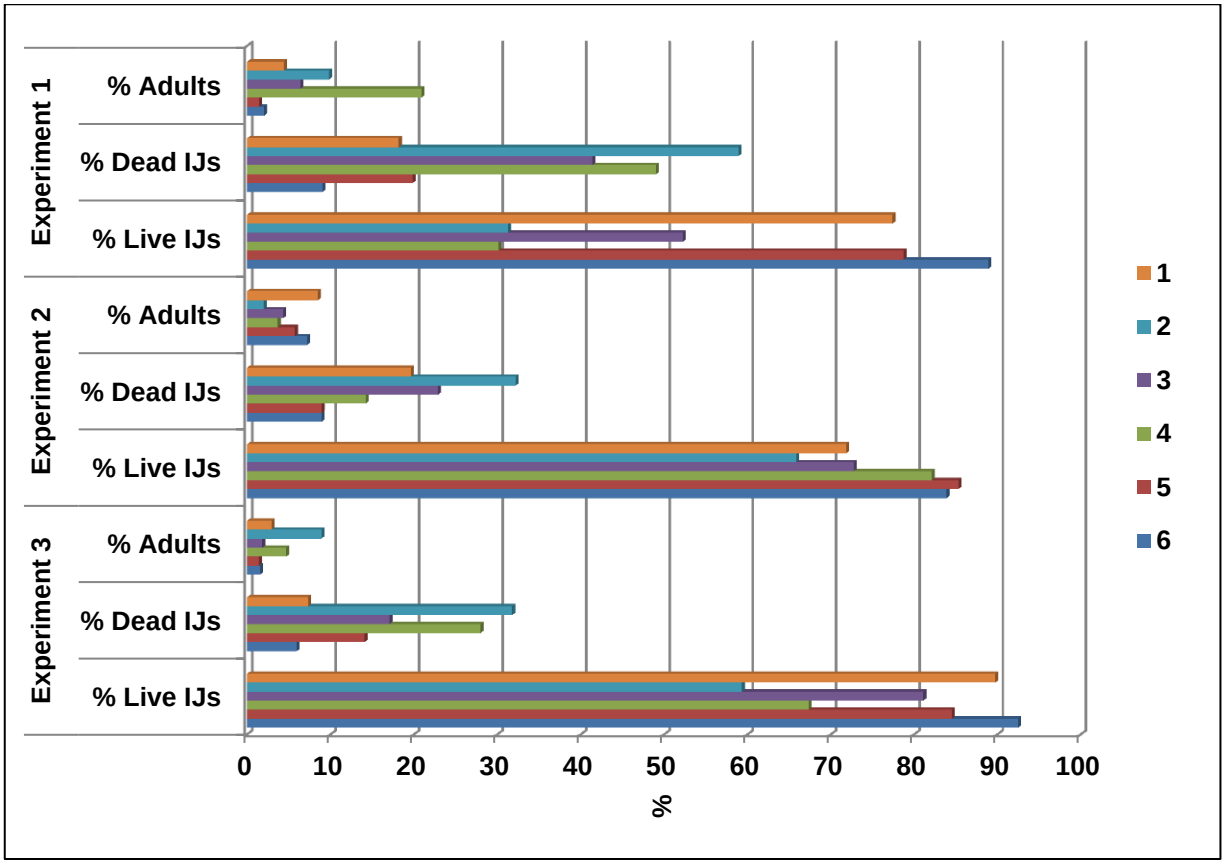

Figure 3.1 Percentage live IJs, dead IJs and adults compared for experiment 1, 2 and 3 at the time of harvest (28 days). In the key, numbers 1 to 6 refer to the six different solid culture media tested.

3.3.3 Infectivity: Experiments 1, 2 and 3

Nematode infectivity was not altered in any of the three experiments (Table 3.4). Mortality of *G. mellonella* was above 90% for all three experiments compared to <10% mortality in the control.

Table 3.4 Infectivity of IJs from three best yielding media against *G. mellonella.*

Media	Experiment 1 % Mortality	Experiment 2 % Mortality	Experiment 3 % Mortality
1. *M. domestica* larval puree + 0.15g canola oil + 5g soy flour + 0.13g yeast*	93	93	100
5. *M. domestica* larval puree	97	93	97
6. *M. domestica* larval puree + 0.15g canola oil	93	100	100
Control	3	7	0

* = Marmite® yeast extract, manufactured by Bokomo Foods

3.3.4 IJ length: Experiments 1, 2 and 3

IJ length was consistent with Medium #6 and Medium #1 with no significant difference (P>0.05) in any of the three experiments (Table 3.5). IJ length ranged from 984µm to 1031µm.

Table 3.5 Mean IJ length for three best yielding media and ANOVA table. Means in a given column followed by a different letter/s differed significantly at the 5% test level.

Media	Experiment 1 Mean IJ length (µm)	Experiment 2 Mean IJ length (µm)	Experiment 3 Mean IJ length (µm)
1. *M. domestica* larval puree + 0.15g canola oil + 5g soy flour + 0.13g yeast*	995.34 ± 62.44 a	1000.34 ± 40.91 a	1031.31 ± 95.32 b
5. *M. domestica* larval puree	1023.20 ± 73.17 a	1026.66±51.54 b	984.06 ± 86.43 a
6. *M. domestica* larval puree + 0.15g canola oil	1004.87 ± 62.04 a	1022.59 ± 41.66 ab	1021.76 ± 74.31 ab
P	0.330	0.093	0.101
LSD	37.80	25.48	45.75
CV%	3.7	2.6	5.5

* = Marmite® yeast extract, manufactured by Bokomo Foods

3.3.5 Modified media experiment (yield, % live, dead and adult, infectivity and IJ length)

Again, production was highest with *M. domestica* larval puree plus 0.15 g canola oil medium (Table 3.6) (781 678 ± 221 420 IJs in 5g of medium). Addition of canola oil to *M. domestica* larval puree improved yield significantly (P>0.05) but did not improve yield when added to soy flour.

Table 3.6 Mean number of IJs (yield) with ANOVA table. Means in a given column followed by a different letter(s) differed significantly at the 5% test level.

Media	Yield
1. 5g soy flour	325,228 ± 173,922 b
2. 5g oil	0
3. 5g *M. domestica* larval puree	476,146 ± 119,001 b
4. 5G soy flour + 0.15g canola oil	361,727 ± 156,121 b
5. 2.5g soy flour + 2.5g *M. domestica* larval puree	599,775 ± 131,840 ab
6. 5g *M. domestica* larval puree+ 0.15g canola oil	**781,678 ± 221,420 a**
7. 2.5g soy flour + 2.5g *M. domestica* larval puree +0.15g canola oil	467,950 ± 97,369 b
F	3.55
P	0.0305
LSD	287.095
CV%	32.78

Production was slow in media with a high concentration of soy flour, and the number of adults was ±10% higher than IJs at the time of harvest (Figure 3.2). The number of dead IJs and adults was below 10% for the highest yielding medium (*M domestica* larval puree + 0.15g canola oil).

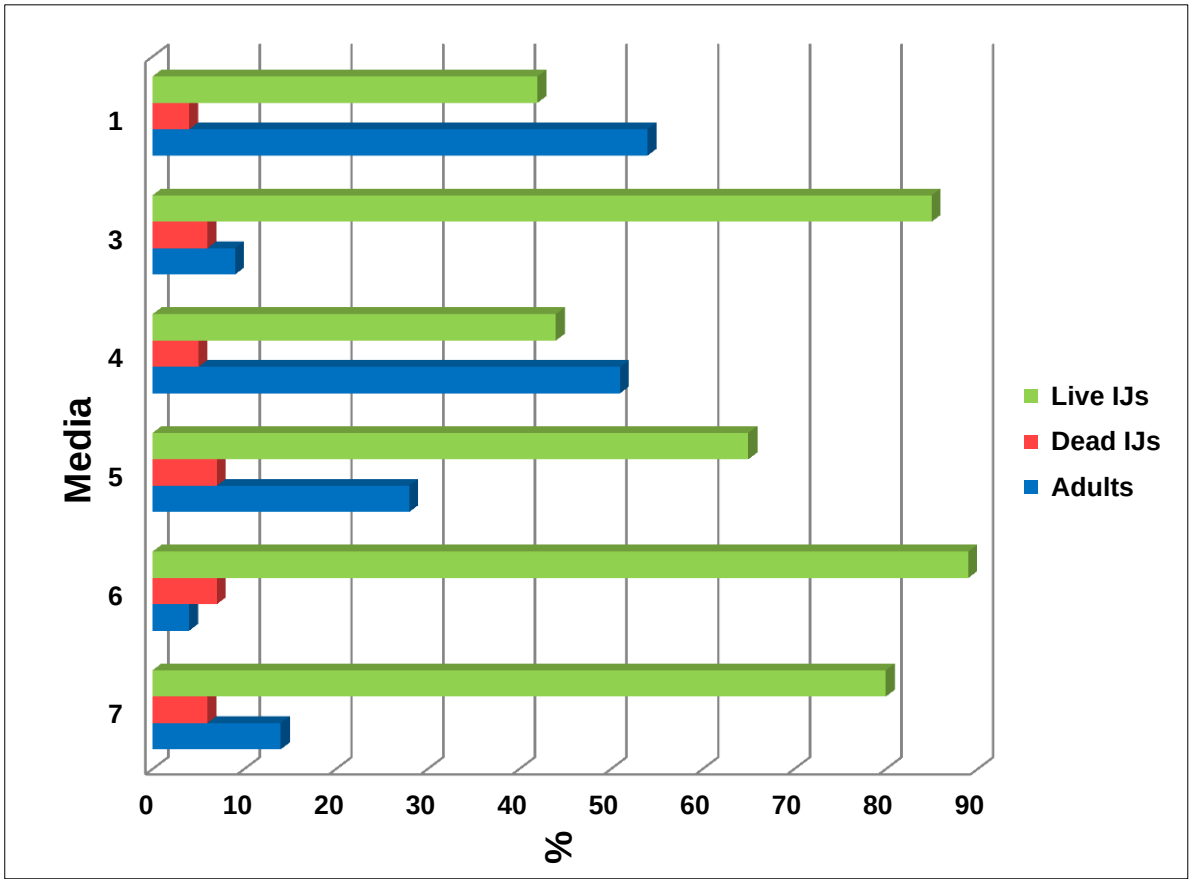

Figure 3.2 Percentage live IJs, dead IJs and adults for the modified media experiment

Table 3.7 Infectivity of IJs against *G. mellonella* after mass production using different media.

Media	% Mortality
1. 5g soy flour	97
3. 5g *M. domestica* larval puree	100
4. 5G soy flour + 0.15g canola oil	100
5. 2.5g soy Flour + 2.5g *M. domestica* larval puree	97
6. 5g *M. domestica* larval puree + 0.15g canola oil	100
7. 2.5g soy flour + 2.5g *M. domestica* larval puree + 0.15g canola oil	97
Control	10

IJs grew significantly longer in Media 1, 5 and 7, measuring 999 ± 64µm, 933 ± 83µm and 1027 ± 83µm, respectively, while measuring shorter in Medium # 6, at 882 ± 124µm (Table 3.8).

Table 3.8 Mean IJ length for three best yielding media and ANOVA table. Means in a given column followed by a different letter/s differed significantly at the 5% test level.

Media	Mean IJ length (µm)
1. 5g soy flour	999.35 ± 64.54 ab
3. 5g *M. domestica* puree larval puree	953.74 ± 94.82 bc
4. 5G soy flour + 0.15g canola oil	1041.17 ± 52.69 a
5. 2.5g soy flour + 2.5g *M. domestica* larval puree	933.72 ± 83.58 c
6. 5g *M. domestica* larval puree + 0.15g canola oil	882.84 ± 124.96 d
7. 2.5g soy flour + 2.5g *M. domestica* larval puree + 0.15g canola oil	1027.33 ± 83.91a
P	<0.001
LSD	48.72
CV%	9.0

3.4 Discussion

The highest concentration of IJs (156, 000/g) obtained with *M. domestica* larval puree plus 0.15g (3%) canola oil is considerably higher than yields achieved by *in vitro* solid production in previously reported studies apart from for 400-542,857 achieved by Bedding (1981 and 1984). El-Sadawy (2011) achieved a maximum of 8,500 IJs/g of medium when seven species of *Steinernema* were mass produced using Wouts solid medium with some modifications. This yield is 18.4 times less than the best yield achieved in our study. In another study, 97,000 IJs/g medium was achieved when three species of *Steinernema* and one species of *Heterorhabditis* were produced in

chicken offal medium (Tabassum & Shahina, 2004). Hara *et al.* (1981) reported 100,000 IJs/g when dog food agar medium was used. Notable difference can be accounted to the balance of nutrients in the production media. Other authors have suggested that lipid components reflecting the nematode's natural host composition are more suitable for *in vitro* production (Han *et al*., 1992). The medium with the maximum yield (*M. domestica* larval puree plus 3% canola oil) in this study closely resembles *in vivo* production with a boost of fat. Some studies show that the quantity and quality of fats in the media increase yield of *in vitro* culture (Abu Hatab *et al.,* 1998; Dumphy & Webster, 1989; Gil, Choo & Gaugler, 2002; Han *et al*., 1992). Dumphy and Webster (1989) compared production of *Steinernema carpocapsae* (Weiser, 1955) when the basal lipid agar was supplemented with different concentrations of butter, halibut oil, cod liver oil, olive oil, corn oil and sunflower oil. Production was highest (±294,000/plate) when sunflower oil was used at a concentration of 0.1ml in 100ml media. Also noted in their study was a drop in yield when the oil concentration was increased: butter, 0.1ml = 31,000/plate and 1ml = 11,000/plate; cod liver oil, 0.1ml = 93,000/plate and 1ml = 54,000/ plate; olive oil, 0.1ml = 13,000/plate and 1ml = 11,000/plate; sunflower oil, 0.1ml = 294,000/plate and 1ml = 90,000/plate. Although only one concentration of canola oil was tested in this study, yield was increased significantly when 3% canola oil was added to a *M. domestica* larval puree medium in all four experiments. It was also noted that production dropped when Marmite® was included as a source of yeast protein in the medium. Medium #2 and Medium #4 had the highest concentrations of Marmite® and gave the lowest yields. It is speculated that the high salt content (11g per 100g) in Marmite® had a negative effect on yield since yield improved considerably (doubled) from 31,092 ± 12,638 IJs/g to 65,045l ± 34,784 IJs/g media when Marmite® was omitted in the soy + *M. domestica* medium. Dumphy and Webster (1989) determined the effect of different salts on nematode production. In their study, they discovered that manganese chloride, ferric chloride and sodium bicarbonate all had adverse effects on nematode production. Ferric chloride inhibited nematode production all together, even at a low concentration of 1mM per 100ml of media. Manganese

chloride reduced yield from 145 x 10^2 to 5 x 10^2 at a concentration of 1mM per 100ml media.

Some reports show that inoculum concentration has an effect on yield, with yield increasing with increase in inoculum size (Han, Cao & Liu, 1993; Hirao & Ehlers, 2010; Wang & Bedding, 1998). However, inoculum dosages did not have an effect on yield during this study, but rather the type of inoculum. When a liquid culture inoculum was used at the concentrations of 225 ± 7, 918 ± 76, 418 ± 79 nematodes/flask, yields did not differ greatly (781,678 ± 221,420, 657,080 ± 118,727 and 733,240 ± 74,761 IJs/flask respectively). In contrast, when solid culture inoculum (on lipid agar) was used at a concentration of 733 ± 160 nematodes/flask, yield dropped to 361,439 0 ± 63,266 IJs/flask. In a liquid medium, nutrients are distributed homogenously and are equally available to each nematode because flasks are incubated with shaking. On the other hand, nematodes on an agar plate are piled up vertically, whereas nutrients and higher oxygen levels are encountered mainly on the surface of the agar.

Production media played a vital role in the length of time of production, and the total yield. Longer culture times can provide higher yields but nematode mortality may also increase with time (Han *et al.,* 1992, 1993; El-Sadawy 2011). Type of protein source may also have a direct effect on nematode production. El-Sadawy (2011) reported that 7 species of EPNs failed to reproduce on dog food agar, but they were all successfully produced on modified Wouts medium containing soy flour as the primary source of protein. In the present study, reproduction occurred on all production media but was significantly slower when soy flour was used without addition of the insect component (Media #1 and #4 in the Modified Media experiment). Canola oil increased yield significantly when added to Medium #6, but production was slow when oil was added to soy flour medium (Medium #4). At the time of harvest, the number of adults was still higher than the number of IJs in the soy flour medium, whereas the number of dead juveniles was lower than 10%. Slow production could be due to an unnatural source of plant protein (Dumphy & Webster, 1989; Han *et al,*

1992). However, the time of harvest was optimal because there was a high number of live IJs and very low numbers of dead IJs and adults in the best yielding medium.

A good production medium or process should yield a high number of IJs, since this is the active ingredient used in nematode formulations (Somwong & Petcharat, 2012). It also indicates nutrient depletion in the medium because nematodes continue with their development to adults as long as nutrients are still available (Gaugler *et al.,* http://oardc.osu.edu/nematodes/video_and_poster.htm). A harvest time of four weeks, as in this study, fall within the range of two to five weeks suggested by Shapiro-Ilan and Gaugler (2002) for *in vitro* production.

The quality of EPNs is often determined by measuring viability or the percentage of live, active IJs in a production batch and/or by testing their virulence against a specific host (Grewal & Peters, 2005). The uneven distribution of nematodes in solid *in vitro* production systems prevents systematic sampling and thus only the above mentioned quality parameters can be assessed at the end of a production cycle (Ehlers & Shapiro-Ilan, 2005). Because IJs are non-feeding, high levels of stored energy compounds such as lipids are needed for survival. Abu Hatab *et al.* (1998) concluded that the lipid composition of EPN is host or medium dependent and can be manipulated in *in vitro* production by adding components similar to natural host nutrition, to boost production numbers and quality.

Quality of nematodes produced using *in vitro* solid medium can be similar to nematodes produced *in vivo* which has the best quality properties (Abu Hatab & Gaugler, 1999; Abu Hatab *et al.,* 1998; Gaugler & Georgis, 1991; Grewal & Peters, 2005; Susurluk, Kongu & Ulu, 2013). Infectivity against *G. mellonella* was above 90% for all media tested. Somwong and Petcharat (2012) also found that the infectivity of *in vivo*-produced IJs against *Spodoptera literal* (Fabricius) (60%) was close to IJs produced on *in vitro* solid production using a medium containing silkworm pupae (62%). Nematode viability should be measured for every batch produced since nematode quality can vary among batches (Caamano, Cloyd, Solter & Fallon, 2008).

Although there was variation between batches, percentage viable IJs was highest for *M. domestica* puree plus 3% canola oil in all four production batches (Batch 1 = 89%, Batch 2 = 84%, Batch 3 = 93% and Batch 4 = 89%). Also noted was a high level of stability with this production medium, with percentage survival consistently above 80% in all four batches. In concurrence with these results are the findings of a study comparing plant protein, animal protein and an *in vivo* production method by Yang, Jian, S. Zhang and G. Zhang (1997). Their *in vivo* method produced the best quality nematodes followed by animal protein and plant protein in terms of respective IJ length (556.6, 514.3 and 497.4µm), dry weight (80.7, 58.6 and 48.2mg) and fatty acid content (15.4 x 10^5 µm v/s, 11.8 x 10^5 µm v/s and 6.8 x 10^5 µm v/s). The shorter IJ length observed with the best yielding media in this study could be as a result of a rapid production cycle, because lipids are known to increase production rate. Nonetheless, IJ length did not affect infectivity of the IJs for any of the media tested.

In conclusion, *M. domestica* larval puree + 3% canola oil was identified as the best production medium for *S. innovationi*. Liquid culture inoculum performed better than solid culture inoculum, irrespective of concentration of the inoculum. Yield was consistently high throughout all four experiments for Medium #6. This medium should be suitable for production of other EPN isolates since it closely resembles a natural host and therefore warrants further evaluation.

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CHAPTER FOUR

Effect of storage temperature and time on survival and infectivity of *Steinernema innovationi*

Abstract

Entomopathogenic nematode species differ in their optimum storage temperature. Infective juvenile (IJ) survival and infectivity of *Steinernema innovationi* was studied at five storage temperatures (5, 10, 15, 20 & 25°C) over a period of 84 days to determine the optimum storage temperature in aqueous suspension (20 000 IJs in 25ml of 0.1% formalin solution). A nematode sponge formulation, at a concentration of 2.5 million IJs in 15ml 0.1% formalin solution, was then stored at the optimum temperature followed by two weeks exposure to 25°C. Survival was highest and most stable at 15°C, ranging from 84% to 88% after 84 days storage. Survival in the sponge formulation improved by 6% from 84% to 90% at 15°C compared to storage in aqueous suspension. Infectivity of IJs against *Galleria mellonella* (Linnaeus) was above 90% for all temperatures except for 5°C at which survival decreased to 10% after 84 days storage. Storage of nematode in a sponge formulation for 14 days at room temperature (25°C) post low temperature (15°C) storage for 84 days did not have a detrimental impact on IJ survival and infectivity (87% & 95%, respectively). A temperature of 15°C was the best storage temperature for *S. innovationi* in these trials.

Keywords: *Steinernema*; infectivity; survival; infective juvenile; temperature

4.1 Introduction

Entomopathogenic nematodes (EPNs) belonging to the genera *Steinernema* and *Heterorhabditis* have been successfully used over decades as biocontrol agents for the management of key agricultural pests because of their ease of culture, high virulence and environmental compatibility (Grewal, 2002). Minimising infective juvenile (IJ) mortality during storage prolongs shelf-life and enables transportation of the product prior to application. IJs are non-feeding and rely solely on energy

reserves for survival and infectivity. Therefore, energy conservation is a vital factor in improving EPN-based biopesticide shelf-life (Andaló, Moino Jr, Maximiniamo, Campos & Mendonça, 2011; Patel, Stolinski & Wright, 1997; Patel & Wright 1997; Qui & Bedding, 2000).

Two methods of improving shelf-life and preservation of stored energy entail the use of low temperature to slow down nematode activity (Goud, Huger & Prabhuraj, 2010; Gülcü & Hazir, 2012; Strauch *et al.,* 2000; Westerman, 1992), and dehydration or desiccation (Divya, Sankar, Marulasiddesha, Sambashiv & Krupanidhi, 2011; Georgis, Dunlop & Grewal, 1995; Hussein & Abdel-Aty, 2012; Strauch *et al*., 2000). Temperature is the most important factor affecting nematode survival in formulations. At elevated temperatures, nematode physiological activity is high, increasing the consumption of stored energy, and resulting in limited shelf-life (Andaló *et al*., 2011). However, even at low temperature EPN species differ distinctly in terms of optimum storage temperature (Andaló *et al*., 2011; Goud *et al*., 2010; Mejia-Torres & Sáenz, 2013; Strauch *et al.*, 2000). Their short shelf-life at room temperature is a major factor limiting EPNs from reaching their full potential as biopesticides (Grewal, 2002). Dehydration presents many challenges including poor nematode survival and difficulty in application because the carriers can block spray nozzles (San-Blas, 2013). For these reasons research focus has recently moved towards improving liquid formulations by using polymer gels (Andaló, Cavalcanti, Molina and Moino Jr, 2010; De Waal, Malan & Addison, 2013; Hussein & Abdel-Aty, 2012; Navon, Nagalakshmi, Levski, Salame & Glazer, 2002; Wilson & Ivanova, 2004). Chen and Glazer (2005) used a combination of calcium alginate granules and glycerol to improve shelf-life of *Steinernema feltiae* (Filipjev, 1934) at room temperature from three months to six months.

In the history of EPN research (San-Blas, 2013), product formulation has been the field with the least publications (about 20% of publications, including quality control, in the period from 1980 to 2010). Furthermore, information about product formulations is usually kept as a trade secret by production companies. A fairly

simple method of storage or formulation is to concentrate nematodes to form a slurry, which is then absorbed into a polyurethane sponge (Bedding, 1984). Besides the two main approaches, some researchers have attempted to formulate infected cadavers by coating them with protectants. Shapiro-Ilan, Lewis, Behle and Mcguire (2001) tested 19 different combinations of starches, clay and flour as coatings to improve cadaver durability. Shapiro-Ilan, Morales-Ramos, Rojas and Tedders (2010) formulated infected host cadavers by enclosing them in masking tape.

The objectives of this study were: 1. To determine the influence of storage temperatures on survival and infectivity of an indigenous *Steinernema innovationi* (Stock, Cimen, Lee, Hatting & Hazir, 2014) originally isolated from Fouriesburg, Free State, South Africa (Hatting, Stock & Hazir, 2009); and 2. To test a concentrated sponge formulation for IJ survival and infectivity following storage at the pre-determined optimum temperature.

4.2 Materials and methods

4.2.1 Optimum storage temperature

IJs of *S. innovationi* were produced *in vivo* by passaging through final instar wax moth larvae (*Galleria mellonella,* Linnaeus) according to the method described by Kaya and Stock (1997). Nematodes were used immediately after harvesting.

To determine optimal storage temperature, 25ml of IJs in 0.1% formalin solution were stored in 550 ml flat angled tissue culture flasks (Lasec SA, www.lasecsa.co.za) at a concentration of 20 000 IJs per flask (Strauch *et al*., 2000). Three flasks were prepared for each test temperature (5, 10, 15, 20 & 25°C) and stored for a period of 84 days in total darkness. For evaluation three samples of 120µl each were pipetted into a 55mm glass Petri dish with 5mm^2 grids drawn on the base with a diamond pen (Lasec SA, www.lasecsa.co.za). Sterile water was then added to the dish to cover the base to a depth of 2mm. Survival was determined by counting, at random, live and

dead IJs to a total of 100 IJs using a dissection microscope at 1.5x magnification (Nikon SMZ800) (Gülcü & Hazir, 2012).

A further 600µl (10 x 60µl) was sampled per flask to conduct the infectivity test. Infectivity was assessed by covering the bottom of wells of a 24 well CELLSTAR culture plate (Lasec SA, www.lasecsa.co.za) with 0.5g sterile loam sand. The wells were each inoculated with 50 IJs suspended in 60µl of 0.1% formalin solution (Hazir, Stock, Kaya, Koppenhöfer, & Keskin, 2001). Controls were inoculated with 60µl of 0.1% formalin solution, only. One final instar wax moth larva was added to each well. A total of 30 larvae (split into three repeats of 10 larvae as single replicates) were used per storage temperature and control. Each culture plate was placed inside a 330 x 215mm zip lock bag (GLAD®, www.glad.co.za) to conserve moisture and incubated at 25°C for 72h in complete darkness.

Sampling was done every 28 days over a period of 84 days as described above.

4.2.2 Storage of nematode formulation at optimum temperature

IJ production: 5g common house fly, *Musca domestica* (Linnaeus), larval puree + 0.15g canola oil was absorbed in 0.5g of 0.5cm^2 sponge cubes placed in a 100ml Erlenmeyer flask and autoclaved at 121°C for 15min. Each flasks was inoculated with 0.5ml of bacterial suspension prepared by selecting a single colony from a NBT (Nutrient agar, bromothymol blue, 2-3-5 triphenyltetrazoliumchloride) agar plate, inoculated 30 ml Nutrient Broth (Biolab, Merck) and incubated at 27°C, with shaking at 100rpm for 24 to 48h. Flasks were incubated at 27°C for 72h. The flasks were then inoculated with 0.5ml of a monoxenic nematode culture and incubated at 22°C for 28 days. Nematodes were harvested by washing the sponges with 50ml distilled water six times. Sponges were squeezed five times during each wash. Three sub-samples were counted per flask to calculate the number of nematodes produced per batch using the dilution method described by Kaya and Stock (1997).

Formulation was prepared by sieving batches of harvested nematodes through two milk filters (www.denvet.co.za) by placing filters in a container with water separate rid of dead IJs. Only live IJs migrate through the filters into the water, leaving behind the dead nematodes in the filters. Live IJs were diluted to 2.5 million IJs in 15ml of a 0.1% formalin solution. This "nematode paste" was then absorbed into a 7.5 x 5.0 x 2.5cm sponge. Three sponges were placed in plastic bags individually and the plastic was sealed using a bag sealer (Verimark, Model: VP5201, www.verimark.co.za). Formulations were stored at 15°C for 84 days in total darkness. Percentage survival was assessed by cutting out five pieces of 0.5cm^2 sponges from the formulated sponge, washing out nematodes from each piece in a separate beaker and counting three sub-samples as described above. This experiment was repeated two times.

Nematode infectivity was assessed by filling wells of a 24 well Tissue Culture (TC) plate with 0.5g sterile loam sand in which 50 IJs were pipetted in 60µl of distilled water per well. One final instar wax moth larva was added per well. Controls were inoculated with 60µl distilled water only. A total of 30 larvae split into three replicates of 10 were used per treatment and a control. Plates were then incubated at 25°C for 72 hours to determine infectivity.

4.2.3 IJ survival and pathogenicity at 25°C, following storage at 15°C for 84 days

Nematode formulation sponges previously stored at 15°C for 84 days were cut into six pieces. Sponges were placed in a plastic bag, sealed with a bag sealer and maintained at 25°C for 14 days, aiming to mimic a scenario from purchase to application where cooling facility may not be available. Nematodes were harvested by washing sponges with 50ml distilled water six times. Sponges were squeezed five times during each wash. Three sub-samples per flask were counted (100 IJs per sub-sample) to determine percentage survival, using the dilution method described by Kaya and Stock (1997). Infectivity tests were performed as described above.

4.2.4 Statistical analysis

A Split-plot analysis of variance (ANOVA), with temperature as main plot and days as sub-plot, was used to analyse optimum temperature data (SAS Institute Inc., 1999). Thereafter, the means were separated using Fisher's unprotected t-test (least significant difference – LSD) at the 5% level of significance (Fisher, 1970). Area under the disease progress curve (AUDPC) analysis was performed using SAS statistic program (SAS Institute Inc., 1999).

4.3 Results

4.3.1 Optimum storage temperature

Percentage survival (Figure 4.1) was the highest ($P< 0.05$; $F= 42.13$; $df= 4.116$) at 15°C reaching 88%, 85% and 84% on sampling Day 28, 52 and 84, respectively. There was no significant difference in percentage survival between the two temperatures on Day 52 and 84. Survival at 20°C was 86% on Day 28 but dropped to 59% after 52 days. At room temperature (25°C) survival dropped to 57% in the first 28 days but remained stable up to Day 84. There was no significant difference between 20°C and 25°C on Day 52 and 84. The lowest percentage survival was recorded at 5°C (35%, 17% & 13% on Day 28, 52 & 84, respectively) and it was significantly different to all other temperatures. AUDPC analysis showed that the area under the survival curve was lowest at 5°C, followed by 10°C, then a peak at 15°C, followed by a decline at 20°C and 25°C (Figure 4.2).

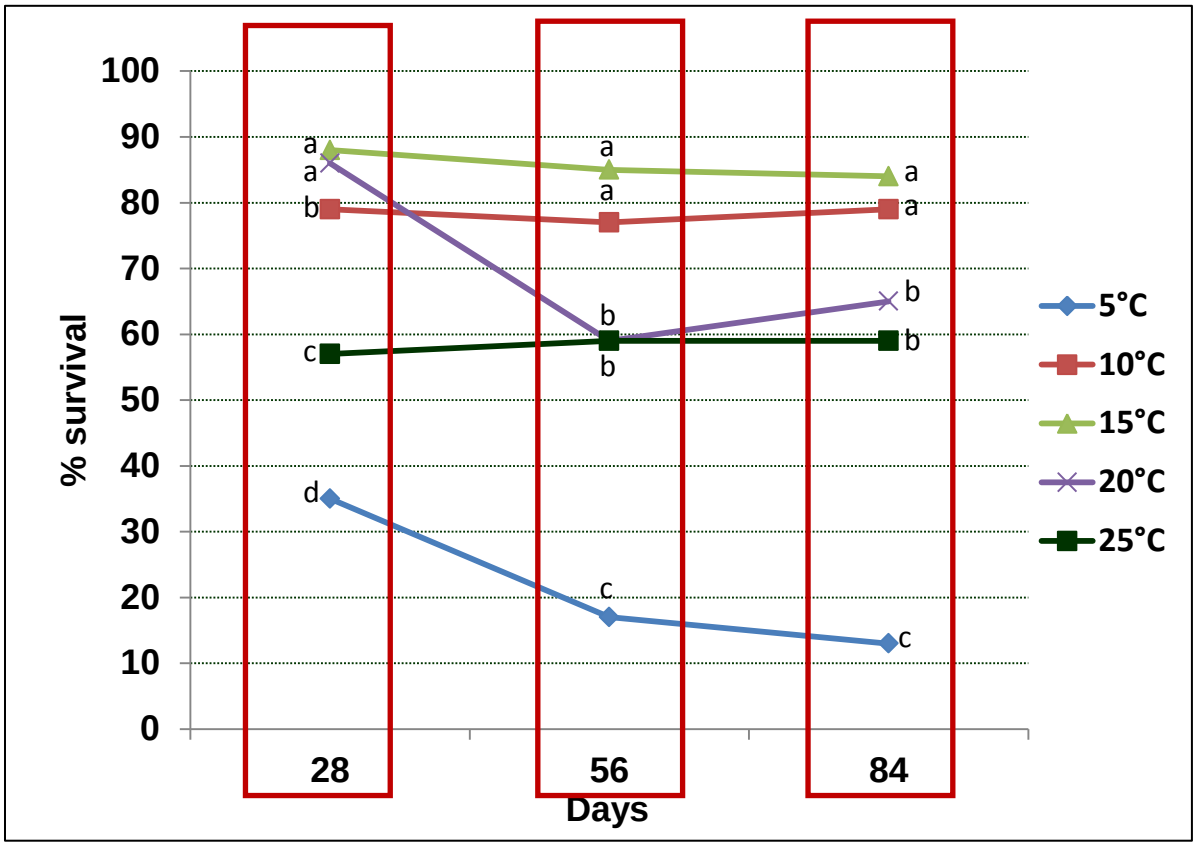

Figure 4.1 Percentage survival of IJs stored in 0.1% formalin solution for a period of 84 days. Lines with different letters within a given column differed significantly at the 5% test level.

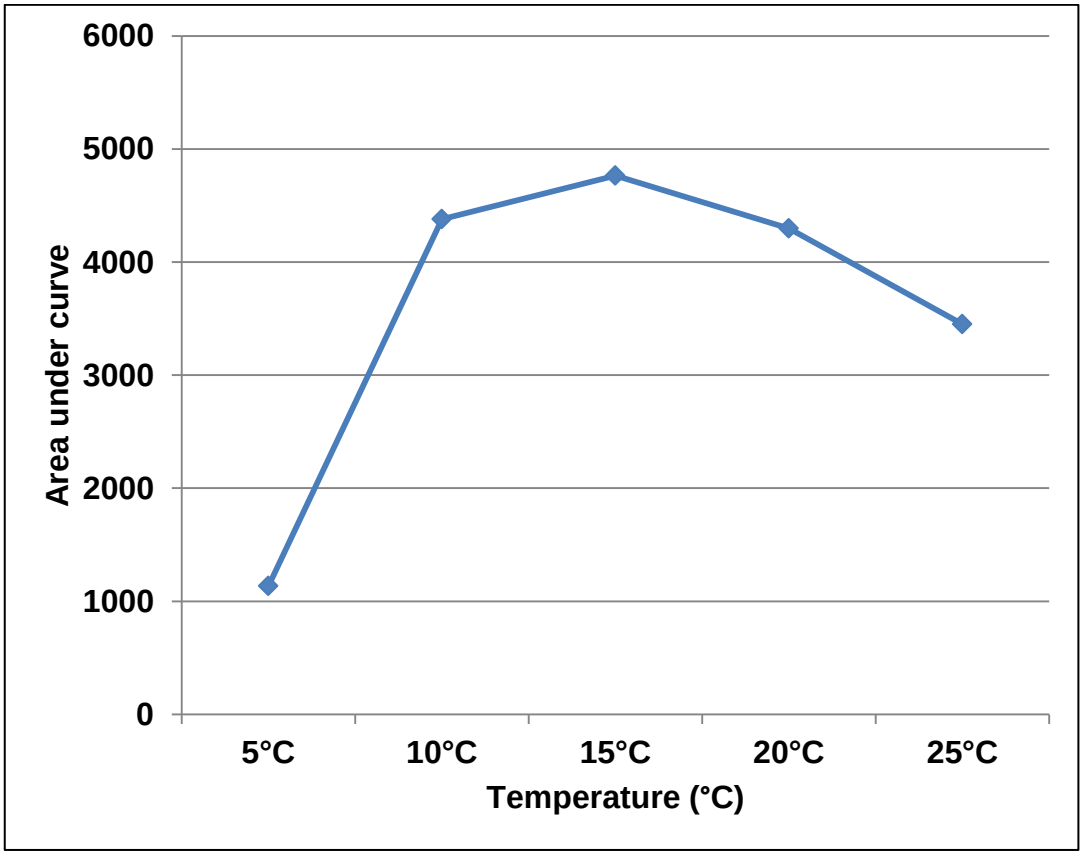

Figure 4.2 AUDPC curve of IJ survival at different temperatures after 84 days storage.

Pathogenicity tests with IJs stored at 10°C to 25°C showed superior virulence (> 90%) against *G. mellonella* on all three sampling dates as compared to 5°C, where mortality dropped to 10% on Day 84 (Figure 4.3).

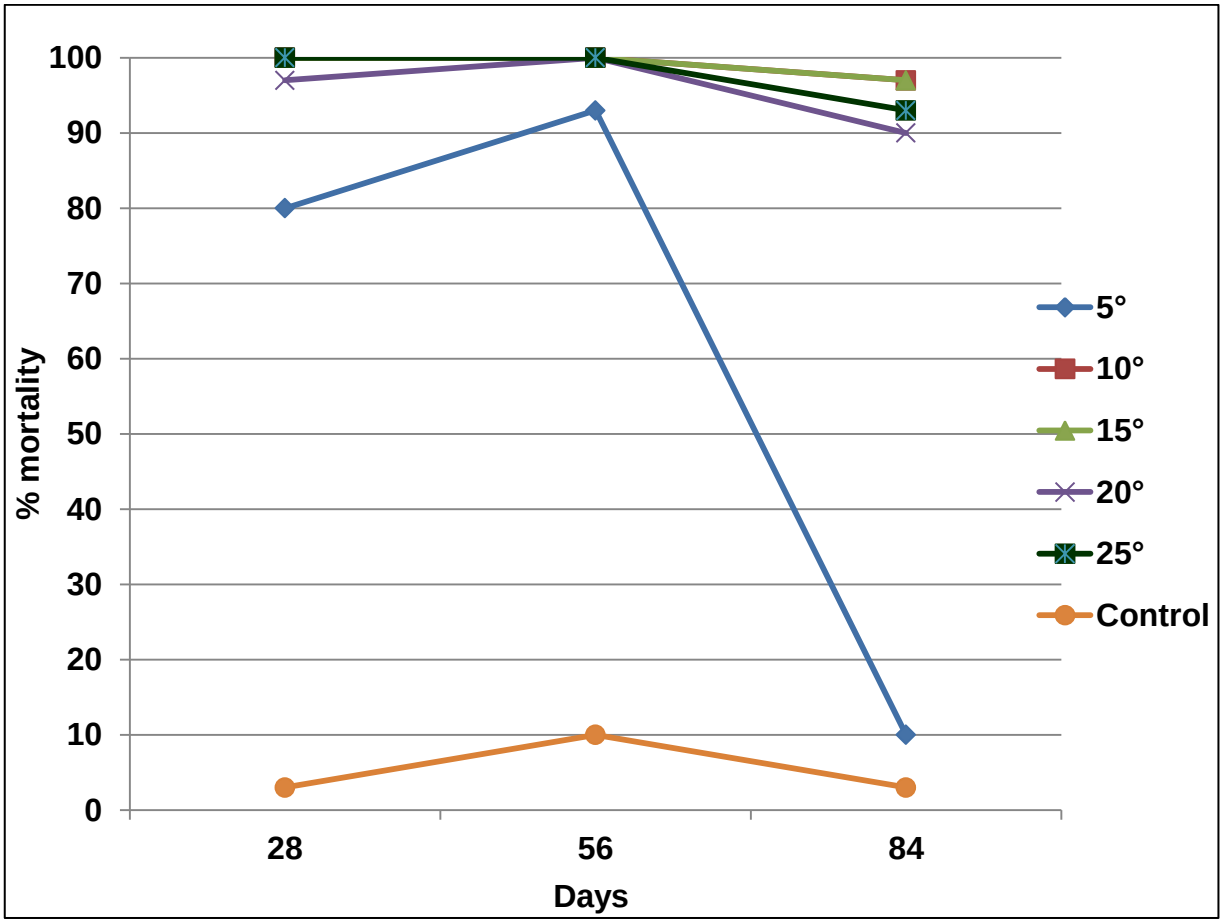

Figure 4.3 Infectivity of IJs against *G. mellonella* post storage at different temperatures over a period of 84 days.

4.3.2 Storage of nematode formulation at optimum temperature

Mean IJ survival in sponge formulation was 92% in the first replication, 90% in the second replication, with a mean survival of 90.5% at 15°C (Figure 4.4). Pathogenicity against *G. mellonella* was 100% in both experiments (Figure 4.5).

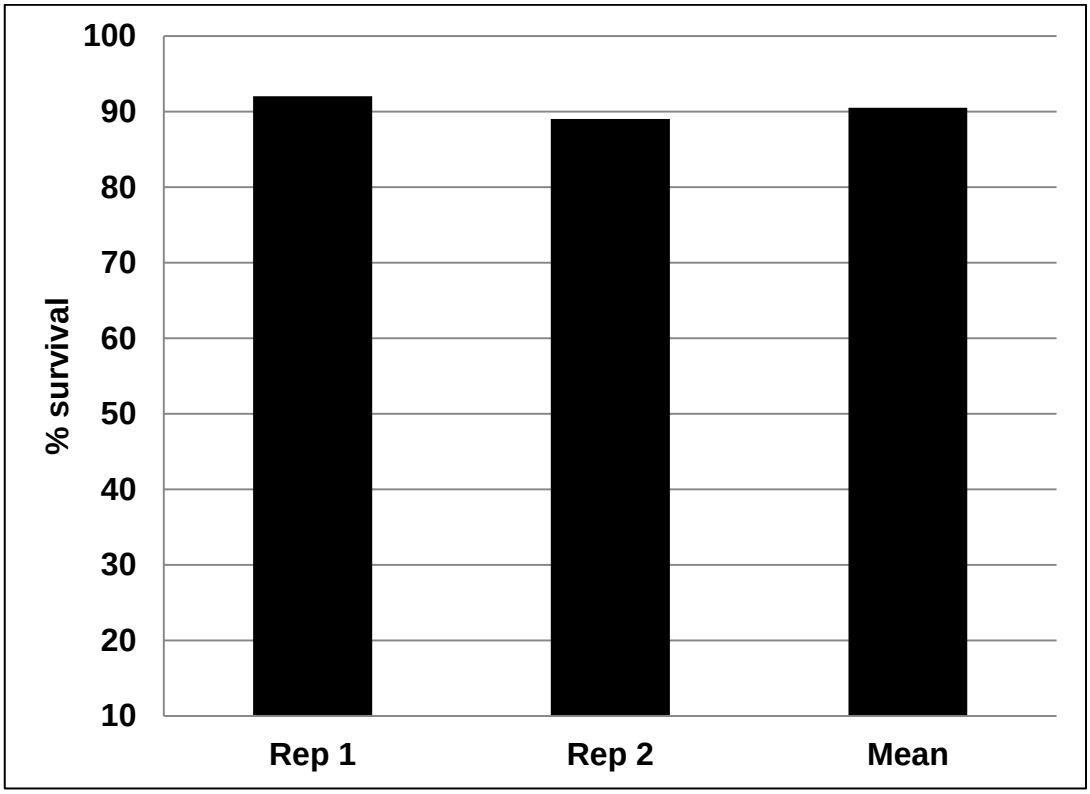

Figure 4.4 Percentage survival of sponge-formulated nematodes after 84 days storage at 15°C.

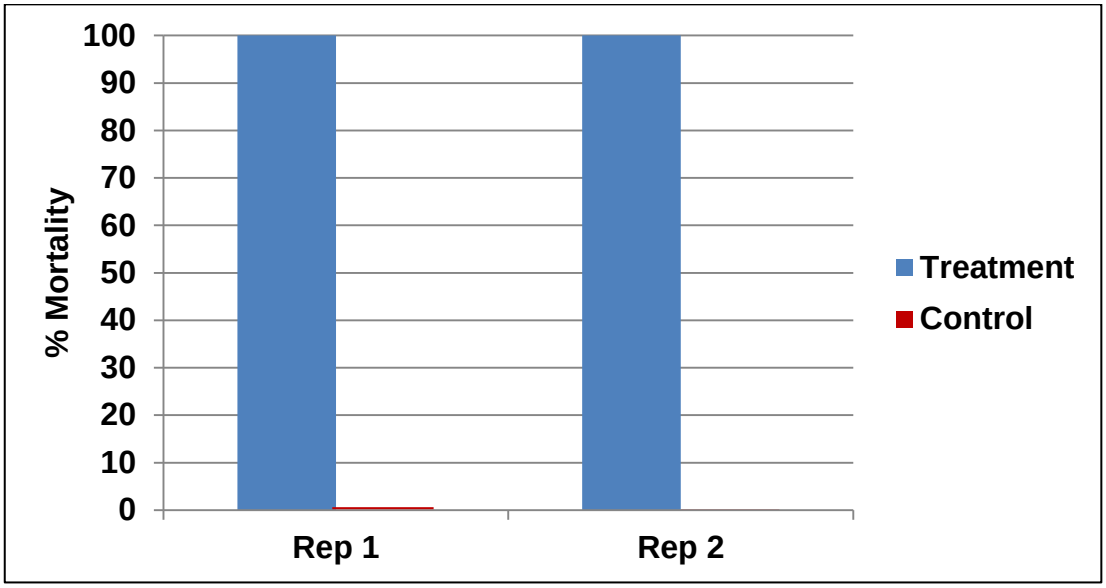

Figure 4.5 Infectivity of IJs against *G. mellonella* post storage at 15°C for a period of 84 days.

4.3.3 IJ survival and pathogenicity at 25°C following storage at 15°C for 84 days

Percentage survival of IJs averaged 90%, 83% and 88% for Replicates 1, 2 and 3, respectively (Figure 4.6). Mean IJ survival of the three replicates was 87 ± 4%. Virulence against *G. mellonella* was above 90% for all replicates (93%, 100% & 93%, respectively) and mean mortality of the three replicates was 95 ± 4% (Figure 4.7).

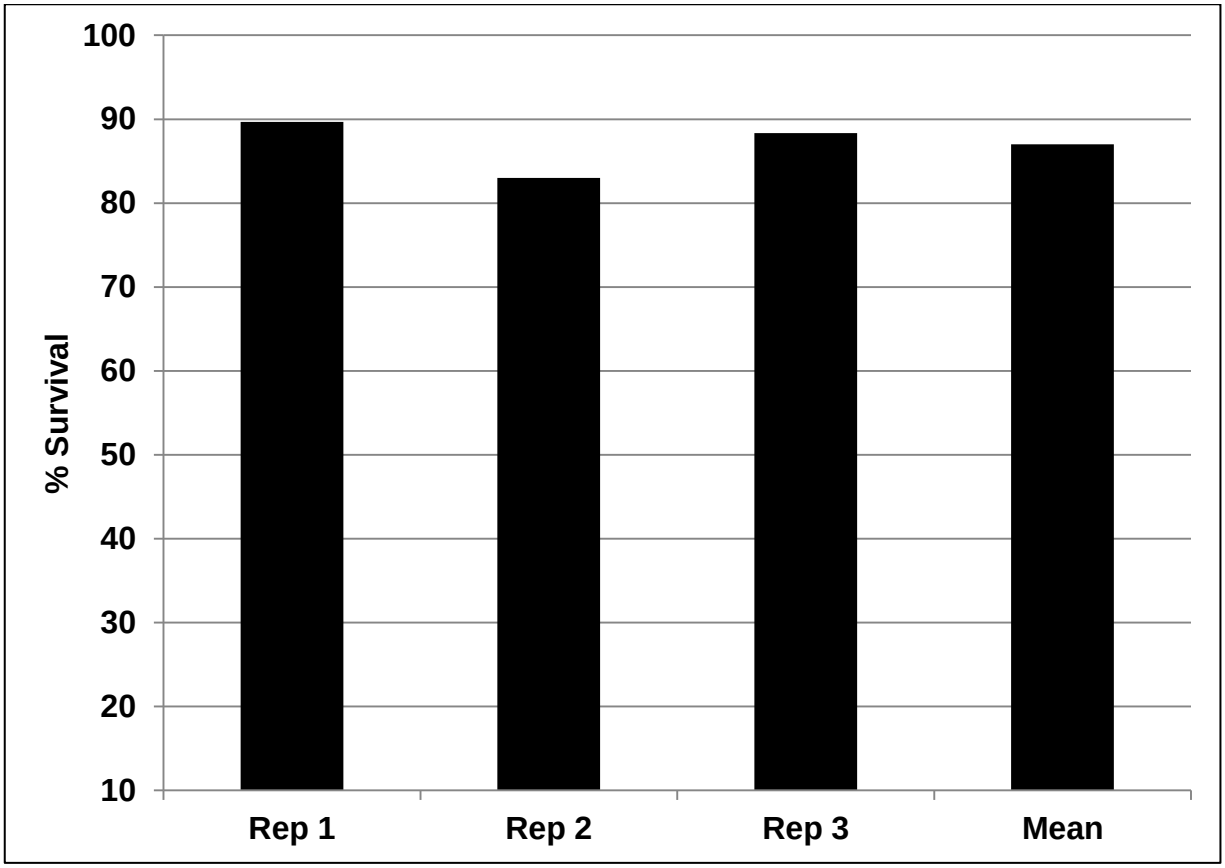

Figure 4.6 IJ survival after 14 days incubation at 25°C following storage at 15°C for 84 days.

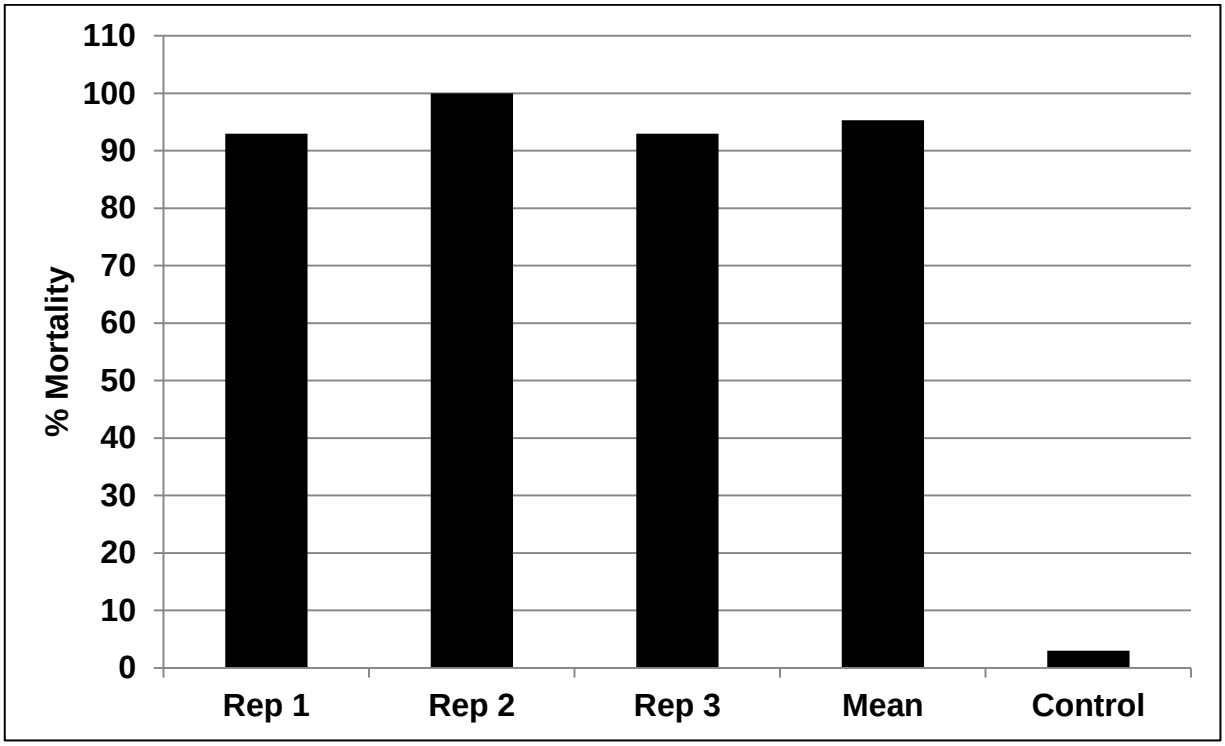

Figure 4.7 Percentage mortality of *G. mellonella* after two weeks incubation at 25°C following storage at 15°C for 84 days.

4.4 Discussion

Temperature had an important effect on IJ storage, survival and infectivity. Survival of *S. innovationi* was better at 10 and 15°C compared to all other temperatures and it was lowest at 5°C. In general, survival decreased with increases in the temperature and duration of storage. Boff (2001) also found that survival and performance was better when *Heterorhabditis megidis* (Poinar, Jackson & Klein, 1987) US strain (NLH-E87.3) was stored at 10 or 15°C than at 5 and 20°C. Low temperatures also reduce the growth of contaminants which compete for available oxygen in storage, thereby causing harmful environmental conditions for the IJs (Strauch *et al*., 2000). Similarly, Cagnolo and Campos (2008) noticed a reduction of IJs activity at 23 ± 1°C after 7 days storage, but they achieved a 95% survival at this temperature compared to 15% survival at 8°C after 12 weeks storage of *Steinernema rarum* (De Doucet, 1986).

However, EPN species differ distinctly in their optimum storage temperature (Andaló *et al.*, 2011; Goud *et al*., 2010; Köppenhofer *et al.,* 2013; Mejia-Torres & Saenz, 2013; Strauch *et al.*, 2000), including their tolerance of low temperatures.

The use of artificial sponges as carriers for IJ storage has been successful in many studies (Andaló *et al.*, 2010; Bedding, 1984; Ley & Mundo-Campos, 2004; Strauch *et al*., 2000). Using a physical carrier as compared to water suspension can reduce stress by simulating a natural environment, because it provides a large surface area for oxygenation through perforation (Andaló *et al.*, 2010). When IJs are stored in aqueous suspension, they sink to the bottom of the container and precipitate from the suspension. This leaves nematodes under a stressful environment of low oxygen supply under water, which may speed up the loss of energy reserves and subsequent low survival or short storage time (Gaugler, Brown, Shapiro-Ilan & Atwa, 2002). Consistent with these results are the findings of Andaló *et al.* (2010) who achieved 89.3 ± 1.3 and 57.5 ± 0.1% in sponge formulation (3000 IJs/ml^{-1}) after 90 and 180 days storage, respectively, at 16 ± 1°C for *Steinernema carpocapsae* All (Weiser, 1955). In their study, foam maintained a higher percentage of live IJs than other substrates tested, namely, fine sand, course sand, agar, soil, starch, expanded clay and phenolic foam. High percentage survival of IJs coupled with high virulence after two weeks at room temperature (following prolonged storage at 15°C) can be considered an advantage for ease of storage/transportation before application by the end user. Previous studies have shown that formulation of active nematodes require refrigeration during transportation and storage, which increases the cost of EPN products (Goud *et al*., 2010; Strauch *et al.*, 2000; Westerman, 1992).

Infectivity against *G. mellonella* remained high for the duration of the experiment except for IJs stored at 5°C, which declined to 10% after 8 weeks storage. Although literature suggest that survival and infectivity is conserved at lower temperatures due to the tendency of IJs to be less active (Grewal, 2002), 5°C did not support *S. innovationi* survival and infectivity. This, however, is probably due to a low percentage of live IJs (13%) rather than a loss of infectivity due to the depletion of

energy reserves. In contrast, Fan and Hominick (1991) recorded a higher percentage survival of *S. feltiae* at 5°C compared to 15°C, but infectivity of IJs stored at 5°C declined to 30% in the first two weeks followed by an unexpected increase to 70% in Week 3 and 4. Similarly, Koppenhöfer *et al.* (2013) showed increases in infectivity occurred when *Steinernema scarabaei* (Stock & Koppenhöfer, 2003) was stored at 8°C over 12 weeks. Low infectivity followed by an increase in infectivity instead of a gradual decrease thereof indicates that the cold temperatures had induced a state of dormancy, followed by switching back to an active 'risk-taking behaviour' when IJs face starvation (Fitters & Griffin, 2004; McNamara & Houston, 1991).

The optimum storage temperature for *S. innovationi* was shown to be 15°C. Other studies have suggested that factors other than temperature and storage time (such as IJs concentration & type of carrier) affect virulence and infectivity of IJs. Therefore, further investigation will be needed to determine how these factors affect *S. innovationi*.

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CHAPTER FIVE

Ecological characterization of the entomopathogenic nematode, *Steinernema innovationi*

Abstract

Entomopathogenic nematode (EPN) species perform differently under a range of environmental conditions and this is dependent on their biological characteristics. This study focused on infectivity traits (time-till-death; number of cadavers with nematodes recycling; and number of established infective juveniles [IJs] per larva) at a range of temperatures (10°C to 35°C) of a new indigenous EPN, *Steinernema innovationi*, isolated from the central region of South Africa. Its foraging behaviour was also studied using sand columns. Persistence of both *in vitro* and *in vivo* cultured IJs was assessed under field conditions. Time-till-death was shortest at 25°C and 30°C (mean 1 day). The highest number of established IJs was recovered at 25°C (mean = 27°C). The nematode infected *Galleria mellonella* larvae were found at all depths. The nematode was capable of covering a distance of up to 15cm in 24 hours. There was no statistical difference between *in vitro* (40%) and *in vivo* (27%) cultured IJ persistence 4 weeks post-application. Furthermore, there was no statistical difference in nematode recovery after 1 and 4 weeks post-application of IJs produced *in vitro* (33% and 40%, respectively) and *in vivo* (60% and 27%, respectively). Thermal activity was optimal at 25°C. The new species was classified as a cruiser EPN and it survived under field conditions for at least 4 weeks.

Keywords: *Steinernema*; infectivity; survival; persistence; infective juvenile; temperature, establishment

5.1 Introduction

A number of entomopathogenic nematode (EPN) species belonging to the families Steinernematidae and Heterorhabditidae have proven to be effective biocontrol agents. These nematodes have been isolated from a range of diverse environments around the globe (San-Blas, 2013). Many species are being isolated, screened and identified. Furthermore, indigenous new species may perform better in the environments that they are adapted to (Hazir, Kaya, Stock and Kestin, 2003). However, some studies demonstrate optimum conditions that are beyond those environmental conditions of the nematodes' origin. For example, a strain of

Steinernema anatoliense (Hazir, Stock and Keskin, 2003) has an optimum temperature of 25°C, which is higher than the temperatures typically encountered in its geographical area of isolation (Gungor, Kestin and & Hazir, 2006). Nevertheless, little information regarding species biocontrol performance are available to accompany descriptions of new species (Çimen, Lee, Hatting & Stock, 2014; Çimen, Lee, Hatting, Hazir & Stock, 2014; Gungor *et al.,* 2006; Koppenhöfer & Fuzy, 2003; Koppenhöfer & Kaya, 1999; Morton & Garcia-del-Pino, 2009). Species performance differ greatly according to their biology (e.g. foraging behavior), artificial manipulation (e.g. *in vitro* mass production and formulation) and the environment (temperature, moisture, soil particle size, UV & pH) (Barbercheck & Duncan, 2004). These intrinsic and extrinsic factors also affect their dispersal and persistence in the field. IJs need moisture or a thin water film for movement in the soil (Kung, Gaugler & Kaya, 1991). Nematode dispersal generally decreases as soil particles become smaller. Small soil particle size and high moisture levels also limit oxygen levels in the soil and therefore cause lower survival of EPNs (Barbercheck & Duncan, 2004).

Steinernema innovationi (Çimen, Lee, Hatting & Stock, 2014) is an indigenous new species isolated from the Free State province in South Africa (Hatting, Stock & Hazir, 2009). The ability of the nematode to infect hosts is crucial for its use as biocontrol agent. Hence, Koppenhöfer & Kaya (1999) requested that the taxonomic description of a new species should be coupled with its ecological characterization. This study focused on three parameters which affect nematode efficacy against insect pests: (i) temperature; (ii) the nematodes' foraging behavior; and (iii) the persistence of IJs under field conditions.

5.2 Materials and methods

5.2.1 IJ production

Nematode production was by *in vivo* and *in vitro* culture. For *in vivo* production, final instar wax moth larvae, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) were used according to the method described by Kaya and Stock (1997). IJs were stored in sterile water in 250ml flat angled tissue culture flasks (Lasec SA, Corning®) at 10°C and were used within two weeks after harvesting.

For *in vitro* production, a 5g sample of a larval puree of common house fly, *Musca domestica* (Linnaeus), + 0.15g canola oil was absorbed in 0.5g of 0.5cm^2 sponge cubes placed in a 100ml Erlenmeyer flask and autoclaved at 121°C for 15min. Each flask was inoculated with 0.5ml of bacterial suspension prepared by selecting a single colony from a NBT (nutrient agar, bromothymol blue, 2-3-5 triphenyltetrazoliumchloride) agar plate, and inoculating 30 ml Nutrient Broth (Biolab, www.merck.co.za), incubated at 27°C, with shaking at 100rpm for 24 to 48h. Flasks were incubated at 27°C for 72h before being inoculated with 0.5ml of a monoxenic nematode culture and further incubated at 22°C for 28 days. Nematodes were harvested by washing the sponges six times with 50ml distilled water. Individual sponges were squeezed five times during each wash. IJs were collected by sieving through a milk filter (www.denvet.co.za).

5.2.2 Effect of temperature on infectivity

The effects of temperature on infectivity was assessed using a Sandwell bioassay system by filling wells of a 24-well tissue culture (TC) plate with 0.5g sterile, air dried loam sand, as per Ricci, Glazer, Campbell & and Gaugler (1996). The plates with sand were incubated for at least 1 hour at 10, 15, 20, 25, 30 and 35°C to acclimatize. Nematode suspensions and sterile water for the control were also incubated at respective temperatures for 1 hour prior to inoculations. After acclimatization, 30 wells per temperature were inoculated by pipetting 50 IJs (produced in vivo) in 60µl of distilled water into each well (Gungor *et al.,* 2006). The control treatment was inoculated with 60µl of sterile distilled water only. One final instar *G. mellonella* larva, of approximately 250 to 300mg (weight difference ≤ 50mg), was added per well. A total of 30 larvae were used per treatment, plus the control treatment. Each culture plate was placed inside a 330×215mm Zip Seal bag (GLAD®, South Africa) to conserve moisture, and was incubated at its appropriate temperature in total darkness. Plates were monitored for larval mortality every 24h for 10 days. Cadavers were washed three times with distilled water to remove IJs from the cuticle, and were then incubated for a further 24h at 25°C followed by enzymatic digestion in pepsin solution (Kaya & Stock, 1997). Parameters measured were: time-till-death;

percentage of larvae with nematode recycling; and number of established IJs per larva.

5.2.3 Foraging behavior

Plastic columns with a depth of 10cm and 5cm diameter were used to assess IJ movement in the sand profile and infectivity of wax moth larvae at different depths (1, 5 and 9cm). The column was filled with moistened (10% moisture) sand in which one immobilized wax moth larva (inside a gauze-type sachet) was positioned at 1cm, 5cm or 9cm in separate columns for each depth. A total of 12 columns were used per treatment and control. Columns were inoculated with 100 IJs in 100µl sterile distilled water dispensed onto the surface of the column. Control columns were treated with sterile distilled water only. All columns were covered with parafilm and incubated at 25°C. After 72h, columns were dissembled and cadavers were washed three times with distilled water to remove IJs from the cuticle followed by enzymatic digestion in pepsin solution (see procedure below) to establish the presence of IJs. Nematode IJs were counted using a hand-held counter under a dissection microscope at 40x magnification.

5.2.4 Distance travelled

This experiment was performed according to the above procedure with few modifications. A total of 125 columns were used, five columns per treatment (depth of 3, 6, 9, 12 and 15cm), for examination every 24h.

5.2.5 Persistence

The experimental layout was 10 plots of 0.5m^2 positioned three meters apart in a linear arrangement. Control plots were positioned 3m from the treatment plots in an outwards direction. Both control and treatment plots were sampled prior to inoculation to check for the natural presence of EPNs. Five treatment plots (replications) were used to inoculate *in vitro* and *in vivo* cultured IJs, respectively. A concentration of 62,500 IJs (P. M. Sharma, A. N. Sharma and Hussaini, 2011) was applied in 2L water over the surface of each plot, excavated to a depth of 5cm. Application was by means of a hand-held trigger sprayer (Fragram trigger sprayer, www.takealot.com/pool-garden/), with the spray nozzle removed, followed by immediate covering with soil.

Post-inoculation sampling was performed by baiting all plots with three caged *G. mellonella* larvae of approximately 250 to 300mg (weight difference ≤ 50mg) per plot, buried at a depth of 5 to 7cm. Larvae were removed on Day 4 and were then washed three times with distilled water to remove IJs from the cuticle, followed by enzymatic digestion in pepsin solution (procedure below).

A representative soil sample was collected from each plot using an auger for soil baiting. Three larvae of 250 to 300mg (weight difference ≤ 50mg) were introduced into each sample and incubated at 25°C, total darkness. On Day 4, cadavers were removed and treated as described for field baiting before enzymatic digestion. Samples were taken weekly over a period of 4 weeks, including an initial sample 1 hour after the nematodes were applied.

5.2.6 Abiotic factors

Soil analyses for each replicate block are listed in Table 6.1. Soil temperature was logged using iButton® data loggers (Coldchain-Thermo Dynamics, www.fairbridgetech.com) buried 5cm under the soil. Rainfall data were retrieved from the Agricultural Research Council-Institute for Soil Climate and Water (ARC-ISCW) weather database. See http://155.240.219.9/agromet/Login_Screen.php?btn_Login=CONTINUE.

Table 5.1 Soil characteristics of the five experimental blocks (A-E).

Block	pH (KCl)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Ca/Mg	(Ca+Mg)/K	CEC $cmol_c kg^{-1}$	Clay %	Density ($g.cm^{-3}$)	S (mg/kg)
A	**5.1**	**31.2**	**124.5 (13)**	**325 (65)**	**65.4 (22)**	**2.3 (0)**	**3.03**	**6.80**	**2.49**	**9**	**1.19**	**6.2**
B	**5.9**	**27.7**	**121.6 (9)**	**491 (72)**	**79.2 (19)**	**3.2 (0)**	**3.79**	**9.99**	**3.43**	**8**	**1.23**	**0.3**
C	**6.0**	**22.7**	**104.7 (6)**	**625 (70)**	**120.1 (22)**	**20.1 (2)**	**3.17**	**15.35**	**4.46**	**10**	**1.08**	**8.4**
D	**5.2**	**20.3**	**112.5 (8)**	**479 (66)**	**112.9 (26)**	**2.7 (0)**	**2.59**	**11.55**	**3.62**	**9**	**1.14**	**7.4**
E	**6.5**	**21.7**	**95.4 (4)**	**756 (63)**	**204.2 (28)**	**61.1 (4)**	**2.26**	**22.36**	**5.96**	**10**	**1.01**	**33.1**

5.2.7 Enzymatic digestion

Nine millilitres of pepsin solution (Kaya & Stock, 1997) were added to a 6.5cm diameter plastic Petri dish, and one cadavers were introduced into each dish. The head of the cadaver was excised and the gut contents were gently pushed out with a forceps. The gut contents were gently teased apart by sucking with a pipette and releasing the content several times to facilitate enzymatic digestion. The Petri dishes were incubated in a shaking incubator at 37°C and 50rpm for 45 min. Contents were transferred to a larger (9cm diameter) glass Petri dish with 1×1cm grids, and diluted with distilled water to render the contents less turbid. The nematodes were counted with a hand-held counter under a stereo microscope at 40x magnification.

5.2.8 Statistical analysis

The effects of different temperatures. The mean number of *G. mellonella* larvae with nematode recycling was subjected to 2x2 Chi-square (χ^2) analysis (Snedecor & Cochran, 1980) using Microsoft Excel, 2013, software. For time-until-death and establishment, means were compared using Fisher's unprotected t-Test at the 5 % level of significance (Snedecor & Cochran, 1980). Regression analysis of time-until-death, and the number of established IJs were performed with TableCurve (SYSTAT Software Inc., 2002). All data were analyzed using SAS statistical software (SAS, 1999).

Foraging behavior. The number of established IJs per depth was analyzed by one way analysis of variance (ANOVA) and the mean counts were separated using Fishers' unprotected t-test at the 5 % level of significance (Snedecor & Cochran, 1980).

Persistence. The number of cadavers with positive nematode recycling was subjected to 2x2 Chi-square (χ^2) analysis (Snedecor & Cochran, 1980) using Microsoft Excel 2013 software to test the dependence ($P<0.05$) of the different treatments (*in vitro* and *in vivo* production) versus number of cadavers with nematode recycling.

5.3 Results

5.3.1 Effect of temperature on infectivity

There were significant differences in the performance of the IJs at temperatures that varied from 10°C to 35°C ($P < 0.05$; df = 1), with highest infectivity measured at 15 to 30°C. Significantly lower infectivity was measured at 10°C (37%) and 35°C (20%) (Figure 6.1).

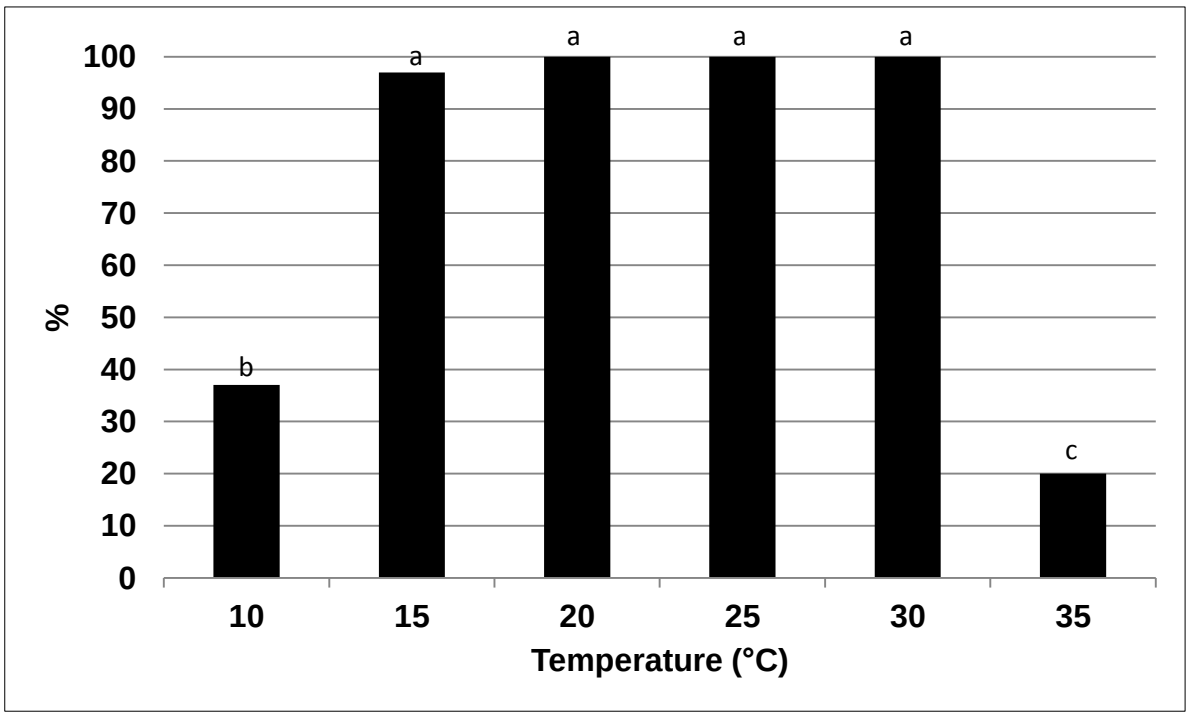


Figure 5.1. Levels of nematode recycling in *G. mellonella* larvae (%) after 10 days incubation at different temperatures. Bars with the same lowercase letter indicate no significant difference (P > 0.05; χ^2 test).

Time-until-death was shortest at 25 and 30°C (P<0.05), averaging one day followed by 20°C, 35°C, 15°C and 10°C after 2, 4, 6. 9 and 8 days, respectively (Figure 6.2).

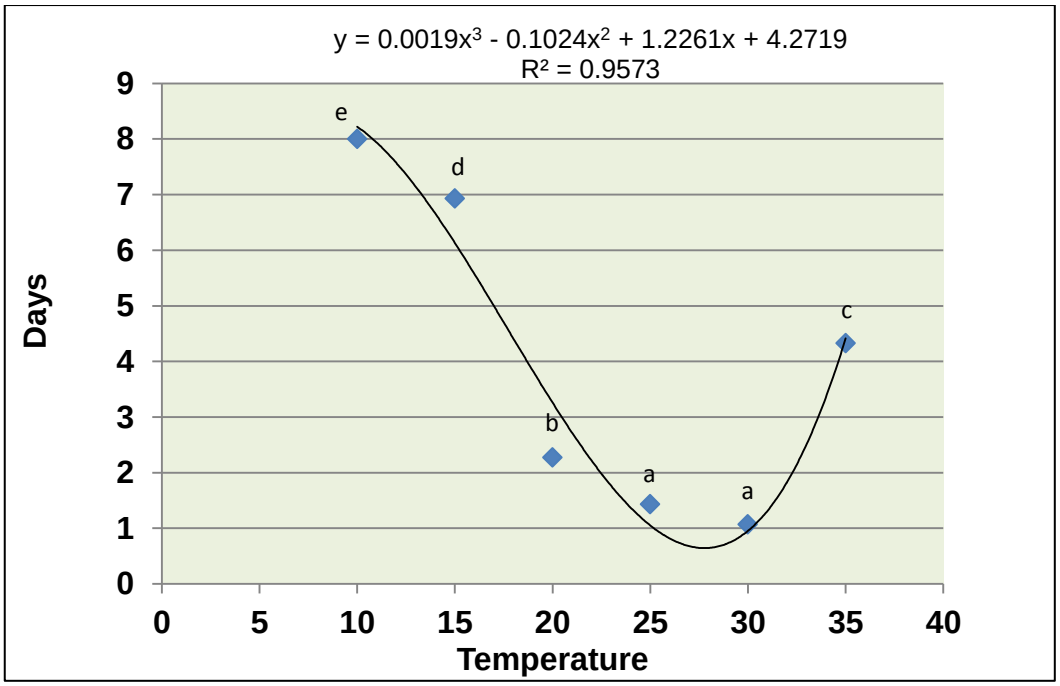


Figure 5.2 Regression analysis of time-until-death vs. temperature performed with TableCurve. Bars with the same lowercase letter indicate no significant difference (P > 0.05; Fisher's unprotected t-Test)

Maximum mean number of established IJs was recorded at 25°C (27.7) followed by 20°C and 30°C (20.3 & 19.2, respectively), then 15°C (9.5) and least establishment at 10°C and 35°C (2.9 & 2.3, respectively) (Figure 6.3).

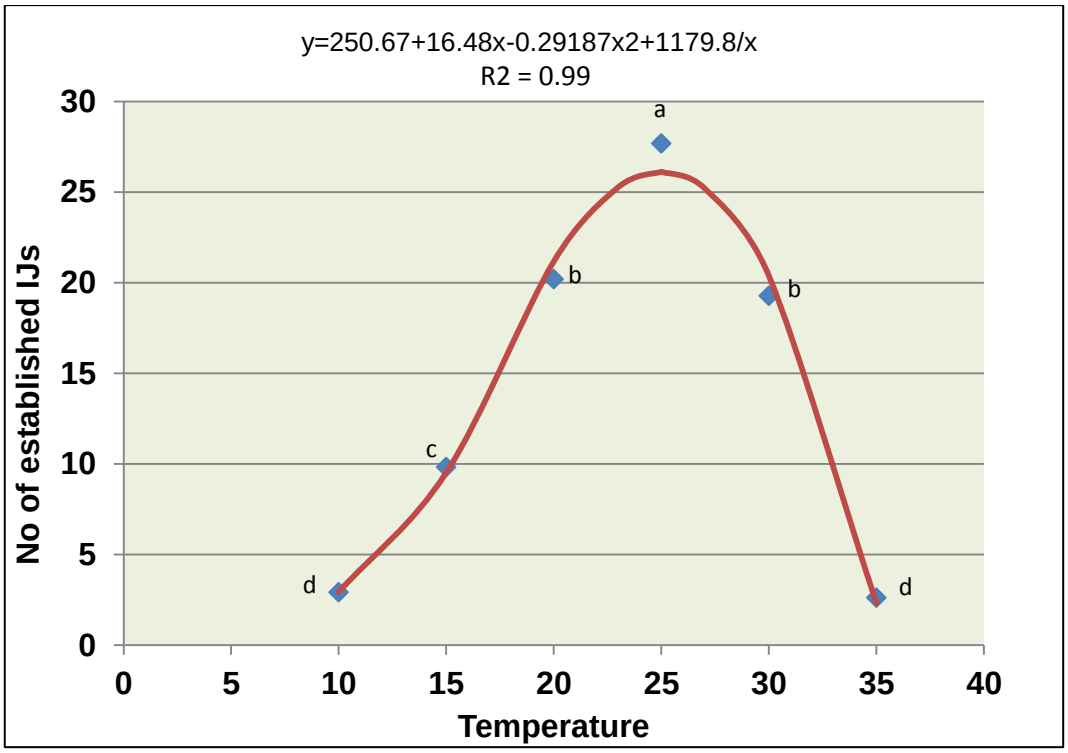


Figure 5.3 Regression analysis of number of established IJs vs. temperature performed with TableCurve. Bars with the same lowercase letter indicate no significant difference (P > 0.05; Fisher's unprotected t-Test).

5.3.2 Foraging behavior

Overall, IJs of *S. innovationi* were found to have infected *G. mellonella* at all the tested depths (Figure 6.4 & 6.5). Non-significant differences (P > 0.05) were observed at 1°C and 5°C in the first experiment (Figure 6.4). In Experiment 2 (Figure 6.5) IJs were able to infect *G. mellonella* at depths up to 15cm within a period of 24h. In this experiment, the number of established IJs was comparable at depths of 9, 12 and 15cm, irrespective of time.

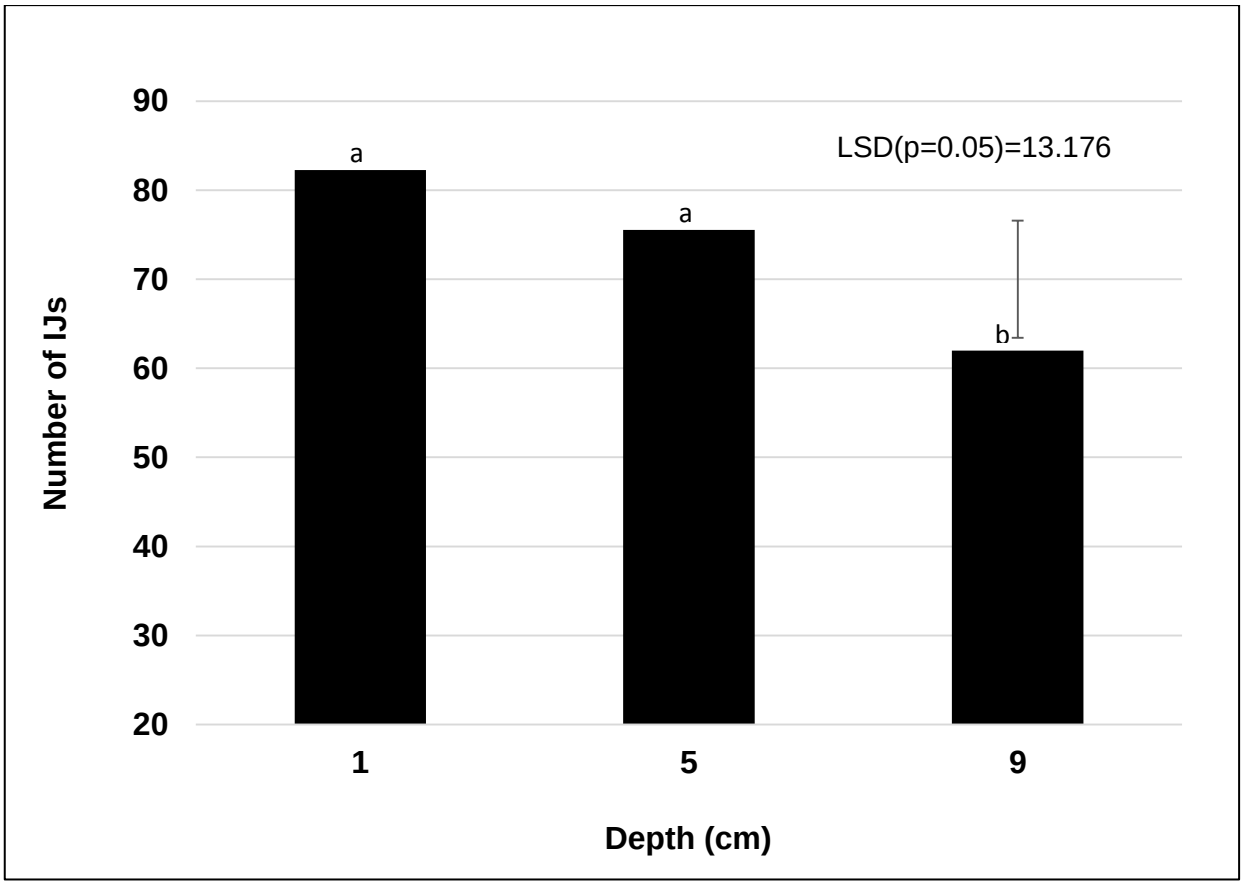


Figure 5.4 Number of established IJs of *S. innovation* at different depths after 72h incubation. Bars with the same lowercase letter indicate no significant difference (P > 0.05; Fisher's unprotected t-Test)

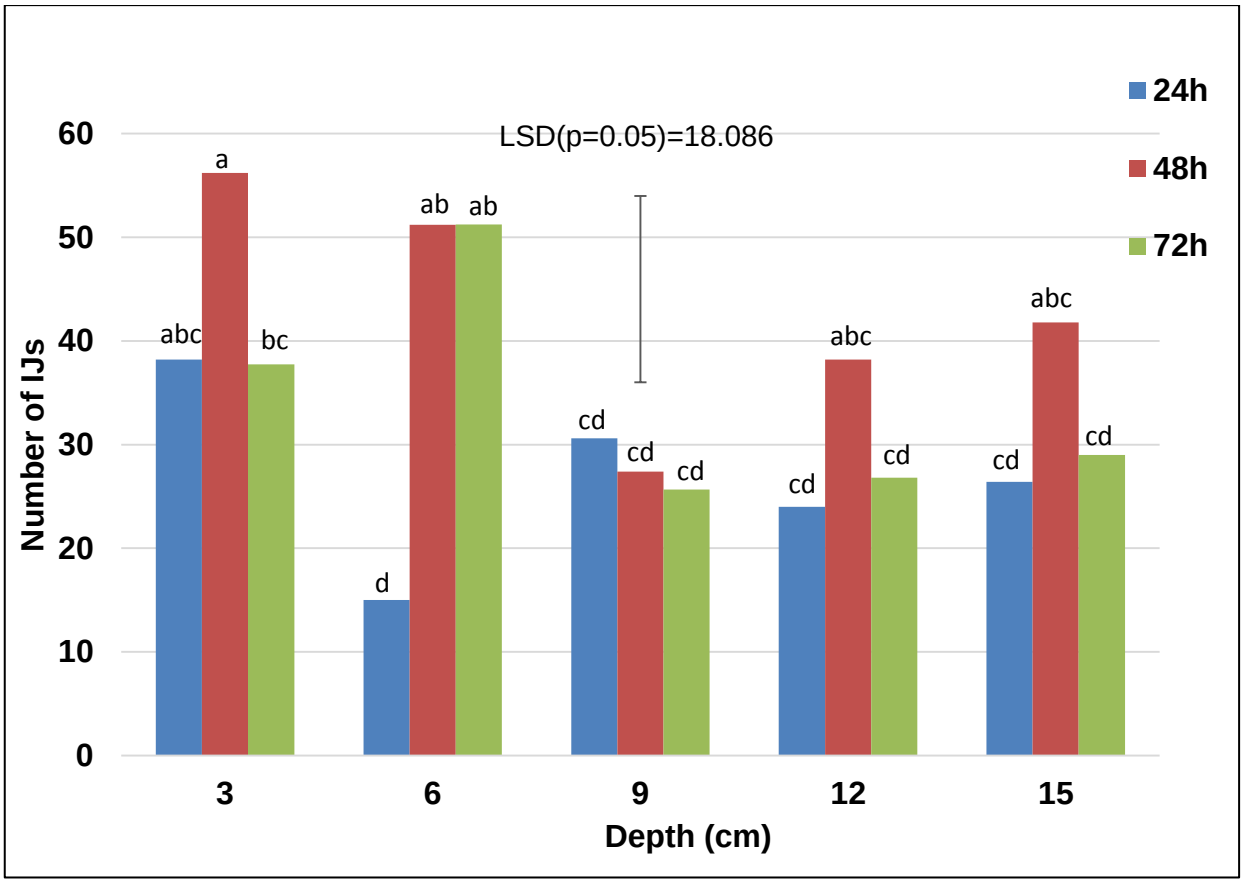
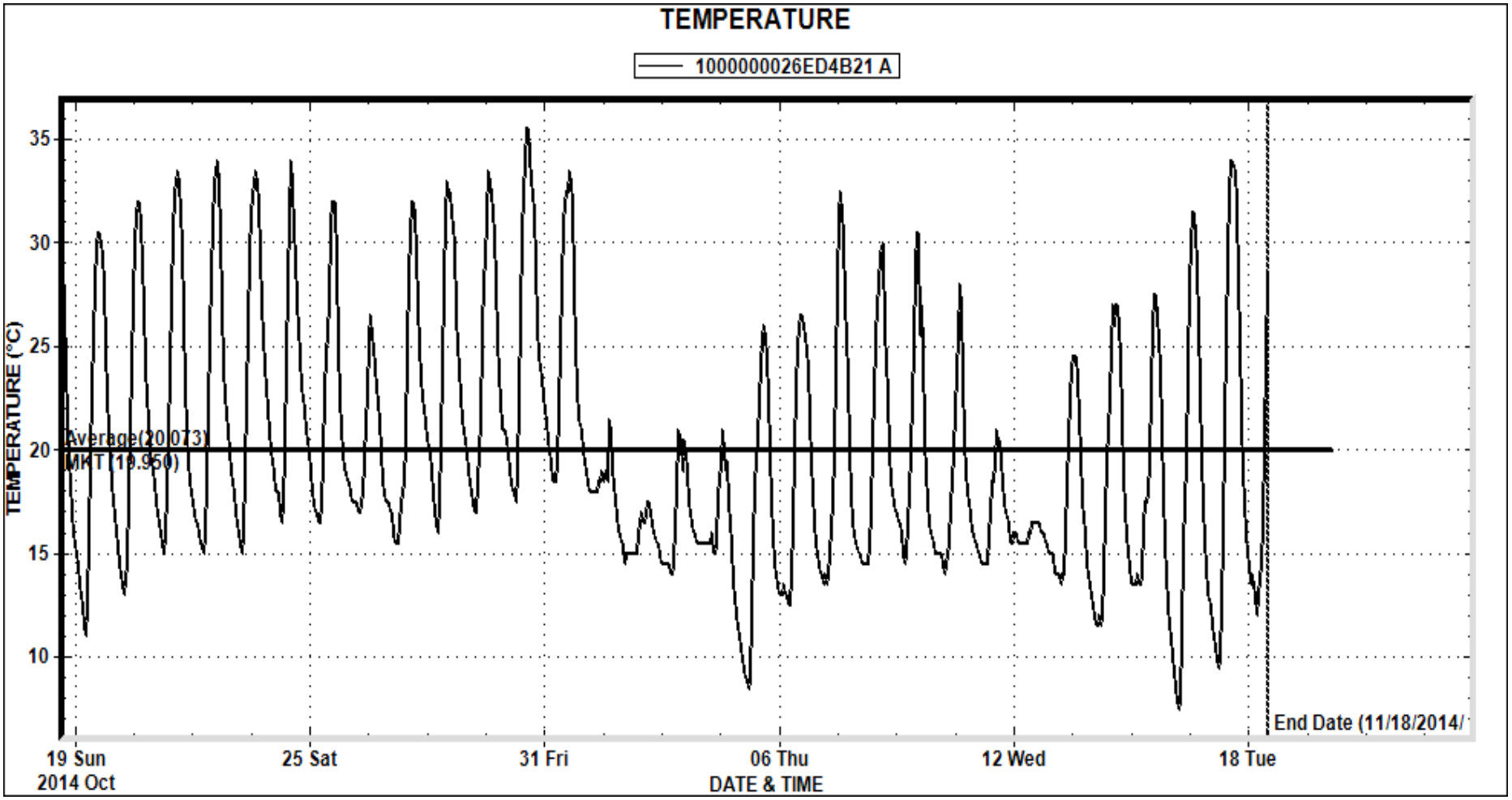


Figure 5.5 Number of established IJs of *S. innovation* at different depths over 72h incubation. Bars with the same lowercase letter indicate no significant difference (P > 0.05; Fisher's unprotected t-Test)

5.3.3 Persistence

Pre-application (natural) prevalence was negative except for one plot which was positive with a *Heterorhabditis* sp. This plot was excluded from the experiment.

There was no significant difference (χ^2 probability = 0.439, df = 1) between *in vivo* and *in vitro* nematode recovery from field plots in Week 4. However, percentage recovery was notably higher (40 % vs. 27%; Figure 6.7) for *in vitro* IJs during Week 4. Furthermore, there was no difference in nematode recovery after 1 and 4 weeks post-application of *in vitro* IJs (33% & 40%, respectively) (χ^2 probability = 1.000, df = 1) and *in vivo* IJs (60% & 27%, respectively) (χ^2 probability = 0.142, df = 1) (Figure 6.7). The level of nematode recovery was lower in Week 2, but recovered after the trial site received 52mm of rain (Figure 6.6).



Rain: 01/11/14 = 52mm; 09/11/2014 = 11mm; 12/11/14 = 29mm

Figure 5.6 Soil temperature and rain during the course of the experiment.

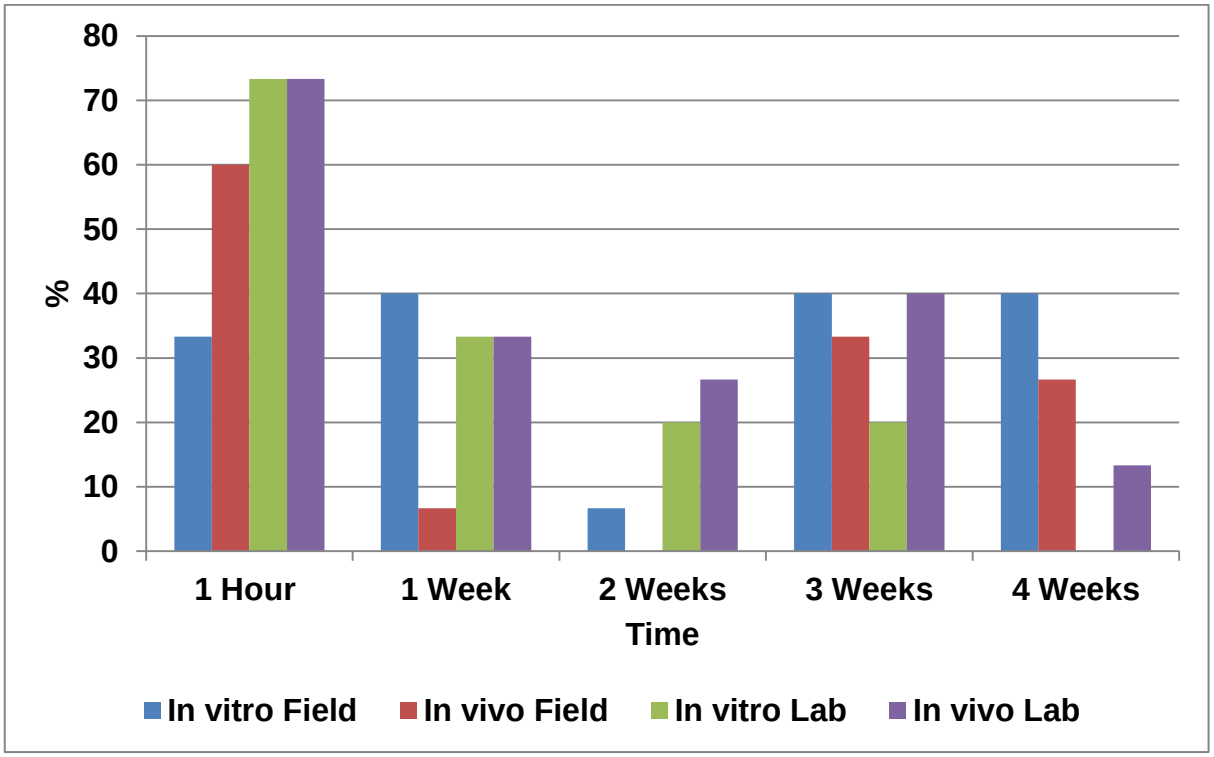


Figure 5.7 Level of *G. mellonella* cadavers (%) with nematode recycling following field and laboratory soil trapping over 4 weeks.

5.4 Discussion

This study documents intrinsic and extrinsic factors that affect performance of *S. innovationi,* which is crucial knowledge for its application as a biopesticide. The temperature experiments showed that this species had an optimum temperature of 25°C. It is important to determine the effects of temperature on their behaviour because EPN species or even strains have been observed to have diverse temperature optima (Grewal, Selvan & Gaugler, 1994; Gungor *et al*., 2006). The activity of IJs of most EPNs is arrested at temperatures below 8°C, and IJs are killed by temperatures above 38°C, depending on the species (Salma & Shahina, 2012). The optimum temperature of most EPN species fall between 20°C to 25°C (Campos-Herrera *et al.*, 2008; Gungor *et al.,* 2006; Koppenhöfer & Fuzy, 2003; Mejia-Torres &

Sáen, 2013; Molyneux, 1985), which correspond with the optimum temperature of this new species. It is believed that EPN isolates or strains are adapted to the environment from which they were isolated (Kung *et al.,* 1991). *S. innovationi* was isolated from the eastern Free State region of central South Africa, with a climate defined as humid subtropical with summer rainfall, and is relatively cool (warmest month < 22°C) (Hatting *et al.* 2009). However, its optimum temperature is higher than the mean temperature of the region from where it was originally isolated. Similarly, *S. anatoliense* has an optimum temperature outside the mean temperature of its region of origin (Gungor *et al.,* 2006). The current study provides another example which contradicts the assumption that EPNs are optimally adapted to the climate of their original environment. However, the higher optimum temperature may have been caused by short term preservation by passaging through an insect at 25°C for several years (Morton and Garcia-del-Pino, 2009; Wang and Grewal, 2002). Or it could be adaptation to warm rearing temperatures. Jagdale and Gordon (1998) demonstrated that the repeated use of a fixed rearing temperature led to the adaptation of a strain to that temperature and improved its performance at temperatures close to the rearing temperature. These authors also showed that infectivity at low temperatures was enhanced by propagating nematodes at such temperatures, whereas infectivity at warm temperatures was enhanced by rearing under warm conditions. Similarly in the current study, mortality, time-till-death and establishment was low at 10°C and was significantly higher at 25°C (the nematode rearing temperature). Number of established IJs also decreased significantly when shifting away from this temperature.

The foraging behaviour of cruiser types is to actively search for hosts, whereas ambushers sit and wait for an insect to pass within close range (Shapiro-Ilan *et al.,* 2009). The intermediate fall between these two categories. Furthermore a recent study of *Steinernema carpocapsae* (known as an ambusher) suggest that this EPN either ambush or cruise depending on the soil type (Wilson, Ehlers and Glazer, 2012). IJs of *S. innovationi* demonstrated their capacity to locate and infect larvae of

G. mellonella at different depths. This nematode species belong to the '*glaseri* group' (Çimen *et al.,* 2014), which is classified as Clade V according to the phylogenetic analysis of the internal transcribed spacer (ITS) and the D2D3 region of the 28rRNA gene (Spiridonov Reid, Podrucka, Subbotin & Moens, 2004). EPNs in this category are classified as cruiser-type (Shapiro-Ilan *et al.,* 2009), which may suggest that S. innovation is a cruiser. Mortality of the target insect larvae within 24h at all depths was encouraged by the relatively large particles of the loam sand used (bulk density = 1.42g.cm^{-3}). Jabour and Barbercheck (2008) observed a rate of movement of 15.4cm.day^{-1} and 33.3cm.day^{-1} for *S. carpocapsae* in soils with bulk densities of 1.4 and 1.5 g.cm^{-3}, respectively. Nematode dispersal is proportional to soil particle size, and IJ movement is slow in soils with a high clay content or small particle sizes (Barbercheck & Duncan, 2004). Cruiser-type EPNs also demonstrate positive attraction towards volatiles emitted by their hosts, in order to locate them (Bilgrami, Kondo & Yoshiga, 2001). However, Morton and Garcia-del-Pino (2009) observed movement of IJs in a sand column to a depth of 20cm without the presence of a host. The nematode *S. innovationi* managed to search for, infect and kill its host within 24h at all depths up to 15cm. Similarly, Bilgrami *et al.* (2001) demonstrated targeted directional movement by *Steinernema glaseri* (Steiner, 1929) towards larvae of *G. mellonella* within 12 hours of incubation. Rapid migration is important for the dispersal of IJs in the field following application.The persistence of EPNs under field conditions is the sum of several parameters including: survival, dispersal, host finding, and reproduction, which are influenced by biotic and abiotic factors (Susurluk & Ehlers, 2008b). The IJ is the only infective, non-feeding, stage in the life cycle of an EPN (Nguyen & Smart Jr, 1992) and the superior persistence of IJs may improve the pathogenicity of an EPN biocontrol agent due to a longer exposure time to detect and infect the host. Prolonged survival may also mitigate the need for subsequent reapplication. There was no significant difference in the survival of IJs as a result of the two production methods. However, mean number of cadavers with nematodes recycling was slightly higher for *in vitro* produced IJs. *In vitro* solid culture is said to produce nematodes of good quality equivalent to those from *in vivo* culture (Shapiro-

Ilan, Han & Dolinski, 2012). In the current study, the high quality of IJs produced *in vitro* could be explained by the addition of 3% oil. Fat increases lipid reserves in the IJs, which are crucial for their survival. Vieux and Malan (2013) also observed no significant difference in infectivity of IJs of *Heterorhabditis bacteriophora* (Poinar, 1976) produced *in vivo* versus *in vitro*. Likewise, Susurluk, Kongu and Ulu (2013) did not observe differences when they tested the virulence of six strains of *H. bacteriophora,* except for one strain. Moreover, they found that reproductive ability of *in vitro* produced IJs was higher or equivalent to those from *in vivo* production. In contrast, Susurluk and Ehlers (2008a) and Ferreira, Addison and Malan (2014) found that *in vivo* produced IJs performed better than *in vitro* produced IJs.

To mitigate the effect of temperature and other abiotic factors, a duplicate set of soil samples was processed in the laboratory at the optimum temperature (25°C) as backup in case the field temperatures were too extreme for nematode infectivity. However, the mean temperature for the duration of the experiment was 20°C, with a minimum of 7.7°C at night and a maximum of 35°C during the day. These temperatures should not have had adverse effects since IJs were exposed to temperatures between 15 and 30°C for the majority of the time. The low night temperature of 7.5°C would have arrested nematode activity until the temperature rose the next day, without killing the IJs (Campos-Herrera *et al*., 2008). While temperatures between 15°C and 25°C are optimal for infectivity, they increase metabolism and fat reserve depletion, which may lead to low persistence (Hazir, Stock, Kaya, Koppenhöfer & Kestin, 2001). Nevertheless, there was no significant difference in nematode recovery between Week 1 and Week 4. The effect of soil type on EPNs can vary amongst species or even strains (Shapiro, McCoy, Fares, Obreza & Dou, 2000). There is a negative correlation between pH and nematode survival: survival and infectivity is greater at a non-acidic pH (Barbercheck & Duncan, 2004). Soil pH in the current study ranged from 5.1 to 6.5, which was not too acidic to affect the survival and infectivity of the IJs. Soil moisture is another important parameter for survival and infectivity of EPNs. The significant difference in survival at Week 2 could

have been caused by dry conditions. An increase in nematode recovery was noted in Week 3 after the trial site received 52mm and 11mm rain during Week 2 and 3, respectively.

This study demonstrated that the optimum temperature for the infectivity of *S. innovationi* was 25°C. Moreover, levels of infectivity and survival, as a measure of persistence under field conditions, was comparable for *in vitro* and *in vivo* produced IJs. This means that the *in vitro* production method described in this study did not detrimentally affect the biology of the nematode. Persistence of IJs in the soil for a period of 4 weeks is sufficient for a commercial biocontrol agent, to target insects entering the soil over a period of time (e.g., pupating larvae).

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CHAPTER SIX

Virulence of *in vitro* solid state mass produced *Steinernema innovationi* against members of Coleoptera, Lepidoptera and Orthoptera under laboratory conditions

Abstract

Entomopathogenic nematodes are mainly used to control soil dwelling insect pests or insects that spend part of their life cycle in the soil. Host susceptibility also differs amongst insects in different orders. Larvae and/or pupae of sugarcane stalk borer, *Eldana saccharina* (Walker); African pink stalk borer*, Sesamia calamistis* (Hampson); spotted stalk borer, *Chilo partellus* (Swinhoe); mealworm*, Tenebrio molitor* (Linnaeus); wax moth, *Galleria mellonella* (Linnaeus); codling moth, *Cydia pomonella* (Linnaeus); diamondback moth, *Plutella xylostella* (Linnaeus), and the house cricket, *Gryllidae acheta* (Linnaeus) representing three orders (Coleoptera, Lepidoptera & Orthoptera) were exposed to a low and high concentrations of 50 and 500 IJs, respectively, to determine their susceptibility to *Steinernema innovationi*. The hosts *G. acheta, C. partellus* and *P. xylostella* showed least susceptibility with maximum mortalities at 500 IJs concentration of 28%, 45% and 92%, respectively. All other hosts suffered 100% mortality. Pupal mortality ranged from 47% to 68%. An LC_{50} and LC_{70} of 3 and 31 IJs/larva, respectively, was calculated for the black cutworm, *Agrotis ipsilon* (Hufnagel). These results provide a broad guideline on the relative virulence of this new species against different hosts.

Keywords: Virulence; entomopathogenic nematodes; infective juvenile; mortality; *in vitro*

6.1 Introduction

Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are exploited globally for biological control of insect pests (San-Blas, 2013). These nematodes offer several advantages over traditionally used chemical pesticides. These include: there is no development of resistance (Divya & Sankar, 2009); they pose no threat to humans, non-target organisms and the environment (Lacey & Georgis, 2012); EPNs can kill their insect host within 48 hours (Shapiro-Ilan, Han, & Dolinski, 2012); no withholding periods; exemption from registration requirements in most countries (Divya, Sankar, Marulasiddesha,

Sambashiv & Krupanidhi, 2011; Ehlers, 2006.); and they can be mass produced easily (Shapiro-Ilan *et al.,* 2012). Although EPNs attack over 200 insect hosts (Hazir, Stock, Kaya, & Kestin, 2003), they are more successful for control of soil dwelling insect pests, or insects that spend part of their life cycle in the soil (Shapiro-Ilan, Gouge & Koppenhöfer, 2002). Host susceptibility differs amongst species, with some EPN species only adapted to particular hosts. For example, *Steinernema scapterisci* (Nguyen & Smart, 1990) is a specialist against Orthoptera with insects in the orders Coleoptera, Lepidoptera and Hymenoptera being poor hosts or non-hosts (Nguyen & Smart, 1992.). This could be attributed to immune responses by the insects (Li, Cowles, Cowles, Gaugler & Cox-Foster, 2007; Wang & Gaugler, 1995). Some insect pests cannot be killed by nematodes because entry portals are not accessible (Eidt & Thurston, 1995). For example, oral filters in the mouth and/or anus may be constricted by a muscle; the spiracle may be covered with a septum; or the spiracle may be too narrow for passage, etc. Nematode infection may also be hindered by aggressive grooming or evasion behaviour such as the formation of impenetrable cocoons (Gaugler, Wang & Campbell, 1994).

Because of their success as bioinsecticides, there growing in commercial interest and many researchers are working on developing EPN products. The development of these products involve mass production of infective juveniles (IJs) (non-feeding free living stage), which may either be achieved by *in vivo*, *in vitro* liquid or *in vitro* solid culture (Ehlers, 2001; Gaugler & Han, 2002; Shapiro-Ilan & Gaugler, 2002). *In vivo* methods produce the best quality IJs because virulence may be altered by *in vitro* production (P. M. Sharma, A. N. Sharma & Hussaini, 2011). Infectivity, measured by virulence potential against host insects, is one of the quality indicators of EPN production (Grewal & Peters, 2005; Susurluk, Kongu & Ulu, 2013). Sand or soil-based bioassays have been developed to simulate natural environmental effects on the nematode and its host under laboratory conditions for the exploration of such infectivity (Ricci, Glazer, Campbell & Gaugler, 1996).

The objective of this study was to determine the efficacy of *in vitro* produced IJs (see method in Chapter 3) of *Steinernema innovationi* (Çimen, Lee, Hatting & Stock, 2014) against different insect hosts. Susceptibility of larvae and/or pupae of *Eldana*

saccharina (Walker), *Sesamia calamistis* (Hampson), *Chilo partellus* (Swinhoe), *Tenebrio molitor* (Linnaeus), *Galleria mellonella* (Linnaeus), *Cydia pomonella* (Linnaeus), *Plutella xylostella* (Linnaeus), and *Gryllidae acheta* (Linnaeus), representing three orders (Coleoptera, Lepidoptera & Orthoptera), was assessed under laboratory conditions.

6.2 Materials and methods

6.2.1 *In vitro* production

A 5g sample of common house fly, *Musca domestica* (Linnaeus), larval puree + 0.15g canola oil was absorbed in 0.5g of 0.5cm^2 sponge cubes placed in a 100ml Erlenmeyer flask and autoclaved at 121°C for 15min. Each flask was inoculated with 0.5ml of bacterial suspension prepared by selecting a single colony from a NBT (nutrient agar, bromothymol blue, 2-3-5 triphenyltetrazoliumchloride) agar plate, and inoculating 30 ml Nutrient Broth (Biolab, www.merck.co.za), incubated at 27°C, with shaking at 100rpm for 24h to 48h. Flasks were incubated at 27°C for 72h before being inoculated with 0.5ml of a monoxenic nematode culture and further incubated at 22°C for 28 days. Nematodes were harvested by washing the sponges six times with 50ml distilled water. Individual sponges were squeezed five times during each wash. IJs were collected by sieving through a milk filter (www.denvet.co.za).

6.2.2 Virulence

The host range tested comprised nine species: *E. saccharina, S. calamistis* and *C. partellus* (supplied by the South African Sugarcane Research Institute [SASRI]); *T. molitor* and *G. mellonella* (reared at the Agricultural Research Council-Small Grain Institute [ARC-SGI]); *P. xylostella* (sourced from ARC-Plant Protection Research Institute [ARC-PPRI]); *A. ipsilon* (supplied by ARC-Grain Crops Institute [ARC-GCI]); *C. pomonella* (sourced from the University of Stellenbosch [US]) and *G. acheta* (sourced from a local pet shop [Paw Pet] in Bethlehem, Free State). Except for *A. ipsilon*, all larvae (mid to final instar) and pupae were exposed to a low and high

concentration of 50 and 500 IJs, respectively (Koppenhöfer & Fuzy, 2003), in 60µl distilled water per host including a control per host insect exposed to 60µl of sterile distilled water only. Medium size *G. acheta* were also exposed to a control treatment. An LC_{50} was determined for *A. ipsilon* by applying IJs at a rate of 0, 1, 5, 10, 25 and 50 IJs/larva. For all lepidopteran and coleopteran hosts, a Sandwell bioassay was used as per Gungor, Kestin & Hazir (2006). The bottom of a 24-well TC plate was covered with 0.5g sterile loam sand per well. IJs (50 or 500) suspended in 60µl of sterile distilled water were pipetted into each well and one larva or pupa was introduced per well. The bioassay of *G. acheta* (adults) was performed in 100ml plastic cups, half filled with 50g of moistened (10% moisture) sand. The insects were fed pieces of potatoes *ad libitum*. In all assays, a total of 30 larvae or pupae (as single replicates) were used per treatment and the control, for all insects. The TC plates and plastic cups were maintained individually inside a 330 × 215mm Zip Seal bag (GLAD®, www.glad.co.za) and incubated at 25°C. Mortality was assessed on Day 3 for larvae (and nymphs of *G. acheta*) and Day 7 for all insect pupae. Each cadaver was dissected to confirm nematode infection (recycling).

6.2.3 Statistical Analysis

Percentage mortality and number of cadavers with positive nematode recycling were classified into two-way frequency (contingency) tables (Snedecor & Cochran, 1980). Chi-square (χ^2) tests (Microsoft Excel, 2013) were used to show the dependence of the different treatments (target pests) versus the mortality / confirmation of mortality. Dependence of the hosts versus mortality / confirmation of nematode recycling in cadaver is shown where the χ^2 probabilities were smaller than 0.05. An LC_{50} for *A. ipsilon* was determined using TableCurve software (SYSTAT Software Inc., 2002).

6.3 Results

6.3.1 Virulence: Mortality

Larval mortality at both 50 and 500 IJs was lowest for *G. acheta* followed by *C. partellus* and *P. xylostella* (Max. 28%, 45% and 92% at 500IJs concentration,

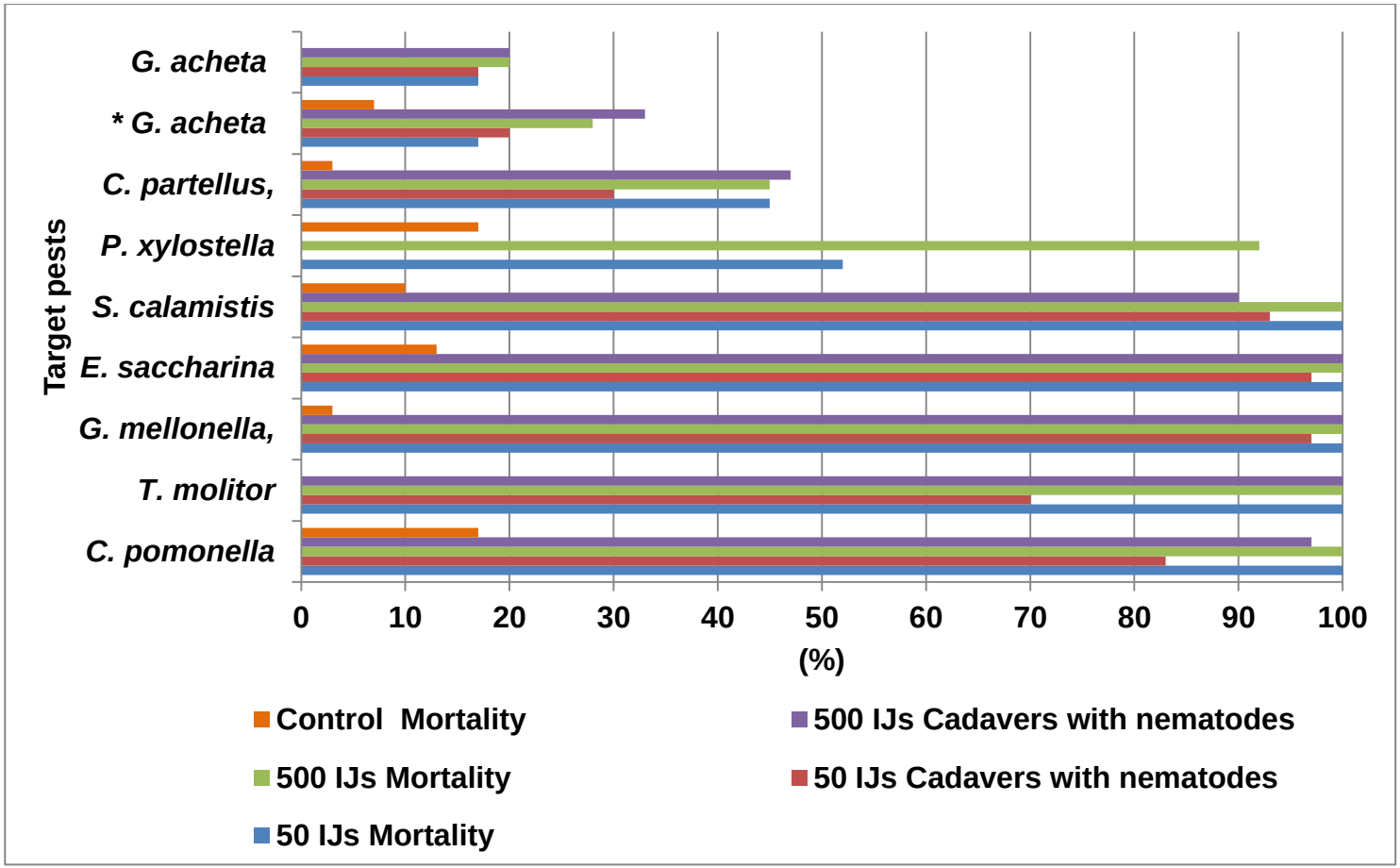
respectively). The rest of the hosts reached 100% mortality. The χ^2 probability for *P. xylostella* (χ^2 probability = 0.002, df = 1) was the only test that showed a significant increase in mortality from 52% to 92% at 50 and 500 IJs, respectively (Figure 6.1).

6.3.2 Virulence: Cadavers with positive nematode recycling

There was no significant difference in the number of larvae with nematode recycling for 50 vs. 500 IJs except for *T. molitor* (χ^2 probability = 0.001, df = 1), which showed an increase from 70% to 100% (Figure 6.1). The number of pupae with nematode recycling was non-significant at 50 and 500 IJs for all host species tested (Figure 6.2).

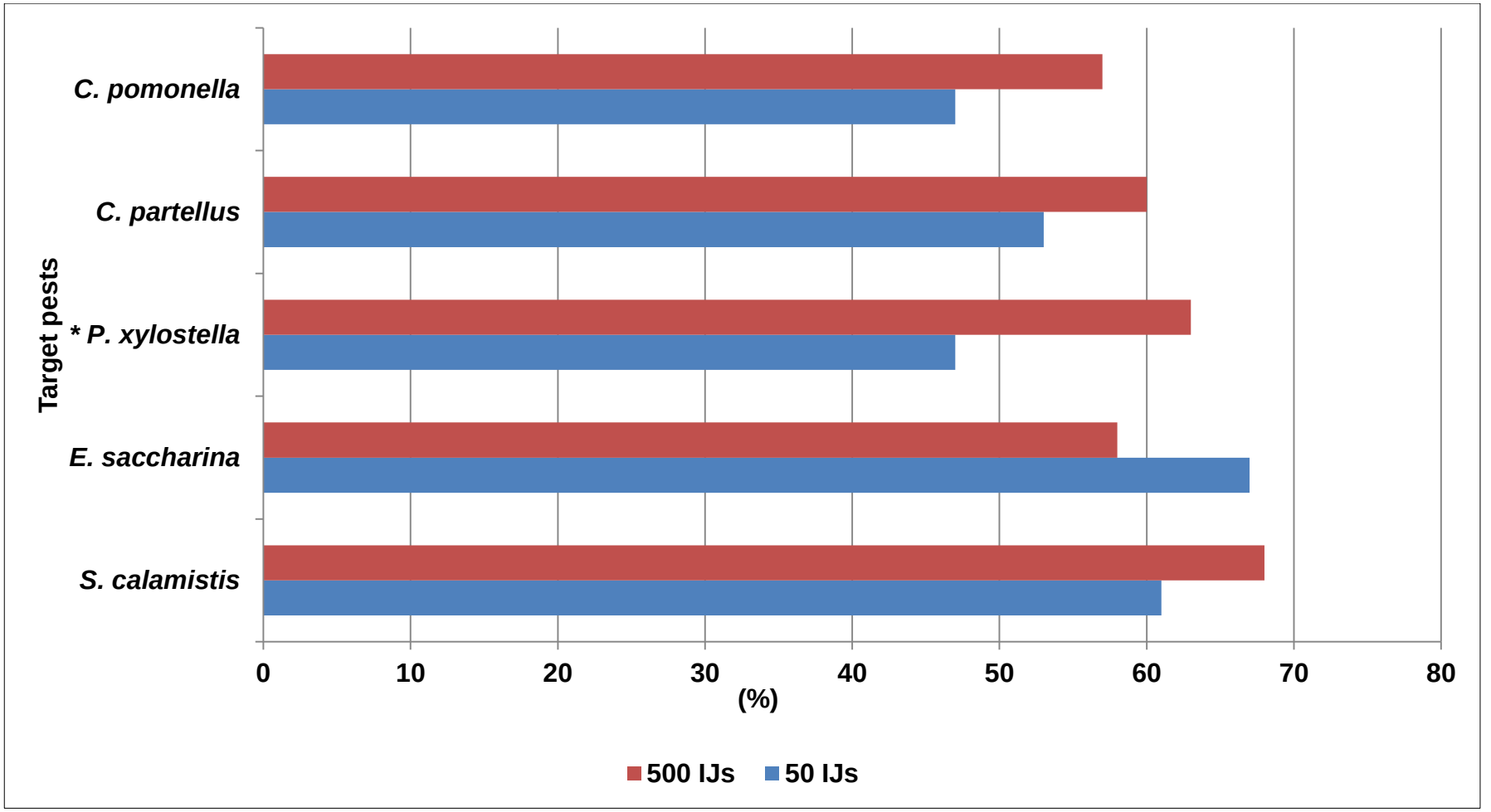
6.3.3 Comparison of number of cadavers (larvae vs. pupae) with nematode recycling (Figure 6.3)

The number of pupae with nematode recycling was lower for *C. pomonella*, *E. saccharina* and *S. calamistis* as compared to larvae (χ^2 probability = 0.003, 0.007 and 0.003, respectively, df = 1). The number of *C. partellus* larvae vs. pupae with nematode recycling was non-significant at the two concentrations (χ^2 probability = 0.067 and 0.301, respectively, df = 1).



* 7 days incubation time.

Figure 6.1 Larval mortality (%) (including *G. acheta*) mortality and cadavers with nematode recycling (%) after inoculation with 50 or 500 IJs/insect. Mortality was corrected using Schneider-Orelli's formula (Püntener, 1981).



* 3 days incubation

Figure 6.3 Number of pupae with nematode recycling (%) following inoculation with 50 or 500 IJs/pupae.

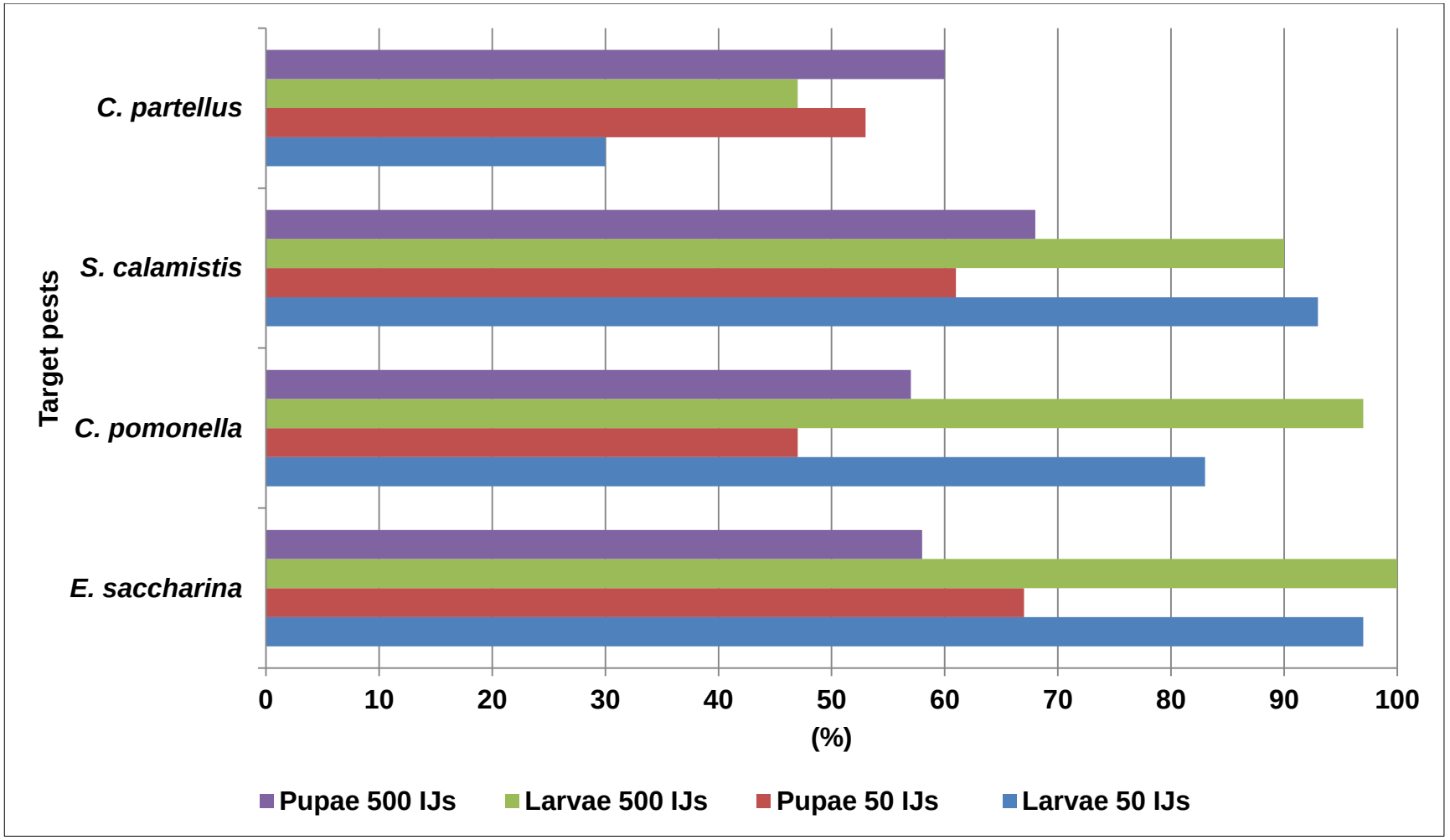


Figure 6.3 Comparison of number of cadavers (larvae vs. pupae) with nematode recycling (%) following inoculation with 50 or 500 IJs/insect.

6.3.4 LC_{50} for *A. ipsilon*

An LC_{50} was reached at a concentration of 3 IJs/larva rising to a maximum LC70 at 31 IJs/larva (Figure 6.4).

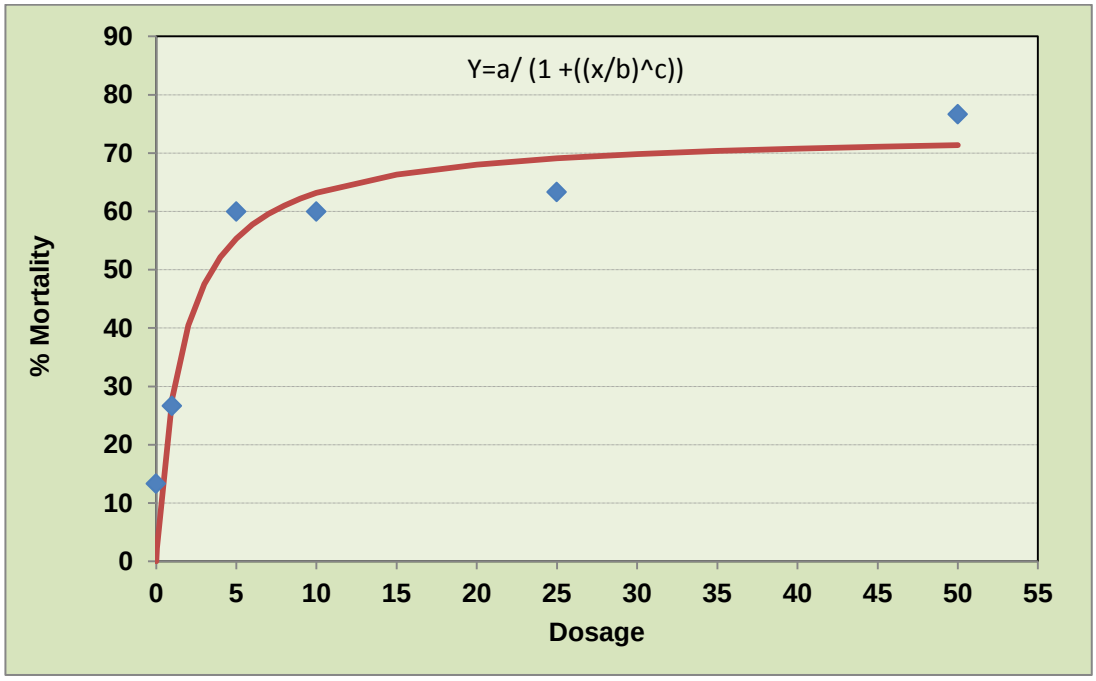


Figure 6.4 $LC_{50\&70}$ of *A. ipsilon* calculated by TableCurve analysis (SYSTAT Software Inc., 2002).

6.4 Discussion

All insects tested were prone to infection by *S. innovationi,* although the level of susceptibility differed amongst hosts, with *G. acheta* suffering only 28% mortality. This could be attributed to the fact that these crickets did not necessarily burrow through the sand in the test containers (they are less exposed) or a 'diluted' IJ distribution in 50g sand as compared to 5g for all other hosts. However, it was positive to observe that 100% of *G. acheta* cadavers showed nematode recycling.

Large variations in efficacy were observed, with mortalities ranging from 28% to 100%, a phenomenon which suggests better adaptation of this species to certain hosts. Variable efficacy may also be related to one or more factors that affect the nematode, its host and/or the environment (McCoy, Stuart, Duncan & Nguyen, 2002). Variation in mortality against different species was also demonstrated by Athanassious, Kavallieratos and Karanastasi (2010) when they tested efficacy of three EPNs against four stored product pests of wheat. In the current study, the larvae of *T. molitor, G. mellonella, E. saccharina, S. calamistis* and *C. pomonella* were the most susceptible (100% mortality). The reason for high mortality of *T. molitor* and *G. mellonella* could be attributed to their inherent high susceptibility to EPN and hence their wide use in laboratory experiments during EPN studies (Shapiro-Ilan *et al.,* 2012). The other three species, *E. saccharina*, *S. calamistis* and *C. pomonella,* may be correspond with *G. mellonella* in terms of susceptibility as they belong to the same order (Lepidoptera). Pillay, Martin, Rutherford and Berry (2009) also achieved 100% mortality at a rate of 250 IJs/larva against *E. saccharina* larvae using two native *Steinernema* isolates from South Africa. Mortality of *C. pomonella* (100%) was higher than that achieved by De Waal (2008) using native (South Africa) EPN isolates at a concentration of 200 IJs/insect. Mortality ranged from 70% to 89.5% for 14 *Heterorhabditis* species and 37.5% to 81.25% for 8 *Steinernema* species. Mortality of *P. xylostella* larvae ranging from 52% to 92% is consistent with 70.7% to 93.3% achieved by Nyasani, Kimenju, Olubayo and Wilson (2007) using a very low concentration of 200 IJs/ml per Petri dish with eight larvae (*i.e*. 25 IJs/larva).

Although Trdan (2005, 2006) concluded that IJ concentration would affect nematode efficacy less than temperature, this study demonstrates (at 25°C) that nematode dose can be manipulated to increase mortality of a particular host. Mortality or percentage of hosts with nematode recycling increased for *P. xylostella* from 52% to 92% and for *T. molitor* from 70% to 100% at the higher concentration of 500 IJs/host. De Carvalho Barbosa Negrisoli, Negrisoli Júnior, Bernardi, and Garcia (2013) also demonstrated a similar trend where mortality of *Analgesia kueniella* (Zeller) was

increased by increasing the dose from 10 IJs/insect to 200 IJs/insect using five strains of *Heterorhabditis* and three strains of *Steinernema*. Similarly, mortality of *A. ipsilon* in this study increased with increased IJ concentration from 1 IJ/larva to reach a LC_{70} at 31 IJs/larva. Although time till death was not tested in this study, Koppenhöfer and Fuzzy (2003) found a positive correlation between time to death and IJ concentration when *S. scarabaei* (Stock & Koppenhöfer) was tested against oriental and Japanese beetles.

Different life stages have also been found to differ in susceptibility to EPNs. In particular, larvae have been found to be more susceptible than pupae (De Carvalho Barbosa Negrisoli *et al.*, 2013; Garcia-del-Pino, Alabern & Morton, 2013; Malan, Knoetze & Moore, 2011; Theunis, 1998). De Carvalho Barbosa Negrisoli *et al.,* (2013) found that mortality of *A. kueniella* larvae was higher than that of pupae. They achieved maximum mortality of 96 and 80% with larvae and pupae, respectively. Similarly, in the current study, larvae of *E. saccharina, S. calamistis* and *C. pomonella* were more susceptible than their pupal stages, at 3 days and 7 days incubation for larvae and pupae, respectively. In another study, Tofangsazi, Cherry and Arthurs (2014) found that the LC_{50} of *H. indica* (Poinar, Karunaka & David) was higher for small larvae as compared to large larvae of *Herpetogramma phaeopteralis* (Guenée). However, Medeiros, Rosa, Tavares and Simoes (2000) suggest that age susceptibility of insects is host specific. The number of larvae and pupae (30% to 60%) of *C. partellus* with nematode recycling did not differ significantly in the current study. The non-significant low mortality against both larvae and pupae may indicate that this species is not suitable to control this species.

These results provide a guideline to indicate relative virulence of this new species against different hosts. More comprehensive studies under glass house and field conditions would be needed to determine the efficacy of *S. innovationi* under unfavourable conditions. Furthermore, these results indicated that the *in vitro* mass production medium used yielded virulent IJs.

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CHAPTER SEVEN

Cost analysis of solid culture production of *Steinernema innovationi*

Abstract

In vitro production of entomopathogenic nematodes (EPNs) can be by solid or liquid culture. The most efficient liquid culture technologies require large capital investments, and considerable expertise to manage the process efficiently. Cost analysis for an *in vitro* solid culture production system was undertaken, including the costs of rearing an insect-based nutrient component. Based on the production costs, an estimated retail price was calculated. This was then compared to the costs of commercial EPN products currently sold on the market. The estimated retail price (R90.61) for *Steinernema innovationi* produced with our solid culture system was considerably lower than the market price for other *Steinernema* species and products (per 50 million *Steinernema* IJs): E~nema (Germany) sells Nemastar® 50 at R309.15; BSAF corporation (USA) sells Nemasys Caterpillar Killer at R312.31; Koppert (Netherlands) sells CAPSANEM at R346.46; BioBest (Canada) sells Carpocapsae-System at R271.50; and Natural Insect Control (Canada) sells NEMS Soil Larva Parasite at R458.55. The production system developed in this study offers a competitive technology to produce EPN products without having to invest in large scale liquid fermentation equipment, by using a relatively cheap production medium and simple solid culture growing conditions.

Keywords: Cost analysis; products; entomopathogenic nematodes; production

7.1 Introduction

Entomopathogenic nematode (EPN) products are commercially successful biocontrol products. They occupy second position in the total use of biopesticides products in Europe and the Netherlands, contributing 14% and 21%, respectively, to biocontrol sales (Ravensberg, 2011). *In vitro* EPN production is either by solid and liquid culture methods (Shapiro-Ilan, Han & Dolinski, 2012). The choice of production method is

influenced by the costs of production. Solid culture methods require a lower capital outlay but it is thought to be feasible only for small scale application. On the other hand, liquid culture involves high levels of capital investment and running cost because of the sophisticated engineering and operating systems/procedures that large volume liquid fermentation units require, and because of the sensitive processes which have to be monitored closely (Lacey & Georgis, 2012). Liquid culture of EPNs is mainly employed by large production companies in developed countries.

Cost analysis in research and development usually involve cost of raw material, medium ingredients and equipment. One challenge is that most of the cost related information is proprietary information within the biocontrol industry (Ravensberg, 2011) and can only be estimated from the retail prices of the EPN products. EPN products are generally more expensive than the alternative chemical pesticides in most markets (Gaugler & Han, 2002). Despite their cost, EPNs are often chosen because of their advantages, which include their safety towards the environment and non-target organisms, the absence of development of resistance by target pests, the absence of withholding periods and their zero registration requirements in most countries (Divya, Sankar, Marulasiddesha, Sambashiv & Krupanidhi, 2011). This chapter summarizes the cost of a solid culture production system, and draws a comparison with the retail prices of products currently on the market.

7.2 Materials and Methods

Prior research identified a solid culture medium on which *Steinernema innovationi* (Çimen, Lee, Hatting & Stock, 2014) grew exceptionally well. This medium is based on a combination of minced bodies of final instar larvae of common house fly, *Musca domestica* (Linnaeus), combined with 3% canola oil.

The ingredients were costed for the production of 50 million IJs, using this approach. These costs were then compared with published retail prices of commercial EPNs offered around the globe. All costs were converted into South African rands (R), given with the exchange rate as on 1st October 2013.

7.3 Results

7.3.1 Cost of rearing *M. domestica*

Table 7.1 Cost for rearing *M. domestica* (Linnaeus) (2kg larvae/ batch) $

Item / Description	Quantity	Cost (R)
Larvae diet		
Bran	2000g~	0.000
Powder milk (Nespray®)	300g	26.665
Sodium benzoate (food grade)	6g	0.135
Dry brewer's yeast (Anchor Yeast®)	20g	4.390
Water	3000ml	0.011
Adult diet		
Powder milk (Nespray®)	10g	0.889
water	250ml	0.000
Glass house operating cost	5cm2 over12 days	0.240
Labour*	3h over 12 days	60.000
Total		**92.330**

$ Cost based on 2013 retail prices (http://retailpricewatch.co.za)

~ By-product of wheat processing quality lab at ARC-SGI

* As per Republic of South Africa labourer minimum wage

7.3.2 Production cost

Table 7.2 Nematode production process costing[$]

Item / Description	Quantity	Price (R)/ 5g batch	Quantity	Price (R)/2KG batch (upscaling)
Medium for bacterial culture				
Nutrient broth	0.5ml	0.006	200ml	2.400
Nematode inoculum medium				
Nutrient agar	3.125ml	0.139	1250ml	55.600
Canola oil	0.005ml	0.000	2ml	0.041
Petri dish (plastic, 65mm)	1⁄8	0.097	50	38.667
Production medium				
M. domestica	5g	0.230	2000g	92.330
Canola oil	0.15ml	0.003	60ml	1.200
Sponge^	27Cm^3	0.000	10800cm^3	0.009
Harvesting				
Water	300ml	0.001	120000ml	0.456
Formulation				
Water	15ml	0.000	6000ml	0.023
Formalin[&]	0.015ml	0.006	6ml	2.230
Sponge^	94cm^3	0.000	1880cm^3	0.002
Glass house operation (28 days)	5cm^2	0.000	2100cm^2	0.252
Labour*	20min	13.334	40min	26.668
Total		**13.816**		**219.878**

[$] Cost based on 2013 retail prices (http://retailpricewatch.co.za)

^ Product code TF 18, Tuf Foam, www.tuffoam.co.za

[&] Product code 252549, Sigma-Aldrich®, www.sigmaaldrich.com

* As per Republic of South Africa labourer minimum wage (×2)

7.3.3 Calculation of production cost per 50 million IJs

- IJ yield / 5g medium = 781 678 IJs
- Upscaling: IJ yield per 2kg medium:
  781 678 IJs × 400 = 3.12×10^8 IJs

- Therefore, Production cost per 50 million IJs:
  $(50 \times 10^6$ IJs) (R219.878) / 3.12×10^8 IJs = R35.20

7.3.4 Breakdown of estimated retail price

Table 7.3 Breakdown of estimated retail price per 50 million IJs

Description	**Cost (R)**	**(%)**
Basic cost of production	35.23	
Marketing	3.52	10
Packaging	5.28	15
Transport	3.52	10
Insurance	1.06	3
Company overhead	7.05	20
Profit margin	10.57	30
Wholesale price	66.23	
Agent's commission	13.25	20
Total cost	79.48	
VAT	11.13	14
Retail Price	**90.61**	

7.3.5 Estimated retail price comparison by different companies

Table 7.4 Price comparison per 50 million *Steinernema* IJs by different companies[$]

Company	Product Name	Cost (R)
ARC (South Africa)*		90.61
E~nema (Germany)	Nemastar® 50	309.15[‡]
BASF corporation (USA)	Nemasys Caterpillar Killer	312.31[‡]
Koppert (Netherlands)	CAPSANEM (*Steinernema carpocapsae)*	346.46[‡]
BioBest (Canada)	Carpocapsae-System	271.50[‡]
Natural Insect Control (Canada)	NEMS (Soil Larva Parasite)	458.55[‡]

* Entomopathogenic nematode developed in the current study.
[$] Cost based on 2013 retail prices
[‡] Price excludes delivery cost

7.4 Discussion

The cost of this production process is considerably lower than the retail prices of the products currently being sold on the market by E~nema (Germany), BSAF corporation (USA), Koppert (Netherlands), BioBest (Canada) and Natural Insect Control (Canada). Moreover, the product developed in this study can compete with the commercial products without investments in major capital equipment, which constitute the most important cost parameter for producing EPNs in bioreactors (P. M. Sharma, A. N. Sharma & Hussaini, 2011), and is therefore the greatest opportunity for cost saving. High capital investment is still a major reason why EPN products are currently limited to developed countries (Kaya *et al.*, 2006). Large companies producing EPNs are backed by other biological products because the demand for EPNs production is seasonal (e.g. E~nema, BSAF corporation & Koppert all sell multiple biocontrol agents) (Ehlers, 2007). Therefore, it may not be advisable to invest a large capital outlay for EPN production only.

Solid culture production of EPNs is associated with high labour costs (Shapiro *et al.*, 2012). However, some small companies in the USA produce EPNs in a similar way on solid media (e.g. Biologic Company, www.biologicco.com) and some follow *in vivo* production using insect hosts (e.g. Hydro-Gardens, http://hydro-gardens.com) (Ravensberg, 2011). South Africa is a developing country in which labour is relatively cheap, compared to developed countries (Geneva, International Labour Office, 2013). Since the production process does not require highly specialized skills, labour costs can be reduced by using unskilled labour paid minimum wages. The production costs presented here exclude once-off capital costs including: registration costs and equipment (autoclave, microscope, laminar flow hood, mincer for the flies, fly cages, packaging machine, labelling machine, growth chambers, etc.) and on-going research and development costs. Scaling up of the production process could reduce production costs significantly (*i.e.,*via economies of scale), as illustrated by upscaling to 2kg batches. Notably, production yields were not significantly different when *M. domestica* larvae were substituted with *Ceratitis capitata* (Wiedemann) larvae (unpublished data). *Ceratitis capitata* larvae can be sourced from a sterile insect technology (SIT) production company in South Africa, which would eliminate the cost of rearing *M. domestica*. Alternatively, Joubert (2012) has described a method in which 10kg of fly maggots can be produced every 3 days in 10L buckets using chicken litter and chicken offal.

In summary, the production system developed in this study offers a highly competitive alternative method to produce EPN products without having to invest in large scale liquid fermentation equipment, by using a relatively cheap production medium and simple solid culture growing conditions. Production costs may be reduced by scaling up, and by using fruit fly larvae or rearing *M. domestica* in a more economical way. Future research will therefore focus on scaling up and reducing production costs.

7.5 References

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THESIS OVERVIEW

Entomopathogenic nematodes (EPNs) have been developed as environmentally friendly biopesticides to aid in the control of insect pests, concurrently reducing the use of pesticides. Development of an EPN strain requires a mass production process that will accomplish high yield, retain virulence and have a reasonable shelf-life (Hazir, Stock, Kaya, & Kestin, 2003). Commercial EPN products are produced mainly in developed countries because of the high capital costs of setting up a conventional liquid culture production facility (Lacey & Georgis, 2012). Presently, there is no such EPN production facility in South Africa. Moreover, the importation of exotic EPN species into South Africa is prohibited by Agricultural Pest Amendment Act 18 of Act 36 of 1983. These two factors have been the forces driving the development of indigenous species of EPNs for commercialization.

The research undertaken in this thesis included the determination of the optimum production and storage temperatures, the optimal nutritional composition of solid mass production media, ecological characterization, determining host range and a cost analysis for the development of an indigenous isolate, *Steinernema innovationi* (Çimen, Lee, Hatting & Stock, 2014). The key outcomes were:

- *S. innovationi* was capable of reproducing at all the temperatures that were tested. There was no significant difference in yield at the two highest yielding temperatures of 22°C and 25°C. The optimum temperature was identified as being 22°C because there was more time for the IJs to build up nutrient stores, and hence there was a significant slower emergence. This allowed the EPNs more time to store critical nutrient reserves, which is the sole energy source for survival of the non-feeding infective juvenile (IJ) stage. The tendency for sub-surface soil temperatures to be lower than ambient temperatures would favour propagation at lower temperatures to prevent

acclimation of the EPNs to higher temperature, which could impair infectivity in the field. A medium containing a puree of *Musca domestica* (Linnaeus) larvae + 3% canola oil outperformed the other media, which contained combinations of plant protein and yeast extract. This yield was also higher than *in vivo* production using the larvae of the wax moth, *Galleria mellonella* (Linnaeus), an insect routinely used for rearing EPNs. In all repeat experiments, liquid culture inoculum performed better than solid culture inoculum, irrespective of the concentration of the inoculum. To the best of our knowledge, the use of *M. domestica* for EPN propagation has not been reported elsewhere.

- When five temperatures were evaluated to determine the optimum storage temperature in aqueous suspension, 15°C was identified as the optimum storage temperature because it ensured a high level of survival (84%) and the greatest virulence against *G. mellonella* (97%).

- Survival of EPN IJs in a sponge formulation was higher than in aqueous suspension, despite the high concentration of IJs in the sponge formulation. A shelf-life of three moths was achieved, which is adequate for small scale production and seasonal marketing systems.

- The new species, *S. innovationi* was classified as a cruiser, with optimum temperature for infectivity of 25°C. *In vitro* produced IJs were found to be comparable to *in vivo* cultured IJs, given no statistical difference between the two production methods for persistence of IJs under field conditions after at least 4 weeks.

- The EPNs did not lose the symbiotic bacterium during the production process, which was demonstrated by the virulence (up to 100% mortality) against nine insect species representing three orders (Coleoptera, Lepidoptera & Orthoptera).

- This study identified an alternative production technology that avoids the extensive capital and technical requirements of liquid fermentation technologies. It also allows for the use of unskilled and semi-skilled labour as it is not technically complex. The estimated retail price for this production system is considerably lower than the cost of current products on the market.

Future prospects

The current study supports the concept of using *M. domestica* as the core nutrient base for the mass production of EPNs. It should be noted, however, that the overall costs excluded research and development, equipment and product registration. There is, therefore, a need for further cost-cutting interventions by scaling up of the process. The retail price could also be reduced by adopting a cheaper method of rearing *M. domestica*, such as that developed by Joubert (2012), who propagated fly maggots on low-cost chicken offal and litter. Moreover, there was no significant difference in IJ yield when the puree of *M. domestica* was compared with a puree of *C. capitata*, a dipteran cultured in mass for use during sterile insect technique (SIT) programmes in the Western Cape. This host may, therefore, be considered as potential alternative without excessive cost implications.

Impregnating sponges with EPNs is a common way to “formulate” active EPNs, although different formulations in which the IJs are immobilized, dehydrated and transported in host insect cadavers may improve their shelf-life. Other studies have suggested that factors other than temperature and storage time, such as IJ concentration and type of carrier, can affect the virulence and infectivity of IJs. Therefore, further research is essential in order to determine how these factors affect *S. innovationi* in storage.

Virulence of *S. innovationi* against larvae and/or pupae of *Agrotis ipsilon*, *Eldana saccharina, Sesamia calamistis*, *Chilo partellus, Tenebrio molitor*, *G. mellonella*,

Cydia pomonella, *Plutella xylostella*, and *Gryllidae acheta* representing three orders (Coleoptera, Lepidoptera & Orthoptera) was confirmed. Acquired knowledge of optimal conditions for infectivity will allow for manipulation of such conditions to enhance efficacy. Further evaluation of *S. innovationi* for control of different insect pests under field conditions is essential for commercialization. Also, the compatibility of EPNs with other pesticides has been reported (P. M. Sharma, A. N. Sharma & Hussaini, 2011) and such compatibility will allow expanded integration of *S. innovationi* into integrated pest management (IPM) programmes.

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