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Appendix IV

GEL ELECTROPHORESIS

Agarose Gel (50ml)

Agarose (0.4g for 0.8% and 0.5g for 1%)

10ml 5X TBE

40ml distilled water

Heat until agarose has completely dissolved

Add 1 µl Ethidium Bromide

5X TBE

54g Tris base

27.5g Boric acid

20ml 0.5M EDTA pH 8.0

Make up to 1L with distilled water and autoclave at 121ºC at 15psi for 20 min

Electrophoresis buffer

100ml 5X TBE

900ml sterile distilled water

2.5µl Ethidium Bromide

Appendix III

ISOLATION OF GENOMIC DNA FROM BACTERIA

(Method from InstaGene matrix, catalogue # 732-6030)

1) Pick an isolated bacterial colony and resuspend it in 1 ml of autoclaved water in a microfuge tube.
2) Centrifuge for 1 minute at 10,000 – 12,000 rpm. Remove the supernatant.
3) Add 200µl of InstaGene matrix to the pellet and incubate at 56ºC for 15-30 min. **Note**: InstaGene matrix mix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. The pipette tip to be used should have a large bore, such as a 1,000µl pipette tip (Bio-Rad's catalogue # 223-9378).
4) Vortex at high speed for 10 s. Spin at 10,000-12,000 rpm for 2-3 min.
5) Use 20µl of the resulting supernatant per 50µl PCR reaction. Store the remainder of the supernatant at -20ºC. Repeat step 5 when reusing the InstaGene DNA preparation.

[a] Hb = *Heterorhabditis bacteriophora*, Hi = *H. indica,* Hm = *H. megidis*, Hmar= *H. marelatus*, Sc = *Steinernema carpocapsae*, Sf = *S. feltiae*, Sg = *S. glaseri*, Sk = *S. kushidai*, Sr = *S. riobrave*

[b] Acccording to Shapiro-Ilan & Gaugler (2002), references indicate just some reports of efficacious control of these pests. For further evidence of efficacy and commercial development are documented in other resources ((e.g.) Georgis & Hague, 1991; Grewal & Georgis, 1998; Kaya &Gaugler, 1993; and Shapiro-Ilan *et al*., 2002).

Appendix II

EXAMPLES OF IMPORTANT PESTS SUCCESSFULLY TARGETED COMMERCIALLY WITH ENTOMOPATHOGENIC NEMATODES

Common name	**Scientific name**	**Commodity**	**Nematode[a]**	**Reference[b]**
Artichoke plume moth	*Platyptilia carduidactyla*	Artichoke	Sc	Bari & Kaya , 1994
Black vine weevil	*Otiorhynchus sulcatus*	Cranberries, ornamentals,	Hb, Hm, Hmar	Bedding & Miller, 1981 Shanks & Agudelo-Silva, 1990
Billbugs	*Sphenophorus* spp.	Turf	Sc	Shapiro-Ilan *et al*., 2002
Blue green weevils	*Pachnaeus* spp.	Citrus	Hb, Hi, Sr	Georgis & Hague, 1991
Black cutworm	*Agrotis ipsilon*	Turf, vegetables	Sc	Georgis & Hague, 1991
Cranberry girdler	*Chrysoteuchia topiaria*	Cranberries	Sc	Georgis & Hague, 1991
Diaprepes root weevil	*Diaprepes abbreviatus*	Citrus	Hb, Hi, Sr	Shapiro-Ilan *et al*., 2002
Fleas	*Ctenocephalides felis*	Household yard	Sc	Silverman *et al*., 1982
Fungus gnats	Sciaridae	Mushrooms, Greenhouse	Sf	Grewal & Georgis, 1998
Mole crickets	*Scapteriscus* spp	Turf	Sr, Sf	Shapiro-Ilan *et al*., 2002
White grubs	Scarabaeidae	Turf	Hb, Sg, Sk	Shapiro-Ilan *et al*., 2002

Appendix I

SOURCES OF CHEMICALS AND SUPPLIES

Chemical/Supply	Source
Agarose	Saarchem
Agar	BioLab
Boric Acid	Saarchem
Calcium Chloride (CaCl2)	Saarchem
Canola oil	Epic Foods
Casein	Oxoid
Chicken fat	Rainbow chickens
Chloroform	Saarchem
Cod liver oil	The Clicks organisation
Corn flour	Tiger Milling (Tiger Brands)
EDTA (ethylene diamine tetracetic acid)	Saarchem
Ethanol (95% and 70%)	Saarchem
Ethidium Bromide	Saarchem
Glycerol	Merck
Honey	Gold Crest foods
Hydrochloric acid	Saarchem
JIK®	Reckitt and Benckiser
MacConkey agar	Merck
NBTA	Merck
Nutrient Broth	Biolab
Nutrient Agar	Oxoid
Parafilm ® M	Sigma
Polyurethane foam	Unilever
Potassium Chloride	Saarchem
ProNutro	Bokomo foods
Ringer's solution	Merck
Sodium Chloride	Saarchem
Spray dried egg yolk	Sigma
Safflower oil	Massmart Holdings
Tris base	Roche
Whole milk powder	Nestle foods
Yeast extract	Merck

alternative to formulating the larvae. On the other hand, further studies need to be carried out before insinuating the afore-mentioned supposition.

Another appreciable observation was that the non-formulated *H-indica*-infected *T. molitor* larvae did not rupture or stick together. Now, whether this observation is characteristic of the examined EPN, or if the same can be said for other EPN-infected cadavers remains to be bared in future research. Nevertheless, the finding hints that *T. molitor* larvae may not need to be formulated before storage and application; the achievability of which should also be explored further.

It seems that the storage and application of EPNS in infected cadavers is a hopeful move towards pest control. Field trials with EPN-infected cadavers have shown considerable management of several pests with *Heterorhabditis spp.* (Shapiro-Ilan *et al.,* 2001). Also, laboratory studies have indicated better dispersal activity (e.g.) in *H. bacteriophora* and *S. carpocapsae* (Shapiro-Ilan & Glazer, 1996) and greater infectivity (e.g.) *H. bacteriophora* (Shapiro-Ilan & Lewis, 1999), when these nematodes were applied in cadavers compared with nematode application in aqueous suspension. Although commercialization of nematode infected cadavers has been hampered by constraints in storage and application (Koppenhöfer, 2000), this study confirms that formulating the cadavers is achievable; a step forward towards improving ease of nematode handling and storage.

Apparently, when exposed to EPNs under similar conditions, mortality is higher in the pupae than in the larvae of *T. molitor*. Moreover, the pupae produce more, or at worst, an equivalent number of IJs compared to the larvae (Ngoma, unpublished). It is unsure if Ngoma made this observation from an experiment designed to test the hypothesis that *T. molitor* pupae would produce more IJs than larvae, or if the observation was merely picked up in the course of research. Nonetheless, assuming the former was the case, and then one could elucidate that the pupae being non-mobile, are easier target hosts than the larvae. Also, in comparison to the pupae, the larvae have a waxier cuticle, thus making nematode attachment more difficult. In addition, the nematode(s), following entry into the non-feeding pupae, utilize the lipid-rich food resources, set aside for use by the pupae during metamorphosis to the adult stage. In contrast to the *T. molitor* larvae, it was obvious that the formulations adhered much better to the pupae; probably because of their rugged morphological features and less waxy cuticle. Assuming that the findings of Ngoma were tested appropriately, one could theorise that formulating *T. molitor* pupae may be a better

outer layer, which is effectively dry. Certain formulations did not adhere to the *H. indica*-infected *T. molitor*. One problem was apparent when the dipping agent did not sufficiently wet the insect cuticle and the rolling agent did not completely coat the cadaver, which was enhanced in the case of *T. molitor* larvae because of their waxy and smooth cuticle This was mostly noted with the maizena-based dipping agents. However, it is worth noting that the formulations adhered better to the pupae than the larvae probably because the pupae are less smooth, and also because of their rugged body structure.

In other cases, the cadavers were coated, but there was little binding between the insect cuticle and the coating. After drying, these formulations chipped or cracked off easily from the cadaver (Fig 7.1A & 7.1C). The cracking was the result of shrinkage of the coating material while drying. Shapiro-Ilan *et al.* (2001) suggested that shrinkage may be minimised by the inclusion of elasticizers in the formulation or by selecting materials that are less prone to swelling. In addition, some of the coating agents soaked up the moisture from both the dipping agent and the cadaver, causing it to nematodes to dehydrate faster (fig 7.1A & 7.1B). This may be overcome by coating the cadavers in an oil emulsion following the dipping agent, before coating with the adsorbent (such as clay), according to the method of Quimby *et al.* (1994).

Some of the formulations adhered to the cadaver but nematode reproduction was poor or failed completely. For example, formulations that included higher concentrations of instant FTD starch (>5%) adhered to the cadaver's surface but nematode reproduction and emergence of IJs was not completed. These higher concentrations of dipping agents made a thicker coating on the cadavers, which may have resulted in the nematodes becoming oxygen deprived. Reduced oxygen levels will lower nematode survival (Kung *et al*., 1990) and consequently reproduction (Shapiro-Ilan *et al*., 2001).

space. Also, if the male to female ratio is altered, such that there are more males than females, it is likely that the inference of Worden & Parker (2001) would be corroborated.

The second part of the study investigated 12 combinations of dipping and rolling agents for formulating EPNs. The most promising formulation tested may be considered the 2.5MC, which was the only formulation to produce a significantly higher number of IJs both 4 and 8 days PI. However, the other three formulations did seem to have potential. A similar formulation study was performed by Shapiro-Ilan *et al*. (2001), using G. *mellonella* and *H. bacteriophora* as the model host and nematode respectively. The number of IJs produced in cadavers formulated 4 and 8 days PI were similar to the number produced in the non-formulated control. Their most successful formulations were 1MC, 10GC, and 2.5MC [where: M, mirasperse- a starch (1 or 2.5 %); 10G, 10% gluten; C, clay]. The 1MC formulation produced up to 300,000 IJs/host. *T. molitor* hosts coated with 2.5MC formulation in this study produced a maximum of only 80,000 IJs. Several explanations may account for this observation. Clearly, the insects and EPNs used in the two studies are completely different. As noted during the one-on-one bioassays (chapter 4) the number of IJs produced per cadaver, from a group of hosts that received the same treatment, may still be significantly different. Furthermore, host size has a significant effect on the number of IJs produced. Having made this argument, the non formulated control in this experiment still produced up to 200 000 IJs on average. This observation suggests that the formulations tested herein may be inferior to those of Shapiro-Ilan *et al*. (2001) and need to be optimised by reviewing the materials and methods. Accordingly, it is suggested that the formulation of EPN-infected cadavers be investigated further in future studies.

The role of the dipping agent in the formulation was to maintain high water concentrations at the interior while the coating agent was meant to provide a relatively inert

A study was done to determine if sieving bran cultures of *T. molitor* would improve its yield (where the sieving action was to separate the eggs from the adults). The results (Fig 7.2) indicated that sieving to separate the eggs from the adults was valuable towards improving *T. molitor* yield. A probable explanation as to why yield was much lower in cultures that were not sieved is that the adult beetles and newly hatched larvae may have eaten some of the eggs while feeding since the eggs were laid in the bran. In addition, movement in the culture disturbed and possibly damaged some of the eggs. On the other hand, the eggs in the sieved cultures were undisturbed and stayed warm.

From Fig 7.2, it is apparent that cultures with more adults produced the least numbers of offspring. This trend is obvious for both the sieved and non sieved cultures. The obtained results were unexpected. The hypothesis here was that more offspring would be produced by cultures with more adults. This premise was based on a study by Worden & Parker (2001), suggesting that females may delay or reduce oviposition or may be incapable of achieving maximal fecundity until they have gained the material and/or genetic benefits of mating with multiple males. Their results showed that female beetles that mated with different male beetles produced up to 32% more eggs than females that mated an identical number of times with only one male. For this reason, adults were placed in cultures in increments of 10 so as to increase the variability of male partners available to mate with female beetles. A probable explanation for this observation is that the cultures were overcrowded. This could have resulted in reduced longevity and fecundity as competition for food and a place to lay eggs undisturbed increased. Notwithstanding, it seems that the supposition of Worden & Parker (2001) is not completely disputed because cultures with less than 60 adults had an increasing yield (fig 7.2), suggesting that there is a limit as to how many beetles may occupy a certain

comparison test performed post ANOVA revealed the differences were between the non-formulated control and all four formulations (in all cases, $P < 0.0001$).

The two-sample t test with equal variances was used to check if the mean numbers of IJs per cadaver was different for those formulated 4 days PI compared to cadavers formulated 8 days PI across the different formulations as well as the non-formulated control. These results are presented in Table 7.2 below. The differences in the mean numbers of IJs per cadaver were significant for all formulations including the controls, except the 2.5MC formulation.

Table 7.2:
T test results comparing numbers of infective juveniles per cadaver formulated 4 days and 8 days post inoculation by treatment.

Treatment	***P*- Value**	**95% CI**		**t**
1SC	0.0008	-38277.88	-11922.12	-4.0016
2.5SC	0.0047	-22968.64	-4831.358	-3.2202
1MS	<0.0001	-3.1314.92	-14085.08	-5.5359
2.5MC	0.8609	-24885.33	-29485.33	0.1777
Control	0.0001	-118402.6	-47197.38	-4.8861

7.4 Discussion

The objectives of the research in this chapter were to produce the EPN *H. indica in vivo* in *T. molitor* larva, and then to formulate the EPN-infected cadavers so as to overcome the hindrance of handling.

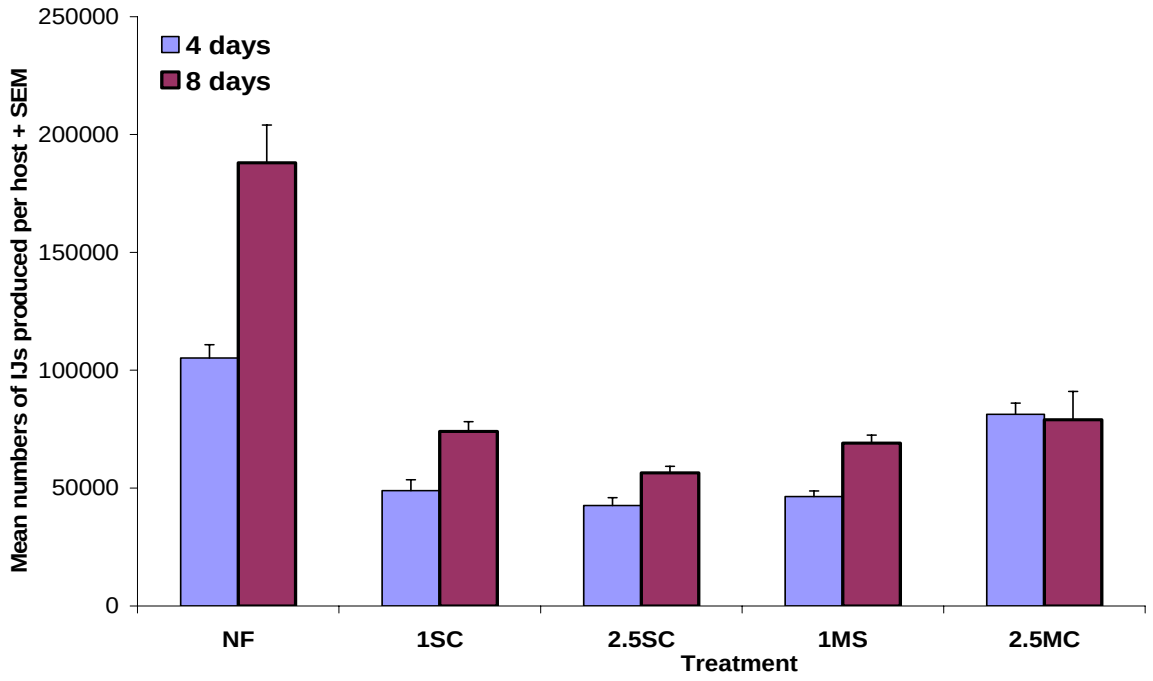


Fig 7.3: Numbers of *Heterorhabditis indica* infective juveniles (IJs) produced in *Tenebrio molitor* cadavers, which were formulated 4 and 8 days post inoculation. NF, non-formulated control; S, starch - Instant FTD 176 (1 or 2.5 %); M, Maizena (corn starch); C, Clay. Bars are mean + standard error of the mean.

Interaction was detected between time of formulation and treatment ($F = 33.21$; $df = 4, 95$; $P < 0.0001$); thus data were not combined across these factors. ANOVA was used to compare the numbers of IJs per hosts for the four different formulations by day. For cadavers formulated 4 days PI, there were significant disparities in the numbers of IJs produced per host amongst the different formulations ($F = 40.03$; $df = 4, 45$; $P < 0.0001$). Bonferroni's multiple comparison test post ANOVA revealed the disparities were between 1SC and 2.5MC, 2.5SC and 2.5MC, 2.5MC and 1MS, as well as between the control compared to all four formulations (in all cases, $P < 0.0001$). In the case of cadavers formulated 8 days PI, there were significant differences in the numbers of IJs produced per host amongst the different formulations ($F = 32.92$; $df = 4, 45$; $P < 0.0001$). Bonferroni's multiple

and 100 adults ($P \leq 0.05$); between cultures with 50 adults compared to those with 100 adults ($P = 0.008$); and between cultures with 60 adults compared to those with 100 adults ($P = 0.008$).

7.3.2 Screening of Formulations for Adhesion and Reproduction

While some of the formulations adhered to the *T. molitor* cadavers and allowed nematode reproduction to proceed, others did not (Table 7.1). The numbers of IJs produced in the non-formulated control was much higher than in all formulations in both 4 days and 8 days PI (Fig 7.3). With all formulations, there were more IJs produced by cadavers that were formulated 8 days PI compared to those formulated 4 days PI, except in the case of 2.5MC where the IJs produced per host were similar for cadavers formulated 4 and 8 days PI (fig 7.3). For 1SC, 2.5SC and 1MS, the numbers of IJs produced by cadavers formulated 4 days PI are similar (Fig 7.3). The 2.5SC treatment produced the least number of IJs compared to the other three formulations (Fig 7.3).

there were variations in the number of hatchlings in cultures that contained 10 adults compared to those with 20 or 30 (etc); But, “sieve A+B” and “ do not-sieve” cultures were compared separately.

After testing for equal variances using Bartlett’s test ($P = 0.263$; $\chi^2 = 11.19$), ANOVA revealed significant differences between groups in the “sieve A+B” cultures ($F = 15.25$; $df = 9, 49$; $P < 0.001$). Bonferroni’s multiple comparison test was performed post ANOVA to spot the disparities. The significant differences in the number of hatchlings were between cultures containing 10 adults and those with 30, 40, 50, 60 and 70 adults ($P \leq 0.005$); between cultures with 20 adults and cultures containing 40, 50, 60 and 70 adults ($P \leq 0.001$); between cultures with 30 adults compared to cultures containing 50 and 60 adults ($P = 0.41$ and $P = 0.024$ respectively); between cultures with 40 adults, compared to those with 100 adults ($P = 0.015$); significant differences between cultures with 50 adults, compared to those with 80, 90 and 100 adults ($P \leq 0.002$); between cultures with 60 adults compared to those with 80, 90 and 100 adults ($P \leq 0.001$); and between cultures containing 70 adults compared to those with 100 adults ($P = 0.024$).

For data on “do not sieve” cultures, Bartlett’s test showed the variances to be unequal ($P = 0.009$; $\chi^2 = 21.83$), thus the data were log transformed (Bartlett’s test: $P = 0.131$; $\chi^2 = 13.77$). A one-way ANOVA comparing the mean number of hatchlings per culture by the number of adults in the cultures revealed significant disparities ($F = 12.20$; df: 9, 49; $P \leq 0.0001$). Bonferroni’s multiple comparison test performed post ANOVA showed the variations were between cultures with 10 adults compared to those with 20, 30 and 40 adults ($P \leq 0.001$); between cultures with 20 adults compared to those with 70, 80, 90, and 100 adults ($P \leq 0.003$); between cultures with 30 adults compared to those with 70, 80, 90 and 100 adults ($P \leq 0.002$); between cultures with 40 adults compared to those with 70, 80, 90

In the statistical analysis comparing sieved and not-sieved cultures, a variance ratio test performed at a 95% confidence level, rejected the null hypothesis that data obtained from "sieve A+B" and "do not-sieve" cultures had equal variances (F = 6.91; *df*: 49, 49; $P < 0.0001$). Following this observation, a two-sample t test with unequal variances was used to test if the differences in the number of hatchlings between "sieve A+B" and "do not-sieve" cultures were statistically significant. Satterthwaite's degrees of freedom were used to compute *P*. Overall, in all cultures combined ((i.e.) regardless of how many adults had been placed in the container), the differences in mean number of *T. molitor* hatchlings were significantly different between sieved and not-sieved cultures ($P < 0.0001$; 95% CI: 180.2 - 272.2; t = 9.83). The numbers of hatchlings from "sieve A+B" and "do not-sieve" cultures were then compared after stratifying by the number of adults per container. The two-sample t test with unequal variances showed that there was no disparity in the mean number of hatchlings observed in "sieve A+B" and "do not-sieve" cultures, in containers which had 20 adults ($P = 0.9385$; 95% CI: -120.1 – 112.8; t = -0.082). Besides the containers with 20 adults, the mean difference in the number of hatchlings observed in all the other containers were statistically significant [(10 adults: $P = 0.0008$; 95% CI: 39.55 – 84.45; t = 7.04); (30 adults: $P = 0.0048$; 95% CI: 57.52 – 223.68; t= 3.94); (40 adults: $P = 0.0005$; 95% CI: 170.14 – 379.86; t = 6.25); (50 adults: $P < 0.0001$; 95% CI: 326.8 – 503.61; t = 11.68); (60 adults: $P = 0.0010$; 95% CI: 274.16 – 575.04; t = 7.46); (70 adults: $P = 0.0010$; 95% CI: 232.64 – 475.36; t = 7.90); (80 adults: $P = 0.0006$; 95% CI: 135.4 – 285.8; t = 6.96); (90 adults: $P = 0.0014$; 95% CI: 129.40 – 275.80; t = 7.59); (100 adults: $P = 0.0055$; 95% CI: 83.52; t = 4.87)].

A one-way ANOVA was used to test if the number of hatchlings were dissimilar amongst the different cultures according to number of adults. In other words: to check if

7.3 Results

7.3.1 Culture of *T. molitor*

From fig 7.2, it is noticeable that there were more *T. molitor* hatchlings in cultures that were sieved compared to those that were not, regardless of how many adults were present in each container.

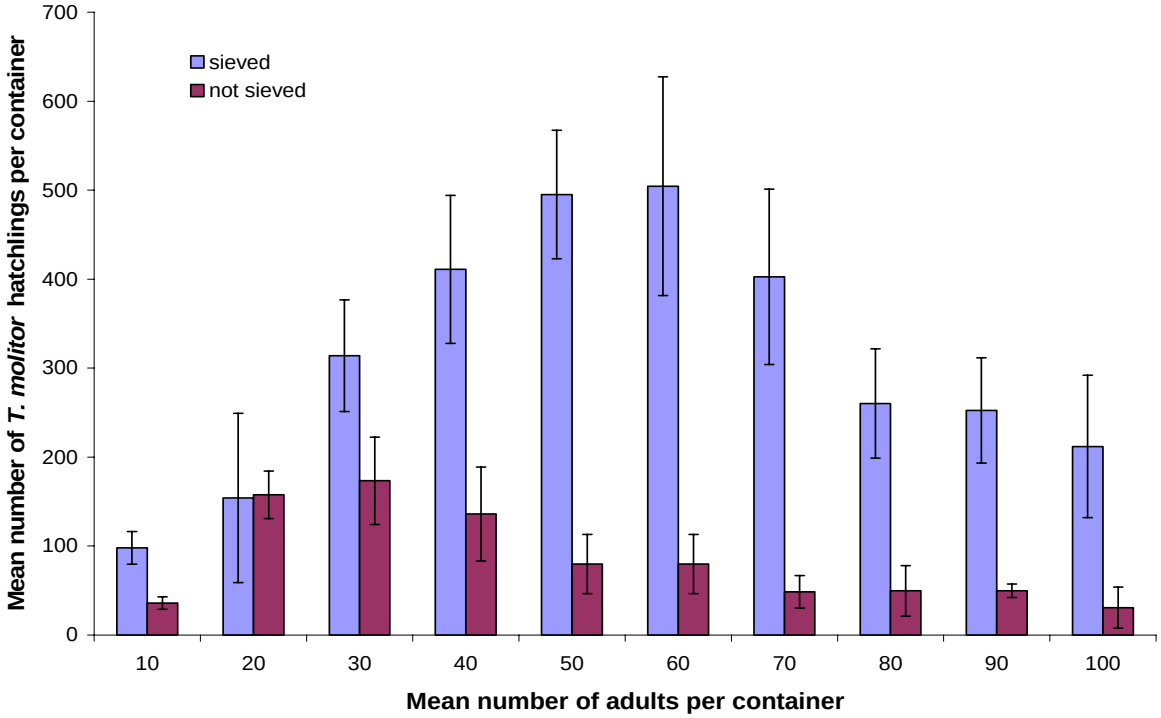


Fig 7.2 Mean number of *T. molitor* hatchlings counted in sieved and non-sieved cultures of *T. molitor*. (Bars are mean ± standard deviation, n = 10 containers).

These differences are more evident as the number of adults per container increase. Another obvious trend that is noticeable especially in the sieved cultures is that the numbers of hatchlings increased with the number of adults per container, but start to decrease with larger numbers of adults.

Table 7.1

Combinations of Rolling and Dipping Agents

Tested for Formulating *Heterorhabditis indica*-Infected *Tenebrio molitor*

Dipping Agent	Rolling Agent	Abbreviation	Result***
1% Instant FTD 176*	Clay	1 SC	pa
2.5% Instant FTD 176*	Clay	2.5 SC	a, r
5% Instant FTD 176*	Clay	5 SC	a, nr
7.5% Instant FTD 176*	Clay	7.5 SC	a, nr
1% Maizena**	Clay	1 MC	a, r
1% Maizena**	Instant FTD 176	1 MS	a, r
2.5% Maizena**	Clay	2.5 MC	a, r
1% Gelatine	Clay	1 GC	pa
2.5% Gelatine	Clay	2.5 GC	a, nr
2.5% Gelatine	Instant FTD 176	2.5 GS	a, nr
1% Cassava Starch	Clay	1 CSC	pa
2.5% Cassava Starch	Clay	2.5 CSC	a, nr

*Instant FTD 176 is a pregelatinised, modified waxy maize starch used for thickening soups, sauces, desserts and pie fillings

** Maizena is corn starch.

*** pa, poor adhesion to host surface; a, formulation adhered to host surface; nr, no reproduction (i.e. no infective juveniles emerged from cadavers); r, reproduction observed

7.2.4 Statistical Analysis

Statistical analysis was performed using Stata SE 9, Statacorp. Differences among treatments ("sieve A+B" and "do not sieve") in mean *T. molitor* count (in the insect experiment; and nematode reproduction (formulation experiment) were evaluated with the t test, ANOVA and Bonferroni's multiple comparison test ($P \leq 0.05$). Similar groups in the insect experiment were also compared with the two-sample t-test with unequal variances. All *P*-values are two-tailed, and means are provided with 1 standard error.

disintegration of the larval integument. The formulation method, taken from Shapiro-Ilan *et al.*, (2001), entailed immersion of nematode-infected cadavers in a liquid suspension ("dipping agent") for approximately 3 sec and then rolling of the cadaver in a powder until adhesion of the powder was maximized. The combination (dipping and rolling agents) was intended to provide structure and prevent rupture.

The results of the different formulation procedures and their suitability are given in Table 7.1 (with their associated abbreviations). Three of the formulations failed to adhere to either 4- or 8-day-old cadavers, thus were not investigated further (Table 7.1). The remaining 9 formulations were tested for their effects on nematode reproduction. Cadavers were formulated at 4 days post inoculation (PI) and 8 days PI and placed in White traps (section 2.3). Progeny IJs were then collected and, after emergence ceased (>15 days PI), the number of IJs produced per cadaver was determined through dilution counts according to the method of Glazer & Lewis (2000) as outlined section 2.5.2. There were 5 replicates (5 insects) per treatment, 10 treatments in all, including a non-formulated control. No progeny IJs emerged from the cadavers in 5 of the formulations, so were not investigated further (Table 7.1).

Fig 7.1 Formulated *T. molitor* larvae and pupae: A, dipped in 2.5% instant FTD 176 and rolled in clay; B, Dipped in 1% Maizena and rolled in instant FTD 176; C, Dipped in 2.5% gelatine and rolled in maizena.

with the bran in "sieve B" for each replicate, and then incubated further. During incubation, bran and apple slices were added to all containers ("sieve A + B" and "do not sieve") as needed. Counting commenced when the larvae were large/visible enough. Larvae were removed from the containers every 3 days and the counts recorded. This procedure was repeated till no more hatchlings were noticeable. The cultures were incubated for another fortnight, with adequate moisture to ensure that all eggs had hatched. All new hatchlings were included in the counts, after which the cultures were discarded.

7.2.2 *In vivo* production of *H. indica* in *T. molitor*

H. indica LN2 was cultured *in vivo* in *Tenebrio molitor* larvae according to the method of Kaya & Stock (1997) at 25 ^{o}C, (section 2.2). IJs were recovered using White traps (section 2.3). In all experiments, infected *T. molitor* cadavers were produced by the exposure of each insect weighing about 0.20 ± 0.05 g to approximately 50 IJs in 90-mm Petri dishes lined with filter paper (Whatman No. 1). Nematode infection, development and reproduction were carried out at approximately 100% relative humidity and 25 ^{o}C.

7.2.3 Screening of Formulations for Adhesion and Reproduction

After *in vivo* production of *H. indica* in *T. molitor* larvae and pupae, the influence of coating agents in promoting stability or longevity of IJs within the infected larval cadavers was evaluated. Twelve combinations of coating agent and modes of application were tested as procedures for encapsulating the larval cadaver. It was assumed that the encapsulation of the larval cadavers with a coating agent would achieve the following: prevent desiccation and

7.2 Materials and Methods

7.2.1 Culture of *T. molitor*

For the *in vivo* culture of *H. indica*, *T. molitor* was cultured in the laboratory as explained in section 2.1.2. Pupae were collected and sexed by the morphology of the eighth abdominal segment according to the method of Prof Byrne of the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg (pers. comm.) - (his method adapted from Bhattacharya *et al*., 1970). Batches of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 newly emerged adults were transferred into plastic containers (40 X 120 mm) filled with wheat bran and apple slices. The containers were then covered with perforated lids for aeration, and labelled accordingly. The male to female sex ratio per container was 50:50. There were 5 replicates per batch. Newly emerged adults were used so that all mating and egg-laying would initiate in the new culture environment. Three such sets of 50 (making 150 containers in total) were prepared, but no pupae or fruit slices were added to the third set up. The set of 50 containers without any insects was labelled "sieve B". One set of 50 containers with pupae was placed in a box labelled "sieve A". The other set of 50 containers with pupae was placed in another box labelled "do not sieve". All three boxes were kept at room temperature till the pupae had developed to adults. The bran and apple slices were replenished as consumed in the containers containing adult beetles.

The bran in the box labelled "sieve A" was passed through a tea sieve every 2 days into the corresponding containers in the third set ("sieve B"), so as to separate the eggs if any, from the adults. The adults were returned to their original containers of "sieve A". The sieving continued till all the adults in the containers from the box "sieve A" were dead. Dead adults from all containers were discarded. The remaining bran in "sieve A" was combined

commercialisation of nematode-infected cadavers has been prevented due to problems in storage and application. Shapiro-Ilan *et al*. (2001) explained that nematode-infected cadavers may stick together or rupture during transportation or application, which may result in reduced efficacy. Furthermore, delaying IJ emergence until time of application is a challenge (without reducing the number of IJs released). Hence, formulation of nematode infected cadavers may overcome these problems (Shapiro-Ilan *et al*., 2001).

In view of the afore-mentioned, *Heterorhabditis indica* and *Tenebrio molitor* were chosen as the "model" nematode and host respectively. *H. indica* is a versatile nematode and has been marketed against economically important pests such as grubs of *Holotricha serrata* and *Leucoppholis lepidophora* (Scarabeidae and Coleoptera respectively), two major soil pests of sugarcane in India (David & Ananthanarayana, 1986). *T. molitor* was chosen over *G. mellonella* mainly because they are cheaper and easier to produce on a large scale and are more widely available in South Africa; but also because like *G. mellonella*, they are very susceptible to EPN infection, produce high nematode yields and have shown no co-evolution with their EPN hosts thus far. Firstly, *T. molitor* was produced on a large scale for *in vivo* culture of *H. indica*. A study was done to determine if sieving bran cultures of *T. molitor* would improve its yield. Secondly, the feasibility of formulating the EPN-infected *T. molitor* cadavers was tested to overcome the hindrances of storage and handling; more specifically, to determine the effects of various formulations applied to cadavers on nematode fitness and reproduction. Additionally, the timing of formulation was investigated; i.e. the number of IJs produced by cadavers that were formulated 4 days after nematode inoculation was compared with cadavers formulated 8 days after nematode inoculation.

provides a suitable shelf life, stability of product from transport to application and ease of handling (Georgis *et al*. 1995). Increased shelf life, in most EPN formulations, is obtained by reducing nematode metabolism and immobilization, which may be accomplished through refrigeration and partial desiccation (Georgis, 1990; Georgis *et al*. 1995). However, the desiccation process can reduce nematode fitness and longevity (Womersley, 1990; Grewal, 2000), and the potential for using desiccation for long-term storage is limited because EPNs apparently cannot reach a true cryptobiotic (fully arrested metabolism) state upon desiccation (Womersley, 1990). Nonetheless, desiccation tolerance can be enhanced by preconditioning the nematodes (Solomon *et al.* 1999). A variety of formulations are available to facilitate nematode storage and application (Ehlers, 2001; Shapiro-Ilan & Gaugler, 2002; Hazir *et al,* 2003). These include activated charcoal, alginate and polyacrylamide gels, baits, clay, peat, polyurethane sponge, vermiculite and water disposable granules (Georgis, 1990; Georgis *et al.* 1995). The water disposable granule was cited as a breakthrough in formulation technology, in which Steinernematids enter a partially anhydrobiotic state allowing them to survive up to 6 months at 4 – 25 ^{o}C (substantially longer than previous formulations) (Georgis *et al*. 1995). Unfortunately, no formulations comparable to water disposable granules have been reported for heterorhabditids, which tend to be less desiccation tolerant then Steinernematids (Shapiro-Ilan & Gaugler, 2002). Albeit, studies have shown that EPNs may also be applied in infected insect cadavers (Creighton & Fassuliotis, 1985; Jansson *et al.,* 1993; Shapiro-Ilan *et al*., 2001). In this method, nematode-infected cadavers are dispersed and repression of insect pests is later accomplished by the progeny IJs that egress from the cadavers. In keeping with laboratory studies by Shapiro-Ilan & Glazer (1996) and Shapiro-Ilan & Lewis (1999), nematode application in infected hosts may be superior to application in aqueous suspension. But Koppenhöfer (2000) hypothesized that

Chapter 7

IN VIVO PRODUCTION AND FORMULATION OF ENTOMOPATHOGENIC NEMATODES

7.1 Introduction

In vivo mass production of EPNs is carried using the wax moth larva (*Galleria mellonella*) as a bioreactor system (Ehlers, 1996). This system takes advantage of the IJs natural migration away from the host cadaver upon emergence. Insects are exposed to EPNs on a dish lined with absorbent filter paper. Two to five days later, infected insects are transferred to White traps in order to harvest the emerging progeny IJs (Grewal *et al.* 1994). This method of EPN culture has been successfully accomplished at the cottage industry level. It is advantageous in that it requires a minimum initial investment, and depends on labour intensive processes involving very simple equipment (Friedman, 1990). However, this *in vivo* mass production system is not without limitation. The process is laborious and cannot achieve the economy of scale necessary for EPN mass production to be economically viable, especially in first world countries where labour is expensive. Nonetheless, the method is simple and reliable and it results in high quality nematodes (Shapiro-Ilan *et al.* 2001). Consequently, industrial-scale *in vivo* production may be applicable in developed countries, and some cottage industries in developed also use this technology (Hazir *et al*. 2003).

Commercially produced EPNS, regardless of culture method, must be formulated for delivery and application (Georgis, 1990; Georgis *et al*. 1995). An effective formulation

S. feltiae complete their development inside a host cadaver located in the unfavourably dry soil, they probably have no choice but to endure the cadaver for two reasons. First, if they manage to exit the cadaver, they are instantly exposed to the low moisture without time to adapt physiologically into a quiescence state. Under such rapid desiccation regimes, IJs survive only a few days or even hours (Kung *et al*., 1991). Second, the cuticle of the host dries out and hardens at a rate and to a degree that probably restricts the escape of the IJs from the cadaver until it is rehydrated (Koppenhöfer *et al*., 1997). By retaining moisture and functioning as a buffer, the host cadaver may serve as a means for nematode populations to persist through dehydration conditions (Koppenhöfer *et al*., 1997). It is not clear how commonly this occurs under field conditions where moisture and temperature conditions are more variable and different insect species serve as hosts. For short periods with insufficient moisture, staying inside the host cadaver could be an efficient mechanism for EPN survival (Koppenhöfer *et al*., 1997).

Although not tested in this study, it seems that anhydrobiosis has no negative effect on nematode infectivity (Koppenhöfer *et al*., 1997; Grewal, 2000), but, more importantly, that EPNs from desiccated host are more infectious than their control counterparts (Shapiro-Ilan & Lewis, 1999; Serwe-Rodriguez *et al*., 2004). However, the issue of infectivity must be considered separately from survival as virulence depends upon the synergistic effect of both nematode/bacterial factors. An increase in infectivity under conditions of host desiccation may be the result of the effect of anhydrobiotic cues on bacterial gene expression and/or EPN fitness (Serwe-Rodriguez *et al*., 2004). The ability to tolerate desiccation is important for the field application of EPN, in attaining effective control of target pests (O'Leary *et al*., 2001) hence, this question needs to be addressed in future studies.

desiccation than were IJs of *S. feltiae*, and they advocated that the slower rate of water loss which they observed in *H. megidis* was most likely die to the sheath (the retained second stage juvenile cuticle) which adheres more tightly to heterorhabditid than to steinernematid IJs (Menti *et al*., 1997).

As depicted by Fig 6.2, species and strains differences in EPN IJ desiccation tolerance do exist. Patel *et al*. (1997) found that IJs of *S. carpocapsae* were more tolerant to rapid desiccation and those of *S. feltiae*, *S. glaseri* or *S. riobrave*. Solomon *et al.* (1999) compared the desiccation tolerance of three species of *S. feltiae* and found one strain, IS-6, isolated from a desert region in Israel exhibited the highest desiccation tolerance. Data presented by O'Leary *et al*. (2001) indicated that species and strain differences in desiccation tolerance occur in heterorhabditid IJs too, as is the case with this study. In addition, the authors found that IJs of *H. megidis* and *H. bacteriophora* were more desiccation tolerant than those of *H. zealandica* and *H. indica*. This was not the case with this study. In contrast, *H. indica* showed the highest desiccation tolerance compared to the other heterorhabditids, while and *H. zealandica* showed desiccation tolerance comparable to *H. bacteriophora*. Perhaps these results should not be compared because the desiccation study by O'Leary *et al*. (2001) was conducted directly on the heterorhabditid IJs, whereas this study was conducted on EPN-infected cadavers. Once again, these differences may be attributable to the role of host on the desiccation tolerance of the EPNs in question.

It is not clear whether IJ persistence within the cadaver is an adaptation to low soil moisture conditions or whether the nematodes are simply trapped in the cadaver. IJs of certain EPNs can survive as individuals in the soil (Kung *et al.,* 1991). For example, IJs of the ambusher *S. carpocapsae* are better adapted to soil moisture conditions close to the soil surface. However, if IJs of a forager such as *H. bacteriophora* or an intermediate cruiser like

the authors concluded that desiccation was not a promising option for the long term storage of this nematode. Their findings may well negate the afore-mentioned explanation (that the persistence of *H. zealandica* in desiccating conditions may be attributable to its size). Notwithstanding, it is imperative for one to note that the study by Surrey & Wharton (1994) was carried out on free-living *H. zealandica* IJs, while this study examined IJs in their host cadavers. This observation supports the premise that the host cadaver can offer a protective environment for nematode development in sand (Perez *et al*., 2003) and under conditions of environmental stress (Lewis & Shapiro-Ilan, 2002), but more importantly, from the effects of desiccation (Koppenhöfer *et al*., 1997). In addition *H. zealandica*-infected cadavers along with the cadavers infected with the other Heterorhabditids, namely: *H. bacteriophora* and *H. indica*, had a gummous consistency; this may have contributed to the better moisture retention of these cadavers.

In accordance with results of other parts of this study, it was hypothesized that *S. feltiae*-infected cadavers would produce the highest number of IJs at all RH levels. Although this seems to be the case at higher RH levels, IJ emergence dropped rapidly as RH decreased. The experiments described in this chapter were carried out in spring, which caused the room temperature to increase almost steadily over the two-week experimental period. It seems that this unexpected drop in IJ emergence from *S. feltiae*-infected cadavers may be attributable to the nematode's poor desiccation survival at warm temperature (Grewal, 2000). *S. feltiae* is a temperate species (Hominick *et al*., 1996) that does not reproduce above 25 ^{o}C, with optimum reproduction occurring at 19 ^{o}C (Grewal *et al.,* 1994). Therefore, it is possible that the cold-adapted *S. feltiae* survives poorly under anhydrobiosis at warm temperatures. Another probable explanation for this observation could be that put forward by Menti *et al*. (1997). The authors established that IJs from two strains of *H. megidis* were more tolerant to

6.4 Discussion

This study showed that emergence of EPN IJs from host cadavers is influenced by RH; that IJs can survive for considerable lengths of time within desiccating host cadavers at RH levels as low as 70.4% and different EPN species are affected in different was by the drying conditions (Fig 6.2). There was a proportional decrease in numbers of IJs produced per host cadaver as RH levels dropped for all four EPN species (Fig 6.3 A-D).

There appears to be some correlation between persistence within cadavers and the ecology of the different EPN species. *H. indica* was originally isolated from a sugar cane plantation in Tamil, India where the climate is very hot, and the soils are more likely to dry out. *H. bacteriophora* was isolated from a dry sandy soil, obtained from an uncultivated farmland that was exposed directly to the heat of the sun, thus the nematode was more open to the elements of desiccation. *H. zealandica* though from a hot area, was isolated from a moist sandy soil under a mango tree. Certainly, sandy soils are more exposed to dangers of desiccation because of their little water retention capacity (Hara *et al*; Stock 1995; Strong *et al*., 1996), but the tree had shaded the nematodes from this danger. Moreover, *H. zealandica* is a forager and is found deeper in the soil. Thus in this case, one could insinuate that *H. zealandica* is heat tolerant but not necessarily as forbearing when it comes to it desiccation tolerance. *H. zealandica* was the largest nematode (in terms of IJ size) examined in this study. It is possible that water loss was reduced from its IJs, hence reducing its vulnerability to desiccation. In a study by Surrey & Wharton (1994), the desiccation survival of *H. zealandica* IJs was tested by exposure to various RH levels on membrane filters and agar plates. After a screening experiment indicating that *H. zealandica* survival would not be improved by adjusting the treatment temperature, the IJ source, or the method of rehydration,

were significantly different to RH treatments of 70.4, 73.8, 77.2, 80.2 and 82.9% ($P \leq 0.001$). The same was true for RH treatment of 95.7% when compared to RH set at 70.4, 73.8, 77.2 and 80.2% ($P \leq 0.016$); and RH 91.4% compared to 70.4, 73.8 and 77.2% ($P \leq 0.041$); as well as RH 87.6% compared with 70.4, 73.8, 77.2 and 80.2% ($P \leq 0.048$); along with RH treatment of 85.2% compared to 70.4 and 73.8% ($P \leq 0.005$). Also, the difference in the mean number of IJs produced by cadavers held at 82.9% RH was significant compared to the number IJs produced by cadavers held at RH of 70.4% ($P = 0.03$).

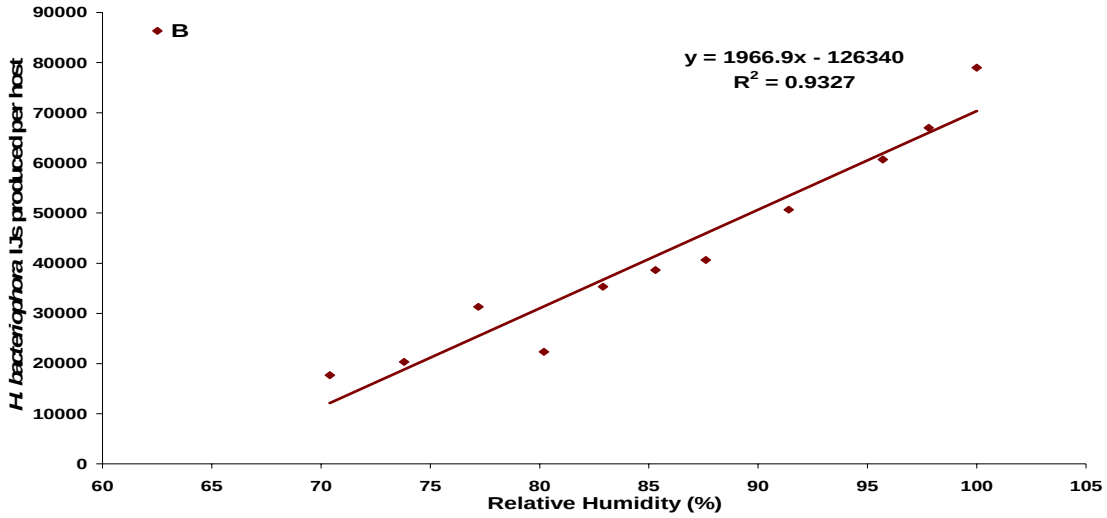
Following ANOVA, differences in the mean number of IJs produced per *S. feltiae*-infected cadaver were very highly significant ($F = 35.77$; $df = 10, 22$; $P < 0.0001$). After Bonferroni's multiple comparison test was performed post ANOVA, the differences RH treatment of 100% and all other treatments ($P \leq 0.001$), except RH treatments of 97.8 and 95.7%. The mean number of IJs produced by cadavers at RH 97.8% was significantly different to all other treatments, except RH 100% ($P < 0.001$). There were also significant differences in the mean number of IJs produced by cadavers held at RH 95.7% compared to the number of IJs produced by cadavers held at RH of 70.4, 73.8, 77.2, 80.2 and 82.9% ($P <0.001$). Similar significant differences in IJs produced per cadaver were perceptible between RH treatment of 91.4% compared with 70.4, 73.8, 77.2, 80.2 and 82.9% ($P \leq 0.004$); as well as between RH 87.6% and RH 70.4% ($P = 0.036$).

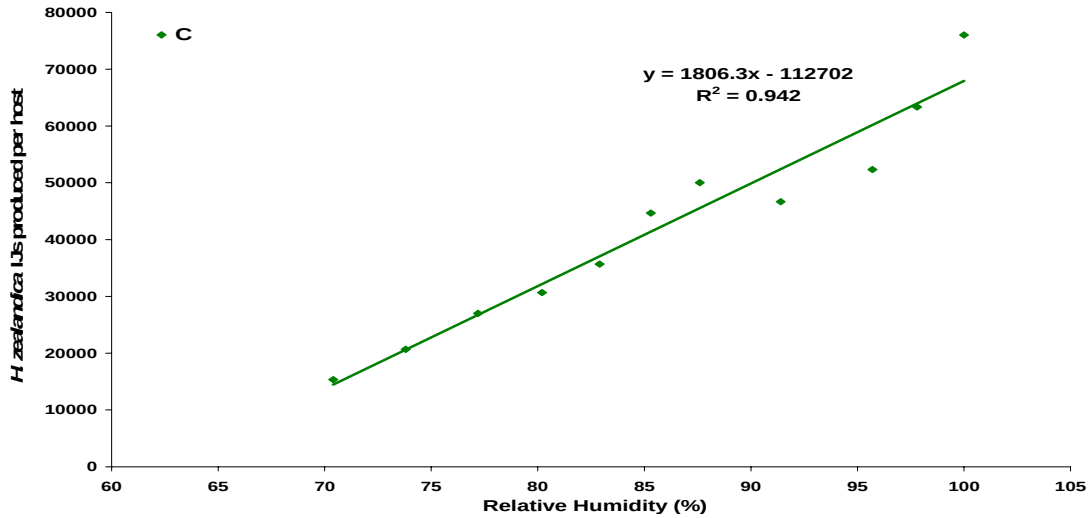
relative humidity treatments. A, *Heterorhabditis indica* strain LN2; B, *Heterorhabditis bacteriophora;* C, *Heterorhabditis zealandica;* D, *Steinernema feltiae* strain SN.

Data were subjected to ANOVA to compare the mean numbers of EPN IJs emerging from the cadavers within EPN species. The mean number of IJs that emerged from *H. bacteriophora*-infected larvae were significantly different among RH treatments ($F = 2.93$; $df = 10, 22$; $P = 0.0170$). When means were compared with Bonferroni's multiple comparison test post ANOVA, the only significant difference was between RH treatment of 70.4% and 100% ($P = 0.07$).

For *H. indica*-infected cadavers, the mean number of IJs produced were also significantly different amongst RH treatments ($F = 14.14$; $df = 10, 22$; $P < 0.0001$). Bonferroni's test, comparing means post ANOVA showed the significant differences were between treatment of 70.4% RH and 91.4, 95.7 and 97.8% RH ($P \leq 0.15$); RH set at 73.8% compared to RH at 95.7 and 97.8% ($P \leq 0.001$); as well as RH at 77.2% compared to RH of 91.4%, 95.7% and 97.8% ($P \leq 0.03$). Differences in the mean number of IJs produced were also noticeable at RH at 80.2% compared with RH at 91.4, 95.7 and 97.8% ($P \leq 0.204$); along with RH was 82.9% was different to the number produced at 97.8% ($P = 0.017$). In addition, there were differences in number of IJs produced between cadavers held at 100% RH when compared with those held at 70.4 up to 85.3% RH ($P \leq 0.38$).

Data of mean number of IJs produced by *H. zealandica*-infected host were subjected to ANOVA and the results showed that differences in the average number of IJs produced per cadaver were highly significant between RH treatments ($F = 26.69$; $df = 10, 22$; $P < 0.0001$). IJs produced by cadavers held at 100% RH were significantly different to all other RH treatments ($P \leq 0.006$), except RH 97.8%. Cadavers held at RH 97.8% produced IJs that





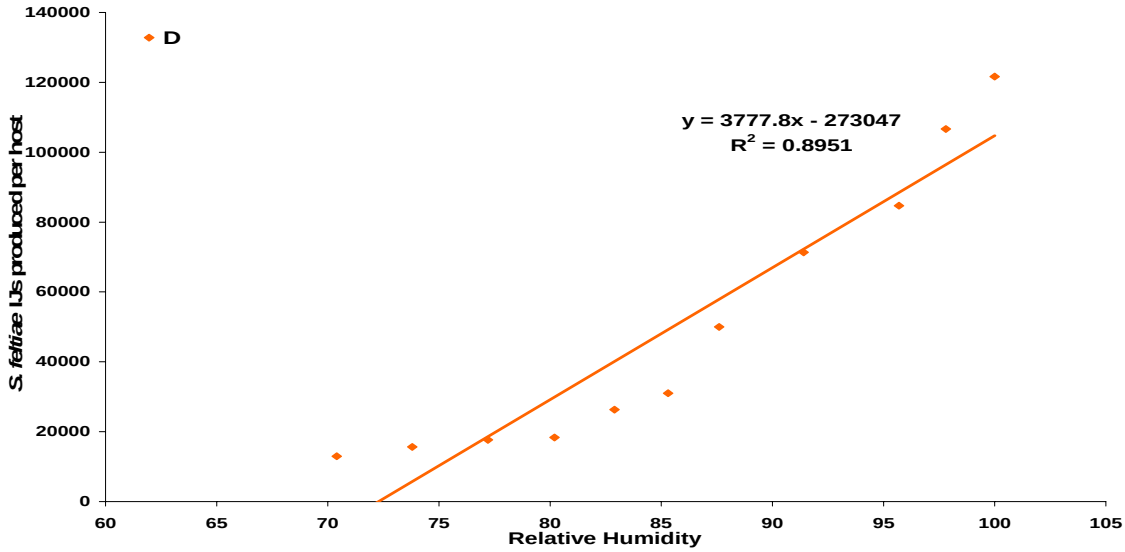


Fig 6.3: Correlation between infective juvenile yield per EPN-infected *T. molitor* cadaver and

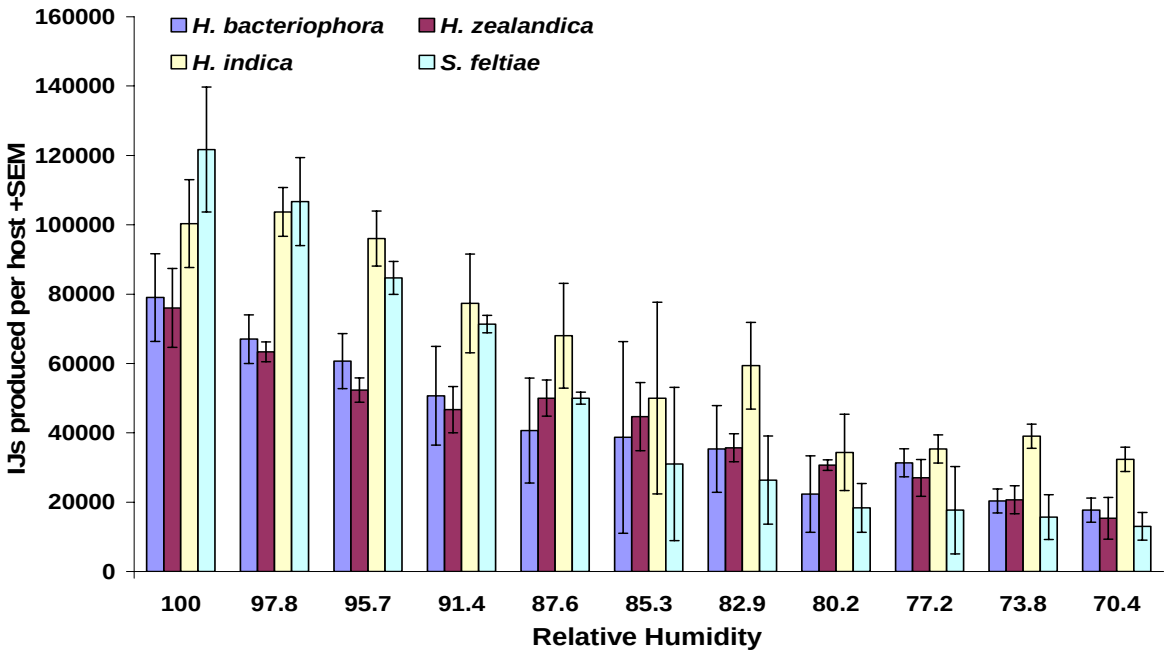
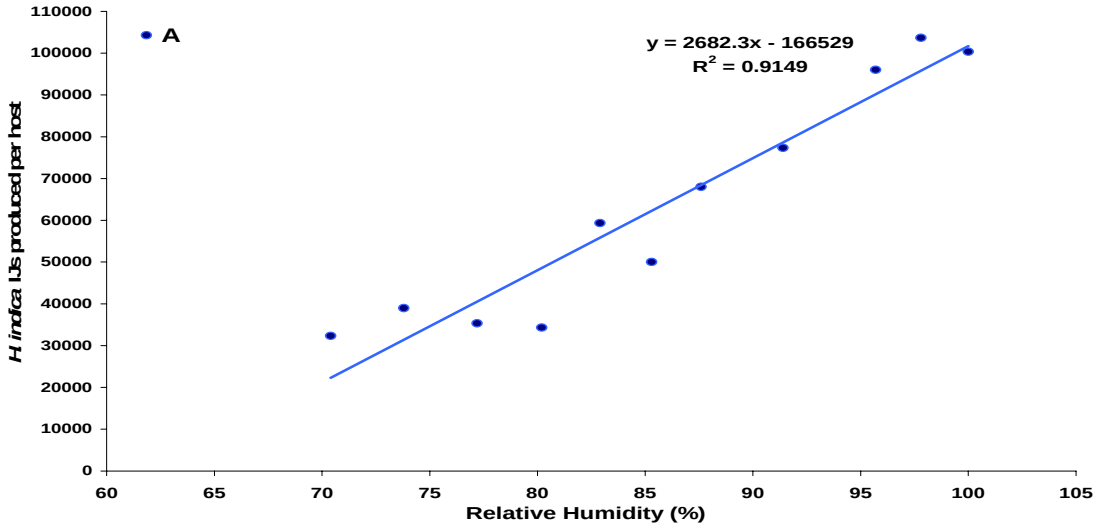


Fig 6.2: Mean infective juvenile production (±SEM) per EPN-infected cadaver subjected to different relative humidity levels.

The relationship between RH treatment and the number of IJs produced per cadaver is linear $P < 0.001$ for all EPNS; $r^2 = 0.97$, 0.94, 0.94 and 0.90 for *H. indica*, *H. bacteriophora*, *H. zealandica*, and *S. feltiae* respectively, (fig 6.3).



6.3 Results

Note:

1. By the days 10 and 12, IJs were observed exiting the *S. feltiae*-infected and *H. indica*-infected *T. molitor* cadavers respectively, which were held at ≤ 95.7% RH.
2. This experiment was carried out at room temperature over a two week period, at the beginning of spring. Room temperature increased steadily from 17 ± 1 ^{o}C at the start of the experiment, to 26 ± 1 ^{o}C at it conclusion, with small fluctuations in between of circa ± 2 ^{o}C.

Fig 6.2 illustrates the average number of IJs produced per host at the different RH for the four EPN species. (*H. indica*-infected cadavers produced more IJs compared to the other EPNs on the whole, except at RH ≥ 97.8% where *S. feltiae*-infected cadavers produced more IJs. However, the number of IJs produced by *S. feltiae* drastically reduced at lower RH ≤ 85.3 (Fig 6.2). The number of IJs produced per host infected by either *H. bacteriophora* or *H. zealandica* was similar across all RH (Fig 6.2).

Fig 6.1: EPN-infected *T. molitor* 3 days post infection; A, infected by *S. feltiae* strain SN, the insects change colour from golden yellow (ref D) to orange-yellow; B, infected by *H. indica* strain LN2, insects are brick red in colour; C, infected by *H. zealandica*, insects change colour to gray; D, (reference) uninfected – insects are still alive and healthy.

6.2.3 Statistical Analysis

Statistical analysis was performed using Stata SE 9, (Statacorp). The mean numbers of EPN IJs emerging from the cadavers were compared among RH treatments within species. Data was evaluated by analysis of variance (ANOVA) after checking for equal variances using Bartlett's test ($P \leq 0.05$). Means were compared by Bonferroni's multiple comparison test ($P \leq 0.05$).

6.2 Materials & Methods

6.2.1 Insects and Nematodes

Infective juveniles of four EPNs, namely: *H. indica* LN2, *H. bacteriophora, H. zealandica* and *S. feltiae*, were used to infect *Tenebrio molitor* larvae weighing about 0.20 ± 0.05 g, at a ratio of about 50 IJs/insect (section 2.2). Nematode infection was carried out at 25 ^{o}C, and approximately 100% RH. Three day old cadavers were then exposed to desiccating conditions. Three days was enough time to establish that host mortality was due to IJ infection. This was determined by a pale brown colour for *S. feltiae* SN, brick red for *H. indica* LN2 and *H. bacteriophora,* and greyish green for *H. zealandica*, indicating death due to bacterial sepsis (Fig 6.1).

6.2.2 Preparation of Relative Humidity Environments

The RH environments were prepared as explained in section 2.8.

environment for nematode development in sand (Perez *et al.*, 2003) and under conditions of environmental stress including freezing (Lewis & Shapiro-Ilan, 2002) and desiccation (Koppenhöfer *et al*., 1997). EPNs can survive dry conditions for extended periods if they remain inside a host cadaver (Koppenhöfer *et al*., 1997). For example, Shapiro-Ilan *et al*., (2001) found that *H. bacteriophora* survived at 0% RH in infected *G. mellonella* cadavers for at least 1 week, conditions that would be lethal to IJs outside a cadaver.

Interest in the promotion of EPNs as biocontrol agents for agricultural insect pests (Georgis & Manweiler, 1994) has provoked a number of studies on optimization of EPN inoculum and developmental conditions (Zervos *et al*., 1991). Desiccation (anhydrobiosis) is considered an important means of achieving storage stability of EPNS by decreasing nematode metabolism (Georgis *et al*., 1995). Given that EPNs are capable only of partial (quiescent) anhydrobiosis, earlier efforts on direct contact desiccation with the aid of absorbents such as clay to achieve storage stability have had limited success (Georgis *et al*., 1995; Grewal, 1998). If dehydrating EPN-infected cadavers do not adversely affect EPN development, reproduction and infectivity significantly, then the feasibility of achieving storage stability of EPNs by dehydrating EPN-infected cadavers for use against insect pest could be explored. Accordingly, this study examined the consequence of desiccation on the reproduction of four EPN species, by determining the number of IJs emerging from the EPN-infected host cadavers at different levels of RH.

megidis at 98% relative humidity for 4 days increased its survival at 57% RH (O' Leary *et al.,* 2001). At the initial slow rate of dehydration, the nematodes synthesize low molecular weight substances including tetrahalose and glycerol (O'Leary *et al.,* 2001; Womersley, 1990). These low molecular weight polyoles play a critical role in the survival of EPNs during dehydration and rehydration when they exit from anhydrobiosis (Womersley, 1990). Such gradual drying conditions are common in soil because the RH in the interstitial spaces stays close to 100% and drops drastically only in extremely dry conditions (Womersley, 1990). Soil desiccation is a gradual process and so may provide EPN IJs enough time to adapt physiologically into a partially immobilized quiescent anhydrobiotic state (Womersley, 1990).

When an insect host is infected in the soil by an EPN, development and reproduction within the cadaver may take between one and three weeks (Stock, 1995), during which the external environmental conditions may change drastically. EPNs are very frequently isolated from sandy to sandy loam soils, such as *H. bacteriophora* and *H. zealandica,* the two indigenous EPN species examined in this study. Rapid decline in soil moisture can occur in the upper soil layers, especially sandy soils with little water retention capacity (Hara *et al*., 1991; Stock 1995; Strong *et al.,* 1996). Moreover, the infected host may move to areas with low soil moisture or RH before succumbing to the infection. In such situations, the host cadaver may retain moisture better than the surrounding soil and protect the IJs from dehydration until the moisture conditions become conducive for IJ dispersal and host finding (Koppenhöfer *et al*., 1997). Indeed, it has been demonstrated that the host cadaver exerts a positive influence on EPN infectivity (Shapiro-Ilan & Lewis, 1999) and offers a possible vector for EPN application as biocontrol agents against agricultural pests (Shapiro-Ilan *et al.*, 2001). Previous "in host" studies indicate that the host cadaver can offer a protective

Chapter 6

THE ANHYDROBIOTIC POTENTIAL OF ENTOMOPATHOGENIC NEMATODES

6.1 Introduction

The sensitivity of IJs of Steinernematid and Heterorhabditid nematodes to desiccation reduces their efficacy in the field (Kaya & Gaugler, 1993), because low moisture adversely affects nematode activity and survival (Womersley, 1990). IJs exposed directly to rapid desiccation conditions in laboratory experiments under low relative humidity (RH) regimes generally do not survive (Womersley, 1990), yet, a considerable number of terrestrial nematode species can undergo anhydrobiosis to some degree for considerable lengths of time in dry soil (Kung *et al.,* 1991). Anhydrobiosis is a general term for a reversible physiologically arrested state of dormancy that results from the absence of water. True anhydrobiotes can lose up to 95-98% of their body water and while in anhydrobiosis, they have virtually no metabolism, thereby conserving energy (Barrett, 1991). Remarkably, a limited degree of dehydration resistance (i.e.) quiescent anhydrobiosis has been observed with EPNs (Womersley, 1990), when the dehydration process is gradual (Koppenhöfer *et al*., 1997). It seems that the pre-acclimation of EPNs at 97% RH subsequently enhances their desiccation tolerance at lower RH. For example, conditioning *Steinernema feltiae*, *S. carpocapsae* and *Heterorhabditis bacteriophora* at 97% RH for 3 days enhanced their survival at 94% RH (Solomon *et al.,* 1999; Womersley, 1990). Similarly, acclimation of *H.*

less effective as biological control agents. This could explain the low yields observed the cultures supplemented with cod liver and safflower oils.

egg yolk liquid culture medium with three different lipids, namely: canola oil, cod liver oil and safflower oil, on IJ yield. The results are presented in Figs 5.3 and 5.4, along with Table 5.2. IJ yields were highest in cultures supplemented with canola oil for both *H. bacteriophora* (Fig 5.6) and *H. zealandica* (Fig 5.4). Both *H. bacteriophora* and *H. zealandica* IJ yield increased almost constantly in the cultures supplemented with cod liver oil, but capriciously in those supplemented with safflower oil (Figs 5.3 and 5.4). In the absence of adequate nourishment, EPN stage-three juveniles develop into IJs, which stop feeding and survive on stored reserves, until they find and infect a new host. Accordingly, optimal physiological fitness of the non-feeding stage necessitates storage of adequate and balanced energy reserves. Contrary to the results of this study, Abu Hatab & Gaugler (2001) found that *H. bacteriophora* culture time, along with IJ yields were not significantly different in cultures supplemented with canola or cod liver oil. The discrepancy in results could be due to the different methods in which the nematodes were culture. The authors used a solid medium made from polyurethane foam infused with growth medium, but egg yolk medium was used in this study. Notwithstanding, Abu Hatab & Gaugler (2001) further reported that *H. bacteriophora* produced better quality IJs (they were lower in poly-unsaturated fat) when cultured in canola oil-supplemented medium compared to those cultured in cod liver or safflower oil-supplemented growth media. It seems that eukaryotes and prokaryotes can protect themselves against temperature extremes by changing their membrane lipid composition. In addition, an increase in the proportion of polyunsaturated fatty acid levels will result in increased membrane fluidity (Hakomori, 1986). Cod liver and safflower oil may increase EPN IJ membrane fluidity (Fodor *et al.,* 1994), which in turn may cause the IJs to be less active, at elevated temperatures (Abu Hatab & Gaugler, 2001). It is plausible that IJs that are less active would be slower in growth and reproduction, and may produce IJs that may be

was used for inoculating both solid and liquid cultures. However, during this experiment, the nutrient broth bacteria cultures used to inoculate the egg yolk media were prepared with bacterial colonies, which have variable sizes, hence resulting in a variable number of bacterial cells in the broths used to inoculate the liquid media. IJ recovery can be influenced by the bacteria pre-culture, (i.e.) the higher the bacteria density, the higher the food signal concentration (Strauch & Ehlers, 1998; Ehlers, 2001). It is possible that the food signal in cultures B20 and B25 were much higher than the others, due to higher bacterial densities. Furthermore, with similar parameters in the same medium, nematode yield can still vary considerably (Ehlers *et al*. 1998; Strauch & Ehlers, 2000). Ehlers (2001) noted that even in the presence of the bacterial food signal, only 18% up to at most 90% of all IJs recover within a period of several days once inoculated into media - hence, the source of unpredictable and unsynchronised IJ recovery *in vitro* cultures (Ehlers, 2001). Consequently, it becomes obvious that a major source for variability is the IJs themselves. It seems that the difference may be due to variable fat reserves of the IJ (Strauch & Ehlers, 1998; Jessen *et al*., 2000). The lower the energy reserves, the higher would be the predisposition of the IJ to recover (Ehlers, 2001). This could also explain why some cultures (B20, B25 and B30) still had adults present (results not shown) at the end of the culture period. In other words, those cultures had been growing for longer before being supplemented with bacteria. Therefore, there is the likelihood that the IJs were already depending on their fat reserves.

The nutritional composition of a medium especially the lipid content is an important component in nematode production and could determine the final yield (Friedman, 1990). Since the highest nematode yields are produced *in vivo* (Abu Hatab *et al.* 1998), fatty acid composition of a medium that resembles the host composition should improve IJ yields produced *in vitro*. The third experiment aimed to explore the outcome of supplementing the

media lack any kind of food signal that could trigger recovery. Fortunately, the symbiotic bacteria produce such food signals and they therefore enable the production of EPN *in vitro* (Ehlers, 2001). This however, was not the case in this study. A likely explanation for this finding is that the IJs inoculated in to the medium were not completely bacteria-free. The IJ inoculum had been cultured on monoxenic lipid and or egg yolk agar plates. Although the IJs were washed in sterile distilled water, the inoculum of symbiotic bacteria IJs carry in their gut was still present at the time of inoculation. It is plausible that the IJs released the bacteria in to the culture medium. The bacteria would then have proliferated. The food signal, which induces IJ recovery, originates from the presence of the symbiotic bacteria. The intensity of the food signal increases with increasing bacterial density, and consequently improves nematode yield (Strauch & Ehlers, 1998). Since the bacterial density was low in the axenic cultures, it is a possible reason for the low *S. feltiae* IJ yields.

The advent of artificial media incorporating the nematodes' symbiotic bacteria (House *et al.* 1965; Bedding, 1981; Wouts 1981) was a major step toward commercial production of these nematodes for insect control. The second experiment investigated the result of adding symbiotic bacteria to culture medium at different times. Compared to the control, the results (Fig 5.5) indicate that the bacteria added to the culture medium post nematode inoculation had in fact improved the IJ yield. But, the increase in IJ yield was more noticeable towards days 20 and 25. Jessen *et al*. (2000) used IJ inocula from different cultures and observed a high variation between the different batches. This has probably not affected the result of this investigation as the IJ inoculum, for the entire experiment, originated from one batch of nematode culture. Nevertheless, this was not the case with the symbiotic bacteria inoculum. Throughout this project, stock cultures of bacteria were prepared and frozen in 1.5 ml Eppendorf tubes (section 2.10); which were used to inoculate the nutrient broth that in turn

The results of the experiments which sought to determine the effect of bacteria IJ recovery, provide evidence that the EPN symbiotic bacteria are not only significant, but are also crucial to IJ recovery and nematode growth and development. *H. bacteriophora* did not produce any offspring in axenic culture. Live IJs were also observed in the axenic cultures of *S. feltiae*, with a few adults (results not shown). It is plausible that the adults could have come from the nematode inoculum (section 5.2.1). Nonetheless, nematode counting prior to inoculation had been performed under a microscope at 40X magnification; there were hardly any adults in the inoculum, or if any, the numbers were negligible. This finding suggests that the IJs had indeed recovered. The increase in mean *S. feltiae* IJ yield in the axenic cultures (Fig 5.4), though minor, is of significance. The cultures, both axenic and monoxenic, had been inoculated with 2500 IJs/ml of egg yolk medium (see methods, section 5.2.2.1). The highest mean IJ yield in the axenic cultures that was 4900 IJs/ml was recorded on day 30 (Fig 5.4). This result indicates that reproduction had indeed occurred and the IJs counted were progeny from the adults. It is probable that the IJ yield would have increased if counts had been done after 30 days. Research performed by Poinar & Thomas (1966) revealed that bacteria-free *S. carpocapsae* IJs were able to penetrate and kill axenic *G. mellonella.* Also, the IJs recovered and developed to adults. Their findings are in accordance with the results of this experiment. Furthermore, the authors discovered that the *S. carpocapsae* adults were only capable of reproducing once the symbiont (*X. nematophilus*) was added Thomas and Poinar, 1966). This was not the case with *S. feltiae,* the EPN examined in this study. In fact, as previously mentioned, reproduction still transpired even in the absence of its symbiont. The results are in agreement with those of Han & Ehlers (2000), who reported that steinernematids produced a limited number of offspring in bacteria-free insects. Nonetheless, 100% of IJ recover within 2 day after having entered the haemocoel of an insect, liquid

22	0.0002	0.5427786	0.768781	16.1125
25	0.0106	0.1529034	0.6363994	4.5325
28	<0.0001	0.5174805	0.5983477	38.3102
30	0.0111	0.1775366	0.7623081	4.4623
35	0.0044	0.3094305	0.8796373	5.7898
Cod liver oil				
4	0.0002	0.4832766	0.754976	12.6535
10	0.0132	0.3326444	1.592368	4.2427
12	0.0017	0.8654632	1.883157	7.4987
14	0.0003	1.35348	2.164985	12.0379
16	0.0005	1.273232	2.199755	10.4072
18	0.0001	1.443039	2.142534	14.2319
20	<0.0001	1.263337	1.704554	18.6760
22	0.0002	1.092993	1.723689	12.3996
25	<0.0001	1.078742	1.241674	39.5412
28	<0.0001	0.8287177	0.9629878	37.0490
30	0.0028	0.3497181	0.8675557	6.5266
35	0.0050	0.3774341	1.116916	5.6107
Safflower oil				
4	0.5507	-0.1757071	0.1089849	-0.6507
10	0.0020	0.3024064	0.6840133	7.1769
12	0.0015	0.4883504	1.041395	7.6798
14	0.0006	0.7123144	1.258229	10.0219
16	0.0025	0.4340026	1.039472	0.0025
18	0.0335	0.0329137	0.4841902	3.1814
20	0.2376	-0.4645407	0.1549545	-1.3875
22	0.8362	-0.1610178	0.1373171	-0.2206
25	0.4541	-0.1404017	0.2597566	0.4541
28	0.9932	-0.2433755	0.2418013	0.9932
30	0.2733	-0.4183443	0.1559185	-1.2688
35	0.3848	-0.222219	0.1067246	0.3848

5.4 Discussion

The key to improving *in vitro* liquid culture of EPNs is a reproducible and high IJ recovery (Ehlers, 2001). Yet, major problems related to EPN liquid culture remain unsolved. Two key factors that affect the outcome, namely: the symbiotic bacteria and type of lipid used in the growth medium were investigated in this study.

For *H. bacteriophora*, ANOVA revealed a significant difference (P = 0.0017; df = 2, 105; F = 6.81). The difference was found between IJ yields in medium supplemented with cod liver oil and that supplemented with canola oil (P = 0.001). In the case of *H. zealandica*, no significant differences were observed in the IJ yield from the media supplemented with different lipids (P = 0.9833; df = 2, 105; F = 0.02).

An unpaired two-sample t-test assuming equal variances was used to compare the mean *H. bacteriophora* IJ yield to that of *H. zealandica* in the different lipid-supplemented cultures. Data was combined across all days for this comparison. In the cultures supplemented with canola oil and those with cod liver oil, the difference was significant. [(canola: P = 0.0016; df = 70; 95% CI = 0.2730, 1.1216; t = 3.2775); (cod liver: P <0.001; df = 70; 95% CI = 0.6658, 1.7589; t = 4.4237)]. But, this was not the case with safflower oil (P = 0.3334; df = 70; 95% CI = -0.2539, 0.7387; t = 0.9740)].

Comparing data for *H. bacteriophora* and *H. zealandica* irrespective of culture time (days) revealed a lot of differences. The t-test was used to locate the differences on particular days for each lipid. The results are presented in Table 5.2 below.

Table 5.2: T-test results comparing IJ yield in liquid culture medium supplemented with either canola oil, cod liver oil or safflower oil (n= 3, df = 4).

Day	***P*-value**	**95% CI**		**t**
Canola oil				
4	0.0147	0.1353611	0.6989109	4.1102
10	0.0201	0.1489572	1.007225	3.7402
12	0.0132	0.1432366	0.6850162	4.2445
14	0.0004	1.137392	1.900861	11.0490
16	0.0005	1.273232	2.199755	10.4072
18	0.0001	1.443039	2.142534	14.2319
20	0.0017	0.3617283	0.7923626	7.4408

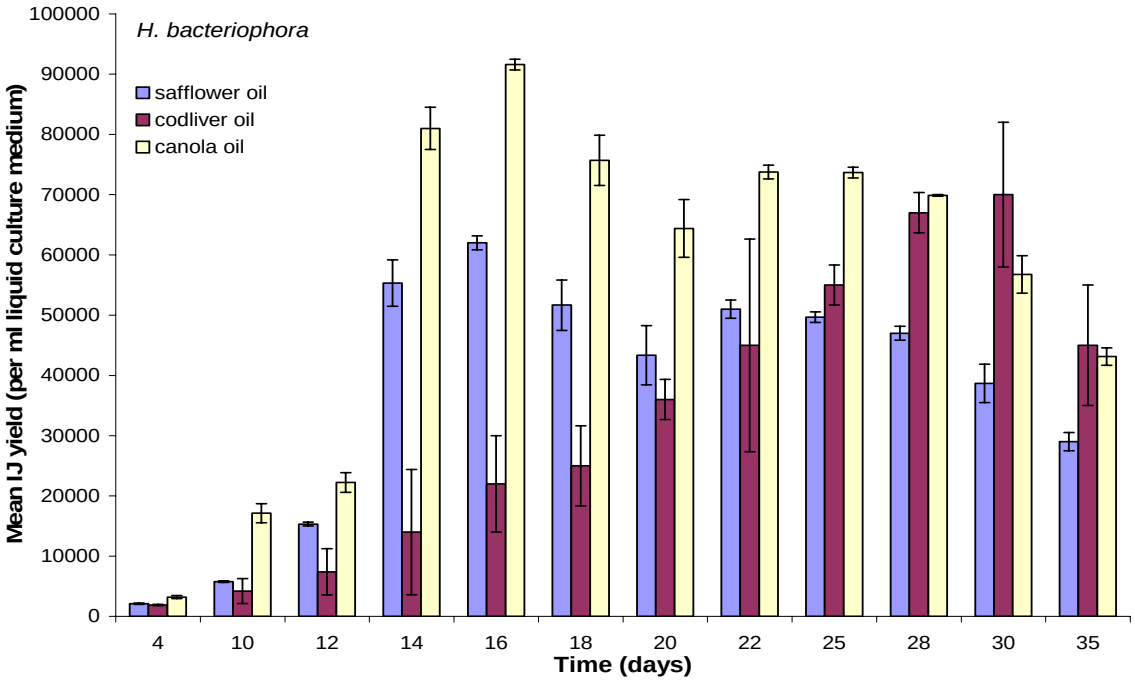


Fig 5.6: Mean *H. bacteriophora* IJ yield in egg yolk liquid medium supplemented with different lipids. Each value (represented by a bar) is a mean of 5 means from 3 experiments, n = 3 (N=9). Error bars are ± standard error of the mean.

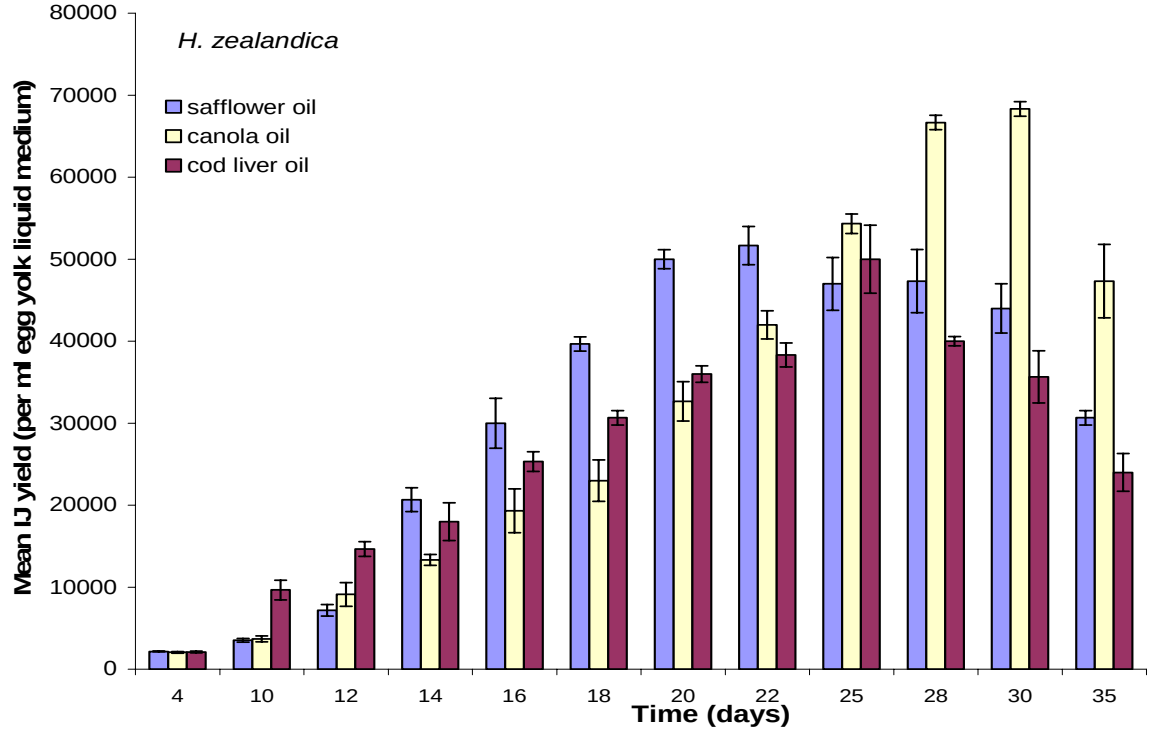


Fig 5.7: Mean *H. zealandica* IJ yield in egg yolk liquid medium supplemented with different lipids. Each value (represented by a bar) is a mean of 5 means from 3 experiments. n = 3 (N=9). Error bars ± are standard error of the mean.

5.3.2 Lipid composition

The outcome of supplementing the egg yolk agar growth medium with canola, cod liver and safflower oils on *H. bacteriophora* and *H. zealandica* IJ yield was examined. The results of this experiment are presented in Fig 5.6 (*H. bacteriophora*) and Fig 5.4 (*H. zealandica*) below. Overall, the IJ yields increased over time and then started to decline towards day 35. Both *H. bacteriophora* and *H. zealandica* IJ yield increased almost constantly in the cultures supplemented with cod liver oil (Figs 5.3 and 5.4). However, the IJ yield was highest in the cultures supplemented with canola oil for *H. bacteriophora* (Fig 5.6), and *H. zealandica* (Fig 5.4). Over all, *H. bacteriophora* cultures had higher IJ yields (Fig 5.6).

A one-way ANOVA was used to compare differences in *H. bacteriophora* and *H. zealandica* IJ yields, both within and between cultures supplemented with the 3 lipids in query. Prior to ANOVA, Bartlett's test was used to test if the data to be compared in ANOVA had equal variances, but they did not. So, the data were log transformed, as it was the only transformation that gave a normal distribution, and the *P*-values ($P = 0.344$ for *H. bacteriophora*; $P = 0.249$ for *H. zealandica*; after data were log transformed) were not significant.

The significant differences in IJ yield were separated by a post ANOVA test (Bonferroni's multiple comparison test). The differences observed on day 15 were between B10 and B15; plus B10 and B25; along with B10 and B30 (P = 0.015; 0.0304 and 0.0304 respectively).

Those observed on day 20 were between the control and B10 (P = 0.030); B10 and B15; plus B10 and B25; as well as between B10 and B30 (P = 0.011; P = 0.002; and P <0.001 respectively). Also, there was a significant difference between B20 and B30 (P = 0.046).

The significant differences detected on day 30 were between the control and B10, B15, B20 and B25 (P <0.001, P = 0.001, P = 0.005 and P <0.001, in that order). There were also differences between B10 and B30 (P <0.001); B15 and B30 (P <0.001); B20 and B30 (P = 0.002); as well as B25 and B30 (P <0.001).

Day 35 differences in mean IJ yield were spotted between the control, B10, B15, B20, B25 and B30 (P = 0.007, P = 0.020, P <0.001, P <0.001 and P = 0.033, correspondingly). Moreover, differences were spotted between B10, B20 and B25; between B15, B20 and B25; also between B20 and B30; as well as B25 and B30. All the aforementioned differences had a P-value <0.001.

Day 40 was also not without significant differences. In fact, the highest number of significant differences in IJ yield amongst the different cultures was spotted on this day. Besides the control and B10, B10 and B15, along with B20 and B25, which were not significantly different from each other (P >0.05), all the other pairs were (P <0.05).

variances were transformed before being used in the comparisons. ANOVA revealed no significant differences in mean IJ yield between cultures on days 2, 5 and 10 [(day 2: P = 0.2036; df = 5, 12; F = 1.72); (P = 0.4755; df = 5, 12; F = 0.97); (day 10: P = 0.1028; df = 5, 12; F = 2.37)]. There were significant differences between the mean *H. indica* IJ yield on days 15, 20, 25 and 30. [(day 15: P = 0.0065; df = 5, 12; F = 5.67); (day 20: P = 0.005; df = 5, 12; F = 10.26); (day 30: P <0.001; df = 5, 12; F = 30.65); (day 35: P < 0.001; df = 5, 12; F = 74.13); (day 40: P <0.001; df = 5, 12; F = 129.33)].

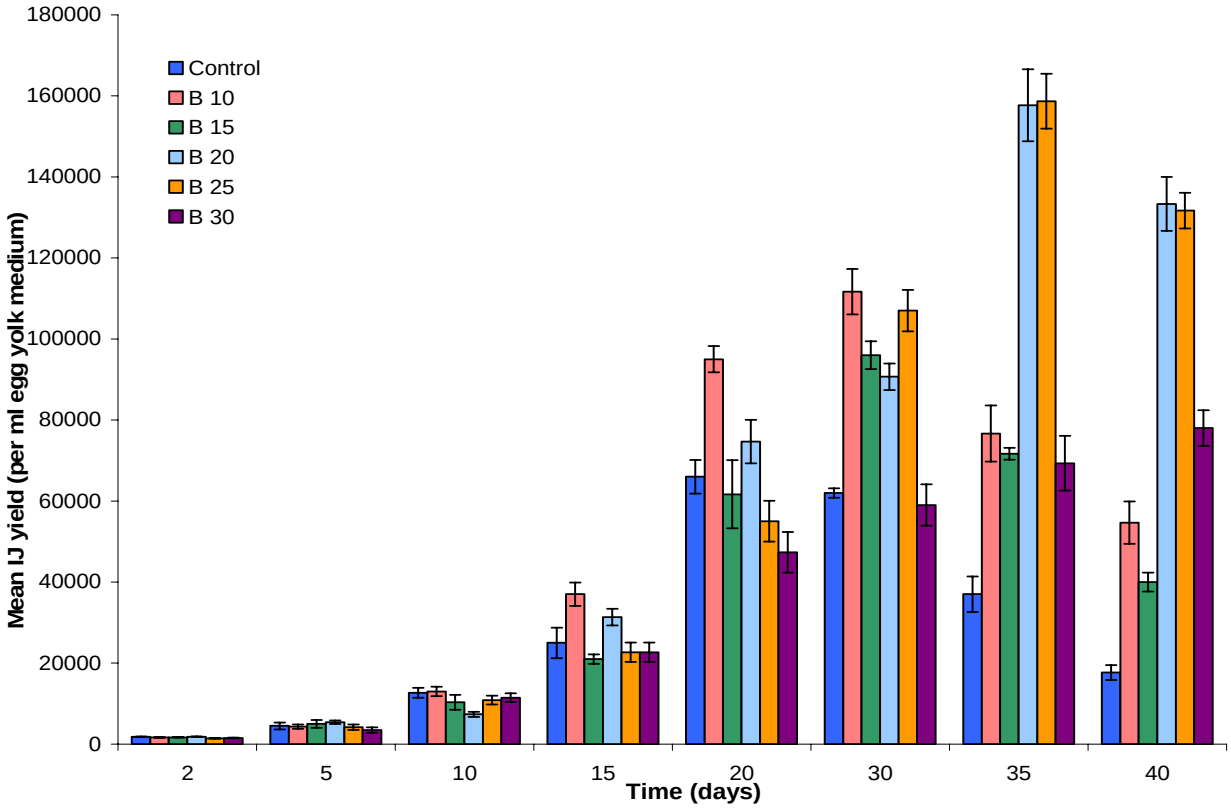


Fig 5.5: *H. indica* IJ yields per ml of egg yolk medium on different days, following the addition of bacteria at specific times. Data are means 15 counts (5 counts per replicate). Bars are ± standard error of the mean. On the key, B means the bacterium added, and the numbers that follow represent the days on which the bacterium were added.

5	0.0033	-19021.71	-7378.286	-6.2952
6	0.0019	-21246.21	-9487.126	-7.2565
7	0.0027	-21996.86	-9003.145	-6.6240
8	0.0005	-23018.73	13381.27	-10.4864
9	0.0002	-22407.44	-14859.23	-13.7077
10	0.0005	-23900.98	-13832.35	-10.4050
20	0.0020	-96737.9	-42728.77	-7.1696
25	0.0001	-123501.8	-84698.19	-14.8970
30	<0.001	-150340.4	-113259.6	-19.7372

5.3.1.3 Influence of adding symbiotic bacteria during culture time on IJ yield.

The aim of this experiment was to determine if adding bacteria at different times into monoxenic cultures of *H. indica* would improve the IJ yield. As described in the methods (section 5.2.2.2), IJs were counted on days 2, 5, 10, 15, 20, 30, 35 and 40, following inoculation with nematodes. From Fig 5.5 below, the IJ yield was almost identical in all cultures up to day 10.

Compared to the other cultures, those that received another inoculum of bacteria on 'B 10' had a higher IJ yield on day 15, and a considerable increase in IJ yield on days 20 and 30, before the yields began to decline. Although the IJ yields are not similar, this trend is also noticeable with the other cultures, especially with the 'B20' and the 'B25' cultures. It is worth mentioning that culture B30 had a lot of adult stage nematodes present on days 35 and 40. In fact, majority of the nematodes present on day 40 in the B30 culture were in the adults (results not shown).

A one-way ANOVA was used to compare the mean IJ yield in the different cultures, both between and within, on days 2, 5, 10, 15, 20, 30, 35 and 40. However, before the ANOVA, Bartlett's test for equality of variances was performed. Data with unequal

A two-sample paired t-test was used to compare IJ yield on the different days (Table 5.1). At a significance level of 5%, no significant differences were observed in the mean IJ yield between monoxenic and axenic cultures on day 2. As early as day 3, till day 30, significant differences were apparent. These differences, although significant, were minor in the early days. However, the differences were highly significant from day 8 up until day 30.

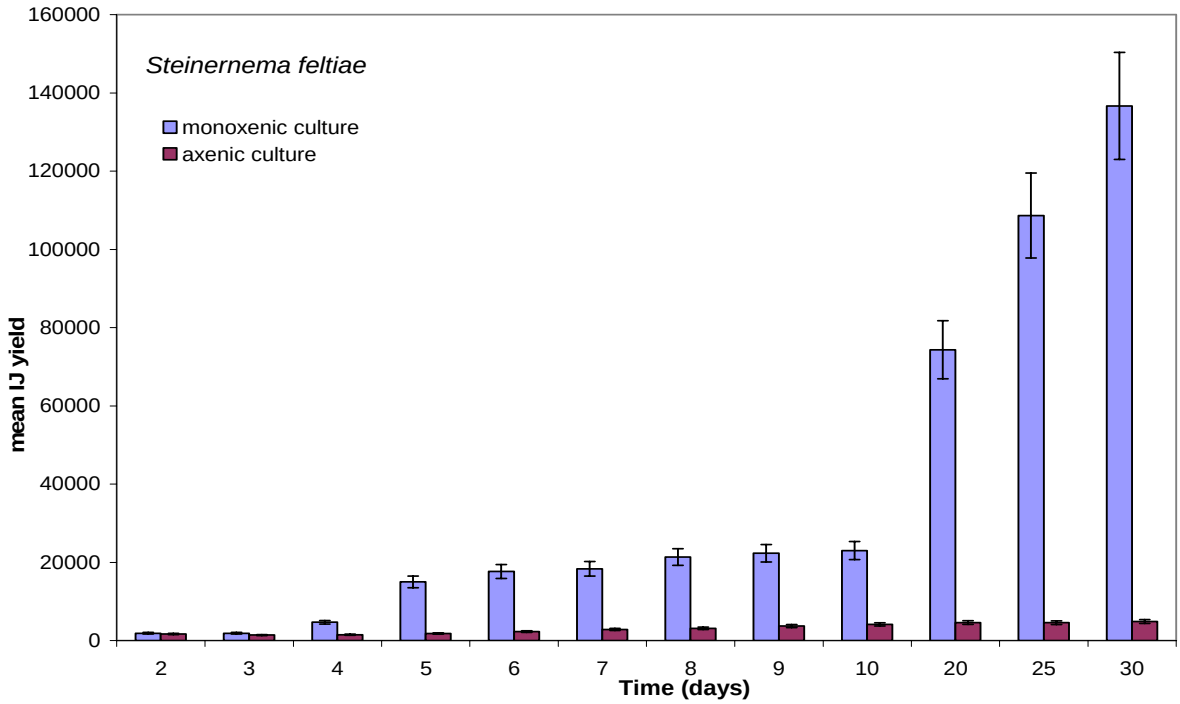


Fig 5.4: Mean *S. feltiae* IJ yield per ml egg yolk in medium (monoxenic and axenic cultures). Data are means of 15 counts (i.e. 5 counts per replicate). Error bars are ± standard error of the mean.

Table 5.1: t-tests results of IJ yields in monoxenic and axenic cultures of *S. feltiae*

Day	*P*-value	95 % CI		T
2	0.6920	-1245.955	912.6212	-0.4287
3	0.1050	-759.3297	-174.0037	-4.4272
4	0.0114	-5105.766	-1227.567	-4.5341

red cultures, *H. indica* & *P. luminescens arkhurstii*; Creamish brown cultures, *S. feltiae* & *X. bovienii*. (NB: photo does not show *H. zealandica* & *P. luminescens* cultures)

5.3.1.2 The influence of symbiotic bacteria on IJ recovery, growth and reproduction.

The experiment sought to determine the implication of the EPN symbiotic bacteria on IJ recovery, growth and reproduction. *H. bacteriophora* (symbiotic bacteria: *P. luminescens laumondii*) and *S. feltiae* (symbiotic bacteria: *X. bovienii*) were used in the experiment.

The *H. bacteriophora* IJs used to inoculate the axenic cultures did not recover. By the day 5, following inoculation of the growth medium with IJs, all the inoculated IJs were dead and had disintegrated; thus no growth or reproduction occurred. Conversely, there was recovery, growth and reproduction in the monoxenic culture. Since the aim of the experiment was to compare IJ yield in monoxenic and axenic cultures, the results of this experiment for *H. bacteriophora* are not shown. *H. bacteriophora* was cultured monoxenically in the same way lipid composition experiment (results in section 5.3.2).

Recovery, growth and reproduction were observed in the monoxenic cultures of *S. feltiae*. Live IJs were also observed in the axenic cultures, with a few adults. However, the IJ numbers remained very low and almost constant. In the monoxenic cultures, the IJ yield was stable, but started to increase gradually till day 10. The numbers had more than doubled by day 20 and continued to increase. These results are presented in Fig 5.4. It is worth mentioning here that the results presented represent only the IJ counts on the specified days. There were a lot of adults observed in the growth, especially from day 5, up until day 10. By day 30, there were hardly any adults left in the growth medium.

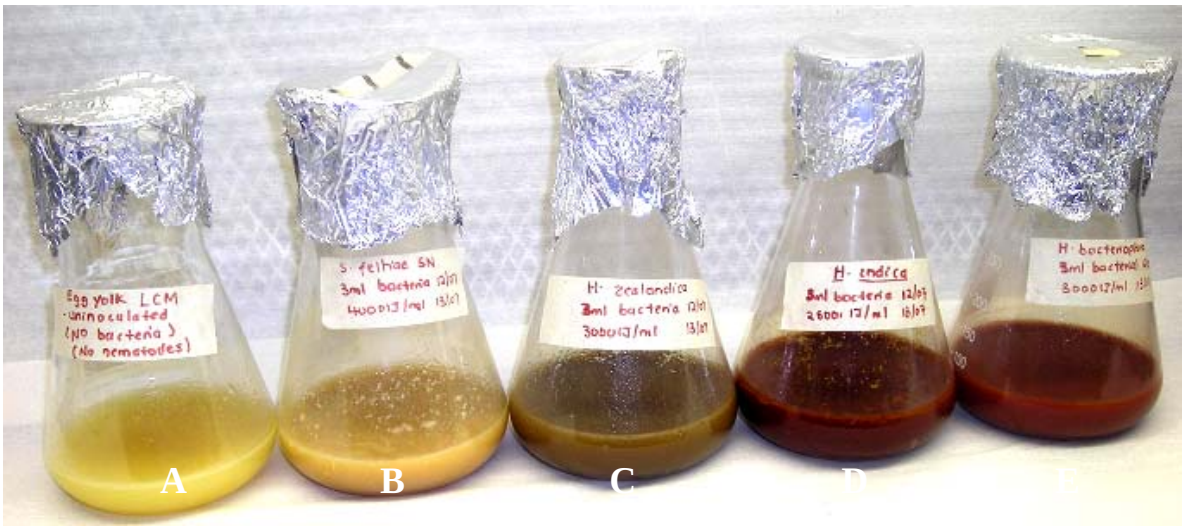


Fig 5.2: Shake flasks containing monoxenic liquid cultures of EPNs. Flasks B to D had been inoculated with a 24 h symbiotic bacterium and incubated on a shaker for 24 h prior to inoculation with nematodes at different IJ densities. The different colours are attributable to the symbiotic bacteria. All cultures were 5 days old when photo was taken. A: uninoculated egg yolk medium, B: *S. feltiae* (symbiont: *X. bovienni*), C: *H. zealandica* (symbiont: *P. luminescens sp*), D: *H. indica* (symbiont: *P. luminescens arkhurstii*), E: *H. bacteriophora* (symbiont: *P. luminescens laumondii*).

Fig 5.3: EPNs in shake flasks on a rotating platform shaker. The intense colors are due to the symbiotic bacteria. Deep pink cultures, *H. bacteriophora* & *P. luminescens laumondii*; Brick

5.3 Results

5.3.1 Experiments with the symbiotic bacteria

5.3.1.1 Culture of bacterial symbionts

The following figs show the nematode symbiotic bacteria growing in lipid and egg yolk agar (fig 5.1) and in shake flasks (figs 5.2 & 5.3).

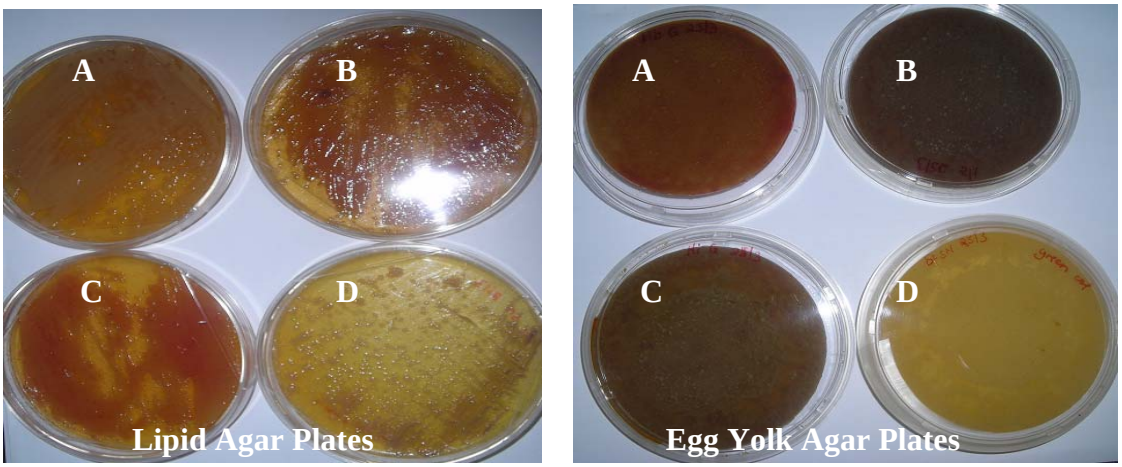


Fig 5.1: EPN symbiotic bacteria growing in a lawn on lipid and egg yolk agar plates. The different colours are characteristic of the bacteria. A: *P. luminescens laumondii*, (isolated from *H. bacteriophora*); B: *P. luminescens sp* (isolated from *H. zealandica*); C: *P. luminescens arkhustii* (isolated from *H. indica*); D: *X. bovienii* (isolated from *S. feltiae*).

(prepared as in section 5.2.1) were aseptically inoculated into the shake flasks at a density of 4000 IJs/ml for both nematode isolates. The flasks were then replaced on the shakers set at 150 rpm and 25 ^{o}C. On days 4, 10, 12, 14, 16, 18, 20, 22, 25, 30 and 35, five 50 µl aliquots were aseptically drawn from the flasks for counting.

5.2.4 Counting

For all experiments, the IJ yield was estimated by counting (section 2.5.2) under a microscope at 40 X magnification. The count for each flask on a specific day is the mean of 5 replicates with the mean counts of five 50 µl aliquots from each of the three flasks.

5.2.5 Data Analysis

STATA SE 9, (Statacorp) was the statistical program used to analyse all data. IJ yield was compared using either the T-test (paired or unpaired) if 2 groups were being compared or a one-way ANOVA (if more 3 or more groups were being compared). However, Bartlett's test for equality of variances was performed prior to ANOVA. Data with unequal variances were transformed. The statistical analysis included control data. Differences in mean IJ yield were separated post ANOVA using Bonferroni's multiple comparison test. The level of significance was set at 5%.

akhurstii), which had been cultured in nutrient broth. The flasks were incubated on a platform shaker set at 180 rpm and 28 ^{o}C for 24 h. All flasks were inoculated with *H. indica* IJs at a density of 2300 IJs/ml (as described in section 5.2.1). The flasks were replaced on the shaker now set at 150 rpm and 25 ^{o}C. Ten days following inoculation with nematodes, the 3 flasks labelled 'day 10' were supplemented with 5 ml of 24 h *P. luminescens arkhustii* culture, which had been had in egg yolk medium. All other flasks received 5 ml of bacteria free egg yolk medium. The same procedure was repeated on days 15, 20, 25 and 30. Thus the control only had one inoculation of the symbiotic bacterium (day 1) while all the other sets of flasks had received 2. On days 2, 5, 10, 15, 20, 25, 30 and 35, following inoculation of nematodes into the shake flasks, five 50 µl aliquots were drawn from each flask for counting.

5.2.3 Lipid composition

The two indigenous nematode species were used in this experiment, namely: *H. bacteriophora* (symbiotic bacterium: *P. luminescens laumondii*) and *H. zealandica* (symbiotic bacterium: *P. luminescens sp.*). Three lipids, namely: canola oil, cod liver oil and safflower oil were used in the investigations. The egg yolk liquid culture medium was prepared as outlined in section 2.6.2 but without any oil. Two millilitres of each lipid was added per shake flasks. There were 18 flasks in total. Of the 18 flasks, 6 were supplemented with canola oil, 6 with cod liver oil, and 6 with safflower oil, before autoclaving. Three flasks with each lipid were aseptically inoculated with 3 ml of 24 h symbiotic bacteria (cultured in nutrient broth) of either *P. luminescens* subsp. *laumondii* or *P. luminescens sp*., such that there were 9 flasks per symbiont. All 18 flasks were labelled accordingly. The bacteria were allowed to multiply on a shaker set at 180 rpm and 28 ^{o}C for 24 h. Nematode suspensions

5.2.2 Experiments with the Symbiotic Bacteria

5.2.2.1 Influence of EPN symbiotic bacteria on IJ 'recovery' and reproduction.

The first experiment was to determine the implication of the EPN symbiont in IJ 'recovery', growth and reproduction. *H. bacteriophora* (symbiotic bacteria: *P. luminescens laumondii*) and *S. feltiae* (symbiotic bacteria: *X. bovienii*) were used in the experiment. There were 6 flasks per nematode, and were labelled accordingly. Three of the 6 flasks were pre-seeded with 3 ml of 24 h their symbiotic bacteria, which had been cultured in nutrient broth. These flasks were labelled 'monoxenic'. The other 3 flasks contained no bacteria so they were labelled 'axenic'. The bacteria were allowed to multiply in the egg yolk medium on a platform shaker (Labcon No 3081U) set at 180 rpm and 28 ^{o}C for 24 h. All the flasks were inoculated with nematode suspension, at a density of 2500 IJs/ml (as described in the preceding paragraph). The flasks were incubated on the same shaker but the temperature was reduced to 25 ^{o}C and the speed to 150 rpm. On day 2, five 50 µl aliquots were aseptically drawn from each flask for counting (section 2.5.2). The procedure was repeated on day 3 through to the day 10 day, as well as on days 20, 25 and 30.

5.2.2.2 The effect of adding symbiotic bacteria during EPN culturing on IJ yield.

The second experiment was to determine if adding bacteria at different times into monoxenic cultures of *H. indica* would improve the IJ yield. Six sets of three, making a total of eighteen shake flasks were used in this experiment. The first set of 3 was labelled control; the second: day 10; the third: day 15; the fourth: day 20; the fifth: day 25; and the sixth set: day 30. All flasks were inoculated with 3 ml of 24 h the *H. indica* symbiotic bacterium (*P. luminescens*

5.2 Materials & Methods

5.2.1 Overview

The nematodes were cultured on lipid agar and egg yolk agar (methods outlined in section 2.6.1). The plates were pre-seeded with 1 ml of 24 h symbiotic bacteria which had been cultured in nutrient broth. The plates were then incubated at 28 ^{o}C for 48 h. After the 48 h incubation period, a bacterial lawn had become established on the plates. The plates were then inoculated with surface-sterilised IJs (section 2.4.1.1) and incubated in the dark at 25 ^{o}C. After 10 - 15 days, the majority of both the lipid agar and egg yolk agar plates consisted of progeny IJs (Fig 5.1). However, IJs from 14 - 20 day old lipid or egg yolk agar plates were used as inoculum for submerged liquid-medium shake-flask culture experiments.

The IJs were obtained by rinsing out the agar plates with sterile distilled water into sterile 50 ml plastic tissue culture tubes. When the IJs had sedimented to the bottom of the tubes, excess liquid was removed using sterile disposable 3.0 ml plastic Pasteur pipettes. All these were done under a laminar flow. The volume of the remaining nematode suspension was recorded. IJ density was estimated by counting (section 2.5.2) under a microscope at 40X magnification. The volume of the nematode suspension was adjusted as to give an IJ density of at least 100,000 IJs/ml up to 200,000 IJs per ml. This was done so that when the shake flasks were inoculated with 1 ml aliquots of nematode suspension, the final inoculum density would be at least 2000 IJs/ml up to 4000 IJs/ml of liquid medium. The liquid culture medium used in this study was the egg yolk medium (method in section 2.6.2). Fifty millilitre aliquots of the medium were transferred to 250 ml Erlenmeyer flasks.

H. bacteriophora and *S. feltiae*. Furthermore, the significance of the symbiotic bacteria in the EPN diet, if it improved *H. indica* IJ yield in liquid culture was also investigated. Actually, *H. indica* is the EPN to have thus far produced the highest number of IJs in liquid culture, reaching maximum average yields of 450,000 IJs/ml (Ehlers *et al.* 2000). To explore the potential of upgrading *H. indica* IJ yields in liquid culture, its symbiotic bacterium (*Photorhabdus luminescens* subsp. *akhurstii*) was added at specific times into the liquid culture medium after inoculation with the nematode.

5.1.2 Lipid Sources

Media composition, particularly lipid content, which is important in determining nematode survival and virulence, is critical to predicting nematode yield (Friedman, 1990) and quality (Abu Hatab & Gaugler, 2001; Yoo *et al*., 2000). Lipids have received greater attention than any other nematode nutritional component (Selvan *et al*. 1993; Abu Hatab & Gaugler, 1999, 2001), because 60% of the total energy from the non-feeding IJs is derived from metabolising lipids (Selvan *et al.* 1993). Towards understanding the influence of dietary lipids on the 2 indigenous nematode species, namely: *H. bacteriophora* and *H. zealandica*, the nematode IJ yields, produced in liquid culture media supplemented with different plant lipids were compared. In fact, such studies have already been carried out on *H. indica* (e.g.) (Ehlers *et al*, 2000) and *S. feltiae* (e.g.) (Lunau *et al*, 1993).

5.1.1 Symbiotic Bacteria

In vitro production of nematodes on solid and liquid media has been successfully accomplished with both *Steinernema* and *Heterorhabditis* species (Ehlers, 2001; Shapiro-Ilan & Gaugler, 2002). As documented by Ehlers (2001), Glaser R.W. was the first to attempt *in vitro* production of an EPN (*Steinernema glaseri*) on a large scale in 1931. Back then, the essential dependency of the EPN on its bacterial symbiont was not yet known, hence, efforts to continuously carry out production were unsuccessful. For instance, EPNs were first grown *in vitro,* on a solid medium axenically (in the absence of the symbiotic bacteria) (Ehlers, 2001). In 1940, Glaser (Ehlers, 2001) and subsequently, Stoll (1952) successfully cultured *Steinernema* spp. in axenic culture but, the low yields and high cost of production made it difficult to exploit the system as a potential method for EPN mass production (Ehlers, 2001). Many years elapsed before House *et al.* (1965), realised that nematode growth and yield increased with the presence of symbiotic bacteria. According to Ehlers (2001), *X. bovienii* was the first observed bacteria inside the IJ of *Steinernema sp.* In 1937; but it was Poinar & Thomas (1966) who described the significance of the symbiotic bacteria *Xenorhabdus nematophilus* in the successful reproduction of *Steinernema carpocapsae*. The symbiotic bacteria in monoxenic cultures produced suitable conditions for nematode reproduction with high numbers of offspring (Ehlers, 2001). Consequently, monoxenicity has since been the basis for *in vitro* culture of EPNs in solid and liquid media, since only the presence of the symbiotic bacterium in monoxenic cultures produces suitable conditions for nematode reproduction with high numbers of offspring (Ehlers, 2001).

The significance of the symbiotic bacteria in the EPN diet, in inducing recovery and reproduction in liquid culture were explored in this study. The nematodes investigated were

equivalent amount of *S. carpocapsae in vivo* (Gaugler & Han, 2002). Nevertheless, liquid culture of EPNs has been difficult to exploit commercially because of unstable IJ yields and prolonged process time (Ehlers, 1996). The main reason for unstable IJ yields from liquid culture is the unpredictable, unsynchronized and low recovery of the inoculated IJs. Low IJ recovery results in low yields and makes necessary, additional scale up steps. In addition, unpredictable recovery prevents the population management that is necessary for maximising yields and decreasing of the process time (Ehlers *et al*. 1998). Consequently, the key to improving the process technology of EPNs is a reproducible and high IJ recovery.

Friedman (1990) argued that mass production using *in vitro* solid culture methods is less likely to produce higher quality nematodes, when compared to the *in vivo* culture method. His argument was validated by Yang *et al.* (1997), who reported reduced quality in *Steinernema carpocapsae* produced in solid culture compared to those produced *in vivo*. On the other hand, Shapiro-Ilan & Gaugler (2002) argued that, no reports have substantiated this claim. Instead, several studies (Abu Hatab & Gaugler, 1998; Abu Hatab & Gaugler, 1999; Gaugler & Georgis, 1991) indicated that *in vitro* solid culture produced nematode quality was similar to nematodes produced by *in vivo*. These positive results were the motivation for the studies examined in this section. The expectation was to obtain *in vitro* liquid culture produced nematodes of quality comparable to those produced *in vivo*. Notwithstanding, the key factors for successful liquid culture of EPNs are media composition (particularly lipid content), symbiotic bacteria, and adequate oxygen (Ehlers, 2001; Gaugler & Han, 2002). Two of these factors namely: the symbiotic bacteria and type of lipid used in the growth medium, were investigated in this study.

developing hermaphrodites, the number of offspring, and the proportion of juveniles which develop to males and females define the final yield of liquid culture (Ehlers, 2001). Consequently, optimisation of *Heterorhabditis* spp. in liquid culture depends largely on the first generation in which all the nematodes are capable of reproducing by selfing (Strauch *et al.* 1994). In contrast, *Steinernema* spp. although amphimictic, are capable of mating in liquid culture. Accordingly, maximizing encounters between males and females of this genus (*Steinernema*) is achievable through bioreactor design and regulation of aeration (Strauch *et al*. 1994).

Culture times which can vary depending on culture medium and specie may be as long as 3 weeks (Surrey & Davies, 1996; Chavarria-Hernandez & de la Torre, 2001); although many species reach maximum production in 2 weeks or less (Friedman, 1990; Ehlers *et al.* 2000; Strauch & Ehlers, 2000; Yoo *et al.* 2000; Neves *et al.* 2001). Other factors that affect EPN nematode yield in liquid culture include nematode inoculum size and species effects (Shapiro-Ilan & Gaugler, 2002). Han (1996) reported optimal *Heterorhabditis bacteriophora* yields with intermediate inoculum sizes, whereas a positive relationship inoculum size and yield exists for *S. carpocapsae*. Ehlers *et al*. (2000), found no effect of inoculum size on *H. indica* yields, which thus far, is the EPN to have produced the highest number of IJs in liquid culture, reaching maximum average yields of 450,000 IJs/ml. Maximum yields reported for other species include 300,000 IJs/ml and 320,000 IJs/ml for *H. bacteriophora* and *S. carpocapsae* respectively (Han, 1996); 138,000 IJs/ml for *H. megidis* (Strauch & Ehlers, 2000), and 71740 IJs/ml for *S. feltiae* (Chavarria-Hernandez & de la Torre, 2001).

In vitro liquid culture is the most cost efficient production method (Ehlers, 2001). For instance, the cost of producing 1 million *Steinernema carpocapsae* in a bioreactor can be as low as US$0.012. This is one-tenth the cost of *G. mellonella* alone required to produce an

easier and more economical than scale-up and separation from solid media (Surrey & Davies, 1996). The development of low-cost liquid culture technology made possible the large-scale outdoor application of these nematodes for insect biocontrol (Ehlers, 1996; Grewal & Georgis, 1998). The liquid fermentation technology methods have economies of scale because the proportion of labour and capital costs decreased in scale as operating costs increased. Consequently, this technology still has the lowest mass-production costs (Hazir *et al.* 2003).

After strain collections, liquid culture media are pre-incubated with the symbiotic bacterium prior to inoculation of the IJs (Ehlers *et al*. 1998; Ehlers *et al*. 2000; Ehlers, 2001; Han & Ehlers, 2001; Strauch & Ehlers, 1998). On a laboratory scale, the liquid cultures are usually contained in shake flasks (Ehlers, 2001). Such flask cultures have been scaled up to 10L, then to 150L and afterwards to 3000L internal loop bioreactors (Ehlers, 2001). The IJs were harvested 12-15 days later, with a separator. The nematode paste was cleaned by passage through centrifugal sifters and formulated in clay, after which they are stored and/or marketed (Ehlers, 2001; Hazir *et al*., 2003). Strategies for maximizing yield in liquid culture for *Steinernema* spp. and *Heterorhabditis* spp diverge due to differing life cycles and reproductive biology (Johnigk & Ehlers, 1999). *Heterorhabditis* spp. are exclusively hermaphrodites in the first generation but subsequent generations can contain amphimictic forms (i.e.) males and females, which cannot mate in liquid culture due to their Y-type copulation behaviour (Strauch *et al.* 1994; Ehlers 2001). Nonetheless, liquid culture is still the preferred method for large scale production of *Heterorhabditis* spp. (Friedman, 1990; Surrey & Davies, 1996; Ehlers, 1996; Ehlers *et al.* 1998). IJs harvested from liquid cultures mainly originate from intrauterine development in the parental hermaphrodites, a process called *endotokia matricida* (Johnnigk & Ehlers, 1999). The IJ recovery, the size of the

establishment of facilities are well underway (Kaya *et al*. 2006). Importing nematodes on a large scale from an industrialised country's mercantile is an option but this will be far too expensive (Bedding, 1990). Consequently, for the implementation of EPNs to be cost effective in South Africa, they must be produced locally. Having established a sound basis from which to proceed, making provision for this means of biocontrol using EPNs in normal agricultural practices would bring several benefits to South Africa.

A significant breakthrough in nematode biotechnology was the development of *in vitro* culture systems on solid media, which initiated their commercial use on in high-value crops, like ornamentals, plants and mushrooms (Strauch & Ehlers, 1998). The 3-dimensional solid media method, first described by Bedding (1981), used crumbed polyether polyurethane foam coated with a nutritive medium. This medium was inoculated first with the symbiotic bacteria, followed the nematodes. This method was expanded later for large scale nematode production (Bedding, 1984). Although labour intensive, it has been the main method of production for some of the commercial producers of nematodes (Friedman, 1990). Accordingly, i*n vitro* solid culture offers an intermediate level of technology and costs compared to *in vivo* and *in vitro* liquid culture. Nonetheless, in the last two decades, nematode production in solid culture has not grown substantially and appears to be used by only two companies (one in the US and the other in China) (Gaugler & Han, 2002). Solid culture may only offer substantial advantages in cost efficiency relative to *in vivo* production but only with sophisticated mechanisation that is less capital-intensive (Gaugler & Han, 2002). Additionally, limited expertise is required, and the logistics of production are flexible (Hazir *et al*. 2003).

The liquid culture of nematodes had been actively researched since the 1980s (Ehlers, 2001). Furthermore, scale-up and separation of nematodes from liquid media was much

Chapter 5

IN VITRO CULTURE OF ENTOMOPATHOGENIC NEMATODES

5.1 Introduction

Entomopathogenic nematology is becoming increasingly commercialised and therefore competitive (e.g.), in the USA, Europe, Asia and Australia (Kaya *et al*. 2006). When field tests and grower trials establish that one or more insect pests can be economically controlled with nematodes, provision is made for introducing this means of control into normal agricultural practices (Bedding, 1990). South Africa has several insect pests that are difficult to control with insecticides (e.g.) the root weevils, sciarids, wood borers, fungus gnats, scarabs, mole crickets…just to name a few, which attack commodities like berries, citrus, mushrooms, ornamentals, turf (etc). These pests have been targeted in other countries using EPNs, with satisfactory results (Appendix II; Bedding & Miller, 1981; Ehlers, 1990; Parkman *et al*., 1993a, 1993b, 1994; Gaugler, 1998).

South Africa has the climatic and humidity conditions conducive to biocontrol of insect pests using EPNs. Moreover, South African based farmers often have a close relationship with their carefully and intensively tended land (Taylor, 2002), making the introduction of EPNs as a biocontrol strategy more feasible. All vegetable and fruit farmers as well as maize, wheat, sugarcane, cotton and groundnut farmers in South Africa can benefit from EPN-based bioinsecticides. Commercial golf course greens-keepers are also a potential market. Thus far, South Africa does not have any EPN producers, but apparently,

environmental factors such as temperature, aeration and moisture could also explain the differences in yield. Generally, optimum culture temperature is related to the nematode's climate of origin (Molyneux, 1986; Grewal *et al*. 1994), and would improve the EPN yield, (e.g.) Grewal *et al*. (1994). Adequate aeration is necessary for nematode development (Friedman, 1990). Moisture level (i.e.) high humidity levels must be maintained throughout the production cycle (Woodring & Kaya, 1988); in the White trap, the substrate must remain moist to prevent cadaver desiccation and allow emerging IJs to migrate, yet too much water will prevent movement and interfere with oxygen exchange (Shapiro-Ilan & Gaugler, 2002).

not find a relationship between penetration rates and mortality. Nevertheless, the penetration ability is an important characteristic for EPNs. High penetration rates would increase the infection rates, which in turn increase toxins produced by developing nematodes (Burman, 1982) and their symbiotic bacteria (Akhurst & Boemare, 1990) and so will enhance septicaemia and kill the insect host more rapidly. Moreover, as previously mentioned above, differences in number of bacteria per IJ as well as behavioural differences between bacteria such as the dynamics of the release of toxins by the symbiont may further account for differences in virulence between EPN species (Gotz *et al.* 1981; Burman, 1982; Akhurst, 1986, Dunphy & Webster, 1986; Ehlers *et al*. 1997; Gerritsen *et al.* 1993, 1997).

The second part of the dose-response bioassay sort to determine if there were differences in the number of progeny IJs emerged from insects exposed to different doses of IJs.; but no differences were observed in the number of emerged IJs at all doses for each nematode species. Apparently, *in vivo* production yields are dependent on nematode dosage (Zervos *et al*. 1991; Boff *et al*. 2000). As was the case with Flanders *et al*. (1996) significant effects of nematode density was not detected in this study. When EPN species were compared, differences were noted in the number of emerged progeny IJs, regardless of the IJ dose used. Shapiro-Ilan & Gaugler (2002) argued that the observations made by Flanders *et al.* (1996), may have been due to a limited range of densities tested, or to the peculiarity of the particular nematode strains that were tested, which is a plausible explanation for the finding in this study. In addition, higher reproductive potential of one nematode relative to another may result from a closer association to the host of its relatives (Shapiro-Ilan *et al*. 1999; Elawad *et al*. 2001). Furthermore, Leite *et al*. (2002) argued that one of the factors that influence reproduction of a nematode within the host is the nematode size, with larger individuals occupying more space, consequently, producing fewer offspring. Likewise,

secondly because this bioassay has been performed on *H. indica* and *S. feltiae* by other researchers (e.g.) (Hazir *et al,* 2001; Leite *et al*. 2002; Phan *et al*, 2005). It seems that the number of nematodes that invade a host is proportional to the exposure concentration (Selvan, *et al*. 1993; Shapiro-Ilan & Lewis, 1999). A dosage that is too low results in low host mortality (this concurred with the results presented in Fig 4.6), while a high dosage often results in a high level of failed infections (this was not the case in this study) due to competition with secondary invaders (Woodring & Kaya, 1988). Differences in mortality were apparent 24 h and 48 h after IJs had been applied; the differences were mostly between the very low and high IJ doses such as 5 and 500 IJs/insect (Fig 4.5 a & b). However, no differences were noted when mortality data was compared between IJ doses at both 72 h and 96 h following IJ application to the insects (Fig 4.5 d & e). These findings suggest that mortality increases with the number of IJs that penetrate an insect host; more IJs probably penetrated the hosts in cases were higher IJ doses were applied per insect, thus the higher mortality trend observed as early as 24 h (Fig 4.5 a). In order to validate this argument, a penetration rate assay would have been appropriate to measure the nematodes ability to penetrate in the insect hosts, but since it is used interchangeably with the one-on-one bioassay (Ricci *et al*, 1996), it was deemed unnecessary in this case. Still, the penetration rate assay would have been suitable for *S. feltiae*, which penetrates in higher numbers (Ricci *et al*., 1996). Conversely, when mortality data in the dose response assay (Fig 4.5 a-e) is compared with mortality data in the one-on-one bioassay (Fig 4.3), the penetration rate argument given above becomes void. Fig 4.3 indicates that single IJs of both *Steinernema* and *Heterorhabditis* species used in this study were capable of killing their hosts. This finding suggests that differences pathogenicity could be explained by differences in the ability to penetrate the insect host. However, Caroli *et al*. (1996) and Ricci *et al.* (1996) did

There was possibly more than one IJ applied to the insect in question. The highest numbers of IJs were observed from *H. indica* and the lowest from *H. zealandica* (Fig 4.4). Leite *et al*. (2002) argued that one of the factors that influence reproduction of a nematode within the host is the nematode size, with larger individuals occupying more space, consequently, producing fewer offspring. This can explain why *H. zealandica,* the largest species (in terms of body size) studied in this bioassay produced fewer descendants.

It is imperative for one to note that the direct contact (one-on-one) bioassay described here excludes host behavioural and ecological barriers to infectivity (Wakelin, 1978). It excludes the influence of infectivity of nematode predators and pathogens and soil structure, moisture, and type (Molyneux & Bedding, 1984). Moreover, it excludes prior invasion and overcrowding effects on infectivity (Anderson & May, 1978). It was a useful bioassay for the 4 EPN species being studied in this research as they all have a lethal level of one for both *G. mellonella* (results not shown) and *T. molitor*; but not for others with a lethal level > 1 IJ/insect e.g. *S. scapterisci* (Converse & Miler, 1999). Although the other assays give a measure of virulence at a consistent dose level, they do not exploit the central thesis of the virulence of only a proportion of the population. A very susceptible host like *T. molitor* was chosen since it has shown no coevolution of coadaptation with the EPNs (Gray pers. Comm.). The direct contact assay thus measured both (a) the virulence of the parasite due to the presence of the symbiont and (b) invasiveness of the nematode, without the interference of any possible host resistance.

The dose-response assay (section 4.3.3) also measured the overall infection process. The assay was carried out in two parts. All 4 EPN isolates were exposed to different doses of IJs. In the first part of the bioassay, mortality data was collected for *H. bacteriophora* and *H. zealandica* only; firstly because the isolates are indigenous and possibly new strains, and

the first generation of heterorhabditids where mating does not occur, reproduction takes place by adult nematode females producing sperm and self fertilising. In accordance with the aforesaid, the data obtained in the exposure time assay for *H. bacteriophora* suggest that this assay may be used to compare different species or production batches with poor penetration ability. However, the biological significance of this measurement and its relationship to nematode activity in the field is yet to be determined (Ricci *et al*. 1996). Glazer (1991) also showed that the larvae of four lepidopteron species was found to be related to virulence, as determined in a dose-response assay.

The one-on-one bioassay (section 4.3.2) measured the overall infection process. The assay examined all 4 nematode species. The highest mortality was observed in *H. indica*-infected insects after 48 h (62.5%). However, *H. zealandica* achieved the highest cumulative mortality (87.5%), recorded after 96 h (Fig 4.3) This finding suggests *H. zealandica* may be more virulent; probably because differences in the number of bacteria per IJ as well as behavioural differences between bacteria such as the dynamics of the release of toxins by the symbiont could have accounted for the differences in virulence between EPN species (Götz *et al*. 1981; Burman, 1982; Akhurst, 1986, Dunphy & Webster, 1986; Ehlers *et al.* 1997; Gerritsen *et al*. 1998). The IJs of EPNs serve as transmission stages (Anderson & May, 1978; Anderson, 1993) in this one-host life cycle, and the success of each nematode species depends upon the transmission efficiency of this stage. The 'lethal level' required to kill an individual host insect in all 4 nematode isolates was found to be 1 IJ, including *S. feltiae* although in this amphimictic nematode a minimum of 1 male and 1 female is needed for reproductive success (Bedding *et al*. 1983; Miller, 1989), thus the reason why offspring was observed in only 1 of 10 White traps. The fact that progeny IJs emerged from only 1 insect can be attributed to experimental error; this datum was excluded from all statistical analysis.

exposure times make it possible for more nematodes to penetrate their insect hosts; and the higher the number of penetrating insects. Therefore, more symbiotic bacteria are released by the nematodes which kill the insects by septicaemia. *G. mellonella* larvae have a softer cuticle, more spiracles and a larger surface area than *T. molitor*. These features allowed nematodes to penetrate *G. mellonella* more easily, thus the reason for this group experiencing the highest mortality of all insect types tested. The second highest mortality was observed in *T. molitor* pupae. This finding is probably due to the facts that: *T. molitor* pupae do not move around as much as the larvae, they have a softer cuticle especially when they are newly moulted, and have a more rugged body structure compared to the larval stage. All these features are believed to have aided penetration into the pupae by nematodes. In addition to the aforementioned, *T. molitor* larvae have a waxy cuticle with more chitin compare to the other two groups tested, suggesting why the lowest mortality at shorter exposure times was observed amongst them.

Only *H. bacteriophora* was examined in this assay. Reason being *H. zealandica*, the other indigenous South African nematode specie was not available at the time the assay was being carried out, and *H. indica* and *S. feltiae* had already been examined in previous bioassays by other researchers (Ricci *et al*. 1996). Although *H. bacteriophora* was not compared to other nematode species in this assay, Ricci *et al*. (1996) observed slow invasion rates in *H. bacteriophora* in an exposure time bioassay. However, the authors indicated that the slow penetration rate did not imply that a nematode had a lower efficacy. For example Georgis & Gaugler (1991) showed *H. bacteriophora* to be most effective against white grubs in many field tests. This may be attributed to the differences in infection strategies.

Mating is essential for further reproduction; thus an invasion of high numbers of individuals increases the probability for mating and further reproduction (Poinar, 1990). In

relation to the IJ doses insects were exposed. However, still looking at Fig 4.6 the observed differences in IJ emergence is noticeable, especially between *Sf* and *Hz* when compare *Hi*. Yet at 95% confidence level, ANOVA still revealed no significant differences when number of IJs emerged was compared between nematode species at all doses: [(5 IJs/insect: $F = 1.30$; $df = 3, 8$; $P = 0.3406$); (10 IJs/insect: $F = 1.44$; $df = 3, 8$; $P = 0.3007$); (25 IJs/insect: $F = 0.80$; $df = 3, 8$; $P = 0.5263$); (50 IJs/insect: $F = 1.06$; $df = 3, 8$; $P = 0.4173$); (75 IJs/insect: $F = 2.21$; $df = 3, 8$; $P = 0.1643$); (100 IJs/insect: $F = 2.38$; $df = 3, 8$; $P = 0.1455$); (200 IJs/insect: $F = 2.66$; $df = 3, 8$; $P = 0.1197$); (500 IJs/insect: $F = 3.83$; $df = 3, 8$; $P = 0.0571$)].

4.4 Discussion

Screening of EPN species is an important step before initiating mass production or field evaluation. Laboratory studies are essential for maximising the chances of success in field experiments (Bedding, 1990). The bioassays used in this study examined nematode activity at some steps in the infection process. From the bioassays, it is clear that the susceptibility of *T. molitor* larvae and pupae and *G. mellonella* larvae to each nematode species is different, thus, suggesting that each complex presents different virulence degrees This is profusely documented in literature (e.g.) (Bedding *et al.* 1983; Fuxa *et al.* 1988; Morris *et al.* 1990; Ricci *et al*. 1996; Rosa *et al*. 2000).

The exposure time assay (section 4.3.1) indicated indirectly how quickly insects were infected by the nematodes. The assay tested *H. bacteriophora* against 3 insect types (*T. molitor* larvae and pupae, and *G. mellonella* larvae). Overall, mortality increased with longer exposure times (Fig 4.2). The highest mortality was observed with *G. mellonella* larvae at all exposure times followed by *T. molitor* pupae (Fig 4.2). These findings suggest longer

The second part of the bioassay sort to determine if there were differences in the number of progeny IJs from insects exposed to different doses of IJs. The results are presented in Fig 4.6 below. The highest number of emerged IJs (mean ± SEM: 172667 ± 53670 progeny IJs) was observed in *Hi*- and the lowest (mean ± SEM: 55333 ± 22423 progeny IJs) in *Sf*-infected cadavers both at a dose of 100 IJs/insect.

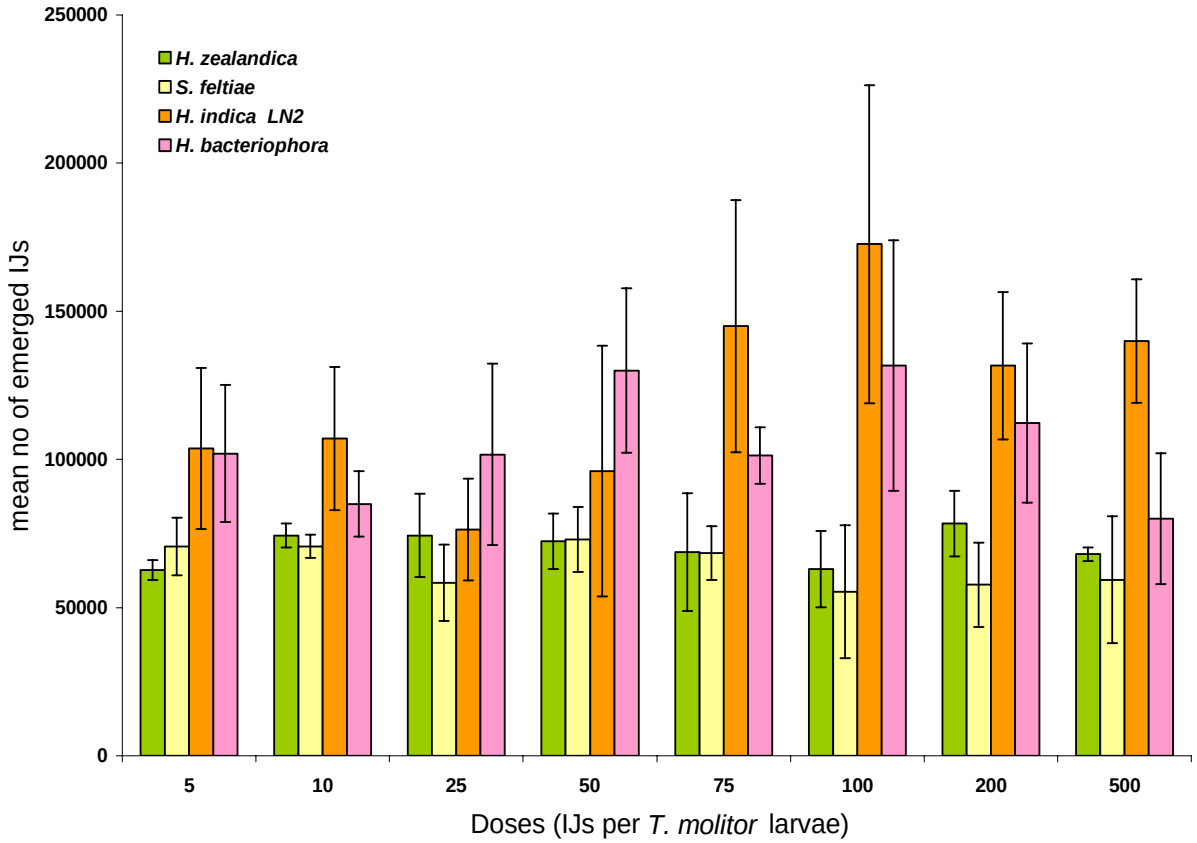
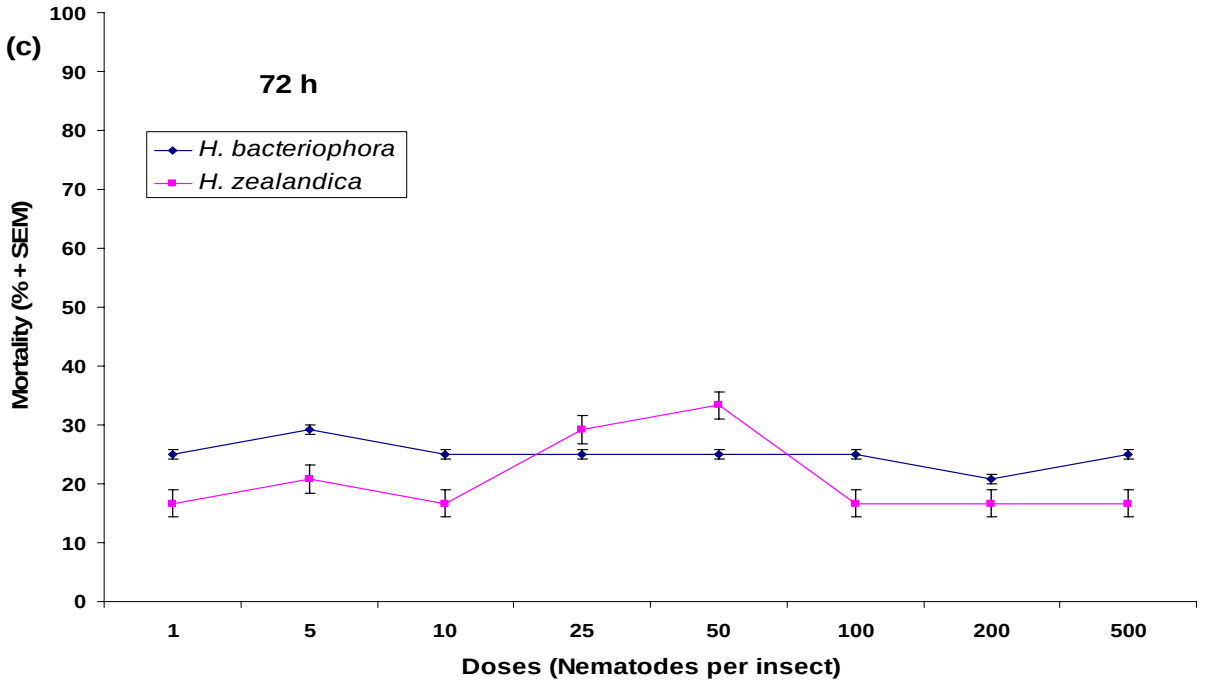


Fig 4.6: Mean number of progeny infective juveniles emerged from *T. molitor* larvae that were exposed to different doses of nematodes. Bars are ± standard error of the mean.

A one-way ANOVA revealed no significant differences in the mean number of emerged IJs when the different doses insects were exposed to were compared within each nematode specie: [(*Hi*: $F = 0.85$; $df = 7, 16$; $P = 0.5614$); (*Hb*: $F = 0.51$; $df = 7, 16$; $P = 0.8117$); (*Hz*: $F = 0.25$; $df = 7, 16$; $P = 0.9642$); (*Sf*: $F = 0.25$; $df = 7, 16$; $P = 0.9652$)]. This concurs with the results presented in Fig 4.5. There is no particular pattern in IJs emerged in



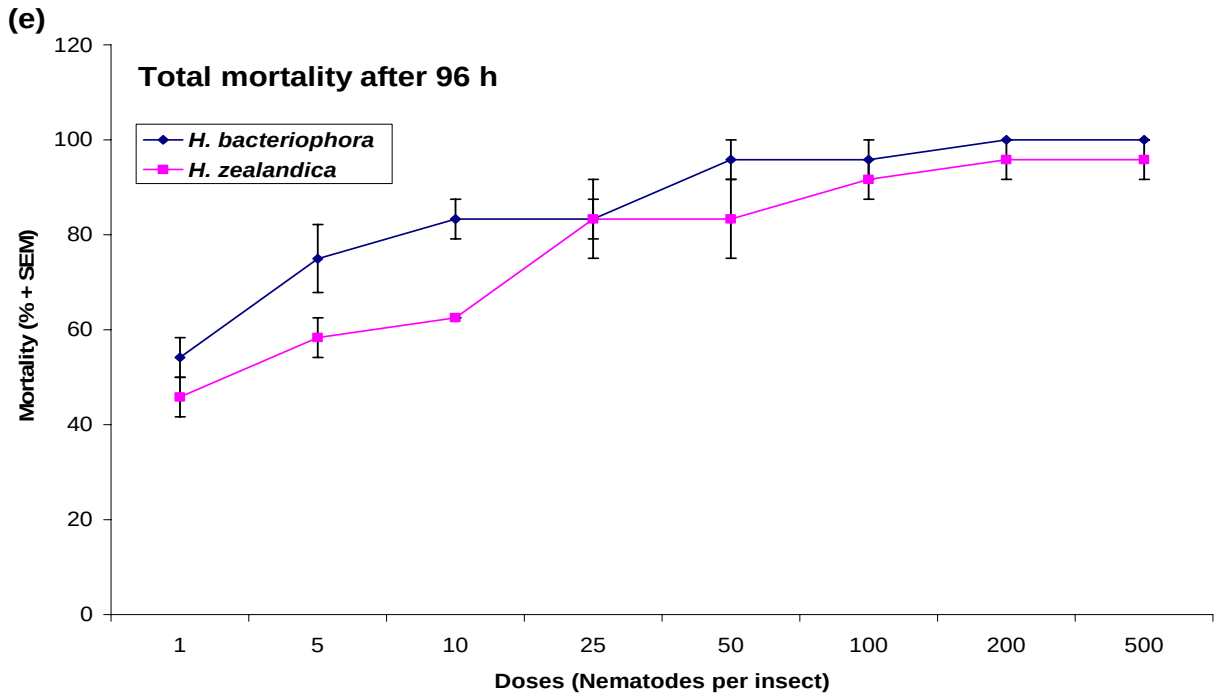
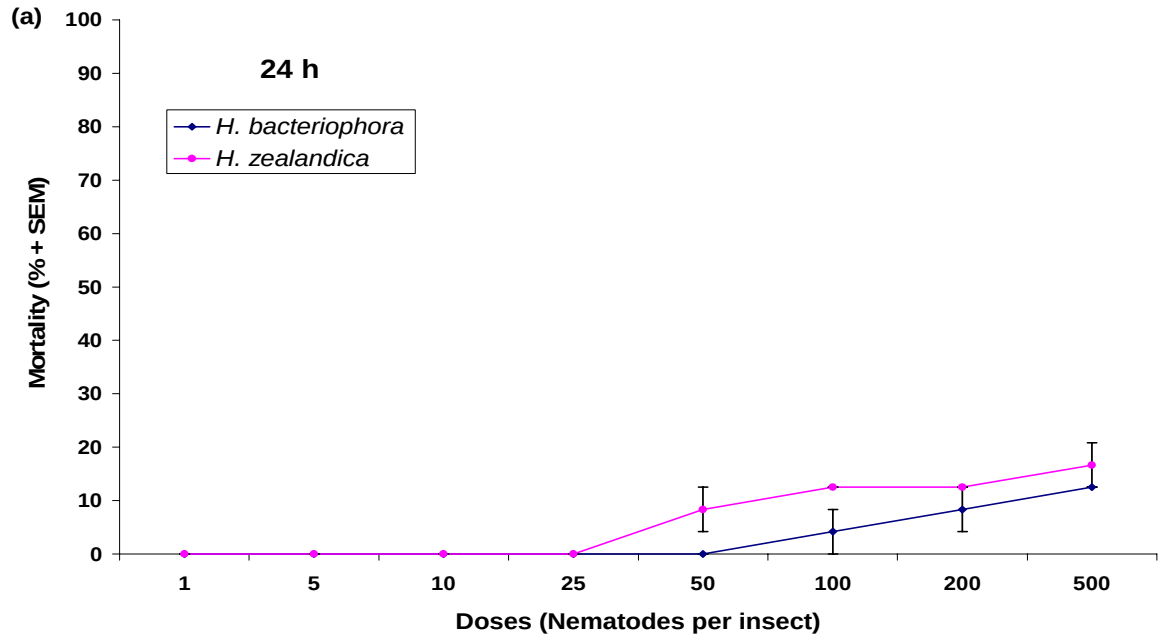
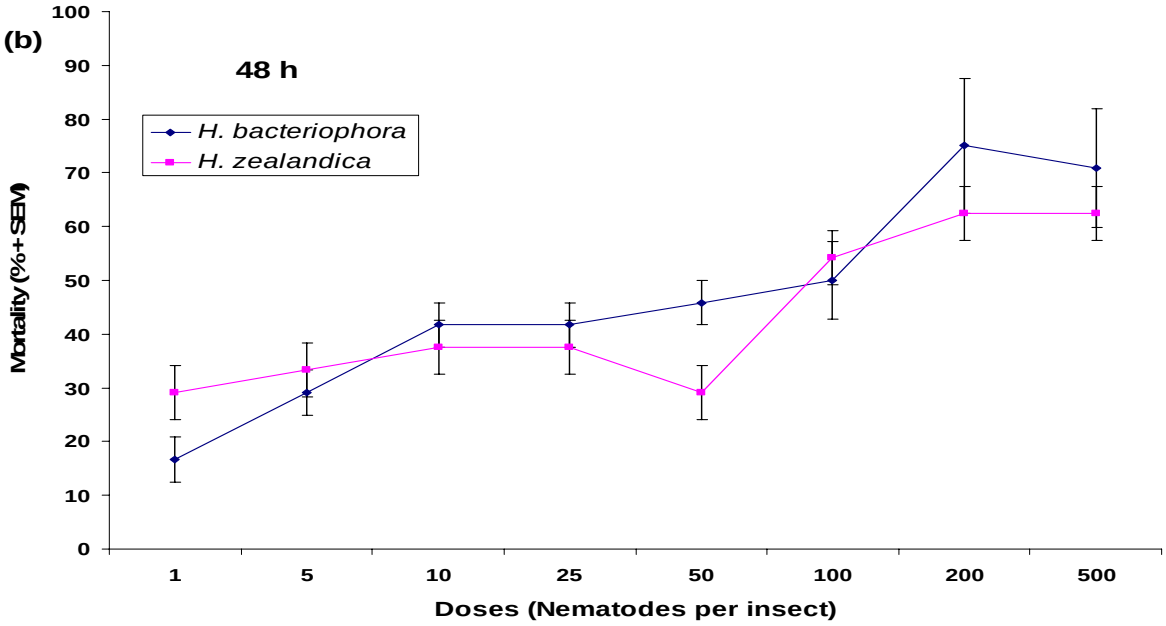


Fig 4.5: Mortality caused by *H. bacteriophora* and *H. zealandica* at 24h intervals. Mortality at: (a) 24 h, (b) 48 h, (c) 72 h or (d) 96 h of exposure; (e) represents the cumulative mortality after 96 h. Bars represent ± standard error of the mean.


(a)
24 h
H. bacteriophora
H. zealandica
Mortality (% + SEM)
100
90
80
70
60
50
40
30
20
10
0
1
5
10
25
50
100
200
500
Doses (Nematodes per insect)


(b)
48 h
H. bacteriophora
H. zealandica
Mortality (% + SEM)
100
90
80
70
60
50
40
30
20
10
0
1
5
10
25
50
100
200
500
Doses (Nematodes per insect)

When mortality data 24 h post exposure (Fig 4.5 a) were analyzed, ANOVA: (*Hb*: $F = 5.43$; $df = 7, 56$; $P = 0.0025$; | *Hz*: $F = 11.43$; $df = 7, 56$; $P<0.001$). Bonferroni's test post ANOVA (*Hb*: differences observed only between 500 IJs/insect when compared to 1, 5, 10, 25 and 50, $P = 0.017$; | *Hz*: differences observed between doses of 1, 5, 10 and 25 when compared to 100 IJs/insect, $P = 0.017$). At 48 h (Fig 4.5 b), ANOVA: (*Hb*: $F = 7.28$; $df = 7, 56$; $P = 0.0005$; | *Hz*: $F = 5.85$; $df = 7, 56$; $P = 0.0017$). Bonferroni's test (*Hb*: differences observed between doses 1 and 200, 1 and 500 IJs/insect, $P = 0.001$ and $P = 0.002$ respectively, and also between doses 5 and 200, 5 and 500 IJs/insect, $P = 0.010$ and $P = 0.024$ respectively. | *Hz*: differences observed between doses 50 compared 200 and 500 IJs/insect, $P = 0.029$). When mortality data obtained at 72 h post exposure to varying nematode doses (Fig 4.5 c), were analyzed using a one-way ANOVA, no significant differences in mortality by dose were observed in either of the nematode species: (*Hb*: $F = 0.06$; $df = 7, 56$; $P = 0.9996$; | *Hz*: $F = 1.21$; df 7, 56; $P = 0.3524$); thus a post ANOVA multiple comparison test was not performed. The same was true for data obtained at 96 h post exposure (Fig 4.5 d) ANOVA: (*Hb*: $F = 2.52$; $df = 7, 56$; $P = 0.0593$; | *Hz*: $F = 1.46$; df 7, 56; $P = 0.2499$).

The cumulative mortality data for every 24 h was then combined across all doses and a t-test assuming equal variances (Bartlett's test for equality of variances had been carried out prior to ANOVA; the t-test assumption was not violated) was used to compare the two nematode species, namely *Hb* and *Hz* (Fig 4.5 e). At 24 h, 48h and 72 h, there were no observed significant differences in the mean percentage mortality between *Hb* and *Hz* [(24 h: $P = 0.1034$; 95% CI = -0.05529 - 0.05291; t = -1.661); (48 h: $P = 0.5691$; 95% CI = -0.6276 - 1.128; t = 0.5734); (72 h: $P = 0.2508$; 95% CI = -0.2435 – 0.9101; t = 1.1632)].

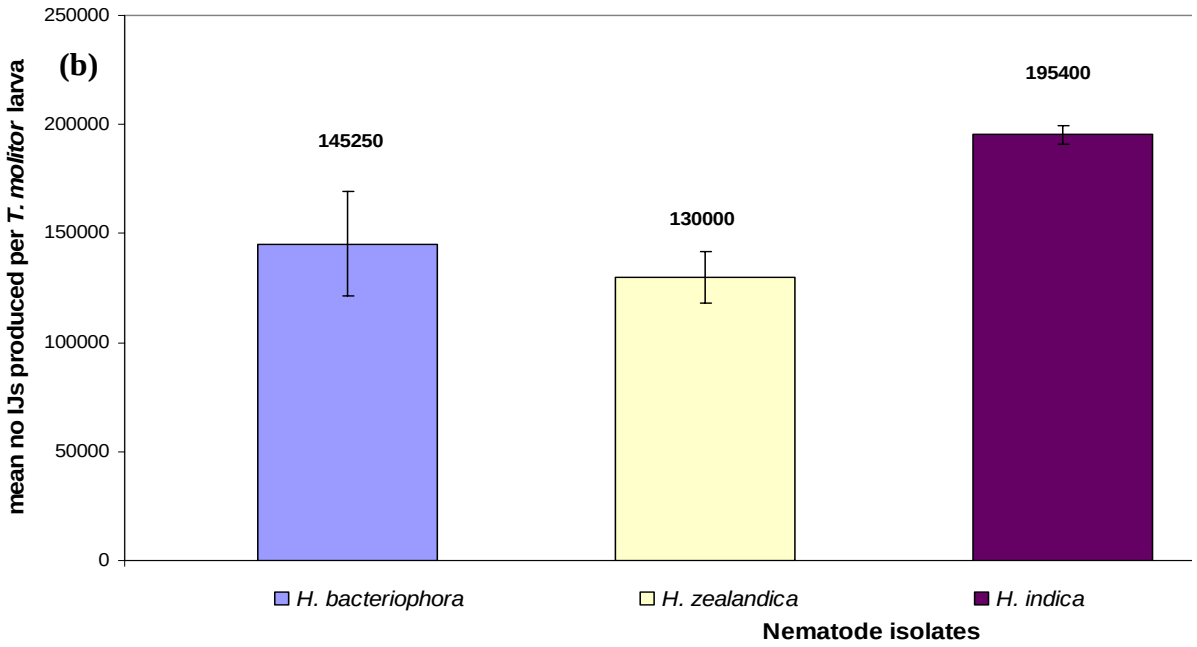


Fig 4.4: Mean number of progeny IJs emerged from one *T. molitor* larva. Bars are ± standard error of the mean; n = 10 for all nematode species.

4.3.3 The dose response bioassay

Please note in this section, *Heterorhabditis bacteriophora = Hb*; *Heterorhabditis zealandica = Hz*; *H. indica = Hi* and *S. feltiae = Sf.*

The dose response assay was carried out in two parts.

In the first part of the dose-response assay, insect mortality increased over time at all dosages for both *Hb* and *Hz* (Fig 4.5 e). Even as early as 24 h after exposure to IJs, a gradual increase in mortality was observed. When mortality was compared at each dose (nematodes per insect) by time, differences were highly significant. Bonferroni's multiple comparison test was performed post ANOVA to spot the differences.

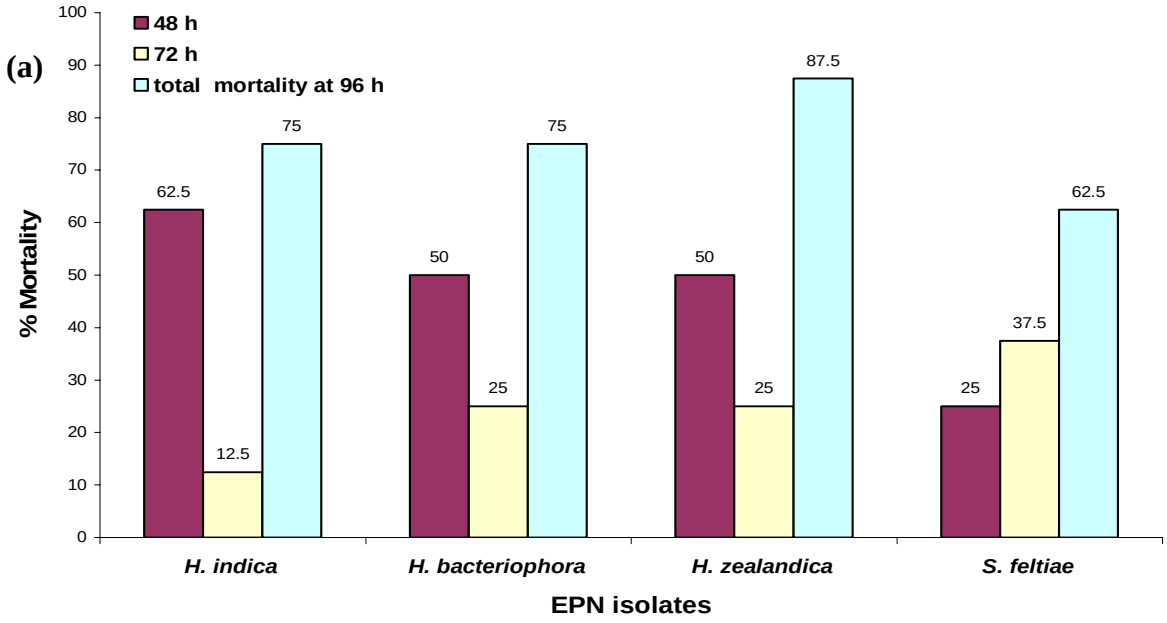


Fig 4.3: Percentage mortality for *T. molitor* larvae in the one-on-one bioassay for various EPNs. Numbers on bars represent mean % mortality for each specie after every 24 h (n = 94).

Fig 4.4 shows the mean number of infective juveniles that emerged from one *T. molitor* larva. Except for 1 White trap, no progeny was observed in any of the *S. feltiae* White traps, n = 1; 210,000 IJs emerged from the *S. feltiae* infected-larva. Since n = 10 in the experimental design, this datum was not included in the analysis. The highest mean IJ count was obtained from *H. indica* (195,400 followed by *H. bacteriophora* (145,250). Differences in mean number of IJs were statiscally significant for all nematode species: (F = 8.56; df = 2, 27; P = 0.0068). Individual means were compared using Bonferroni's multiple comparison test. There were no differences in the mean number of progeny IJs between *H. bacteriophora* and *H. zealandica* (P = 0.953) and *H. bacteriophora* and *H. indica* (P = 0.052); but *H. zealandica* and *H. indica* were significantly different (P = 0.008).

When mortality amongst insect types (*T. molitor* larvae, *T. molitor* pupae and *G. mellonella* larvae) were compared in a one-way ANOVA, there were no differences detected at any of the exposure times: [(2 h: $F = 0.1250$; $df = 2, 6$; $P = 0.1250$); (5 h: $F = 0.78$; $df = 2, 6$; $P = 0.5008$); (8 h: $F = 3.11$; df 2, 6; P = 0.1183); (12 h: $F = 5.44$; df 2, 6; $P = 0.0501$) 18 h ($F = 3.25$; $df = 2,6$; $P = 0.1106$); (24 h: $F = 1.50$; $df = 2, 6$; $P = 0.2963$)].

Differences in exposure times within and between all insect types were statistically significant ($F = 58.96$; df 5, 48; $P<0.01$). The same differences and similarities in exposure times were detected (P-values were about the same) when data were analyzed individually within each insect type; thus data were combined across all insect types. The differences were detected between 2 h and 8, 12, 18 and 24 h respectively ($P<0.01$), between 5 h and 12, 18 and 24 h respectively ($P<0.01$); between 8 h and 12, 18 and 24 h respectively ($P<0.01$); between 12 h and 24 h ($P = 0.007$). No differences were observed between 2 and 5 h, between 5 and 8 h, between 12 and 18 h and between 18 and 24 h ($P = 0.055$; $P = 1.000$; $P = 0.104$ and $P = 1.000$) respectively (Bonferroni's multiple comparison test).

4.3.2 One on one quality bioassay

In the one-on-one quality bioassay, nematode virulence was measured by insect mortality over 96 h (Fig 4.3). *T. molitor* mortality was observed only after 48 h during the bioassay. Mortality was highest (62.5%) for *H. indica* after a period of 48 h. The highest cumulative mortality (time = 96 h) was obtained by *H. zealandica* (87.5%), followed by *H. indica* and *H. bacteriophora* (75%). Data were combined across all 3 experiments after ANOVA showed no significant differences ($F = 0.05$; df 3, 8; $P = 0.987$).

analysis, since insect mortality did not result from any of the control treatments. Means for percentage mortality were separated using the post ANOVA Bonferroni's multiple comparison test. Values of $P \leq 0.05$ were considered significant.

4.3 Results

4.3.1 Exposure time bioassay

Results are presented in Fig 4.2 below. The overall trend showed a gradual increase in mortality with increasing exposure time. The highest mortalities were achieved with *G. mellonella* larvae, followed by *T. molitor* pupae. Complete mortality was achieved for *G. mellonella* larvae only when they were exposed for18 and 24 h.

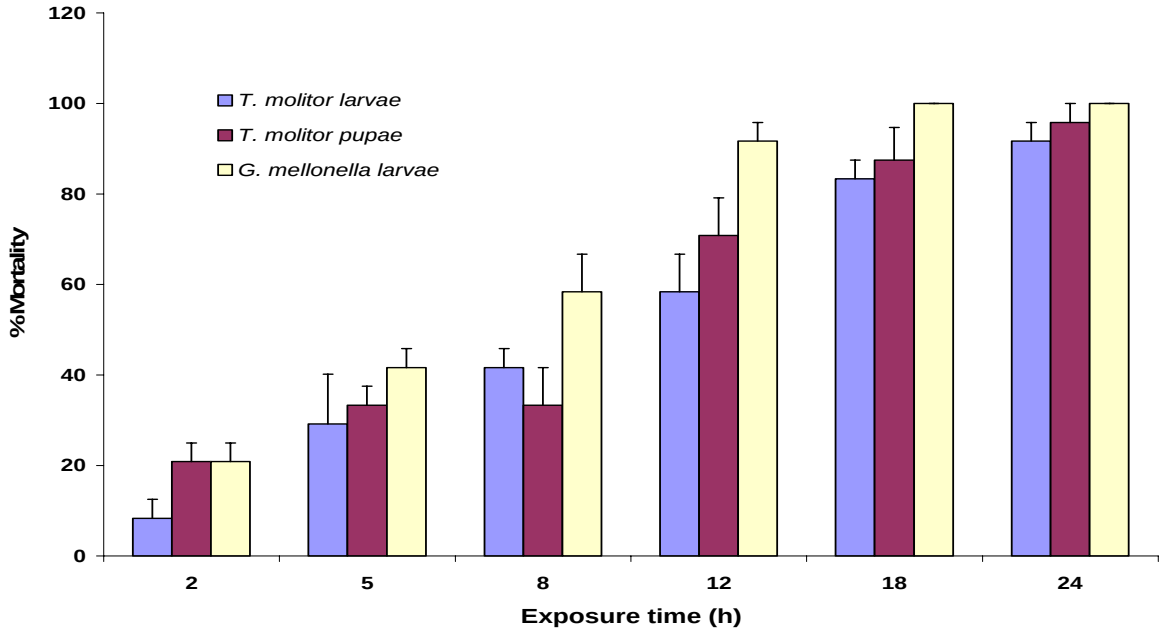


Fig 4.2: Percentage insect mortality following exposure of *T. molitor* larvae and pupae and *G. mellonella* larvae to 200 *H. bacteriophora* IJs/insect, for different time (h) periods

microscope (Olympus) at 40X magnification. In the second part of the assay all 4 nematode isolates, namely: *H. bacteriophora, H. zealandica, H. indica* and *S. feltiae* were treated as described above in the first part of the bioassay, (i.e.) with varying concentrations of IJs in 50 µl of distilled water. All nematode-infected insect cadavers were placed on White traps to collect progeny IJs. For each nematode species, 3 White traps were randomly selected for counting using a dissecting microscope (40X magnification) as described in section 2.5.

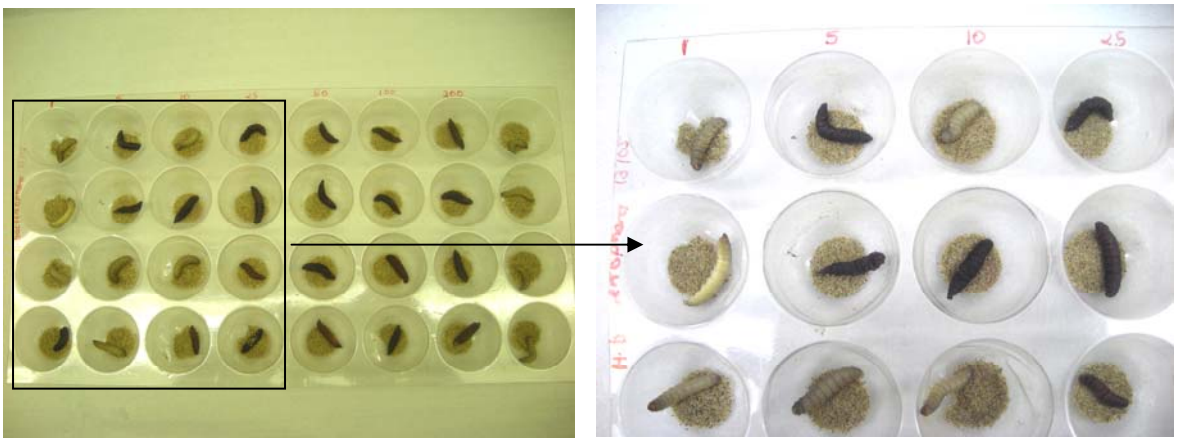


Fig 4.1: Bioassay set up in multi well plates. This dose-response bioassay was set up using *G. mellonella* larvae as hosts. Brick red cadavers are larvae are dead from *H. bacteriophora* infection. The numbers on the plates are the nematode doses that were applied to the wells.

4.2.4 Data analysis

STATA SE 9, (Statacorp) was the statistical program used to analyse all data. Virulence data as well as data on the mean number of progeny IJs emerging per cadaver were analyzed using one-way ANOVA. The assumption of equal variances for was checked using Bartlett's test before ANOVA. Mortality data expressed as percentages were transformed by Arcsin transformations and before statistical analysis. Control data were not included the statistical

in 30 µl of distilled water, followed by another 30 µl of sterile distilled water, which served to to rinse the pipette tip into each well and reduce the retention of nematodes in the pipette tip. One *T. molitor* larva was added into each nematode-laden cell. Thus, there were 32 *T. molitor* larvae per EPN isolate, including 4 controls.The lids were replaced and the plates were sealed with parafilm to prevent desiccation. The plates were incubated for 48 h, at 25 ^{o}C, after which the onset or occurrence of insect mortality due to IJ infection, (determined by a pale brown colour for *S. feltiae* SN, brick red for *H. indica* LN2 and *H. bacteriophora,* and greyish green for *H. zealandica*, indicating death due to bacterial sepsis) was monitored every 24 h for 96 h. All EPN-infected cadavers were placed on White traps (1 cadaver per White trap). Ten White traps per nematode isolate were selected at random for counting. The total number of IJs that emerged from each larva was estimated by counting (as described in section 2.5) using a dissecting microscope (Olympus) at 40X magnification.

4.2.3.3. The dose-response assay

The dose-response assay (Morris *et al*., 1990) was done in two parts; the first part involved only *H. bacteriophora* and *H. zealandica*. The second part involved all 4 nematode species. In the first part of the bioassay, different IJ concentrations (1, 5, 10, 25, 50, 100, 200 and 500) of *H. bacteriophora* and *H. zealandica* were applied in 50 µl of distilled water per *T. molitor* larva, using a micropipette. Eight *T. molitor* larvae were used for each concentration, per EPN species. For *H. bacteriophora* and *H. zealandica* only, mortality was monitored every 24 h over a 96 h period following initial exposure of the larvae to IJs. Every EPN-infected insect cadaver was placed on a White trap (Fig 2.1b). The total number of IJs emerging from each larva was estimated by counting (section 2.5.2) under a dissecting

150 – 354 µm). The nematode IJs were transferred to the wells in 60 - 70 µl of sterile distilled water. Control wells received water only. Plates were then incubated at 25 ^{o}C in the dark. These particular wells were chosen because the wells have a relatively small volume; the insect is thus forced to be in close proximity to the nematodes. Each bioassay was repeated three times in parallel. In addition, different batches of nematodes and insects were used.

4.2.3.1. The exposure time bioassay

In the exposure time bioassay (Glazer, 1992), *T. molitor* larvae and pupae along with *G. mellonella* larvae, were exposed to IJs of *H. bacteriophora*. Two hundred IJs were applied to one insect larva or pupa. The insects were exposed to the IJs for set time intervals: 2 h, 5 h, 8 h, 12 h, 18 h and 24 h. In total, 10 insects were used per exposure time (8 for the experiment, and 2 as controls). As indicated above, the control insects received water only, but every other treatment was exactly as with the experimental insects. After each exposure time, the larvae and/or pupae were rinsed in sterile distilled water to remove any nematodes from the surface, after which the insects were placed in 90 mm Petri dishes lined with 90 mm diameter filter paper (Whatmann Cat No 01001090) and incubated in the dark at 25 ^{o}C. Insect mortality was monitored every 24 h after initial exposure of the insects.

4.2.3.2. The one-on-one quality bioassay

The one-on-one quality bioassay (Miller, 1989), was carried out on all four EPN isolates. Only *T. molitor* larvae were used as insect hosts in this bioassay. Under a dissecting microscope, one nematode was transferred from a Petri dish, by micropipette, into each well

infecting their insect hosts, hence creating a virulence profile for the EPN in question (Miller, 1989). The dose-response assay was done to estimate the IJ lethal dose required to cause death in 100% of the test insects (Morris *et al*. 1990); and to investigate if there was a difference between the numbers of IJs emerging from the insects infected at different doses.

4.2 Materials & Methods

4.2.1 Nematodes

The 4 EPN species used were *H. bacteriophora, H. zealandica H. indica* LN2 and *S. feltiae* SN (section 3.2). All EPNs were cultured *in vivo* in the greater wax moth, *G. mellonella* and or *Tenebrio molitor* larvae according to the method of Kaya & Stock (1997) at 25 ^{o}C, (section 2.2). IJs were recovered using White traps (section 2.3) and kept in aerated water at ambient room temperature (21 - 23 ^{o}C).

4.2.2 Insects

G. mellonella and *T. molitor* larvae and pupae were reared in the laboratory (section 2.1). In all experiments, the insect hosts weighed 0.25 ± 0.05 g and 0.20 ± 0.05 g respectively. *G.mellonella* larvae were obtained from cocoons and starved for 3 days prior to use.

4.2.3 Bioassays

Thirty-two-well round bottomed tissue culture plates (Falcon), of 1.5 cm diameter were used as the experimental arena. In all bioassays, 1 wax moth or meal worm larva was exposed to IJs of the different nematode species on 0.5 g of sterilized air-dried river sand (particle size

Chapter 4

BIOASSAYS

4.1 Introduction

For an EPN isolate to be used successfully in biological control programmes, it must have good pathogenicity to insects and be easy to propagate on a large scale. With the expansion of commercial interest in EPNs, the susceptibility of many economically important insect pests has been tested in a wide range of laboratory assays. Pertaining to pathogenicity, there is evidence from infectivity assays (Griffin *et al*. 1989; Fan & Hominick, 1991; Bohan & Hominick, 1996, 1997), injection assays (Sims *et al*. 1992), and symbiont assays (Selvan *et al*. 1993) that only a certain proportion of any population of EPNs are capable of infecting their insect hosts. Converse & Miller (1999) proposed that a virulence profile would measure that proportion and would be unique for each EPN species and strain due to differences in nematode infectivity between species and stains (Bedding *et al*. 1983; Morris *et al.* 1990, 1991). In view of the afore-mentioned, information on the pathogenicity of the 4 EPN isolates used in this study was obtained through different bioassays using *Tenebrio molitor* and *Galleria mellonella* as insects host. Three types of bioassays, namely: the one-on-one, exposure time, and the dose-response assays were carried out to determine the virulence and or infectivity and stability of the nematodes.

The exposure time bioassay was carried out to determine the minimum time required by the EPNs to penetrate and kill an insect host (Glazer, 1992). The one-on-one bioassay was carried out to measure the proportion of the different nematode isolate populations capable of

EPN symbiont, is not bioluminescent, does not absorb dyes, has no colony form variations (Boemare, 2002)… just too name a few; thus, no conclusions can be drawn from these reports on potential risks related with the use of EPN and their symbiotic bacteria (Akhurst & Smith, 2002). Notwithstanding, growth was observed at 37 ^{o}C for *P. luminescens sp.* and *P. luminescens* subsp. *laumondii*, indicating that they are heat tolerant and may be adapted to grow at mammalian body temperature. This heat tolerance was possibly caused by an adaptation of the bacteria to the higher temperature through the production of temperature stable enzymes of the selection of heat resistant variants. This has been previously reported in a similar study by Ehlers *et al.* (2000) using a *Photorhabdus* sp. isolated from *H. indica* strain LN_2-1, where the symbiont tolerated temperatures up to 40 ^{o}C. On the basis of these results, it cannot be excluded that the symbionts of *H. bacteriophora* and *H. zealandica* would be able to grow at mammalian body temperature. It is therefore recommended that the bacteria are always treated as potentially pathogenic to humans, and to test the safety of these bacteria for vertebrates before it is used commercially.

Table 3.2 shows the growth rates and mean doubling time for the different symbiotic bacteria. Growth rate was smallest for *X. bovienii* (1.99 hour^{-1}), and highest for *P. luminescens sp.* (2.45 hour^{-1}). The contents of *P. luminescens sp.* culture doubled in only 18 min. It was much longer for *P. luminescens* subsp. *laumondii* (19.62 min). Overall, the growth rate and the mean doubling time for all 4 bacteria symbionts were similar to each other.

study were indeed bioluminescent, but since this characteristic was being tested for visually instead of using proper equipment like a photometer or scintillator, the results may have been prone to human error. Although a photometer or other suitable equipment was not used to detect bioluminescence in this study, determining luminescence visually was not considered a limitation in this study, since the objective was to differentiate phase I from phase II colonies; the phase I colonies were luminous enough to be detected by eye. The test for antibiotic activity was not used in this study but it is recommended also to confirm the phase in which a nematode bacterial symbiont is in.

The optimum growth temperatures of the different symbiotic bacteria are presented in Table 3.2. The differences in optimal growth temperatures between the symbionts could be correlated with their ecological origins (Fischer-Le Saux *et al*. 1998). The growth profile of *P. luminescens* subsp. *laumondii* (Fig 3.4) suggests the possibility that its optimum growth may not be 28 ^{o}C; the growth measured using CFU 28 ^{o}C, is closer to the bacterium's growth at 25 ^{o}C, which OD are measurements. But, growth was also observed at 37 ^{o}C. It is possible that the optimum growth temperature for *P. luminescens* subsp. *laumondii* ranges between 25 – 28 ^{o}C. The growth of *P. luminescens sp.* at 25 and 28 ^{o}C are almost similar (Fig 3.5), which implies that its optimum growth ranges between those two temperatures. One prerequisite for a human-pathogenic strain of *Photorhabdus* would be the ability to grow at temperatures of ≥ 37 ^{o}C. According to Boemare (2002), the upper threshold for growth in NB occurs at 35 - 36 ^{o}C and 33 - 35 ^{o}C for *P. luminescens* subsp. *laumondii,* and *P. luminescens sp.* respectively, but this was not the case in our study; growth was observed at 37 ^{o}C for both bacteria. A related group non-symbiotic *Photorhabdus* spp. was reported five times from humans in the USA (Farmer *et al*. 1989), and from five patients in Australia (Peel *et al*. 1999), implying that the bacterium grows at 37 ^{o}C. Although from the same genus, this bacterium is not an

intestines. Furthermore, when other bacterial strains were found associated with *Steinernema* (Aguillera *et al*. 1993), it was shown that they were contaminants of the cuticle (Bonifassi *et al*. 1999) and in all cases the symbiont was found in the IJ gut. This was probably the case with this study, but it is also possible that grinding the IJs using a mortar and pestle increased the chances of contamination. Moreover, the IJs were surface sterilised, but the insect cadaver was sterilised by igniting and plunging. The results also advocate that there is greater success in isolating EPN symbiotic bacteria by streaking the EPN-infected cadaver hemolymph onto MacConkey agar or NBTA. Nevertheless, the purified colonies must have characteristics same as those found in publications (e.g. Kaya & Stock, 1997). This method is only recommended if expected results are known, that is, if the symbiont is known. For uncharacterised EPN isolates, it is suggested that all available techniques be used to isolate the symbiont. This will increase the accuracy and precision of the results.

P. luminescence subsp. *laumondii* and *P. luminescens sp.* phase variants of *Photorhabdus* spp. were easier to identify on MacConkey agar (only cultures > 7 days old) than on NBTA. It was also noted that the colonies on MacConkey agar were more than double the size of those on NBTA, and the colour changes after phase variation was also evident (e.g.) *P. luminescens* subsp. *laumondii* (Fig 3.2b C). Akhurst & Boemare (1986) carried out a study, in which they tested 150 strains of *Photorhabdus* for bioluminescence; all but one isolate was positive for light emission. The results obtained from this study confirm their findings: bioluminescence was detected in all three *Photorhabdus* spp, but light emission was most intense for *P. luminescens sp.* Different *Photorhabdus* spp. show differences in their luminescence intensity (Gerritsen & Krasomil-Osterfeld, 1994). Akhurst & Boemare (1986) also noted light emission in Phase II colony variants. This was not the case with this study. Notwithstanding, it is possible that the phase II colony variants in this

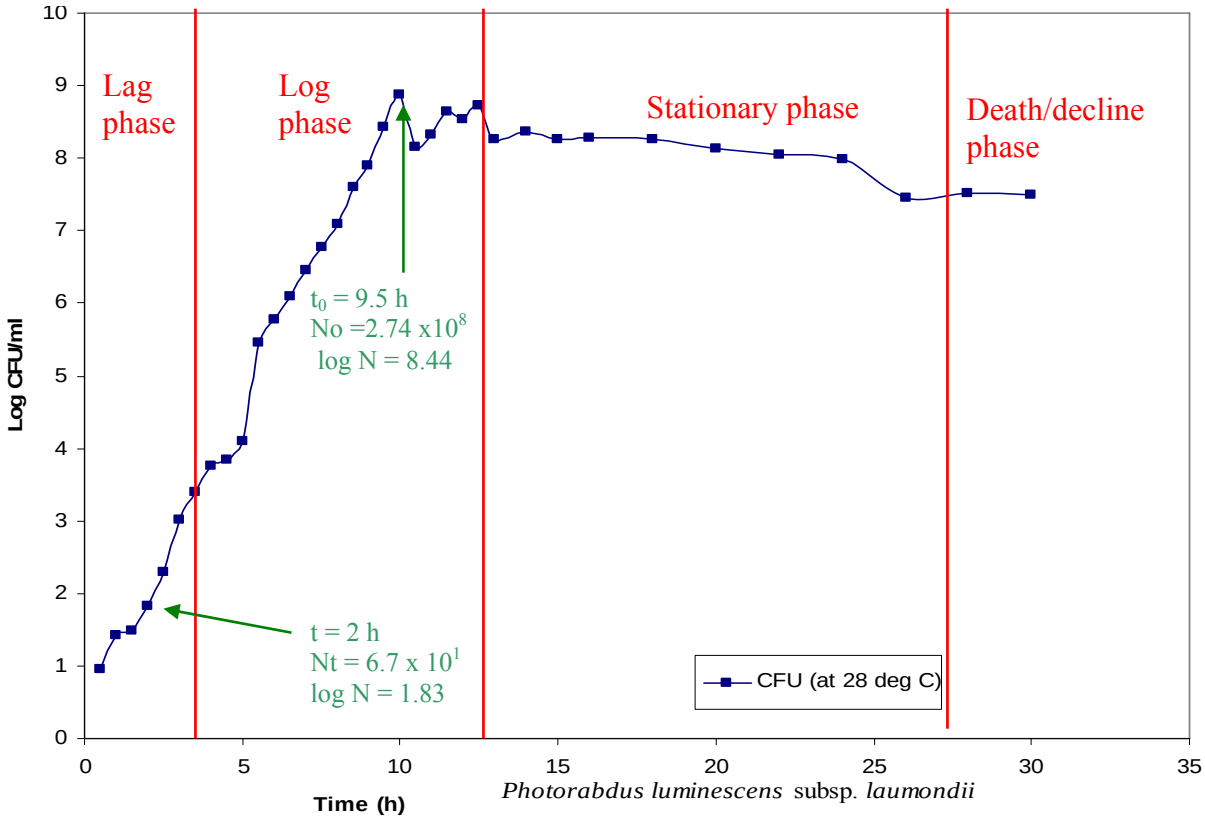


Fig 3.8: Growth profile of *P. luminescens* subsp. *laumondii* (isolated from *H. bacteriophora*) at optimum growth temperature 28 ^{o}C.

3.4 Discussion

The homologous symbiotic bacteria associated with the 4 EPN isolates were successfully isolated and cultured. The method of isolating bacteria directly from the haemolymph proved more successful than the ground-up IJ method. Although the isolations were carried out aseptically, the cultures still had to be purified several times using the streak plate technique. This observation is in agreement with a study by Forst *et al*. (1997). The authors reported that all isolations made during the last 20 years from wild nematodes (about 1000) established the presence of *Xenorhabdus* in *Steinernema* and *Photorhabdus* in *Heterorhabditis* juvenile

3.3.4 The Specific Growth Rate Constant (μ) and Doubling Time (g)

μ and g (section 3.2.2.4) for the optimum growth of each symbiotic bacterium were calculated from the CFU growth curves, as shown below, for *P. luminescens* subsp. *laumondii* isolated from *H. bacteriophora* (Fig 3.8). The results are presented in Table 3.2.

(a) Growth rate constant (μ) from the equation: $\ln N_t - \ln N_o = \mu (t-t_o)$

$\Rightarrow \mu = (\log_{10} N - \log_{10} N_o)\ 2.303 / (t-t_o)$

$= (8.44 - 1.83)\ 2.303 / (9.5 - 2)\ h$

$= 2.03\ h^{-1}$

(b) Mean generation or doubling time (g) from the equation: $\log_{10} N_t - \log_{10} + g \log_{10} 2$

$\Rightarrow g = (\log_{10} N_t - \log_{10} N_o) / \log_{10} 2$

$= (8.44 - 1.83)\ h / 0.301$

= 22.94 generations in 7.5 hours

= 7.5 / 22.94 = 0.33 h (x 60 = 19.62 min)

Table 3.2: Growth rate and mean generation time for the homologous symbiotic bacteria EPNs.

EPN isolate	Symbiotic bacteria	Growth rate	Doubling time
H. bacteriophora	*P. luminescens* subsp. *laumondii*	2.03 h^{-1}	19.62 min
H. zealandica	*P. luminescens sp.*	2.45 h^{-1}	18.00 min
H. indica LN2	*P. luminescens* subsp. *akhurstii*	2.21 h^{-1}	18.82 min
S. feltiae SN	*X. bovienii*	1.99 h^{-1}	18.73 min

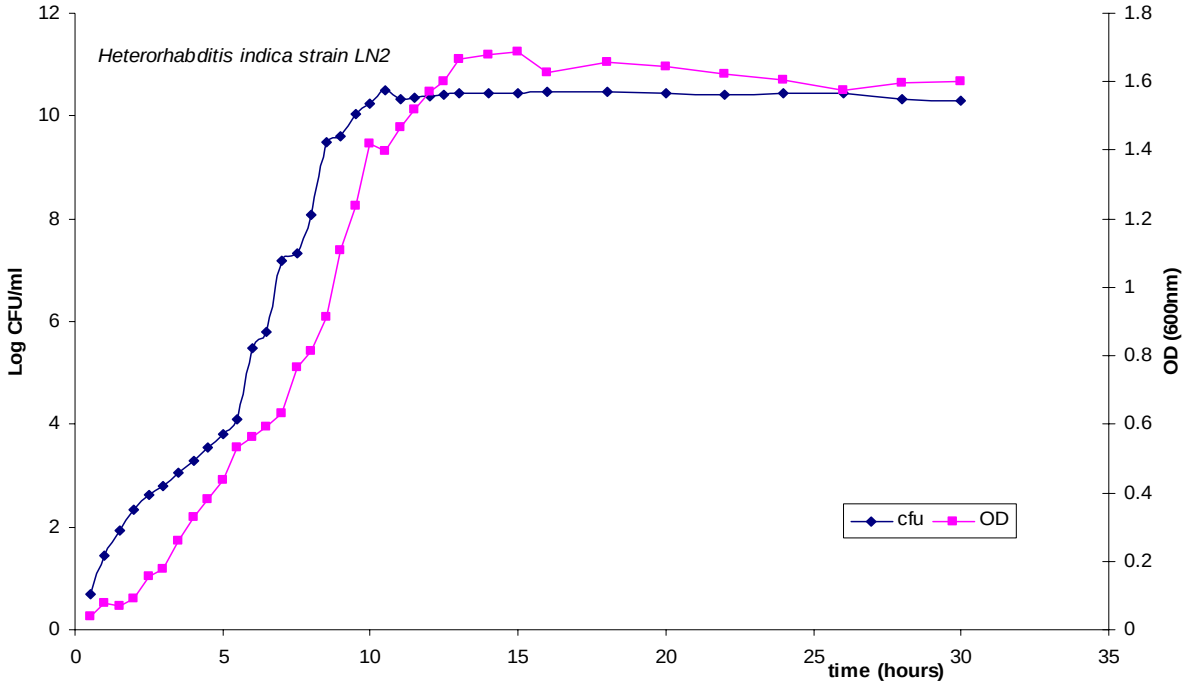


Fig 3.6: Growth profile of *P. luminescens* subsp. *akhurstii* (isolated from *H. indica* LN2) at optimum growth temperature 28 ^{o}C.

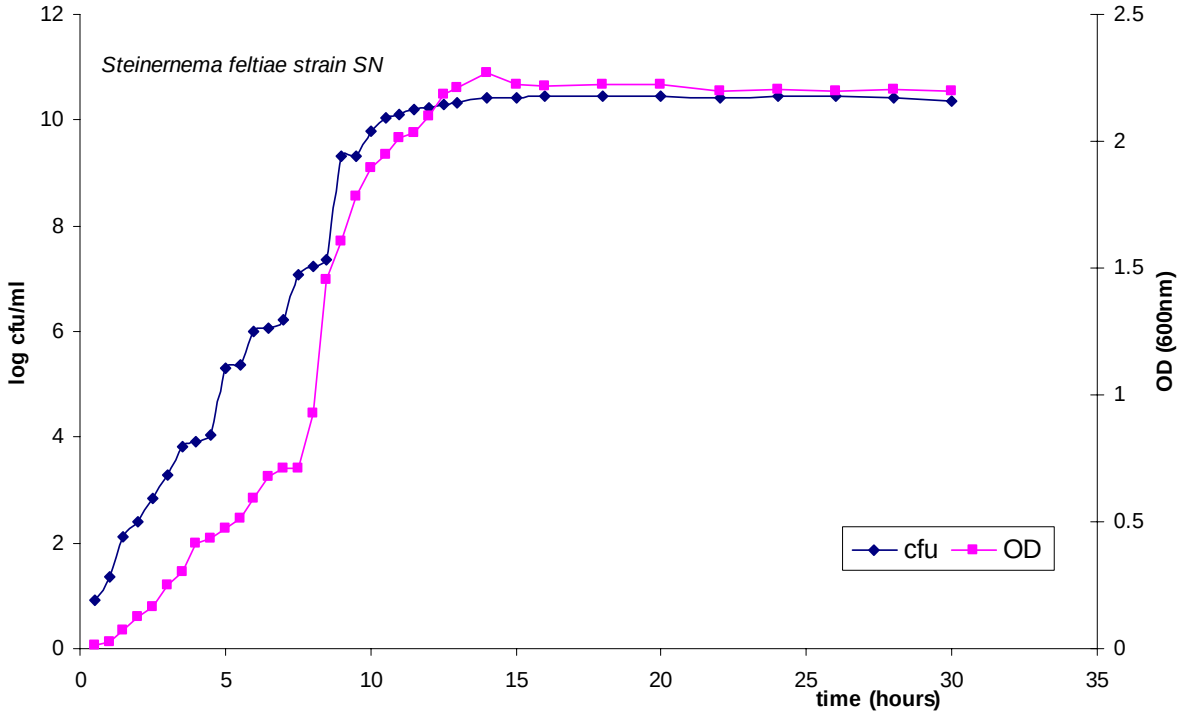


Fig 3.7: Growth profile of *Xenorhabdus bovienii* (isolated from *S. feltiae*) at optimum growth temperature 28 ^{o}C. Data are means of three replicates.

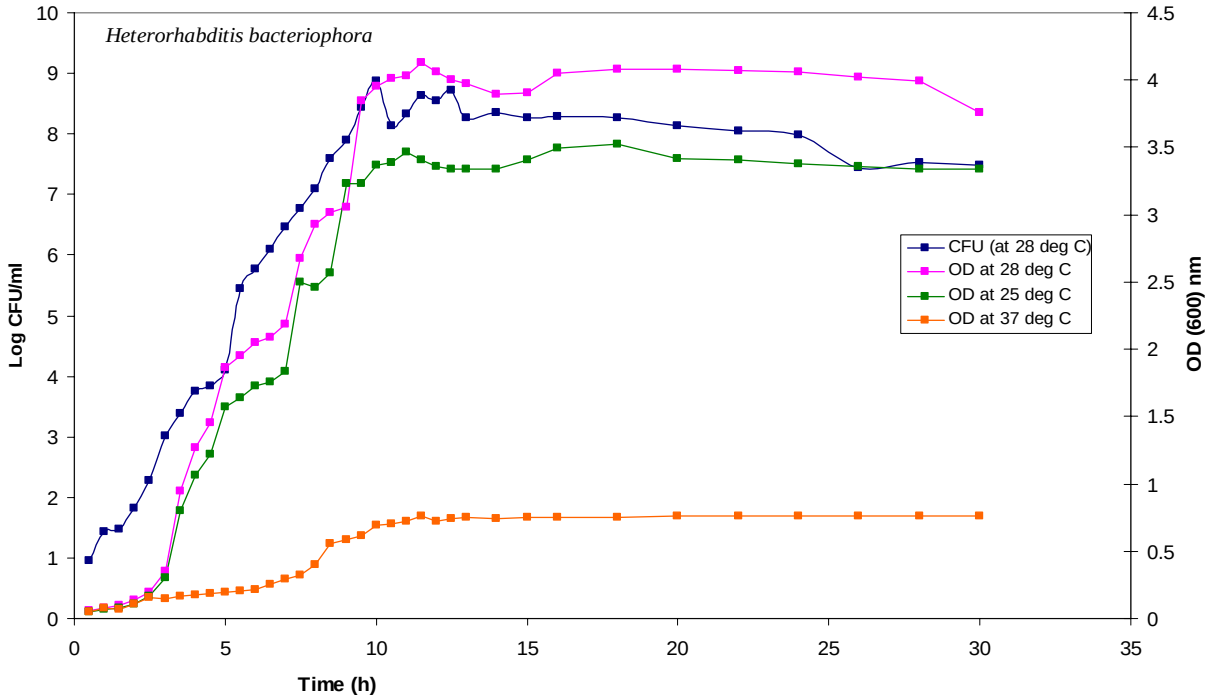


Fig 3.4: Growth profile of *Photorhabdus luminescens* subsp. *laumondii* (isolated from *H. bacteriophora)* at growth temperatures of 25, 28 and 37 ^{o}C. Data are means of three replicates. CFU readings were obtained at growth temperature 28 ^{o}C.

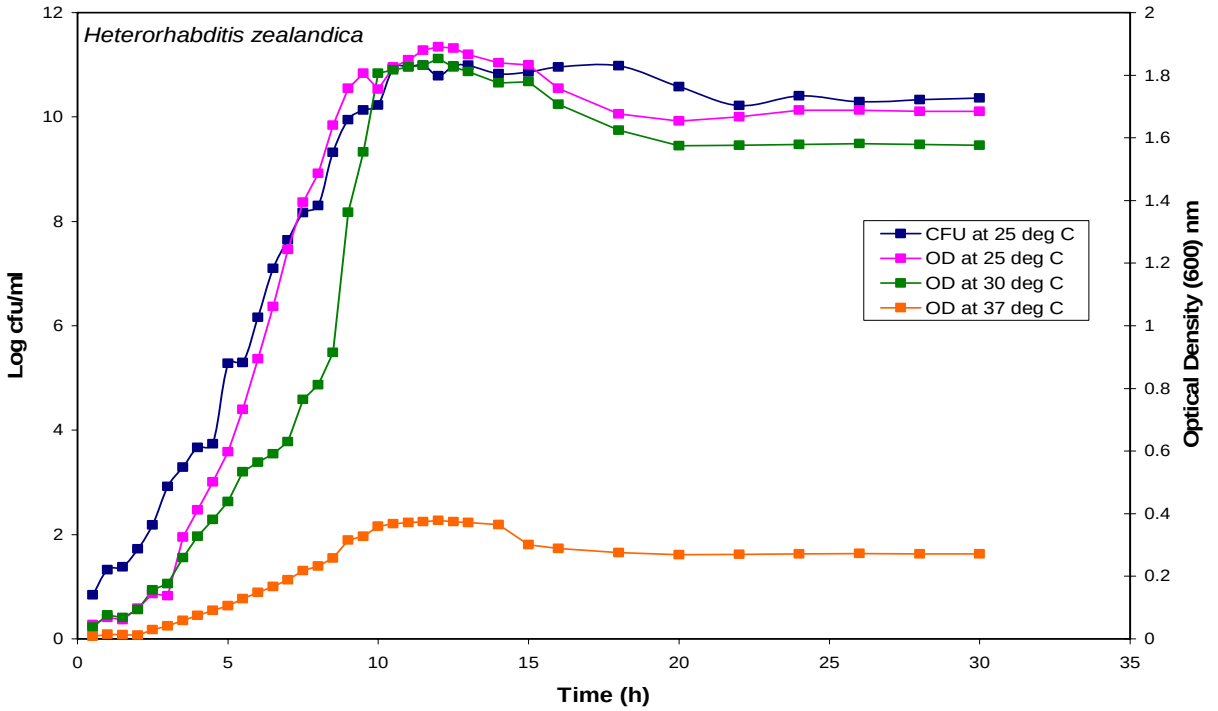


Fig 3.5: Growth profile of *Photorhabdus luminescens sp.* (isolated from *H. zealandica*) at growth temperatures of 25, 28 and 37 ^{o}C. Data are means of three replicates. CFU readings were obtained at growth temperature 25 ^{o}C.

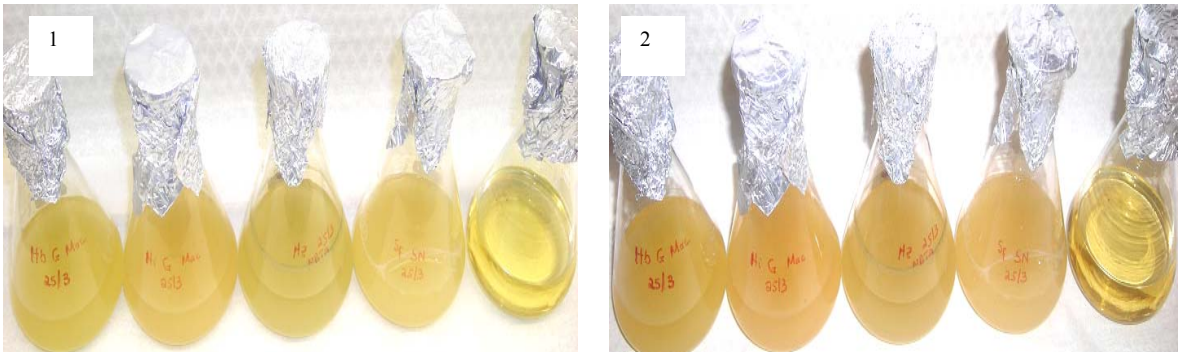


Fig 3.3: Nematode symbiotic bacteria in nutrient broth
Nutrient broth (NB) cultures of symbiotic bacteria associated with EPN
1 & 2: at time 12 h and 30 h respectively. 1A & 2A: P. *luminescens* subsp *laumondii,* 1B & 2B: *P luminescens* subsp. *akhurstii*, 1C & 2C: *P. luminescens sp.*, 1D & 2D: *X. bovienii*, E: Uninoculated nutrient broth (blank)

Table 3.1: Optimum growth temperature for symbiotic bacteria isolated from EPNs.

Nematode isolate	Symbiotic bacteria	Optimum growth temperature
H. bacteriophora	*P. luminescens* subsp. *laumondii*	28 ^{o}C
H. indica	*P. luminescens* subsp. *akhurstii*	28 ^{o}C
H. zealandica	*P. luminescens*	25 ^{o}C
S. feltiae strain SN	*X. bovienii*	28 ^{o}C

3.3.3 Culture of symbiotic bacteria (growth curves and optimum temperature).

The optimum growth temperatures were estimated from the growth curves of the different symbiotic bacteria are reported in Table 3.1. The data were obtained from measuring OD and CFU of nutrient broth cultures (Fig 3.3) grown at set temperatures. The incubator temperatures varied by ± 1 ^{o}C. Optimum growth temperature estimates were based on the length of the log phase and the number of cells produced measured indirectly by their level of light absorbance.

The bacteria growth profiles for the different symbiotic bacteria are shown in the Figs 3.4 – 3.7. The optimum growth temperature for *P. luminescens* subsp. *laumondii* was estimated to be 28 ^{o}C. However, when the growth of this same bacterium was tested at the same temperature using CFU, which is a more direct method of measuring growth, the curve was more similar to its growth pattern at 25 ^{o}C (Fig 3.4). The estimated optimum growth temperature for *P. luminescens sp* was 25 ^{o}C (Fig 3.5). The growth patterns for both *P. luminescens* subsp. *laumondii* and *P. luminescens sp* at 25 ^{o}C and 28 ^{o}C were almost similar, (i.e.) the curves almost touch each other. Both bacteria also managed to growth at 37 ^{o}C, but much fewer cells were produced. The lag phase was about 2 h at 25 ^{o}C and 28 ^{o}C but it was much shorter, about 0.5 h at 37 ^{o}C for both bacteria.

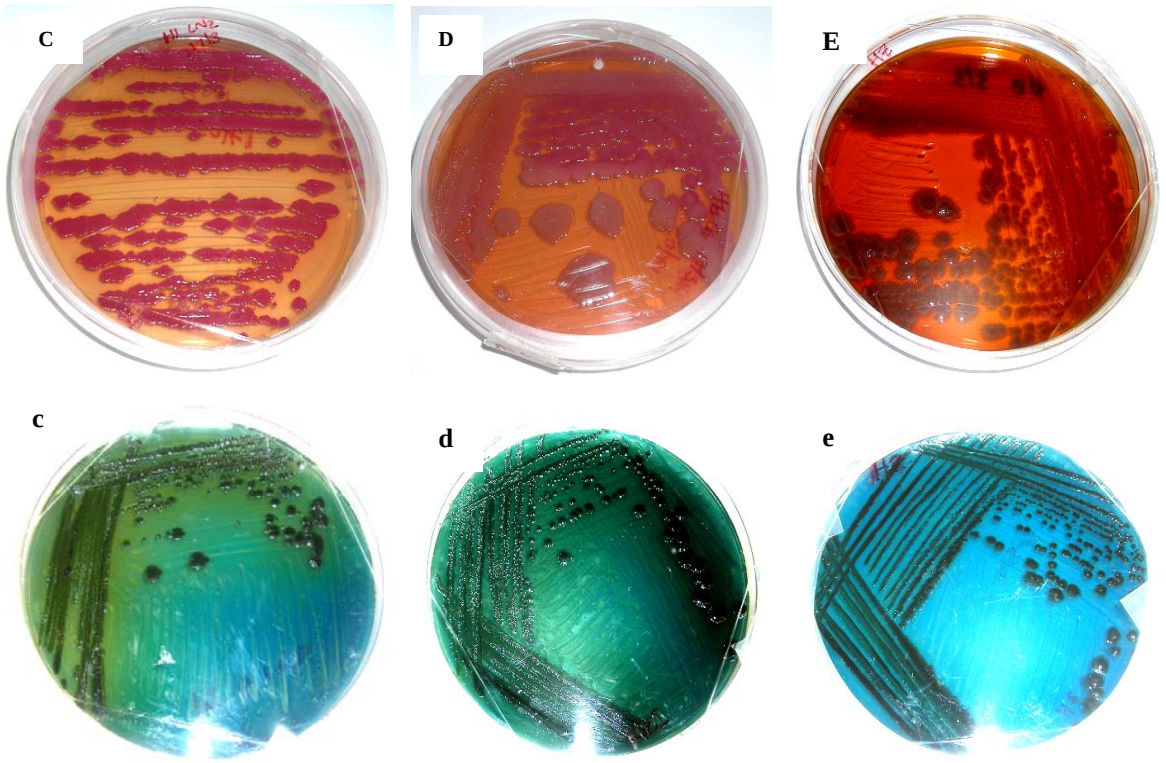


Fig 3.2 b: Symbiotic bacteria on MacConkey agar (red plates) and NBTA (blue plates).
Plates C and c are *P. luminescens* subsp. *akhurstii* isolated from *H. indica* strain LN2.
Plates D and d are *P. luminescens* subsp. *laumondii* isolated from *H. bacteriophora.*
Plates E and e are *P. luminescens sp* isolated from *H. zealandica.*
Colonies are deep red, red and faint red on MacConkey agar (C, D & E) respectively. On the NBTA plates, the colonies are green with a red centre.

3.3.2 Identification of *H. indica* symbiotic bacteria

The amplified 16S rDNA gene produced a PCR product of approximately 1,600 base pairs. When the sequenced product was compared with published sequences on NCBI using the algorithm BLAST, the homologous bacterial symbiont of *H. indica* LN2 showed a 99% similarity to *Photorhabdus luminescens* subsp. *Akhurstii* (Results not shown).

be detected on MacConkey agar cultures for all 3 bacteria. By the tenth day, most colonies had reverted to Phase II on both NBTA and MacConkey agar. By this time, none of the *Photorhabdus* spp. were bioluminescent (Fig 3.2b).

Xenorhabdus bovienii, the symbiont of *S. feltiae* was green on NBTA; its phase I colonies were surrounded by cleared zones due to the absorption of bromothymol blue from the NBTA agar. No bioluminescence was detected for *X. bovienii.*

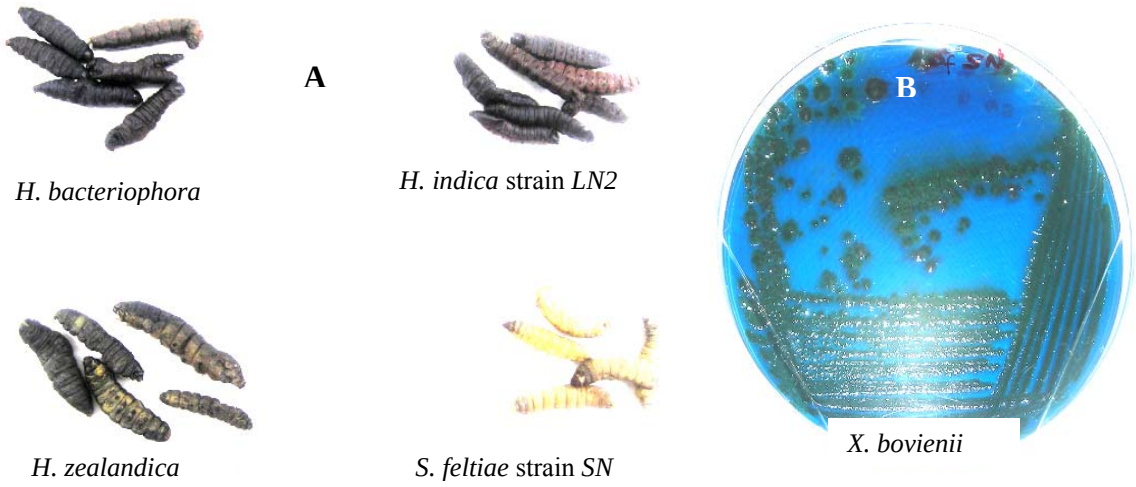


Fig 3.2a:
A: *G. mellonella* cadavers infected by different EPN isolates, those infected by *H. bacteriophora* and *H. indica* LN2 are brick red in colour, while those infected by *H. zealandica* and *S. feltiae* are green and brown in colour respectively.
B: Phase I colonies of *X. bovienii* isolated from *S. feltiae* are blue-green on NBTA agar.

3.3 Results

3.3.1 Characterisation of phase I and II variants

Symbiotic bacteria were isolated using one of two methods: streaking haemolymph or grinding surface sterilised IJs (section 2.3.1). Pure colonies of the bacteria were obtained by using the streak plate technique. On MacConkey agar, *Photorhabdus luminescens* subsp. *akhurstii* (isolated from *H. indica*) colonies were burgundy red (Fig 3.2b C); *P. luminescens* subsp. *laumondii* (isolated from *H. bacteriophora*) colonies were bright pink-red (Fig 3.2b D); while *P. luminescens sp* (isolated from *H. zealandica*) colonies were green-red (Fig 3.2b E). All three *Photorhabdus* species produced green colonies with reddish brown centres on NBTA plates. These pigmentations were also observed in the nutrient broth cultures (Fig 3.3). The colours were faint in the 12 h cultures, but had intensified after 30 h.

The morphology of all Phase I colonies of the *Photorhabdus* spp. were convex, opaque, had irregular margins and a sticky consistency (Fig 3.2b C, D and E); while phase II colonies were flat and translucent with a bigger diameter (e.g.) Fig 3.2b D. It was difficult to differentiate the phase variants in NBTA (Fig 3.2a & 3.2b: B, c, d & e).

The 'eye' technique was applied to detect if any of the symbiotic bacteria were bioluminescent. Bioluminescence was not detected in 24 h streak plate cultures of *P. luminescens akhurstii* and *P. luminescens* subsp. *laumondii*. Conversely, a faint luminescence was noticeable for *P. luminescens sp*. However, after 48 h, a faint luminescence was also detected in the other two bacteria. For all three bacteria, luminous intensity seemed to increase on daily, but was highest on days 3 & 4 on MacConkey agar, and days 4 & 5 on NBTA; although colonies of the *P. luminescens sp* (isolated from *H. zealandica*) were most luminous, when compared to the other bacteria. By day 6, bioluminescence could no longer

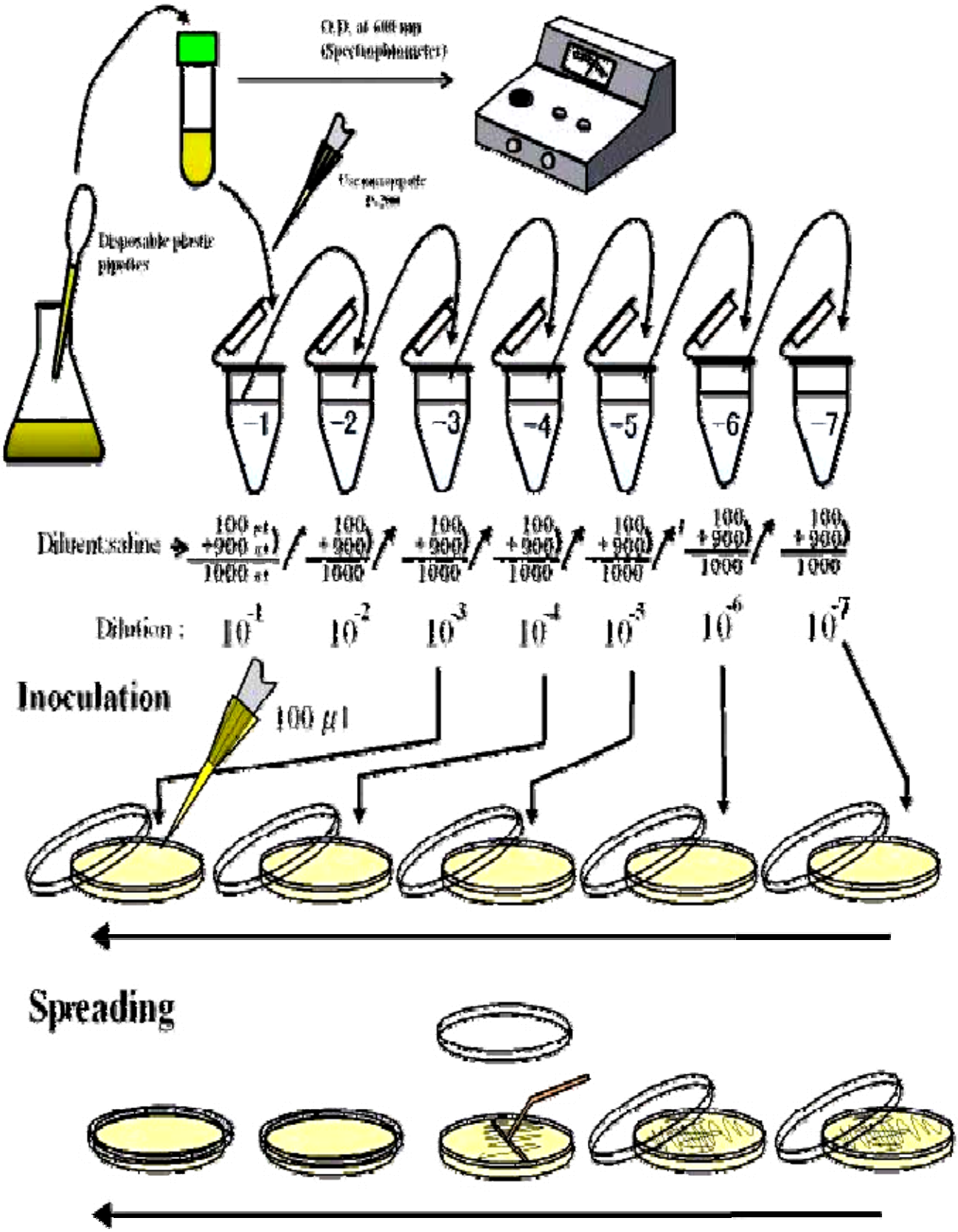


Fig 3.1**:** Enumeration of colony forming units and growth curve of bacteria

Http://wwwmicro.fhw.oka-pu.ac.jp/lecture/exp/exp-index.html

had been obtained indirectly using OD measurements. Thus, CFU was recorded only after the optimum growth temperature had been obtained. The optimum growth temperature was determined by comparing how fast a bacteria culture reached the logarithmic growth phase at the different temperatures. The exponential phase of growth for each symbiotic bacteria isolate was estimated from the growth curves.

3.2.2.4 The Growth Rate Constant (μ) and Mean Generation Time (g)

During the exponential (or logarithmic) growth phase, a bacterial culture mimics a first-order chemical reaction, i.e. the rate of increase of cells is proportional to the number of bacteria present at that time. The constant of proportionality or specific growth rate, μ, is an index of the growth rate and is called the growth rate constant:

Rate of increase of cells = μ x number of cells.

The value of μ can be determined from the following equation:

$\log_{10} N - \log_{10} N_0 = (\mu/2.303)\ (t - t_0)$

In other words, the natural log of the number of cells at time t minus the natural log of the number of cells at time zero (t_0) equals the specific growth rate constant multiplied by the time interval.

The mean generation time or "doubling time" (g) is the average time required for all the components of the culture to double. This is calculated from the following equation:

$\text{Log10}\ N_t = \log_{10} N_0 + g \log_{10} 2$

μ and g are related to each other: $\mu = \ln 2/g = 0.693/g$

participants who had all been given markers prior to the experiment were then asked to select the plates that "glowed in the dark" and mark the "glowing" spots. Drawings depicting the marked areas were made for comparison. The entire experiment was repeated on a daily basis starting with 24 h bacteria cultures on NBTA and MacConkey agar plates over a 5 day period, and then on day 7 for three consecutive days.

3.2.2.2 Identification of the symbiotic bacteria associated with *H. indica* LN2

Total genomic DNA was isolated from the symbiotic bacterium of *H. indica* (section 2.4.2). The 16S rDNA region of the bacterium was amplified by PCR (section 2.4.5). The PCR product was sequenced and compared with published DNA sequences in the NCBI database using the algorithm BLAST (section 2.4.5).

3.2.2.3 Culture of symbiotic bacteria (growth curves and optimum temperature).

Phase I colonies of the isolated symbiotic bacteria (section 3.2.2.1) were inoculated into 50 ml of nutrient broth contained in 100 ml Erlenmeyer flasks. These were allowed to multiply at 25 ^{o}C, on a platform shaker (Labcon) set at 180 rpm, for 30 h (there were three flasks per symbiotic bacteria per temperature). Optical density (OD) measurements at 600 nm were obtained every 30 min using a Diode Array® S2100 spectrophotometer (Biochrome). The symbionts of *H. zealandica* and *H. bacteriophora* were also grown at 28 ^{o}C and 37 ^{o}C respectively. Due to the labour and cost involved in using colony forming units (CFU) as a direct measurement of growth in the bacteria cultures, CFU were recorded (Fig 3.1) every 30 min over a 30 h period, only at the optimum temperature as a confirmation of the data which

Burnell (National University of Ireland, Maynooth, Ireland). It was originally isolated from Easwaramoorthy (Sugarcane Breeding Institute, Coimbatore, Tamil, India), *S. feltiae* strain SN (symbiotic bacteria: *Xenorhabdus bovienii*) was obtained from Ralf-Udo Ehlers (University of Kiel, Germany).

3.2.2 Isolation, identification and culture of symbiotic bacteria

3.2.2.1 Characterisation of phase I and II variants

The symbiotic bacteria associated with the above EPNs (section 3.2.1) were isolated as described in section 2.4.1. Streak plates of NBTA and MacConkey agar were prepared to obtain pure individual colonies. Phase I and Phase II colony variants (section 1.4) were selected/characterised based on colony morphology (Bleakley & Nealson, 1988; Kaya & Stock, 1997). Phase I colonies of symbiotic bacteria isolated from the *Heterorhabditis* spp. were checked for bioluminescence on a daily basis. There was no equipment available to measure bioluminescence, so it was determined visually. In order to confirm and improve the accuracy of the results, three other people were enrolled in the experiment to help identify bioluminescent bacteria; and they were orientated accordingly. Each participant was taken to a dark room at different time intervals, where streak plates of pure *Heterorhabditis* spp. Phase I colonies were being held. The plates had been laid out on a Table and covered with cardboard, such that none of the participants could see the plates prior to the experiment. Each person taken into the dark room was seated before the Table on which the plates had been set, and was left in the dark room for at least 30 min, which was time enough for their eyes to have adjusted to the darkness. The cardboard was then taken off the plates. The

growth temperature for each species was estimated. In addition, the growth rate and doubling time for each isolated was determined from the curves at optimum growth temperature for each species. In a study by Ehlers *et al.* (2000), the optimum growth temperature for *Photorhabdus luminescens* subsp. *akhurstii* was between 25 and 30 ^{o}C. In this study, 28 ^{o}C was used as its optimum growth temperature. For *Xenorhabdus bovienii* the optimum temperature is usually 28 ^{o}C (Boemare & Akhurst, 1988; Fischer-Le Saux *et al*. 1998). Thus these bacteria (*X. bovienii* and *P. luminescens* subsp. *akhurstii*) were grown at their optimum temperature of 28 ^{o}C in order to obtain information on their growth rate and doubling time.

3.2 Materials & Methods

3.2.1 Entomopathogenic Nematode isolates and their symbiotic bacteria

A total of 4 EPN isolates were used during this study. Two of the isolates *Heterorhabditis bacteriophora* and *Heterorhabditis zealandica,* with symbiotic bacteria *Photorhabdus luminescens* subspecies *laumondii* and *Photorhabdus sp.* respectively, obtained from Professor Vince Gray's laboratory (University of the Witwatersrand, Johannesburg, South Africa) were indigenous species. *H. zealandica* was isolated from a cool moist sandy soil collected from under a mango tree, on a farm situated on the north side of the Magaliesburg, near Hartebeesport dam, South Africa. *H. bacteriophora* was isolated from a dry sandy loam soil, in an uncultivated farm land at the ARC Roodeplaat experimental farm near Pretoria, South Africa. The other two nematode strains used in this study, namely: *Heterorhabditis indica* strain LN2 and *Steinernema feltiae* strain SN were exotic EPN species. *H. indica* LN2 (symbiotic bacteria: *Photorhabdus* luminescens *subsp. akhurstii*) was obtained from Ann

Phase I and Phase II forms of *Xenorhabdus* and *Photorhabdus* species have been isolated from the IJs of their symbiont nematodes, (i.e.) *Steinernema spp*. and *Heterorhabditis spp.* respectively. Apparently, the Phase I *Xenorhabdus spp*. are preferentially carried by the IJs which are attracted to the insect host (Bedding & Akhurst, 1975). According to Grunder (1997), the Phase I symbiont associates with the nematode IJ naturally, while the Phase II symbiont occurs after prolonged *in vitro* culturing, or *in vivo* when the nematode IJs emigrate from the cadaver. The Phase I form is superior to the Phase II form in its ability to support nematode propagation (Akhurst, 1980; Smigielski *et al*. 1994). Ehlers (2001) suggested that the optimum growth temperature of a new isolate should always be defined prior to mass production, as any deviation surpassing the optimum can induce the formation of the secondary phase, which impedes nematode reproduction. It is therefore important to ensure that the phase I variant is present when producing nematodes *in vitro* (Akhurst & Boemare, 1990).

The surveys of EPNs conducted from 2004-2006 by Gray and Ngoma (unpublished) in the provinces of Limpopo and Gauteng of South Africa yielded both *Steinernema* spp. and *Heterorhabditis* spp. EPNs; two of which were used in this study, namely; *H. bacteriophora* and *H. zealandica*. These indigenous species were used so as to provide the possibility of increasing the genetic variation of EPNs, adapted to the environmental conditions in South Africa. The other two EPN species used in this study were exotic, namely *Heterorhabditis indica* strain LN2 and *Steinernema feltiae* strain SN.

The EPN species used in this study had been donated with samples of their symbiotic bacteria except for *H. indica* LN2. After its isolation, molecular methods were used to confirm that it was indeed the symbiont of *H. indica* LN2. Growth curves at different temperatures were obtained for the different symbiotic bacteria, from which the optimum

Exotic EPNs have also been used in the past but there had been increasing concern about their introduction in many countries, because they may have a negative impact on non-target organisms (Bathon, 1996); but Parkman & Smart (1996) showed that even after the release of an exotic nematode species, no detrimental effects were observed. Additionally, the COST-OECD expert group evaluated and reported no long-term effects on non-target organisms (NTOs) or other environmental impacts following the application of indigenous or exotic EPN (Ehlers & Hokkanen, 1996).

EPNs of the genus *Steinernema* are symbiotically associated with bacteria of the genus *Xenorhabdus spp*. (Thomas & Poinar, 1979; Akhurst 1983) while EPNs of the genus *Heterorhabditis* are associated with bacteria of the genus *Photorhabdus spp*. (Molyneux, 1986; Boemare *et al*. 1993; Gaugler, 1998; Ehlers, 2001). These bacteria have attracted a lot of research. For instance, in the pharmaceutical and agro-forestry industries, EPN symbiotic bacteria, or somewhat their secondary metabolites, have been explored for their commercial potential (Webster *et al*. 1998). Furthermore, the occurrence of protein toxin genes identified in *Photorhabdus* spp. (Bowen *et al.* 1998), has enhanced the use of the bacteria alone for biological control. Nonetheless, the foremost applied interest in studying the symbiotic bacteria of EPN concerns nutritional requirements for improving mass production of the nematodes for the biological control of insect pests. In fact, it is known that the quality of the symbiont inoculum, in terms of viability and preservation from microbial contamination, must be maintained for effective commercial production (Ehlers *et al.* 1990, 1998).

The phenomenon of phase variation is characteristic of the *Xenorhabdus* and *Photorhabdus* species; the two extremes of which are Phase I (primary phase) and Phase II (secondary phase) (Akhurst 1980; Dybvig, 1993; Forst & Nealson, 1996; Owuama, 2001). The reason for the occurrence of the two forms is not known yet (Hazir *et al*, 2003). Both the

Chapter 3

ENTOMOPATHGOENIC NEMATODES AND THEIR HOMOLOGOUS SYMBIOTIC BACTERIA

3.1 Introduction

EPNs are found under diverse ecological conditions (Hominick *et al*. 1996; Kaya *et al*. 2006). Substantial efforts have been made in EPN research to isolate, identify and test a range of EPN against economically important insect pests. No one species of EPN is the best control agent for all or even most insect species. Consequently, it is preferable to examine different species rather than various strains of one or more species because species usually exhibit greater differences in infectivity (Bedding, 1990).

The African continent represents a fertile field for EPN exploration because only a few countries have been surveyed (Kaya *et al*. 2006). In contrast to human modified areas, natural habitats are likely uncontaminated by introduced nematodes and offer increased opportunities for finding native nematode species (Stock *et al*. 1999, 2003). In this respect the isolation of native species of EPNs provides a valuable source not only from a biodiversity perspective but also from a more applicable standpoint (Stock *et al.* 1999). These views support that of Bedding (1990), who suggested that, when developing a strategy for inundative release of EPNs against local insect pests, indigenous nematodes may be more suitable because of their adaptation to local climate and population regulators.

2.10.2 Symbiotic bacteria

Individual colonies of symbiotic bacteria were grown in nutrient broth (Merck) for 48 h at 25 ^{o}C on a 3081U platform shaker (Labcon). Aliquots (1.5 ml) of 24 h old NB bacterial culture were added to 0.3 ml of sterile glycerol in 2 ml safe lock tubes (Eppendorf ®) and mixed on a vortex mixer Vm-1000 (Sturdy). Tubes were stored at -20^{o}C for 1 month.

estimated by counting (section 2.5.2) under a dissecting microscope (Olympus) at 40X magnification.

2.9 Formulation

Several combinations of wetting and dipping agents were tested for their suitability to formulate nematode-infected cadavers. Four- and eight-day-old EPN-infected cadavers were formulated by immersing in a dipping agent for about 1 min to wet them, thus serving as an adhesive and coated in a rolling agent to protect the cadavers from sticking together and/or drying. Formulated cadavers were selected at random and put on White traps, (1 cadaver per White trap). The total number of IJs that emerged from each formulated cadaver was estimated by counting under a dissection microscope (Olympus) at 40X magnification.

2.10 Storage

2.10.1 Entomopathogenic Nematodes

About 1000-2000 IJs/ml were surface-sterilised in 0.1% hypochlorite (section 2.4.1.1) and stored on 1 cm^3 pieces of moist sterile polyurethane foam at 8 ^oC in 50 ml sterile tissue culture tubes (Corning ®) for about 1 month. The EPNs were also maintained *in vivo* in insects and *in vitro* on lipid agar and egg yolk agar plates.

of larvae to 200 IJs (per larva) for set time periods. (Insect mortality was determined by poking still insects; if no movement was noted, it was considered dead. Moreover, straight insects and insects which were already showing colour change as is evident in EPN-infected insects, were considered dead as well). The dose-response assay involved exposing larvae to different nematode concentrations ranging from 1 to 500 IJs/larva; mortality was monitored every 24 h over a 96 h period. Every EPN-infected insect cadaver was placed on a White trap (Fig 2.1b). The total number of IJs emerging from each insect larva was estimated by counting at 40X magnification.

2.8 Anhydrobiotic Potential of EPNs

The impact of the insect host cadaver on development and reproduction of the EPNs under desiccating conditions was tested by exposing EPN-infected insect cadavers to decreasing levels of relative humidity (RH). Thirteen 50 ml solutions composed of distilled water and glycerol (w/w) solutions with different RH levels ranging from 70.4% to 100% were prepared in 150 mm glass Petri dishes. Sixty five 48 h old EPN-infected insect cadavers were placed in the bottom a 90 mm Petri dish (BioLab), which was floated without the lid in the 100% RH environment. After 24 h, 60 of the larvae were moved to a lower RH environment, leaving behind 5 larvae at 100% RH. Every 24 h, all but 5 larvae were moved to a lower RH environment such that at the end of 13 days, there were 5 larvae per RH environment. On day 14, 3 EPN-infected cadavers per RH were chosen at random and placed individually on White traps at ambient laboratory temperature (ca 22-25 ^{o}C) to collect progeny IJs. This was done for each nematode species. The total number of IJs that emerged from each larva was

medium (excluding canola oil which did not mix well with other ingredients) were transferred to 250 ml Erlenmeyer flasks. After this step, 2 ml of canola oil was added to each flask using a 3 ml plastic Pasteur pipette. The flasks were stopped with cotton wool and covered with heavy duty aluminium foil. The flasks were autoclaved at 121 ^{o}C and 15 psi for 20 min. When the medium had cooled down, the flasks were pre-seeded with 3 ml of 24 h symbiotic bacteria NB cultures. The bacteria were allowed on a platform shaker (Labcon No 3081U) set at 180 rpm and 25 ^{o}C for 24 h. The flasks were inoculated with surface-sterilised IJs (section 2.4.1.1) at densities of about 2500-4000 IJs/ml.

2.7 Bioassays

Three types of bioassays, namely: the one-on-one bioassay (Miller, 1989), the exposure time assay (Glazer, 1992), and the dose response bioassay (Morris *et al*., 1990), were carried out to determine the virulence/infectivity and stability of the EPNs. All bioassays were conducted in multi-well plastic tissue culture trays (BD Falcon ^{TM}). The one-on-one bioassay was carried out to measure the proportion of the different nematode populations capable of infecting their insect hosts, hence creating a virulence profile of the EPNs in question. Individual insect larvae were exposed to one IJ for 72 h, after which the onset or occurrence of insect mortality was monitored every 24 h over a 4-day period. Every EPN-infected insect cadaver was placed on a White trap (1 cadaver per White trap). The total number of IJs that emerged from each larva was estimated by counting (section 2.5) under a dissection microscope (Olympus) at 40X magnification. The exposure time bioassay was carried out to determine the minimum time required by the different EPN isolates to penetrate and kill an insect host. The evidence of insect mortality was monitored every 24 h after initial exposure

48 h, a bacterial lawn had become established on the plates, which were then inoculated with surface-sterilised IJs (section 2.4.1.1). The majority of the lipid and egg yolk agar cultures consisted of progeny IJs after 14 days.

2.6.2 Liquid culture

Sterile IJs from 14- to 20- day old lipid agar EPN cultures were used as the inoculum for submerged liquid-medium shake-flask culture experiments. The IJs were obtained by washing the lipid agar plates with sterile distilled water. The IJs were collected into sterile 50ml polystyrene tissue culture tubes containing Ringer's solution. When the IJs had sedimented to the bottom of the container, excess liquid was removed using sterile disposable 3.0 ml plastic Pasteur pipettes. The remaining volume was recorded; IJ density was estimated by counting and then the volume was adjusted as to give an IJ density of 50,000 to 250,000 IJs per ml. Aliquots of sterile IJs (density between 2000 - 5000 IJs/ml) were added to 250ml Erlenmeyer shake flasks containing 50ml of egg yolk liquid culture medium adapted from Surrey and Davies, (1996).

Ingredients, egg yolk liquid culture medium (in 1 L of distilled water):

- 12.5 g of spray dried egg yolk;
- 23 g of yeast extract;
- 2.3 g of non-iodised sodium chloride
- 40 ml of corn oil*

**This was replaced with 40 ml canola oil.*

Preparation:

The egg yolk culture medium was prepared without canola oil because it was immiscible with the other components of the culture medium. Fifty millilitre aliquots of the egg yolk

2.6 *In Vitro* Culture of EPNs

2.6.1 Solid culture

EPNs were cultured on lipid agar adapted from Kaya & Stock, (1997) and egg yolk agar adapted from the egg yolk liquid culture by Surrey & Davies (1996).

Ingredients per 1 L of distilled water:

Lipid agar

- 10 g corn syrup*
- 5 g yeast extract
- 25 g nutrient agar
- 2.5 ml cod liver oil
- 2 g $MgCl_2.6H_2O$

**10 g of honey was used in place of corn syrup*

Egg yolk agar

- 12.5 g of spray-dried egg yolk
- 23 g of yeast extract
- 2.3 g of non-iodised sodium chloride
- 40 ml of corn oil*
- 10 g of agar bacteriological**

** Only 3 ml cod liver oil used instead of 40 ml corn oil*

*** Was added to make a solid medium*

Preparation:

Although the recipe is for 1 L, the ingredients were weighed out for a 0.8 L recipe to prevent the media from boiling out during autoclaving. The components of either medium were usually weighed out into1 L Schott bottles (Duran), and mixed on a magnetic stirrer (No MSH10, Labcon). When the ingredients had blended together, the medium was autoclaved at 121^{o}C and 15 psi for 20 min and about 20 ml aseptically poured into sterile 90 mm Petri dishes (BioLab) under a laminar flow (Scientific). Lipid and egg yolk agar plates were pre-seeded with 0.5 ml of 24 h symbiotic bacteria NB culture and incubated for 48 h 25 ^{o}C. After

dishes were counted under a dissecting microscope. Final nematode concentration (yield) per ml was calculated by multiplying the average of the three 50 µl counts by 20.

2.5.3 Adjusting nematode concentrations

A defined number of nematodes were needed to inoculate insects for bioassays or the liquid culture media. For smaller numbers, nematodes were counted individually because, at low concentrations (<50 IJs) estimation of nematode numbers by the procedure described above (section 2.5.2) would have been inaccurate due to large variation in sampling. Therefore, counts of individual nematodes of each sample are required. Conversely, this method was not reasonable for counting >50 IJs, and is almost impossible as nematode numbers become larger. As such, to adjust to any concentration, the following formula (Glazer & Lewis, 2000) was used: $[(i/c) - 1] \times V = V_a$

Where i = initial concentration 50 µl-1, c = final concentration 50 µl-1, V = volume of the suspension (ml), and V_a = the amount of water (ml) to be added if (positive) or removed (if negative) from the suspension. If a higher concentration was needed, the nematodes were allowed to settle to the bottom of the container for at least 30 min, after which excess water (determined by the above formula) was removed. The final concentration was checked by repeating steps (a) and (b) above (section 2.5.2). The final count was usually within ± 10% of the needed concentration.

using the program BioEdit, and then compared to published sequences in the NCBI database by using the algorithm BLAST, in order to identify the most similar 16S rDNA sequences.

2.5 Cleaning & Counting of Nematodes

2.5.1 Cleaning the nematode suspension

IJ of various nematode species/strains were obtained from different sources: from *in vivo* cultures as described above (section 2.2) or from solid or liquid *in vitro* cultures as described below (section 2.6). Some nematode suspensions included media (e.g. liquid cultures), dead non-infective and some dead infective stages. The IJ suspensions were cleaned by physical means, that is, they were washed and settled several times in sterile distilled water. To avoid pre-selection of active nematodes, separation techniques based on nematode behaviour such as migration through a modified Baermann funnel was not used.

2.5.2 Estimating final nematode yield

Counting large numbers of nematodes was impractical, so the following serial dilution method described by Glazer & Lewis (2000) was commonly used during the course of this study: (a) the nematode suspensions were properly shaken in the 50 ml tissue culture tubes they were contained in. A 50 µl aliquot was withdrawn with a micropipette and transferred to a 5-cm Petri dish. Three such samples were taken from each suspension and placed into 3 different Petri dishes; 15 ml of water was added to each Petri dish. (b) The nematodes in the

a) Initial denaturation: 92 ºC for 2 min

b) 35 cycle amplification series:

   i. 95ºC for 30 sec (denaturation)

   ii. 57ºC for 30 sec (reannealing)

   iii. 72ºC for 1 min (extension)

c) Final extension: 72ºC for 10 min

2.4.4. Agarose gel electrophoresis of the PCR products

The PCR products (section 2.4.3) were separated in 1% (w/v) agarose gels (Sigma) in electrophoresis buffer (appendix III) at 80V. Four µl of the PCR product was first mixed with 1µl of tracking dye (Promega) on a square of Parafilm®, after which the mixture was loaded into the gels. Five µl of a 1 Kb DNA Molecular Weight Marker with tracking dye (Promega) was also loaded into the centre slots of the gels. The separated DNA bands in the agarose gels were viewed under ultraviolet light and photographed with a Polaroid camera (UVP GelDoc).

2.4.5 Nucleotide sequencing of PCR-amplified 16S rDNAs

The PCR-amplified 16S rDNAs of the bacteria were sequenced. Single-strand sequencing was performed by Inqaba Biotechnological Industries (Pty) Ltd.), using the two aforementioned PCR primers (Table 2.1). The 16S rDNA sequences obtained were aligned

2.4.3 PCR amplification of the region of the 16S rRNA gene

One DNA segment containing the 16SrRNA gene (approximately 1.6 kb) from each bacterial isolate was amplified using two eubacterial-specific oligonucleotide primers (Table 2.1), synthesized by Inqaba Biotechnological Industries (Pty) Ltd.

Table 2.1: Oligonucleotide primers used for the amplification of a region of the bacterial 16S gene (Brunel *et al*, 1997; Liu *et al*., 2001)

Oligonucleotide	Sequence	T_m (^oC)
Sense primer *E. coli* numbering 6–25	5'-GGAGAGTTAGATCTTGGCTC-3'	62
Anti-sense primer *E .coli* numbering 1540–1521	5'-AAGGAGGTGATCCAGCCGCA-3'	66

PCR tubes (Whitehead Scientific 0.2ml thin wall PCR® tube with domed cap) were set up as follows: 25µl of 2X PCR Master Mix (Promega), 1µl of each primer (sense and anti-sense), 20.5µl nuclease free water (Promega) and 2.5µl of template DNA which was always added last to avoid contamination. A positive control with *E. coli* was prepared. A negative control was also prepared, containing all the above components except the template, which as replaced with nuclease-free water. All the PCR tubes including the controls were centrifuged at 10000 rpm for 1 min. The following cycling file was used to amplify the DNA in a Gene Amp® PCR express 2700 thermocycler (Applied Biosystems):

by adding 31 g of nutrient agar (NA) and 0.025 g bromo-thymol blue (BTB) and made up to I L using distilled water in a 2 L Schott ® bottle. The mixture was autoclaved for 20 min at 121 ^{o}C and 15 psi. When it had cooled to 50 ^{o}C, 0.04 g triphenyl tetrazolium chloride (TTC) (Sigma) was dissolved in 0.5 ml cold sterile distilled water, and added to the NA-BTB mixture after being filtered through a 0.2 μm filter (Cuno AMF Zetaphor). TTC was added last because it breaks down at temperatures higher than 50 ^{o}C.

2.4.1.2 Isolation of bacteria from insect haemolymph (Kaya & Stock, 1997).

G. mellonella or *T. molitor* larvae were exposed to 100 IJs/insect in 90 mm Petri dishes (BioLab) lined with moist Whatmann® filter paper (Cat No 1001090). After 48 h, dead insects infected by EPNs were surface-sterilised by dipping in absolute alcohol (96% concentration), ignited for 2 sec and cooled down by plunging into sterile distilled water under a laminar flow. The cadavers were carefully cut open aseptically with a sterile scalpel (Saltex), taking precaution to avoid rupturing of the mid-gut. Haemolymph was streaked on to MacConkey, NBTA or nutrient agar, using a heat-sterilised inoculating loop.

2.4.2 Bacterial genomic DNA extraction

Total genomic DNA was extracted from the symbiotic bacteria as follows using Instagene matrix (Bio-Rad cat # 732-6030) as outlined in appendix II. The isolated DNA was analysed for purity (260/280 nm absorbance ratio) and quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

2.4 Isolation & Identification of Symbiotic Bacteria

2.4.1 Isolation of symbiotic bacteria

The symbiotic bacteria associated with the EPNs in this study were isolated either from the haemolymph of infected insect hosts or from surface sterilised IJs.

2.4.1.1 Isolation of bacteria from surface-sterilised IJs (Kaya & Stock, 1997).

Working under a laminar flow (Scientific), IJs collected from White traps (section 2.3). These were transferred into 50 ml plastic tissue culture centrifuge tubes (Corning) and allowed to settle by gravity, after which the excess Ringers solution they were contained in was removed from the tubes. 20 ml of 0.1% (v/v) sodium hypochlorite (28.6 ml JIK ® in 1000 ml sterile distilled water) was added to the tubes to sterilise the IJs for 1 h, and by this time the IJs had settled to the bottom of tube. Using a 3 ml disposable sterile plastic Pasteur pipette (Corning), the IJs were transferred to fresh 0.1% hypochlorite solution contained in another 50 ml plastic tissue culture centrifuge tubes for a further 3 h. Excess hypochlorite solution was removed from the tube and IJs were rinsed three times in sterile distilled water. The sterilised IJs were then homogenised in a sterilised mortar and pestle, and the homogenate transferred to a 100 ml Erlenmeyer flask (Duran) containing 50ml of nutrient broth on a platform shaker (No 30814, Labcon) set at 180 rpm and 25^{o}C for 48 h, for the bacteria to multiply. A sample of the bacterial culture was plated on to nutrient broth-bromothymol blue-triphenyltetrazolium chloride-agar (NBTA) and/or MacConkey agar plates to obtain pure single colonies of the symbiotic bacteria. Pure colonies were selected from the plates and re-plated onto fresh NBTA and MacConkey plates. NBTA was prepared

Fresh Ringer's solution was added to the White traps until IJs ceased to emerge from the cadavers, after which the cadavers were discarded.

Two methods were used in setting up White traps depending on the experiment. If the IJs were to be used for inoculating *in vitro* cultures (section 2.5), then the White trap was set up with about 5-10 EPN-infected insect cadavers as shown in Fig 2.2a. In bioassay experiments where IJs were counted per insect, the White traps were set up with only one insect (Fig 2.2b)

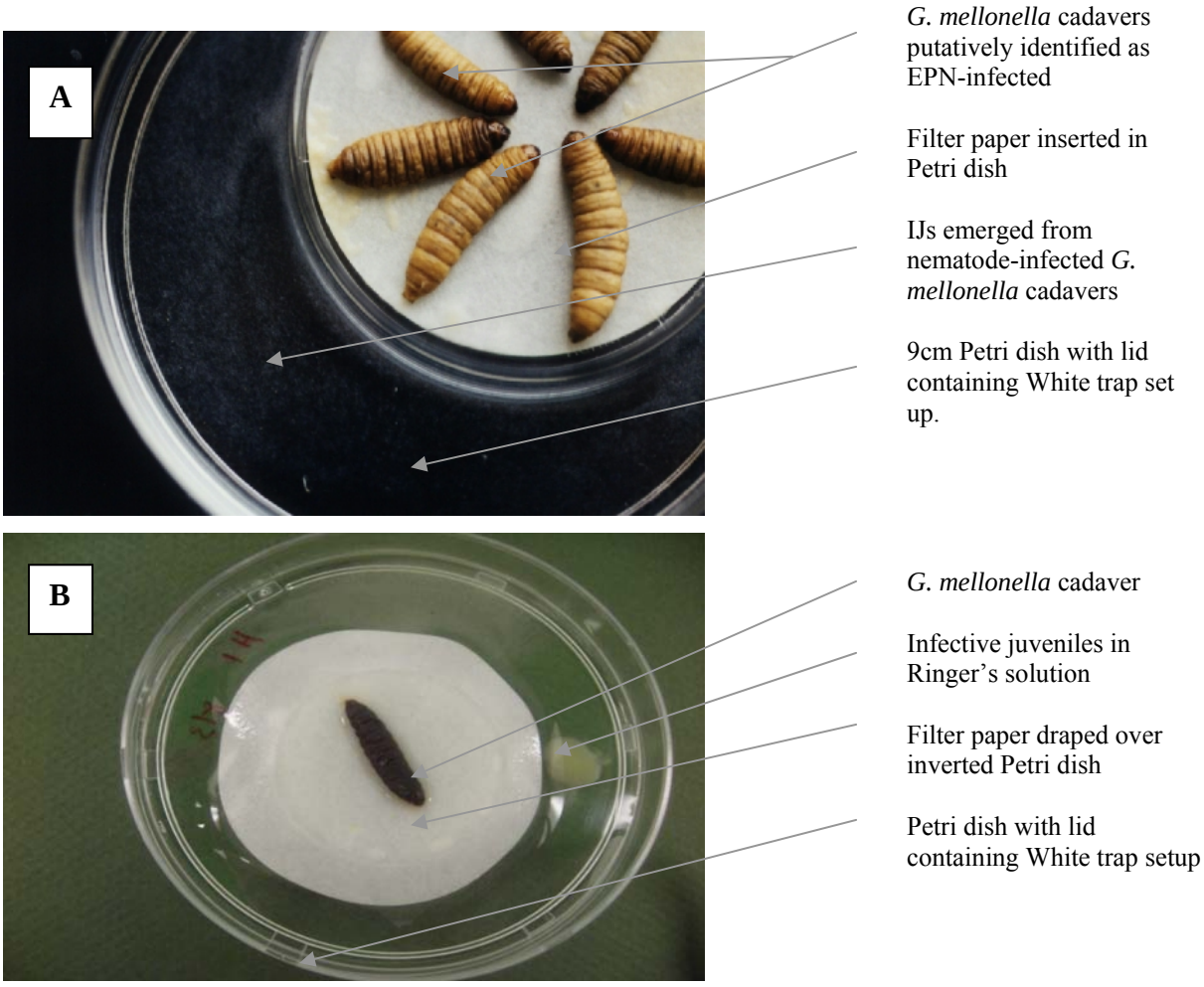


Figure 2.2: Illustration of White traps used to collect IJs from many (2.2 A) and one (2.2 B) EPN-infected *Galleria mellonella* wax worm insect cadaver(s).

undisturbed in the dark. Fresh bran and fruit or vegetable pieces were added to the adult population as well to keep the cultures thriving.

2.2 *In Vivo* Culture of EPNs

EPNs were maintained as *in vivo* cultures in all three insect species namely *G. mellonella* and *T. molitor*. Insect larvae were placed on a thin layer (2 – 4 mm) of sterilized river sand in Petri dishes. About 50 IJs in sterile distilled water were applied per larvae to the sand, such that the sand was moist (about 8.0% w/w) but not wet. After 7 – 10 days following infection of the larvae, progeny IJs were collected from the insect cadavers using the White trap procedure described below (section 2.3).

2.3 White Traps

The White trap was first introduced by White (1927). The insect cadavers (section 2.3) recovered from the soil samples were placed on modified White traps as described by Kaya & Stock (1997). A 5 cm diameter inverted Petri dish was placed in the bottom half of a 9 cm Petri dish half-filled with Ringer's solution. Filter paper of about 5.5 cm diameter (Whatmann) was draped over the inverted Petri dish with its edges touching the Ringer's solution. The cadavers were placed on the filter paper and covered with the Petri lid. After 10-15 days, the IJs emerged from the cadavers leaving behind the cadaver tissues, migrated over the filter paper and ended up in the Ringers solution from which they were collected.

- 1.5 ml calcium propionate**
- 2 g of methyl-4-hydroxybenzoate***

**Multivitamin bran was replaced with 500g ProNutro original*

*** Calcium propionate was replaced with 1M HCl*

****This served as a preservative to prevent fungal/bacterial growth*

Method: The honey, glycerol, and 150 ml of distilled water were combined together in a 1 L beaker. The contents were warmed in a microwave oven to make the liquids less viscous. Meanwhile, the ProNutro, yeast extract powder, preservative and 1M HCl were combined together in a mixing bowl. The honey-glycerol-water mixture was then added to the dry ingredients and mixed properly. The remaining 150 ml of water was boiled and used to rinse out the beaker into the mixing bowl with the rest of the ingredients. The resulting mixture was cooked on high in a microwave oven until the medium formed a sticky consistency. The medium was then cooled and broken into little pieces, which were then transferred to the *G. mellonella* rearing containers.

2.1.2 *Tenebrio molitor* (meal worm) rearing

A starter culture of meal worm larvae was purchased from a pet shop. They were fed with autoclaved wheat bran and kept in the dark at 25 °C. Apple, carrot and or potato slices were placed on top of the bran to provide moisture for the growing larvae. The adults (darkling beetles) were moved to new breeding containers to copulate and lay eggs. The bran was sieved every week to separate the eggs from the adults, and the eggs were also incubated

2.1.1 *Galleria mellonella* (wax worm) breeding

The wax worms were reared in 3 L volume Consol® glass jars (11 cm diameter and 15 cm height) at 25-28 ^{o}C on an artificial medium. The method was adapted from Woodring & Kaya (1988). The metal Consol ® jar lids were modified by cutting an 8 cm diameter circular opening into the lid. A 9 cm diameter circular stainless steel mesh screen (350 μm) was placed on the inside or underside of the modified lid. The metal screen was clamped in place sealing off the opening by screwing the lid tightly onto the jar. The screen facilitated heat and air exchange while preventing larval or moth escape from the jar. Adult moths were placed in the containers ensuring that there was at least a 1:1 male to female ratio to enable mating of the females. Wax paper was pleated or crumbled into loose balls and placed in the containers to serve as oviposition papers. The papers were checked for eggs regularly; eggs were removed and placed in other glass jars set up as described above. Fresh food was added to the containers fortnightly to keep the cultures thriving. In case of fungal/bacterial growth or mite infestation, the culture in question was discarded, and the glass container washed with soapy water and sterilised in an autoclave.

The following *G. mellonella* growth medium recipe used in this study was adapted from Woodring & Kaya (1988):

Ingredients:

- 200 ml honey
- 200 ml glycerol
- 300 ml distilled water
- 500 g multivitamin bran *
- 5 teaspoons yeast extract powder

Chapter 2

MATERIALS AND METHODS

The following flow chart (Fig 2.1) depicts the sequence of experimental activities and experimental methodology that was followed in these investigations:

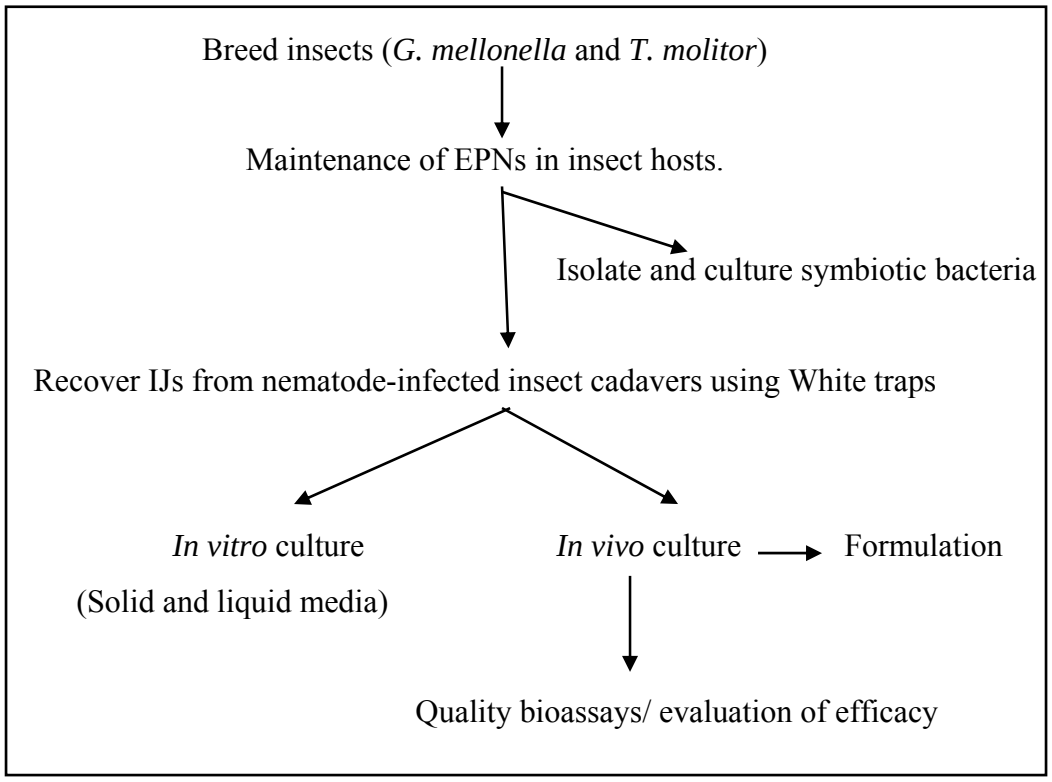


Fig 2.1: Overview of methodology

2.1 Insect Hosts

Two insect species were used during this study; namely: *Galleria mellonella* and *Tenebrio molitor.*

The rationale behind this research was to assist in the development of a method for cost effective production of EPNs for biological control. It is note worthy that not all aspects towards developing a mass production system for EPNs could be examined in a study of this magnitude. Nonetheless, some features have been explored effectively.

This dissertation has been structured as follows: The previous sections of this Chapter (1), was a literature review covering some of the important aspects surrounding biological control as a whole. A background on EPNs and their symbiotic bacteria, as well as the insect hosts used in the study was also included. The chapter concluded with the objectives of the study. Chapter 2 presents a detailed break down of the materials and methods employed during this study. Chapter 3 presents information on the sourcing of two local and two exotic EPN species, as well as the isolation, culture and maintenance of the bacterial symbiont isolated from each EPN. Chapter 4 describes a series of bioassays that were carried out to evaluate the virulence and efficacy of the EPNs, as well as to determine their stability and potential for mass production. In chapter 5, various parameters and considerations in the *in vitro* production of EPNs which investigated and experimented with at laboratory level are presented. Results of the anhydrobiotic potential of the nematode species used in this study are presented in Chapter 6. Chapter 7 covers the laboratory scale production of the host insect (*T. molitor*), the *in vivo* production of *H. indica* strain LN2, and formulation of nematode-infected cadavers in several combinations of rolling and dipping agents.

(Spaull, 1991). Taylor (2000) isolated EPNs from South African soils in the greater Johannesburg area, but did not characterise any of the isolates. More recently, Nguyen *et al*. (2006) reported a new species- *Steinernema khoisanae* n. sp from South African soils while Malan *et al*. (2006) described EPNs from the south western parts of South Africa.

It seems that very little work has been conducted on EPNs in South Africa, yet the EPNs have a great possibility in being used for biological control. In view of the aforementioned, the current study sought to determine whether indigenous nematode isolates, in particular, can be used to mitigate the destructive behaviour of local target pests. Consequently the ultimate goal of this research was to develop a process technology, for the production of highly effective, low-cost nematode-bacteria products, utilising both indigenous internationally sourced isolates for the biological control of insect pests that attack plants. In this study, 4 EPN species were investigated, two were indigenous and the other two were already being used as biocontrol agents elsewhere. The objectives were to:

- To investigate the growth dynamics of the symbiotic bacteria associated with 4 EPNs
- To evaluate efficacy and pathogenicity in terms of IJ density in bioassay studies.
- To investigate the growth dynamics and yield of indigenous EPNs under *in vitro* culture on conditions in a liquid medium using shake-flasks.
- To determine the significance of lipid composition on IJ yield and production time.
- To test the feasibility of formulating nematode-infected insect cadavers to overcome the hindrances of storage and handling.
- To explore the role of the insect host cadaver on survival of EPN isolates under desiccating conditions.

near soil line; and are pests to many types of stored grain (http://insected.arizona.edu/mealinfo.htm).

1.6 Aims and Objectives

In almost all countries, EPNs are exempted from registration requirements as bio-control agents of insect pests (Kaya & Gaugler, 1993). This enables small and medium sized enterprises to develop nematode-based plant protection products (Ehlers, 2001). EPNs are mainly produced commercially in Asia, Europe and USA by very few companies. As a result, the prices of EPNs are still too high to permit their application on low value crops (Kaya & Grewal, 2002). *Kaya et al*. (2006) reported eight producers of EPNs in the USA and five in Europe in 2004. This total of 13 did not include distributors.

The African continent represents a fertile field for EPN exploration because only a few countries have been extensively or systematically surveyed (Kaya *et al.* 2006). In the broadest geographic sense, EPNs are widespread. *Steinernema* and *Heterorhabditis* species are ubiquitous in distribution and have been recovered from soils throughout the world (Hominick *et al*. 1996), except Antarctica (Hominick *et al.* 1996; Hominick, 2002). Hence, there is opportunity for the discovery of new nematode species and strains adapted to local environmental conditions and pests.

A few surveys for entomopathogenic nematodes have been documented with a number of new species reported e.g. *S. karii* from Kenya (Waturu *et al.* 1997) and *H. taysaerae* from Egypt (Shamseldean *et al.* 1996). New strains of *H .indica and H. bacteriophora* were reported from Kenya (Burnell & Stock, 2000) and Egypt (Stack *et al*. 2000; Hominick, 2002). Some EPNs have also been recovered from South African soils (e.g.)

and are widely available from many commercial sources (Kaya & Stock, 1997; Ehlers, 2001; Hazir *et al,* 2003).

1.5.2 *Tenebrio molitor*

Tenebrio molitor more commonly known as the mealworm is the larval form of a species of darkling beetles. They are of importance to many predators like rodents, lizards, predatory beetles, spiders, and birds. Other than *G. mellonella,* the most commonly used host for *in vivo* culture is *T. molitor,* but little research had been reported on IJ production in this host (Shapiro-Ilan & Gaugler, 2002). In recent years, there are more reports citing the use of *T. molitor* as susceptible hosts (e.g.) Bruck *et al.* 2005; Shapiro-Ilan *et al.* 2006; Ramos-Rodriguez *et al.* 2006; etc. Shapiro-Ilan *et al.* (1999) reported average number of IJs produced per *T. molitor* larva ranging from 51875 for *H. marelatus* to 115538 for *H. bateriophora* strain TF. Clearly, nematode yield in *T. molitor* varies among nematode strains and species (Shapiro-Ilan & Gaugler, 2002). Like all holometabolic insects, *T. molitor* (the mealworm) goes through four life-stages: egg, larva, pupa, and adult. Larvae typically measure about 2.5cm or more, whereas adults are usually between 1.25 - 1.8 cm in length (http://en.wikipedia.org/wiki/Mealworm). The larvae and adults (beetles) eat decaying leaves, sticks, grasses and occasionally new plant growth. As general decomposers, they also eat dead insects, faeces and stored grains. Mealworms live under rocks and logs, in animal burrows and in stored grains. They prefer darkness and to have their body in contact with an object. Mealworms have a positive impact on the ecosystem in that they promote the degradation and recycling of organic materials not readily used by others and are food for other animals. On the downside, mealworms sometimes feed on seedlings and clip plants off

chloride. The exception is *Xenorhabdus poinarii* whose colonies are red for both phase I and II because they do not adsorb BTB (Kaya & Stock, 1997).

1.5 Insects used for rearing EPNs

1.5.1 *Galleria mellonella*

Galleria mellonella belongs to the order Lepidoptera (moths and butterflies). It is commonly known as the greater wax moth. It causes the greatest damage in apiaries, leading to material and financial losses every year (Http://www.Apis.admin.ch/English/pdf/Deseases/mottenschaedene.pdf).

G. mellonella development goes through 4 consecutive stages: egg, larva, pupa and adult. The cycle can last anywhere between 6 weeks - 6 months depending on the temperature and the availability of food. The adult moth requires no food or water (Williams, 1978). The mated female moth lays about 300-1000 eggs usually in batches of about 100. They are laid in cracks or corners. The eggs are dirty white in colour, oval, and about 0.5 mm across. They are inconspicuous and easily overlooked. At lower temperatures, the life span of the adult is greatly increased, taking up to 30 days to hatch. At temperatures between 30-35^{o}C which is optimum, the eggs hatch 3-5 days after oviposition. Variations that occur in the body size and colour of the insect depend on the diet of the larvae (Williams, 1978).

The late instar of larvae of *Galleria* is used extensively as the host(s) of choice for the *in vivo* culture and study of EPNs. Hazir *et al*. (2001) reported 80,000 IJs while Shapiro-Ilan *et al.* (2001) reported up to 300,000 IJs emerging from one *G. mellonella* cadaver. *Galleria* larvae are eminently suitable because they are susceptible hosts, are easily reared in the lab

EPN symbiotic bacteria phase variants differ in their biochemical and physiological properties; and vary in their colony morphology. Differences between the primary and secondary forms are mostly biochemical: the primary form produces antibiotics, adsorbs certain dyes, and develops large intracellular inclusions composed of crystal proteins; whereas, the secondary form does not or only weakly produces antibiotics. It does not absorb dyes and production of intracellular inclusion bodies is inefficient (Forst & Nealson, 1996). Kaya & Stock (1997) described the colony morphology of phase I, when grown on MacConkey agar or NBTA as granulated, convex, opaque and circular with irregular margins. The colonies also have a sticky consistency. The cells are small to middle sized and the majority have ovoid and or rhombic or rectangular inclusion bodies. They also show positive antibiotic activity. Phase II cells of *Photorhabdus* spp. exhibit only about 1% of the bioluminescence exhibited by Phase I cells in the dark (Boemare & Akhurst, 1988). On MacConkey agar, *Photorhabdus* spp. Phase I colonies are red, bright pink or red-brown due to the dye absorbed from the medium, while Phase II colonies are flat, translucent and have a yellow-brown colour. On nutrient broth-bromothymol blue-triphenyltetrazolium chloride-agar (NBTA), phase I colonies vary in colour from blue to green to purple to red depending on the species (Kaya & Stock, 1997). Therefore, NBTA is a less reliable indicator of phase variants since several Phase II *P. luminescens* show differences in their luminescence intensity (Gerritsen *et al*., 1992). *Xenorhabdus* spp. usually has mucoid colonies and produce pigments (Boemare & Akhurst, 1988; Givaudan *et al*. 1995). As such, the Phase I colonies are surrounded by cleared zones in NBTA because bromothymol blue (BTB) has been adsorbed from the NBTA to produce blue to green colonies (Akhurst, 1986); whereas Phase II colonies are shaded from red to rust from adsorption and reduction of triphenyltetrazolium

The phenomenon of phase variation is characteristic of the *Xenorhabdus* and *Photorhabdus* species and appears to be a response to environmental change. The two extremes are Phase I or the primary phase, and Phase II or the secondary phase (Akhurst 1980, Dybvig, 1993). There is evidence to suggest the existence of other phase variants or intermediate phases (Wang & Dowds, 1993; Gerritsen & Smits 1997). Both the Phase I and Phase II forms of *Xenorhabdus* and *Photorhabdus* species have been isolated from the IJs of their symbiont nematodes, (i.e.) *Steinernema spp*. and *Heterorhabditis spp.* respectively. Apparently, the Phase I *Xenorhabdus spp*. are preferentially carried by the IJs which are attracted to the insect host (Bedding & Akhurst, 1975), because they support nematode reproduction better than the phase II (Smigielski *et al*. 1994). According to Grunder (1997), the Phase I symbiont associates with the nematode IJ naturally, while the Phase II symbiont occurs after prolonged *in vitro* culturing, or *in vivo* when the nematode IJs emigrate from the cadaver . The phase II variant is not retained by the IJs of *Heterorhabditis bacteriophora* (Han & Ehlers, 2001). The reason for the occurrence of the two forms is not known yet (Hazir *et al*, 2003).

The primary form is superior to the secondary form in its ability to support nematode propagation (Akhurst, 1980); (e.g.) Phase I *P. luminescens* in association with *Heterorhabditis spp*. usually produce three times more IJs, but over seven times more are produced when *Steinernema* spp. associate with Phase I *Xenorhabdus* spp. than when the nematodes carry the Phase II forms (Akhurst, 1982). It is therefore important to ensure that the phase I variant is present when producing nematodes *in vitro* (Akhurst & Boemare, 1990). However, other reports have indicated that to a limited extent, *Steinernema* spp. can grow and reproduce under axenic *in vivo* and *in vitro* conditions (Lunau *et al.* 1993; Han & Ehlers, 2000).

Heterorhabditis are associated with bacteria of the genus *Photorhabdus* spp. (Molyneux, 1986; Boemare *et al*. 1993; Gaugler, 1998; Ehlers, 2001). Both EPN genera are closely related to *Caenorhabditis elegans* (Ehlers, 1996). *C. elegans* is the current model organism for studying animal genetics and development (Riddle *et al.* 1997), and its genome of has been completely sequenced (De la Torre, 2003).

Bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus* are Gram-negative motile rods in the family Enterobacteriaceae, with an average size of 0.2-2 x 4^{-10} μm. They are also facultative anaerobes, asporogenous, and give a positive oxidase test (Thomas & Poinar, 1979; Forst *et al*. 1997).

Any given EPN is specifically associated with only one symbiotic bacterial species; but the symbiotic bacterial species may be associated with more than one EPN (Hazir *et al.* 2003). According to Akhurst & Boemare (1990), the best EPN reproduction occurs with their natural symbiont, but in some cases, the EPN can develop on other bacterial species; (e.g.) Aguillera & Smart (1993) successfully cultured *Steinernema scapterisci* in nine different bacteria including *E. coli* monoxenically.

The relationship between the EPN and bacterium complex is symbiotic because: the nematode is dependent on the bacteria for (i) killing its insect host, (ii) creating a suitable environment for its development by producing antibiotics that suppress competing micro-organisms, (iii) transforming the host tissues into food source, and (iv) serving as a food resource. On the other hand, the bacteria need the nematodes for (i) protection from the external environment, (ii) penetration into the host's haemocoel, and (iii) inhibition of the host's antibacterial proteins. Thus, the bacteria depend on the nematode as an intermediate vector for locating its terminal host- a susceptible insect.

hosts (Poinar, 1990; Ehlers, 2001); (e.g.) Hazir *et al*. (2001) reported 80,000 IJs while Shapiro-Ilan *et al.* (2001) reported up to 300,000 IJs emerging from one *Galleria mellonella* cadaver.

The EPN lifecycle from entry of IJs into the host until emergence of new IJs is dependent on temperature and varies amongst species and strains (Hazir *et al.* 2001). It generally takes approximately 6-18 days at temperatures ranging from 18-28 ^{0}C in *G. mellonella* (Poinar, 1990; Nguyen & Smart, 1992).

1.4 The Symbiotic Bacteria

Bacteria play a key role in the commercial control of insect pests, (e.g.) *Bacillus thuringiensis* and *B. sphaericus* have been used in controlling groups of insects like diptera, lepidoptera and coleoptera (Burges, 1982; Aronson *et al*. 1986). The mechanism by which these bacteria infect and kill target insects is well documented (Knowles *et al.* 1989). Invariably, this group of bacteria is only effective after a susceptible insect has ingested their toxins. The resulting passive infection which follows, apparently limits the degree of efficiency of the bacteria to control the pests. Conversely, active infection occurs with another group of insect pathogenic bacteria- those mutually associated with EPNs (Aronson *et al*. 1986). Their pathogenicity is based on their ability to multiply within the haemocoel of a host insect and kill it by septicaemia (Kaya & Gaugler, 1993), as described in detail in the preceding paragraph (section 1.3).

EPNs of the genus *Steinernema* are symbiotically associated with bacteria of the genus *Xenorhabdus* spp. (Thomas & Poinar, 1979; Akhurst 1983) while EPNs of the genus

Analogous to the description of the lifecycle of the closely related nematode *Caenorhabditis elegans*, this process is called 'recovery' and the recovery inducing signal, the 'food signal' (Woodring & Kaya, 1988; Strauch *et al*. 1994; Strauch & Ehlers, 1998; Johnigk *et al*. 2002). The bacteria produce toxins, protein-destroying enzymes and other metabolites; which defeat the host's innate immune or defence mechanism. Finally, the insect host is killed by septicaemia, usually within 2-3 days. The bacteria then proliferate on the hosts' resources (Gerritsen & Smits, 1993; Lunau *et al.* 1993, Johnigk & Ehlers, 1999, Johnigk *et al.* 2002). The IJs feed on the proliferated bacterial cells and the degraded liquefying host tissues, moult and resume development to the fourth stage juvenile, which later mature in to adults (Poinar, 1990; Zioni *et al.* 1992; Kaya & Gaugler, 1993). *Steinernema spp*. IJs become amphimictic adults (i.e.) males or females (Poinar, 1990), whereas the *Heterorhabditis spp*. develop into self-fertilizing hermaphrodites, with later generations producing two sexes (Johnigk & Ehlers, 1999). Strauch *et al.* (1994) reported that offspring of the first generation hermaphrodites can either develop into amphimictic adults or into automictic hermaphrodites simultaneously. These authors also reported that development to self-fertilising hermaphrodites is induced by low concentrations of nutrients, while development into amphimictic adults is induced by favourably nutritional conditions. One to three generations can be completed in a cadaver (Poinar, 1996; Surrey & Davies, 1996).

When food resources in the host become scarce, juveniles developing at the time, retain an inoculation of mutualistic bacteria in their anterior gut, received from the internal host environment (Boemare, 2002; Johnigk *et al.* 2002), and remain developmentally arrested once they reach the J3 stage, which is adapted to withstand the outside environment. At this stage, hundreds of thousands of infective juveniles emerge from the cadaver in search of new

saprotrophs. Thus, nematode growth and reproduction depend upon conditions established in the host cadaver by the bacterium (Gaugler, 1998).

The life cycle of EPNs (*Steinernema spp.* and *Heterorhabditid spp*.) begins in the soil. As shown in Fig 1.1 below. The IJs search for and invade insect hosts in the soil. They gain entry into the insect through natural openings like the mouth, anus and spiracles, or through soft cuticular parts (Surrey & Davies, 1996; Kahel-Raifer & Glazer, 2000).

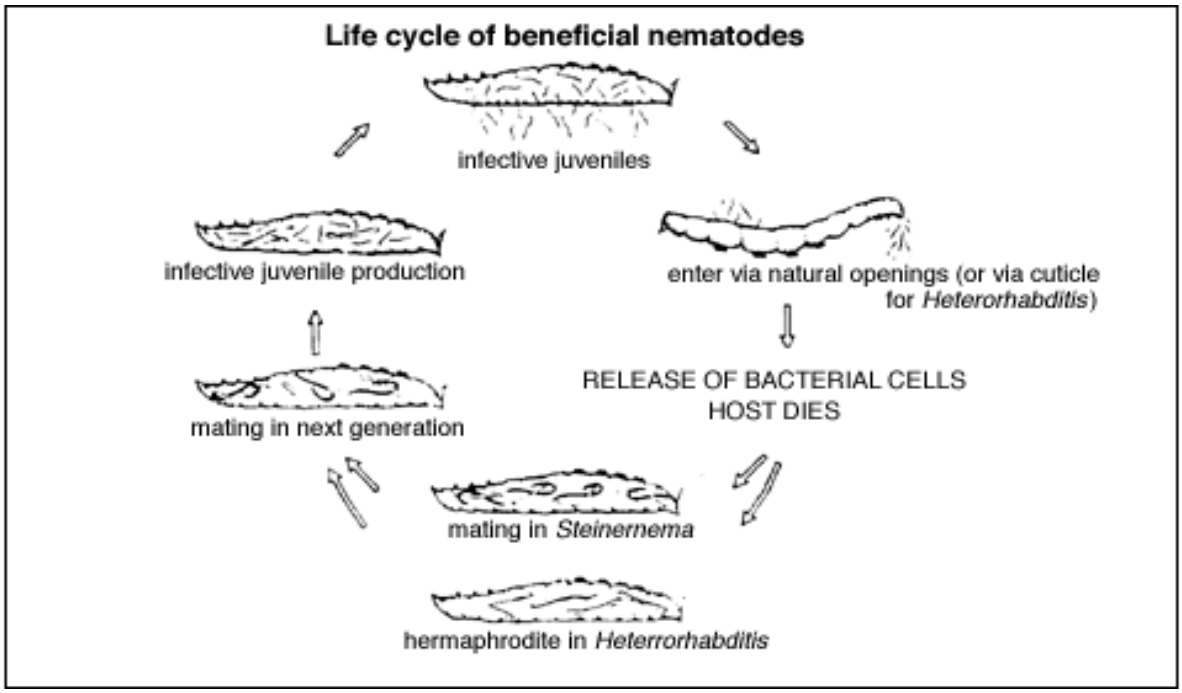


Figure 1.1: Life cycle of beneficial nematodes
Taken from (http://www.nysaes.cornell.edu/ent/biocontrol/pathogens/nematodes.html)

Once in the insect cavity, the IJs release symbiotic bacteria from their gut into the host haemolymph, causing death within 48 hours by septicaemia (Gotz *et al.* 1981). Once in the host haemolymph, the IJs find optimal conditions for reproduction. They respond to yet unknown food signals in the insect (Strauch & Ehlers, 1998) and exit from the developmentally arrested IJ (J3) stage. The IJ moults to initiate growth to the J4 stage.

and digestive tract are greatly reduced while the mouth and anus are closed (Poinar, 1990). IJs can be used for insect control purposes due to their ability to survive without any exogenous source of energy for weeks or even months, and they depend exclusively on their accumulated energy reserves (Johnigk & Ehlers, 1999). Where conditions are otherwise favourable, the duration of survival is determined by the amount and rate of utilization of the reserves; these are mainly neutral lipids and may account for a considerable proportion of the body contents (Barrett & Wright, 1998). For example, the IJ stage of the EPNs *Steinernema* and *Heterorhabditis* lipids constitute 32% to 43% of total body weight (Fitters *et al.* 1999; Selvan *et al.* 1993).

EPNs are a nematode-bacterium complex. The IJs carry between 200 cells (Strauch & Ehlers, 1998) to 2000 cells (Johnigk *et al*. 2002) of their specific (homologous) bacterial symbiont (Strauch & Ehlers, 1998; Han & Ehlers, 2000; Johnigk *et al.* 2002). IJs and adults of *Steinernema spp.* preferentially carry monoxenic cells of their bacterial symbionts in special vesicles (Poinar & Thomas, 1966; Bird & Akhurst, 1983), while *Heterorhabditis spp*. nematodes carry their symbiotic bacteria cells throughout the entire intestine (Poinar & Thomas, 1966; Bird & Akhurst, 1983, Endo & Nickle, 1991; Boemare *et al,* 1993).

The nematode may appear as little more than a biological syringe or vector for its bacterial partner, yet the relationship between these organisms is one of classic mutualism. The symbiotic bacteria lack invasive powers and are dependent upon the nematode to locate and penetrate suitable hosts. Without the nematode the bacteria cannot survive well in the natural environment and are generally not pathogenic when ingested by a host (Akhurst & Boemare, 1990; Morgan *et al*. 1997). The bacteria then contribute anti-immune proteins to assist the nematode in overcoming the hosts' defences, and anti-microbials that suppress colonization of the cadaver by competing secondary invaders such as opportunistic microbial

can persist in the soil (Ehlers, 1996), hence are capable of causing long term sustainable control of the pest populations (Georgis & Gaugler, 1991).

EPNs are versatile in being useful against many soil and cryptic insect pests in diverse cropping systems (Begley, 1990). Over 90% of insect pests spend part of their life cycle in the soil, as a result; the potential to use EPN is very promising. Furthermore, the environment offers excellent conditions for nematode activity and survival since soil is the natural reservoir for EPNs (Gaugler, 1988). Several EPN species are currently under evaluation for mass production and field efficacy for the biological control of insect pests (Ehlers, 2001). Appendix II shows some examples of insect pests that have been successfully targeted with commercially produced EPNs.

1.3 Biology and Life Cycles of Entomopathogenic Nematodes

Steinernema spp. and *Heterorhabditis spp.* nematodes are not closely phylogenetically related, but they share similar life cycles through convergent evolution (Poinar, 1993). The life cycle of EPNs have several developmental stages, the egg, four juveniles stages (denoted as (J for juvenile) J1, J2, J3 and J4) and the adult stages (male, female and hermaphrodite in the case of *Heterorhabditis spp.*). The developmentally arrested non-feeding third stage infective juvenile (J3), in both *Steinernema spp.* and *Heterorhabditis spp.* is the only free-living stage formed as a response to depleting food sources and adverse conditions. The J3 infective juvenile (IJ) also known as the dauer juvenile, is adapted to long term survival in the soil for extended periods of time. The term dauer, which means enduring in German, was first introduced by Fuch in 1915 (Bedding, 1984; Molyneux, 1986; Ehlers, 2001). This free-living dauer or IJ stage is non-feeding with a non-functional alimentary canal; the lumen

alongside many types of pesticides (Nishimatsu & Jackson, 1998; Koppenhöfer *et al*. 2000). In addition, their effects are non-detrimental to non-target organisms (Ehlers, 1990; Georgis *et al.* 1991), which allows exemption from US federal pesticide registration by the EPA (Kaya & Gaugler, 1993).

EPNs and their associated bacteria cause no detrimental effect to mammals, to plants (Kaya & Gaugler, 1993, Bathon, 1996) or to human beings (Boemare *et al*. 1996; Akhurst & Smith, 2002). A joint workshop supported by the EU COST Action 819 "Entomopathogenic Nematodes" and the OECD Research Programme "Biological Resource Management for Sustainable Agriculture Systems", which met in 1995 to discuss potential risks related with the use of EPN in biological control, concluded that EPNs are safe to production and to application personnel, as well as consumers of agricultural products treated with EPNs (Ehlers & Hokkanen, 1996). There are no reports documenting pathogenic effects of EPN symbiotic bacteria on humans. Nonetheless, a related group of non-symbiotic *Photorhabdus* spp. has been reported five times from humans in the USA (Farmer *et al*. 1989), and from five patients in Australia (Peel *et al*. 1999). Still, no conclusions could be drawn from any of the reports; or, on the pathogenic effects non-symbiotic *Photorhabdus* spp. has on humans. This is with regards to the potential risks associated with the use of EPN and their symbiotic bacteria (Akhurst & Smith, 2002).

EPNs have chemo-receptors and are motile (Boemare *et al*. 1996). They also have the ability to seek their host actively (Campbell & Lewis, 2002). Different species exhibit different search strategies (varying from ambush strategy, to cruise foraging, with many intermediate types) to increase the probability of finding a host (Campbell & Gaugler, 1997; Campbell & Kaya, 2002; Grewal *et al*. 1994). EPNs are mobile, reproduce in the host and

exoskeleton or internally in the reproductive, respiratory, digestive or excretory system. Commensal nematodes may also be found in the haemocoel where they do very little, if any, damage to their host. Most of these nematodes, as well as free-living ones, may utilize a dead insect as a nutrient resource, feeding on the microflora or microfauna associated with the cadaver. Thus, examination or dissection of living hosts of cadavers may disclose nematodes that may be erroneously classified as parasitic (Kaya & Stock, 1997). Many of the parasitic species found within the phylum Nematoda cause important diseases of plants, animals and humans. Some of these nematodes belonging to the genera *Steinernema* and *Heterorhabditis* have symbiotic relationships with bacteria that kill their hosts quickly. Because of this rapid kill, the term entomopathogenic is used to describe these nematodes (Kaya & Stock, 1997).

Entomopathogenic nematodes (EPNs) are parasites of soil-inhabiting insects and are found in a variety of soil habitats. Only EPNs possess an optimal suite of biological control attributes (Gaugler, 1998). Besides microbial pathogens and arthropod bio-control agents of pest insects, the potential exists for the use of EPNs to protect vegetation in natural and agro-ecosystems from invasive insect pest species. Moreover, the exploitation of EPNs as biocontrol agents will further reduce reliance on chemical pesticides (Ehlers, 1996).

EPNs have been most widely used as biological control agents in soil environments (Gaugler & Kaya, 1990; Gaugler, 2002, Kaya *et al*., 2006). They are highly effective and often surpass the control results achieved with chemicals as they possess an optimal balance of desirable insect pest control attributes (Ehlers, 1996, Ehlers, 2001). For instance, EPNs kill a broad range of insect hosts within 24 to 48 h of application (Georgis & Manweiler 1994; Ehlers & Peters, 1995; Ehlers & Hokkanen, 1996; Gaugler, 1998; Hazir *et al*. 2003; Ramos-Rodriguez *et al*. 2006). Furthermore, they can be applied easily using standard equipment (Georgis & Kaya, 1998; Hayes *et al*. 1999). Also, they can tolerate or can be applied

alternative to continued reliance on pesticides (Ehlers, 1996; Mathre *et al*. 1999; McSpadden *et al*. 2002). Accordingly, the topic of biological control attracts researchers from many different disciplines, ultimately aiming to develop alternatives to the deleterious effects of chemical pesticides to non-target organisms and the natural environment (Burges & Hussey, 1971; Perkins, 1982; Graves *et al.* 1999). The most common description for the subject can be noted as 'the influence of natural predators, parasites and pathogens applied by man against a pest', with distinctions being made between macrobial and microbial control (Burges & Hussey, 1971; Dixon, 2000). Microbial control agents include bacteria, fungi, nematodes, protozoa and viruses (Evans, 2000). In the case of their control of arthropods such as insects, the interactions most relevant are those termed the entomogenous relationships, where the microbes cause a lethal (or fatal) infection of the insect (Boucias & Pendland, 1998). The degree of control obtained through use of any biological control agent is however difficult to determine without relevant base-line data (Burges & Hussey, 1971); nevertheless, data that can be obtained by meticulous selection under controlled environments. Entomopathogenic nematodes are one such of microbial agents tested for their ruinous effects against insect and ultimately for use in biological control.

1.2 Entomopathogenic Nematodes

Nematodes are colourless and unsegmented simple roundworms with no appendages. They may be free-living, predaceous or parasitic. Many nematode species are associated with insects, and these insect-nematode relationships may range from fortuitous to parasitic (Kaya & Stock, 1997). Commensalism is one of the most common associations between nematodes and insects. The nematodes may be found externally on various areas of the insects'

protozoa, viruses and fungi (McSpadden *et al*. 2002); but today the application has expanded to methods targeted at other invertebrates and plant pathogens (Van Driesche & Bellows, 1996).

There is an increasing demand by governments today for safe pesticides with low toxicity, short term persistence, low mobility in the soil to avoid ground-water contamination and limited effects on non-target organisms. These concerns about environmental health and safety have led to increased restrictions on a variety of chemical pesticides (Ehlers, 1996; Strauch & Ehlers, 1998); including those used to suppress plant diseases (McSpadden *et al*. 2002). There have also been substantial regulatory changes in the past several years, (e.g.) the Food Quality Protection Act (FQPA, Montreal Protocol) of 1996 (http://www.epa.gov/pesticides/regulating/laws/fqpa/). These new regulations have resulted in a decline in the availability, effectiveness, or desirability of insecticidal compounds registered for soil application and older compounds have been banned (Strauch &Ehlers, 1998; Ramos-Rodriguez *et al*. 2006). Furthermore, numerous reports of failures with traditional soil insecticides have been documented- such as development of resistance; insecticides destroying other beneficial insects like natural predators and parasitoids, thus resulting in the resurgence of pest problems; and also emergence of new crop complexes (Racke & Coats, 1990; Subramanyam & Hagstrum, 1995). Likewise, there is growing concern about chemical residues, worker safety, and shifting consumer demands that favour the adoption of more environmentally favourable management tools for insect pests (Ramos-Rodriguez *et al*. 2006).

With emphasis being placed on the disadvantages regarding the use of chemical pesticides, biological control may be an alternative effective strategy for insect pest management. This approach fits into an overall pest management program, and represents an

Chapter 1

INTRODUCTION

1.1 Biological Control

Ehler (1990) defined biological control as the action of natural enemies (arthropod predators, insect parasitoids and microbial pathogens) that maintain a host population at levels lower than would occur in the absence of these enemies. While the concept of biological control is based on the assumption that naturally occurring biotic mortality factors restrain populations of living organisms (Ruberson *et al.* 1999), additional pest control tactics counted as biologically-based control strategies such as plant host resistance, mating disruption, pheromone trapping, genetic controls and biological toxins are excluded from the concept for the purposes of this review (Ruberson *et al.* 1999). The authors agree that these methods of pest control are equally essential, yet they insist that such methods have little in common with biological control or with each other outside the point that they may be integrated and used together (Ruberson *et al.* 1999). Biological control can be divided into two categories, namely: natural- and applied biological control. Natural biological control which is what occurs much of the time; occurs where co-evolved or natural enemies reduce populations of potential pests without human intervention, whereas applied biological control requires human intervention, rather than simply letting nature take its course (Ehler, 1990). Biological pest control was first used for insects, mites and weeds (Van Driesche & Bellows, 1996); and was based on highly specific naturally occurring insect diseases caused by bacterial,

SD	Standard Deviation
sec	Second(s)
SEM	Standard Error of the Mean
spp.	Species (plural)
sp	Species (singular)
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TTC	Triphenyl tetrazolium chloride
µ	Micro
V	Volts

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BTB	Bromothymol blue
cm	Centimeter(s)
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra-Acetate
EPN(s)	Entomopathogenic Nematode(s)
EtBr	Ethidium Bromide
EY	Egg Yolk
Fig	Figure
g	Gram(s)
h	Hour
IJ(s)	Infective Juvenile(s)
L / l	Liter
LA	Lipid Agar
min	Minute(s)
n	Sample size
NA	Nutrient Agar
NB	Nutrient Broth
NBTA	Nutrient broth-bromothymol blue-triphenyltetrazolium chloride-agar
P	Probability
PCR	Polymerase Chain Reaction
PI	Post inoculation
rDNA	Ribosomal Deoxyribonucleic acid
RH	Relative Humidity
rRNA	Ribosomal Ribonucleic acid
rpm	Revolutions per minute

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number of IJs produced per cadaver was linear $P < 0.001$ for all EPNS; $r^2 = 0.97$, 0.94, 0.94 and 0.90 for *H. indica, H. bacteriophora, H. zealandica*, and *S. feltiae* respectively. Overall, IJ yield was significantly different between very high (≥ 97.8%) and very low RH levels (≤ 77.2%).

The feasibility of formulating EPN-infected *T. molitor* larvae to overcome the hindrance of handling and storage was tested using *H. indica*. Firstly, *T. molitor* was cultured in bran. The different bran cultures had variable numbers of adults ranging from 10 to 100 (in increments of 10), with a male/female sex ratio of 50:50. Half of the cultures were sieved to separate the eggs from the adults, but other cultures were not sieved. The results indicated that sieving was useful in improving *T. molitor* yield. Also, it was perceptible that cultures with too many (≥ 80) or too few (≤ 30) adults per container produced fewer offspring in both cultures that were sieved and those that were not. Secondly, 12 combinations of dipping and rolling agents were tested for formulating 4 and 8 day old *H. indica*-infected *T. molitor* larvae. The dipping agent coated the surface of the cadaver and served as an adhesive for the powder, which was intended to prevent cadavers from sticking together. Three of the formulations failed to adhere to either 4- or 8-day-old cadavers. The remaining 9 formulations were tested for their effects on nematode reproduction. Cadavers were formulated at 4 days post inoculation (PI) and 8 days PI and placed in White traps. There were no progeny IJs from 5 of the formulations. Of the remaining 4, the most promising formulation was the 2.5MC because the cadavers coated with the formulation produced IJs that were significantly higher in yield than other formulations for both 4 and 8 day old cadavers. The other three successful formulations were 2.5SC, 1SC and 1MS.

luminescens arkhustii were added into different *H. indica* egg yolk cultures, 10, 15, 20, 25 and 30 days following inoculation of 2300 IJs/ml. The cultures were incubated on a platform shaker set at 150 rpm and 25 ^{o}C. Progeny IJs were counted on days 2, 5, 10, 15, 20, 30, 35 and 40 following inoculation of IJs in the cultures. The results showed that adding bacteria to monoxenic cultures boosted IJ yield. IJ yield was highest on day 35, in cultures that had received bacteria on days 20 and 25. *H. bacteriophora* and *H. zealandica* were inoculated at a density of 4000 IJs/ml into monoxenic egg yolk cultures supplemented with either canola oil, safflower oil or cod liver oil. Progeny IJs were counted on days 4, 10, 12, 14, 16, 18, 20, 22, 25, 30 and 35 following IJ inoculation. IJ yield increased constantly in cod liver oil-supplemented cultures, but IJ yield was highest in cultures supplemented with canola oil for both nematodes. Overall, *H. bacteriophora* cultures had higher IJ yields.

The consequence of desiccation on the reproduction of all four nematode species was determined by the number of IJs emerging from EPN-infected host cadavers at different levels of relative humidity (RH). Thirteen different humidity environments with levels ranging from 70.4 – 100% RH were prepared using glycerol and water. EPN-infected *T. molitor* larvae were exposed to any of the 13 RH levels, but the cadavers were preconditioned at a higher RH. More precisely, preconditioning occurred at the RH preceding that for which it was intended, for 24 h before being exposed to the intended lower RH. Cadavers were randomly selected from all 13 RH environments and placed on White traps and progeny IJs counted, for all four nematode species. *H. indica*-infected cadavers had the highest IJ yield, except at RH $\geq$ 97.8% where *S. feltiae*-infected cadavers produced more IJs. However, the number of IJs produced by *S. feltiae* drastically reduced at lower RH levels $\leq$ 85.3. The number of IJs produced per host infected by either *H. bacteriophora* or *H. zealandica* was similar across all RH levels. The relationship between RH treatment and the

examined in the one-on-one bioassay. Individual insects were exposed to single IJs of the 4 EPNs. *H. indica* killed 62.5% of insects after only 48 h of exposure, but *H. zealandica*-infected insects experienced the highest mortality (87.5%) at the end of the 96 h of exposure. *H. indica*-infected cadavers produced the highest number of IJs (195,400 IJs) while the least (130,000 IJs) was produced by *H. zealandica*-infected cadavers. In the dose response bioassay, different IJ concentrations (1, 5, 10, 25, 50, 100, 200 and 500) of the four EPNs were applied per *T. molitor* larva. The bioassay was carried out in two parts. In the first part, mortality data was collected for *H. bacteriophora* and *H. zealandica*. Significant differences in insect mortality were apparent as early as 24 h post nematode application; the differences were mostly between the very low and high IJ doses such as 5 and 500 IJs/insect. However, no differences were noted when mortality data were compared between IJ doses at both 72 h and 96 h following IJ application to the insects. In the second part of the dose response bioassay, the number of progeny IJs emerging from EPN-infected cadavers was determined for all four EPNs. No significant differences were noteworthy in the number of emerged IJs at all doses for each nematode species.

The role of two factors in EPN recovery, growth and reproduction in liquid culture were investigated, namely type of lipid and the role of EPN symbiotic bacteria. *H. bacteriophora* and *S. feltiae* were inoculated at densities of 2500 IJs/ml into monoxenic and axenic egg yolk liquid cultures, incubated on a platform shaker set at 25 ^{o}C and 150 rpm. Progeny IJs were counted on days 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25 and 30 following IJ inoculation. *H. bacteriophora* did not produce any offspring but *S. feltiae* recovered and produced very few IJs in axenic culture. However, recovery, growth and reproduction were observed in monoxenic cultures of both EPNs. The difference between IJ yield in monoxenic and axenic cultures were highly significant from days 4 up to 30 ($P < 0.05$). Five millilitre aliquots of *P.*

ABSTRACT

Several entomopathogenic nematode (EPN) species of the genera *Steinernema* and *Heterorhabditis* are currently under evaluation for mass production and field efficacy for biological control of insect pests. Two indigenous EPNs, namely *Heterorhabditis bacteriophora* and *H. zealandica*, and two exotic EPNs, namely: *H. indica* LN2 and *Steinernema feltiae* SN were examined in this study. Their symbiotic bacteria, namely: *Photorhabdus luminescens* subspecies *laumondii, P. luminescens, P. luminescens* subspecies *arkhustii* and *Xenorhabdus bovienii* respectively, were isolated from EPN-infected insect host haemolymph and cultured successfully in NBTA, MacConkey agar and nutrient broth. The growth curves, mean generation times and growth rate constants were calculated for each of the symbiotic bacteria. *X. bovienii* had the smallest growth rate constant (1.99 hour-^{1}) while *P. luminescens* had the longest (2.45 hour-^{1}). Doubling time was similar for all bacteria (range 18 – 19.62 min). Optimum growth temperature was determined to be 28 ^{o}C for all EPNs; except for *P. luminescens* isolated from *H. zealandica*- it had an optimum growth temperature of 25 ^{o}C. Bioluminescence was detected in all phase I *Photorhabdus* species (symbionts of the Heterorhabditid nematodes).

Three kinds of bioassays- namely: the exposure time bioassay, the on-on-one bioassay and the dose-response bioassay were performed to determine the infectivity of the four nematodes. In the exposure time assay, *T. molitor* larvae and pupae and *G. mellonella* larvae were exposed to IJs of *H. bacteriophora*. Overall, insect mortality increased with longer exposure times. *G. mellonella* larvae experienced the highest mortality. All four EPNs were

DEDICATION

~~~~~~~~~~~~~ To my BB, Michael and Alice~~~~~~~~~~~~~

As an expression of strong personal regard, and a deep gratitude for their great loving kindness, I dedicate this work to my first, best and most consistent patrons and protectors. I thank God for you. You gave me hope to see this through.
~~~~~~~~~~~~~

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at any other university.

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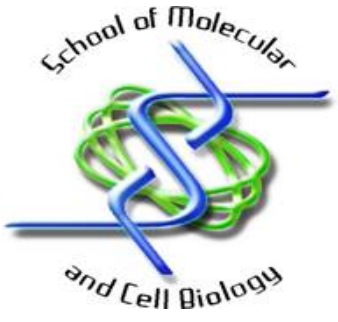
Mirabel A Nyamboli Date



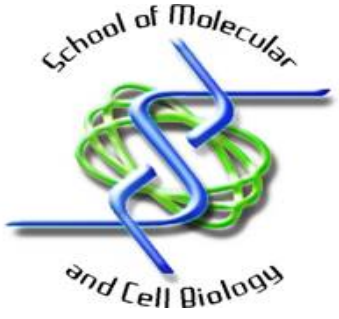
MASS PRODUCTION OF ENTOMOPATHOGENIC NEMATODES FOR PLANT PROTECTION

MIRABEL A NYAMBOLI

0415275H

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg; in fulfilment of the requirements for the degree of

Master of Science



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