Characterization, Host Bioassay, and In vitro Culture of Indigenous Entomopathogenic Nematodes and their Bacterial Symbionts.

Lubanza Ngoma
0413449k

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.

October 2008
DECLARATION

I declare that this research report 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.

----------------------------------------------
Lubanza Ngoma     Date
ABSTRACT

The prevailing use of chemical pesticides has generated several problems including insecticide resistance, outbreak of secondary pests, safety risks for humans and domestic animals, contamination of ground water and decrease in biodiversity among other environmental concerns (Webster, 1982). These problems and the non-sustainability of control programs based mainly on conventional insecticides have stimulated increased interest in the development and implementation of cost-effective, environmentally safe alternatives to chemical pesticides for insect pest control. One of the most promising strategies to help minimize dependence on chemical pesticides has been the recent application of entomopathogenic nematodes (EPNs) as biocontrol agents. EPNs in the families Steinernematidae and Heterorhabdidae have been shown to have considerable potential as biological control agents. As a natural process, biological control has the potential to play an important role in the suppression of field crop pests in agriculture. EPNs as biocontrol agents have the following advantages: high virulence, safety to non target organisms, ability to search for hosts, high efficacy in favourable habitats, high reproductive potential, ease of mass production, ease of application (Ferron & Deguine, 1996).

To isolate the EPNs in South African soil, 200 soil samples were randomly collected from 5 locations in the agricultural research council (ARC) Pretoria, Gauteng province in April 2006; and 5 locations in Brits, North West province in March, 2006. At the different collection sites, soil samples were obtained from soils associated with various types of vegetation. The nematodes were collected from sandy soil by the insect-baiting technique and maintained successfully in vivo for 12 months on Galleria mellonella (G. mellonella), 4 months on Tenebrio molitor (T. molitor); 2 months Pupae and in vitro (lipid agar) for 2 weeks in the laboratory. Out of a total of 200 soil samples that were baited, 2 were found to be positive for EPNs.
In addition to completing Koch’s postulates, the colour of cadavers infected by the putative EPNs were also used as a diagnostic characteristic for categorizing the nematode isolates. Characterization and identification of the EPN isolates were based on morphological characters, as well as on a molecular marker (18S rDNA).

On the basis of the morphological and molecular data that was obtained both of the EPNs isolates were placed in the family Heterorhabdidae: *Heterorhabditis bacteriophora* (*H. bacteriophora*) and *Heterorhabditis zealandica* (*H. zealandica*). Also from the phylogenetic trees generated from the 18S rDNA sequence, the indigenous putative *H. bacteriophora* was shown to be closely related to *H. bacteriophora* (accession number EF690469) and indigenous putative *H. zealandica* to *H. zealandica* (accession number AY321481). The two EPNs were found associated with Gram negative rod-shaped bacteria. The bacterial symbionts of the two isolates were isolated and a region of the 16S rDNA gene was sequenced. National Center for Biotechnology Information (NCBI-BLAST) results of the 16S rDNA sequence obtained showed the endosymbiotic bacteria to be *Photorhabdus luminescens laumondii* (*P. laumondii*) (*H. bacteriophora*) and *Photorhabdus sp* (*H. zealandica*). Results of the tree showed that isolates from *H. bacteriophora* appeared to be closely related to *P. luminescens subsp laumondii strain TT01 Ay 278646*. The isolates from *H. zealandica* appeared to be most closely related to *Photorhabdus sp* (Accession number: Q 614 Ay 216500).

Bioassays were used to determine the infectivity of the two EPNs. In this experiment different infective juvenile (IJ) concentrations (5, 10, 25, 50, 100,200 400 and 500) of the two EPNs were applied per *G. mellonella; T. molitor* larva and pupae. The bioassay was carried out in two parts. In the first part, mortality data was collected for *H. bacteriophora* and *H. zealandica*. The results showed that the degree of susceptibility of *G. mellonella, T. molitor* larvae and pupae to each nematode species was different. When 24 h post-exposure mortality data for larvae exposed to the IJs of *H. bacteriophora* and *H. zealandica* were analyzed, ANOVA showed no differences
in mortality between insects exposed to different *H. bacteriophora* IJ doses (Fig: 8.1 ABC). However, there were significant differences in mortality between insects exposed to different IJ doses of *H. zealandica* such as 5 and 500 IJs/insect (Fig: 8.2 ABC) Therefore, 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. The highest susceptibility was observed with *G. mellonella* followed by *T. molitor* pupae and then *T. molitor* larvae. According to Caroli et al., (1996), the total mortality of insect such as *G. mellonella* and other lepidopterans, was reached within 24-72 h of exposure to nematodes at concentrations such as those tested here. In this study similar results were observed with high concentration of nematodes (100, 200 and 500). In the second part of the dose response bioassay, the number of progeny IJs emerging from EPN-infected cadavers was determined for all two EPNs.

The results indicate that IJ progeny production differed among the three insect hosts used, the IJ doses they were exposed to, as well as the EPN species (Figs 8.3 & 8.4). The highest number of emerged IJs of *H. zealandica* was produced by *G. mellonella* (mean ± SEM: 220500 ± 133933 IJs), followed by *T. molitor* larvae (mean ± SEM: 152133 ± 45466 IJs) and the lowest then *T. molitor* pupae (mean ± SEM: 103366 ± 56933 IJs).
ACKNOWLEDGEMENTS

First, I would like to thank God for bringing me this far and for giving me the wisdom, the courage and the strength to go on.

I thank my supervisor Professor Vince Gray for his academic and technical guidance and the time he sacrificed with discussions, improvements and draft readings. I am very grateful and I appreciated his enthusiasm and interest in my work.

Two year Msc bursary from Innovation Fund made this work possible, for which I am extremely grateful.

My appreciation goes to the School of Molecular and Cell Biology for their financial support and for providing me with the much needed facilities used during this project.

I thank all my family, friends and colleagues for their interest in me as a person as well as in this project. I especial wish to mention my parents, Stephan Tshimanga and Emerence Lubuya, for all your support and encouragement. Without them I would not have come this far. Parents-in-law, Prof Patrice Munabe and Susan Tshiame, it wonderful to be part of your family.

To my wife, Susan Thiame, I send out unlimited heartfelt gratitude for her encouragement throughout the past two years, in times of frustration and excitement and for believing in whatever I wanted to do. Without her support and love this dissertation would never have seen the light. I love you, now and forever.
DEDICATION

This thesis is dedicated to my daughter Divine Lubuya and my wife Susan Tshiame. They have been my inspiration and motivation throughout this work. I love you both.
TABLE OF CONTENTS

DECLARATION-------------------------------------------------- II
ABSTRACT----------------------------------------------------- III
ACKNOWLEDGEMENTS--------------------------------------------- VI
DEDICATION---------------------------------------------------- VII
TABLE OF CONTENTS ------------------------------------------- VIII
LIST OF FIGURES----------------------------------------------- XIII
LIST OF TABLES----------------------------------------------- XVI
LIST OF ABBREVIATIONS----------------------------------------- XVII

Chapter 1 Literature Review

1.1 Introduction----------------------------------------------- 1
1.1.1 Insect pests---------------------------------------------- 1
1.1.2 Chemical control of insect------------------------------- 3
  Disadvantages of pesticide chemicals------------------------ 4
  Pest resistance to pesticides------------------------------- 4
1.1.3 Biological control----------------------------------------- 6
1.1.4 Entomopathogenic nematodes as biological control agents-- 8
1.1.5 Biology and life cycle of EPNs--------------------------- 11
1.1.6 Ecology of EPNs------------------------------------------ 13
1.1.7 Bacterial symbionts------------------------------------- 14
  Xenorhabdus and Photorhabdus----------------------------- 14
1.1.8 Molecular and morphological characterization of EPNs----- 16
1.1.9 Motivation----------------------------------------------- 16
1.1.10 The main objectives------------------------------------ 17
1.1.11 Research strategy--------------------------------------- 17

Chapter 2: Materials and Methods------------------------------ 20
2.1  *Galleria mellonella* breeding----------------------------------------------- 20
2.2  *Tenebrio molitor*----------------------------------------------------------- 22
2.3  Sampling strategy------------------------------------------------------------- 23
2.4  Origin of the EPN isolates----------------------------------------------------- 24
2.4.1  Brits----------------------------------------------------------------------- 24
2.4.2  Agricultural research council (ARC)------------------------------------------ 25
2.5  EPNs isolation  -------------------------------------------------------------- 26
2.5.1  Live-bait method------------------------------------------------------------ 26
2.5.2  White traps---------------------------------------------------------------- 28
2.5.3  Harvesting of nematodes----------------------------------------------------- 29
2.5.4  Maintenance of EPNs---------------------------------------------------------- 29
2.5.4.1  *In vivo* method--------------------------------------------------------- 29
2.5.4.2  *In vitro* solid method--------------------------------------------------- 30
2.6  Koch’s Postulates-------------------------------------------------------------- 31
2.7  Identification of nematode isolates-------------------------------------------- 32
2.7.1  Molecular identification of EPNs-------------------------------------------- 32
2.7.1.1  Extraction of DNA-------------------------------------------------------- 32
2.7.1.2  Polymerase chain reaction (PCR)----------------------------------------- 32
2.7.1.3  Agarose gel electrophoresis--------------------------------------------- 34
2.7.1.4  DNA sequencing---------------------------------------------------------- 35
2.7.1.5  Sequence alignment and phylogenetic analysis--------------------------- 35
2.7.1.6  Morphological observations--------------------------------------------- 36
2.7.1.7  Light microscopy-------------------------------------------------------- 36
2.7.1.8  Scanning electron microscopy------------------------------------------- 37
2.8  Identification of symbiotic bacteria----------------------------------------- 37
2.8.1  Isolation of symbiotic bacteria------------------------------------------ 37
2.8.2  Molecular identification of symbiotic bacteria--------------------------- 38
2.8.2.1  Extraction of DNA----------------------------------------------------- 38
2.8.2.2  PCR amplification of 16S rDNA--------------------------------------- 38
2.8.2.3  Agarose gel electrophoresis------------------------------------------ 39
Chapter 3: Entomopathogenic Nematode Isolation

3.1 Introduction
3.2 Materials & Methods
3.2.1 Soil sampling
3.2.2 Extraction of nematodes from soil
3.2.3 Koch’s postulates
3.3 Results
3.3.1 Nematode isolation from soil samples
3.3.2 Koch’s postulates
3.4 Discussion

Chapter 4: Molecular Characterization of Nematode Isolates

4.1 Introduction
4.2 Materials & Methods
4.3 Results
4.4 Discussion

Chapter 5: Morphological Classification of Nematode Isolates

5.1 Introduction
5.2 Materials & Methods
5.3 Results
5.3.1 H. zealandica
5.3.2 H. bacteriopohora
5.4 Discussion------------------------------------- 74

Chapter 6: Isolation, Characterization and Identification of Endosymbiotic Bacteria Associated with EPNs H. zealandica and H. bacteriophora----------------------------------- 75

6.1 Introduction ------------------------------------- 75
6.2 Materials & Methods--------------------------------- 76
6.3 Results------------------------------------------- 77
6.3.1 Identification of endosymbiotic bacteria from H. zealandica and H. bacteriophora based on 16 rDNA sequence-------------------------------------- 80
6.3.2 Phylogenetic tree and sequence alignments ------------- 81
6.4 Discussion------------------------------------- 89

Chapter 7: Culture and Maintenance of Putative EPN Isolates------------------------------------ 91

7.1 Introduction------------------------------------- 91
7.2 Materials & Methods---------------------------- 92
7.2.1 In vivo culture of putative EPNs------------------ 92
7.2.2 In vitro culture of putative EPNs------------------ 92
7.3 Results---------------------------------------- 92
7.4 Discussion------------------------------------- 93

Chapter 8: Bioassay Studies------------------------------------- 95

8.1 Introduction------------------------------------- 95
8.2 Materials & Methods---------------------------- 96
8.3 Results---------------------------------------- 96
8.4 Discussion------------------------------------- 103

References--------------------------------------------- 109

Appendix I: Sources of chemicals and supplies-------------------------- 130
Appendix II: Galleria medium------------------------------------------ 132
Appendix III: Nematode genomic DNA extraction-------------------------- 133
Appendix VI: Protocol for the isolation of symbiotic bacteria associated with EPNs------------------------------------- 135
Appendix V: Isolation of genomic DNA from bacterial cells associated with EPNs----------------------------------------------- 136
Appendix VI: Recipes for in vitro culture media--------------------------------- 137
Appendix VII: Gel electrophoresis-------------------------------------------- 139
Appendix VIII: Processing nematodes specimens to glycerin------------------ 140
Appendix IX: Preparation of nematodes for scanning electron microscopy--- 142
Appendix X: Heterorhabditis zealandica and Heterorhabditis bacteriophora 18S rDNA gene partial sequence----------------------- 143
Appendix XI: Photorhabdus sp and Photorhabdus luminescens laumondii, 16S rDNA gene partial sequence-------------------------- 145
Appendix XII: Spectrophotometer determination of DNA concentration------ 147
LIST OF FIGURES

1.1 Life cycle of: A) Heterorhabditis bacteriophora and B) Steinernema carpocapsae (Taken from Wang & Bedding, 1996)---------------------- 11
1.2 Overview of methodology used in this study -------------------------- 19
2.1 Adult wax moths (male and female) and larva kept in 3L glass bottles 21
2.2 A) The greater wax moth, G. mellonella insect B) G. mellonella larvae---------------------------------------------- 22
2.3 T. molitor ------------------------------------------------------------- 23
2.5 Map of North West Province, South Africa-highlighting town around which soils were collected. (Source: www.stayinsouthafrica.co.za/images/northwest.--------------------- 25
2.6 Map of Gauteng Province, South Africa-highlighting town around which soils were collected. (Source: www.africangamesafari.com/gauteng_map--------------- 26
2.7 T. molitor live bait method ------------------------------------------ 28
2.8 A, B & C) illustration of White traps used to collect IJs from G. mellonella, T. molitor and lipid agar ------------------------------------------ 29
2.9 Illustration of the glass slide for permanent mount of EPNs after being processed through glycerin, and B): complete glass slide --------- 36
2.10 Bioassay set up in multi well plates. This dose-response bioassay was set up using A) G. mellonella and B) T. molitor larvae as hosts. The dead insects which are brick red in colour were inflected by nematode isolate--------------------------------------------------------------- 42
3.1 A, B, C & D): G. mellonella and T. molitor cadavers infected by different EPN isolates, those infected by nematode isolate 411 are brick red in colour, while those infected by nematode isolate 386 are green and brown in colour respectively (E & F) Light micrographs of nematode isolate 386 emerging from a Tenebrio molitor larvae and isolate 386 in a white trap respectively------------------ 48
4.1 DNA fingerprints of nematodes isolates 411 and 386 amplified with TW81 and AB28 primers. Lane 1 (Molecular Weight Marker: # SM098, Fermentas) Lane 2-4 (386). Lane 5-6-7-8 (411). -------------------------- 53
4.2 Phylogenetic tree resulting from existing Heterorhabditis nematode database sequences together with highlighting sequences discovered in this study--------------------------------------------------------------- 54
4.3 DNAm an multiple sequence alignment of the ITS1 - rDNA partial sequence obtained for H. zealandica and H. bacteriophora, South African isolate------------------------------------------------------------------- 60
5.1 Light micrograph of anterior region of female showing A) stoma, B) corpus of pharynx and C) lip
5.2 Light micrograph of pharyngeal region of adult female showing A) nerve ring B) isthmus C) and basal pharyngeal bulb
5.3 Light micrograph of young female posterior showing the conical tail with narrow pointed terminus
5.4 Light micrograph of young female posterior showing the rectum
5.5 Light micrograph: Female mid region showing the intestine
5.6 Light micrograph: Female mid region showing vulva lips protruding
5.7 *H. zealandica*. Scanning electron micrographs of IJs. A and B) anterior region showing head with tessellate pattern; C) showing position of vulva; D) Portion of body showing tessellate pattern and longitudinal ridges; E) Posterior region showing anus position and; E) the conical tail with narrow pointed terminus
5.8 Light micrograph of anterior region of young female showing A) transparent lips B) corpus of pharynx, C) nerve ring, D) ) isthmus, E) basal pharyngeal bulb
5.9 Light micrograph of pharyngeal region of adult female showing A) stoma and B) lips
5.10 Light micrograph: Female mid region showing the intestine
5.11 Light micrograph: Female mid region showing vulva
5.12 Light micrograph of young female posterior showing A) anal region slightly protruding and B) tail pointed
5.13 Light micrograph of young female posterior showing visible anus and situated anterior to the tail
5.14 *H. bacteriophora*. Scanning electron micrographs of IJs. A and B) anterior region showing head with tessellate pattern; C) Portion of body showing tessellate pattern and longitudinal ridges; D) Posterior region showing anus position and E) tail pointed; F) Whole IJs
6.1 Flasks A and B had been inoculated with a 24 h symbiotic bacterium and incubated on a shaker for 72 h. The different colours are attributed to the symbiotic bacteria. A) Symbiotic bacteria from *H. bacteriophora* and B) Symbiotic bacteria from *H. zealandica*
6.2 EPN symbiotic bacteria growing in MacConkey and NBTA plates. The different colours are characteristic of the bacteria. A and B): Symbiotic bacteria isolated from *H. zealandica*, colonies are deep green-red on MacConkey agar and blue-green with a red centre on NBTA, C and D) Symbiotic bacteria isolated from *H. bacteriophora*; colonies are deep red on MacConkey agar and green with a red centre on NBTA
6.3 Scanning electron micrograph showing the bacterial cells associated with (A, B, C) Symbiotic bacteria isolate from *H. zealandica* and (D, E, F). Symbiotic bacteria isolate from *H. bacteriophora*
6.4 PCR amplification of 16S rDNA. Lane 1: Molecular Weight marker (# SM098, Fermentas). Lanes: 2-3-4 bacterial strain isolated from *H.
zealandica. Lanes 5-6-7-8: Bacterial strain isolated from H. bacteriophora

6.5 Phylogenetic tree of 16S rDNA of P. laumondii and Photorhabdus sp, symbionts of H. bacteriophora and H. zealandica respectively isolated from South Africa soil

6.6 DNAman multiple sequence alignment of the 16S - rDNA partial sequence obtained from P. subsp laumondii and, Photorhabdus sp, symbionts of H. bacteriophora and H. zealandica respectively isolated from South African soil

8.1 A, B, C) The percentage mortality of T. molitor larvae and pupae and G. mellonella larvae following exposure to different IJ concentrations (5, 10, 25, 50, 100, 200, 400, and 500) of IJs of H. bacteriophora in the dose response assay for 24 h, 48 h, 72 h and 96 h of exposure represents the cumulative mortality after 96 h. Bars represent ± standard error of the mean

8.2 A, B, C: The percentage mortality of T. molitor larvae, pupae and G. mellonella following exposure to different concentrations (5, 10, 25, 50, 100, 200, 400, and 500) of IJs of H. zealandica in the dose response assay for 24 h, 48 h, 72 h and 96 h of exposure represents the cumulative mortality after 96 h. Bars represent ± standard error of the mean

8.3 Mean number of progeny IJs emerged from G. mellonella and T. molitor larvae and pupae that were exposed to different doses of H. zealandica nematodes. Bars are ± standard error of the mean

8.4 Mean number of progeny IJs emerged from G. mellonella and T. molitor larvae and pupae that were exposed to different doses of H. bacteriophora nematodes. Bars are ± standard error of the mean
LIST OF TABLES

3.1 Results of soil samples------------------------------------------ 47
6.1 Characteristics of Photorhabdus sp and P. luminescens laumondii------ 77
7.1 Results of in vivo culture of 2 nematode isolates---------------------- 92
7.2: Results of in vitro culture of 2 nematode isolates--------------------- 93
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>Degrees of freedom</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-Acetate</td>
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<tr>
<td>EPN(s)</td>
<td>Entomopathogenic Nematode(s)</td>
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<tr>
<td>IJ(s)</td>
<td>Infective Juvenile(s)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>IMP</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>RH</td>
<td>Relative Humidity</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet Radiation</td>
</tr>
<tr>
<td>NBTA</td>
<td>Bromothymol blue and triphenyltetrazolium chloride medium</td>
</tr>
<tr>
<td>ARC</td>
<td>Agricultural Research Council</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacers</td>
</tr>
<tr>
<td>TAF</td>
<td>Triethanolamine-formalin</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>Tm</td>
<td>Melting temperature</td>
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