

NEMATODE CONTROL THROUGH BIOLOGICAL MEANS

by

ABHISHEK SHARMA

Centre for Rural Development and Technology

Submitted

In fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

MAY 2013

Dedicated
to
The Almighty
&
My family

CERTIFICATE

This is to certify that the thesis entitled “*Nematode Control through Biological Means*” submitted by **Mr. Abhishek Sharma** has been prepared under our guidance with the rules and regulations of Indian Institute of Technology Delhi, India. The research report and results presented in this thesis have not been submitted for any degree or diploma in any other institute or university.

(Dr. Satyawati Sharma)
Professor & Head
Centre for Rural Development
and Technology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi -110016
INDIA

(Dr. S.N. Naik)
Professor
Centre for Rural Development
and Technology
Indian Institute of Technology Delhi
Hauz Khas, New Delhi -110016
INDIA

ACKNOWLEDGEMENTS

It gives me great pleasure and moment of immense satisfaction to express my sense of reverence and sincere gratitude to my supervisor Prof. Satyawati Sharma for her motherly affection, motivation, enthusiasm, constructive criticism and support. Her academic excellence and constant guidance steered me through the work all the way and all the time. She also contributed a lot to develop my vision and ideas in the direction of rural development. Undoubtedly, it was a fortunate experience to work under her reflective guidance.

I take this esteemed opportunity in expressing my sincere bouquet of gratitude to Prof. S.N.Naik, my supervisor, for his guidance, incessant encouragement and untiring attention, which he bestowed on me right from the inception to the successful completion of this thesis.

I would like to extend my gratitude to the honourable members of my research committee Prof. Saroj Mishra (Chairperson, DBEB), Prof. A.K. Srivastava (External expert, DBEB) and Dr. Anushree Malik (Internal expert, CRDI) for their valuable time, comments, suggestions and moral support to improve my thesis. I am also grateful to faculty members of CRDI for their support and encouragement throughout this work.

I wish to express my cordial thanks to staff members of CRDI, IIT Delhi for their assistance and help related to equipment, information, services and resources. I am extremely grateful to Mr. Ramkumar, who helped me with my fieldwork.

I am especially grateful to Dr. Naved Sabir, National Centre for Integrated Pest Management, IARI, for providing green house facility. I am indebted for his genuine support and valuable guidance. I sincerely thank Dr. Savita Yadav, AIIMS, for providing me her lab facilities for enzyme purification studies. I owe special thanks to Dr. Aditya Mittal, KSBBS, IIT Delhi, for his valuable suggestions and guidance.

I wish to thank my seniors, colleagues and juniors, Dr. Kaviraj, Dr. Monica, Dr. Pratibha, Dr. Madhumita, Dr. Subbalaxmi, Aditi, Sharad, Ritika, Garima, Himanshi, and Shalini for their co-operation and help. I am indebted to my friends Manu, Sachin, Prachi, Abhishek and brothers Siddharth & Suryaveer whose unconditional support and constant encouragement enabled me to finish my work in time. I owe a special debt of gratitude to Kalpana and Naveen for their selfless support and ready gesture to help throughout the research work.

Indeed the words at my command are inadequate to express my deep and emotional feelings towards my wife, Aditi Sharma. I am always indebted to her patience, love, encouragement, continuous support and sacrifices. I owe my loving thanks to my little daughter, Nishka.

I am speechless to express my deep sense of regards to my parents for their contribution and believing in me. It is their love, affection and blessings that helped me to move ahead and made me reach to the finish line. I shall be failing in my duty if I do not acknowledge my in-laws for their eternal fountain of blessings and constant back up during the course of study.

There are many others who have supported and helped me in various ways. I sincerely thank all of them and request their forgiveness for not mentioning them here by name.

In spite of all this valuable guidance and help, errors must remain which are my domain of responsibility.

Abhishek Sharma

ABSTRACT

Root-knot nematode species (*Meloidogyne* spp.) are among the most widespread agricultural pests in the world. Infection by root knot nematode (RKN) occurs in the roots of plants resulting in significant reduction of yield and quality that equates to considerable financial losses for growers in agricultural and horticultural sectors. In developing countries where nematode problems are prevalent, infection by nematodes greatly compounds the burgeoning problems of low agricultural productivity. The need for alternative management systems for the control of plant-parasitic nematodes has increased dramatically over the last decade, mainly because of the banning of the most important nematicides. Therefore, biological control of phytonematodes has received an enhanced impetus and several attempts in the industrial/ commercial sector as well as in academia, have been made to fulfill this need.

Keeping all these facts into consideration, the present work entitled “Nematode control through biological means” was focused on controlling nematode i.e. *Meloidogyne incognita* by a potent fungal biocontrol agent, *Paecilomyces lilacinus* 6029 along with some non-traditional agro-industrial wastes, viz., non-edible oil cakes, biogas slurry and fly ash. These substrates had a dual role: one of controlling nematodes and second as providing nutrition for the growth of antagonist i.e. *P. lilacinus* 6029.

The study was started with the screening of non-edible oil cakes (Jatropha, Neem, Karanja and Mahua) for nematode control. Both aqueous and crude active components of each cake were prepared and bioassayed against J₂ larvae of *M. incognita*. In general, aqueous extracts of oil cakes were superior to crude bioactive components. Among the four selected aqueous extracts of non-edible oil cakes, Mahua cake proved to be weakest nematicidal and thus left out for the next step where oil cakes were tested as a effective substrate of *P. lilacinus* 6029. Karanja cake was selected as a major substrate from other oil

cakes for the growth of fungus as it not only supported the better growth but also showed moderate nematicidal effect. Next, biomass production was maximized by applying response surface methodology to experimental variations in key physico-chemical factors - Carbon/Nitrogen (C/N) ratio and pH. This approach eventually predicted a maximum biomass production of 10.559 g/l with a media having a C/N ratio of 35.88 and pH 5.9. An experimental production of 10.529 g/l biomass was obtained. The remarkable agreement between the predicted and the experimentally obtained biomass confirmed the validity of the approach utilized to maximize production of *P. lilacinus*.

In vitro bioassay of culture filtrate showed that optimized Karanja-sucrose media not only enhanced the growth as compared to commercially available Czapeck's Dox Broth (CDB) but also increased the virulence of *P. lilacinus* 6029. HPLC analysis of fungal filtrate showed that the fungus secreted more amount of nematicidal toxin i.e. leucinostatins, in optimized media than in CDB. Bioassay guided identification confirmed the presence of four phenolic compounds and one aliphatic compound in the culture filtrate. The compounds identified through GC-MS were phenyl ethyl alcohol, benzoic acid, benzene acetic acid, 3,5-Di-t-butylphenol and 2-ethyl butyric acid.

Protease produced by nematophagous fungus was studied because they are important in relation to degradation of the cuticle of nematodes. Protease enzyme secreted by *P. lilacinus* 6029 on Karanja based medium was purified 6.9-fold with 22.6 % yield having specific activity 188.08 U mg⁻¹ after Sephadex G-50 gel filtration chromatography. The apparent molecular mass of the partially purified protease was estimated to be ~35 KDa as seen on SDS-PAGE gel. Fatty acid profiling showed presence of ten fatty acids in fungal filtrate. Out of ten, three well-proven nematicidal acids detected were: Butyric acid (21.75 % of the total), Oleic acid (10.25 % of the total) and Linoleic acid (0.85 % of the total).

Regarding the development of the bionematicide, a novel process was designed to produce the formulation of *P. lilacinus* 6029 using one part of mixed substrate (60 % Karanja cake and 40 % sundried biogas slurry) and one part carrier (flyash). The whole base material was inoculated with the liquid inoculum (from the optimized Karanja-sucrose medium) and was then subjected to solid state fermentation (diphasic SSF). Shelf-life of formulation was tested after storage at different temperature conditions (4 °C, 26 °C and 40 °C) for six months. No change in survivability of spores was observed at 4 °C while at 26 °C, viability of spores remained more than 50 % (53.21 %) even after 6 months of storage.

The human health risk for *P. lilacinus* 6029 was also investigated. Germination and survival of spores from the formulation at 36 °C were studied. There was rapid germination of *P. lilacinus* 6029 conidia (98.9 % in 18 h) obtained from the formulated product at 26 °C but delayed (96.7% in 48 h) when incubated at 36 °C. No spores survived on exposure to 36 °C after 15 days of incubation.

In field-testing, formulation when applied @ 10^7 spores/g of the soil (1g formulation / 100 g soil) not only controlled the root knot infestation effectively but also enhanced the growth, yield and biochemical parameters of tomato crop (*Lycopersicon esculentum*) under green-house conditions. Comparison of the slopes describing the population dynamics of the fungus showed no significant differences between soil without plant and soil from the root zone of the test plant with and without *M.incognita*.

These findings open up the interesting possibilities for ecofriendly management of plant parasitic nematodes. Incorporation of *P. lilacinus* as biocontrol agent along with nutritious as well as nematicidal substrates can act well synergistically to mitigate the use of hazardous chemical nematicides for controlling nematodes along with the enhanced biomass production and soil fertility on sustainable basis.

CONTENTS

	Page no.
Certificate	<i>i</i>
Acknowledgements	<i>ii</i>
Abstract	<i>iv</i>
List of figures	<i>xiii</i>
List of tables	<i>xvii</i>
List of plates	<i>xx</i>
Abbreviations	<i>xxii</i>
CHAPTER 1 INTRODUCTION	<i>1</i>
1.1 Background	1
1.2 Chemical pesticides	2
1.3 Biopesticides	4
1.4 Nematodes.....	8
1.4.1 Plant-parasitic nematodes	9
1.5 Nematode control	11
1.5.1 Physical control.....	11
1.5.2 Chemical control	11
1.5.3 Biological control.....	12
1.6 Mass production of antagonists	16
1.7 Scope of present work.....	17
1.8 Objectives of the study.....	18

CHAPTER 2 REVIEW OF LITERATURE	19
2.1 Root-knot nematode.....	19
2.1.1 Nematode (<i>M. incognita</i>) infestation on tomato (<i>Lycopersicon esculentum</i>).....	21
2.1.2 Symptoms of root knot infestation	22
2.1.3 Biology of root knot nematode	23
2.2 Chemical control of nematodes	27
2.3 Biological control of nematodes.....	29
2.3.1 Organic amendments	29
2.3.2 Mode of action of nematode suppression by organic soil amendments	34
2.3.3 Biological antagonists	37
2.3.4 <i>Paecilomyces lilacinus</i> as biocontrol agent	41
2.3.5 Mode of action of fungal antagonists	43
2.4 Synergistic effect	48
2.5 Mass production of fungal antagonists by SSF	48
CHAPTER 3 MATERIALS AND METHODS.....	51
3.1 Screening of non-edible oil cakes for nematicidal activity and as growth substrate.....	51
3.1.1 Procurement and multiplication of culture of root-knot nematode (<i>M. incognita</i>).	51
3.1.2 Non-edible oil cakes (NEOCs)	54
3.1.3 Characterization of NEOCs	54
3.1.4 Preparation of aqueous (cold and autoclaved) extracts of NEOCs	54
3.1.5 Extraction of crude active components from NEOCs	55
3.1.6 Juvenile mortality (Bioassay)	55
3.1.7 Microorganism and inoculum preparation.....	56

3.1.8 Growth of <i>P. lilacinus</i> 6029 on cake agar medium.....	57
3.1.9 Growth of <i>P. lilacinus</i> 6029 on cake agar broth	58
3.2 Statistical optimization of growth media, using non edible oil cake, for <i>P. lilacinus</i> ...	58
3.2.1 Experimental design for the optimization by one-variable-at-a time method	58
3.2.2 Optimization of critical factors for biomass production.....	58
3.2.3 Light microscopic observations	59
3.2.4 SEM analysis	60
3.3 <i>In vitro</i> bioassay of <i>P. lilacinus</i> 6029 culture filtrate	60
3.4 Identification of nematocidal metabolites	62
3.4.1 Bioassay guided identification of metabolites in culture filtrate from the optimized media.....	62
3.4.2 Detection of leucinostatins in culture filtrate of <i>P. lilacinus</i> 6029	64
3.4.3 Fatty acids analysis.....	65
3.4.4 Production, purification and characterization of a serine protease	65
3.5 Development of bionematicide formulation through diphasic mixed solid substrates fermentation.....	67
3.5.1 Substrates and carriers	67
3.5.2 Mixed solid substrates fermentation.....	68
3.5.3 Optimization of culture conditions to increase spore yield of formulation	69
3.5.4 Shelf life study	69
3.6 Bioefficacy evaluation of the formulation under laboratory conditions	70
3.6.1 Human risk assessment tests (in reference to effect of temperature)	71
3.6.2 Dose-response relationship	72
3.7 Bioefficacy evaluation of the formulation under green house conditions	72

3.7.1 Soil analysis	74
3.7.2 Inoculation of root-knot nematodes	74
3.7.3 Nematode infestation.....	75
3.7.4 Plant growth parameters and biomass yield	76
3.7.5 Persistence study of <i>P. lilacinus</i> 6029 in soil	76
CHAPTER 4 RESULTS	78
4.1 Screening of non-edible oil cakes for nematicidal activity and as growth substrates ...	78
4.1.1 Nematicidal activity of aqueous extracts of NEOCs	78
4.1.2 Nematicidal activity of crude bioactive components	81
4.1.3 Growth of <i>P. lilacinus</i> 6029 on NEOCs as substrate	81
4.2 Statistical optimization of growth media, using Karanja cake, for <i>P. lilacinus</i> 6029 ...	86
4.2.1. One variable at a time analysis for optimization of parameters.....	86
4.2.2 Optimization of critical factors for biomass production.....	88
4.2.3 Relationship between biomass and spore count.....	93
4.2.4 Types of spores	94
4.2.5 Time course study of <i>P. lilacinus</i> 6029 in optimized (Karanja-sucrose) and CDB media.....	95
4.3 <i>In vitro</i> bioassay of <i>P. lilacinus</i> 6029 culture filtrate	96
4.4 Identification of nematicidal metabolites.....	101
4.4.1 Bioassay guided identification of metabolites in culture filtrate from the optimized media.....	101
4.4.2 Identification of compounds by FTIR and GC-MS	107
4.4.3 Detection of leucinostatins in culture filtrate of <i>P. lilacinus</i> 6029	112
4.4.4 Fatty acids analysis secreted by <i>P. lilacinus</i> 6029	114

4.4.5 Production, purification and characterization of a serine protease from <i>P. lilacinus</i> 6029	115
4.5 Development of bionematicide formulation through diphasic mixed solid substrate fermentation (MSSF)	120
4.5.1 Screening of substrates compositions.....	121
4.5.2 Screening of carriers.....	123
4.5.3 Optimization of culture conditions to increase spore yield of formulation	123
4.5.4 Shelf life of <i>P. lilacinus</i> 6029 formulation	130
4.6 Bioefficacy evaluation of the formulation under laboratory conditions	133
4.6.1 Human risk assessment tests (in reference to effect of temperature)	136
4.6.2 Dose-response relationship	137
4.7 Bioefficacy evaluation of the formulation under field conditions (green house study)	141
4.7.1 Characteristics of the soil used in the experiments	141
4.7.2 Effect of formulation on nematode infestation in tomato crop	142
4.7.3 Effect of bionematicidal formulation on the growth parameters of tomato	145
4.7.4 Persistence study of <i>P. lilacinus</i> 6029 in soil	153
CHAPTER 5 DISCUSSIONS	155
5.1 Nematicidal potential of oil cakes	155
5.2 Non-edible oil cakes as growth substrates for <i>P. lilacinus</i> 6029.....	158
5.3 Efficacy of <i>P. lilacinus</i> 6029 filtrate against <i>M. incognita</i>	162
5.4 Identification of nematicidal metabolites of <i>P. lilacinus</i> 6029	166
5.4.1 Identification of leucinostatins.....	168
5.4.2 Fatty acids analysis secreted by <i>P. lilacinus</i> 6029	171
5.4.3 Isolation and characterization of Protease	173

5.5 Development of bionematicidal formulation	174
5.5.1 Shelf life study of formulation	179
5.6 Bioefficacy evaluation of the formulation under laboratory conditions	181
5.6.1 Human risk assessment tests	182
5.6.2 Dose-response relationship	183
5.7 Bioefficacy of formulation under field conditions (green house study)	184
5.7.1 Effect on plant growth parameters	187
5.7.2 Persistence study of <i>P. lilacinus</i> 6029 in soil	188
CHAPTER 6 SUMMARY AND CONCLUSIONS	190
REFERENCES	199
Curriculum Vitae	231