UNIVERSITI PUTRA MALAYSIA

BIOLOGICAL CONTROL OF ROOT KNOT NEMATODES (Meloidogyne incognita) ON BLACK PEPPER (Piper nigrum L.) USING paecilomyces lilacinus and bacillus thuringiensis

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FSPM 2012 5

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By

PAU CHEN GUAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Master of Science

August 2012

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DEDICATION

"HE chose the lowly things of this world and the despised things – and the things that are not – to nullify the things that are, so that no one may boast before HIM."

1 Corinthians 1: 28-29.

I would like to dedicate this thesis to my late mother and late father for the love with which they brought me up. To my late mother I owe my deepest gratitude for her invaluable advice and many life's lessons which I utilize to my advantage. To my late father, I would like to thank him for the love and freedom he had given me to pursue my full time study. It is sad that he couldn't wait long enough to see the completion of my thesis and graduation. I miss them all and believe they will share the joy of my accomplishment from above. I also wish to dedicate this thesis to all my siblings, especially to my sisters, and thank them for their encouragement and moral support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

BIOLOGICAL CONTROL OF ROOT KNOT NEMATODES (*Meloidogyne incognita*) IN BLACK PEPPER (*Piper nigrum L.)* USING *Paecilomyces lilacinus* and *Bacillus thuringiensis*

By

PAU CHEN GUAN

August 2012

Chairman: Stephen Leong Chan Teck, PhD

Faculty: Faculty of Agriculture and Food Sciences (Bintulu)

Black pepper (*Piper nigrum L.*) is an important cash crop of Sarawak. However the plantations are facing challenges due to widespread infestation of root-knot nematode. Development of biological control methods to address this problem is important for sustainable farming as well as consumer health. *Paecilomyces lilacinus* (PL), a saprophytic soil fungus has drawn many research attentions owing to its promising effect in parasitizing and controlling population of phytonematodes. *Bacillus thuringiensis* (BT), a spore forming bacterium with well known insecticidal property also has been reported in demonstrating toxicity towards root-knot nematodes. Ten indigenous strains of PL and a strain of BT carrying Cry6 and Cry14 gene sequences were isolated as an initiative to combat root-knot nematode (RKN) problem. In female nematode bioassay on water agar, PL demonstrated high significant colonization (>90%, $P<0.01$) on female. In egg parasitism test, spore suspension (10^5 spore/ml) of PLA exhibited 78.8% parasitism on eggs. Meanwhile,

hatching rates of nematode eggs incubated in spore suspension of PLA for seven days were significantly reduced; 89% of eggs were hatch-inhibited as compared to control (26%). Pot trials were conducted to evaluate the efficacy of PL and BT in managing RKN infestation on black pepper cuttings in single (PL alone, BT alone) and concomitant treatment (BT + PL) under opened house condition. All treatments manifested significant reduction in root gall index as compared to control in the 70 days treatment. In 140 days treatment, fenamiphos and PL were recorded to produce the lowest number of gall per root system (14.6 and 71.9 galls/root system respectively). The percentages of reduction in nematode reproduction factor (R_f) for these two treatments were at the greatest, 99.8% and 99.2% respectively. BT and concomitant treatments exhibited no significant difference in term of the number of gall per gram of root as compared to control in both 140 days and 180 days of treatments. In experiment II and III, PLA alone appeared to demonstrate better suppression of RKN per root system than in dual combination treatment (BT+PLA). For 180 days treatments, RKN reproduction factor for fenamiphos, PLA, BT and BT+PLA treatments were 0, 47, 113 and 108 respectively. The above investigation provides opportunity to further evaluate PLA's efficacy in field trial with integrated management.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Master Sains

KAWALAN BIOLOGI TERHADAP NEMATOD BENGKAK AKAR (*Meloidogyne incognita*) PADA LADA HITAM (*Piper nigrum L.)* DENGAN MENGGUNAKAN *Paecilomyces lilacinus* DAN *Bacillus thuringiensis*

Oleh

PAU CHEN GUAN

Ogos 2012

Pengerusi: Stephen Leong Chan Teck, PhD

Fakulti: Fakulti Sains Pertanian dan Makanan (Bintulu)

Lada hitam (*Piper nigrum* L.) merupakan tanaman penting yang mendatangkan sumber pendapatan kepada Sarawak. Namun, tanaman ini menghadapi cabaran disebabkan jangkitan penyakit nematod-bengkak-akar yang merebak dengan meluas. Perkembangan kaedah kawalan biologi terhadap masalah ini adalah penting demi kemanpanan pertanian serta kesihatan pengguna. *Paecilomyces lilacinus* (PL), sejenis kulat saprofitik yang berasal dari tanah telah menarik banyak perhatian dalam penyelidikan kerana kesan positif dalam menjangkiti dan mengawal populasi fitonematod. *Bacillus thuringiensis* (BT), sejenis bakteria pembentuk spora yang terkenal dengan nilai keracunan terhadap serangga, juga telah dilaporkan bahawa la menunjukkan ketoksikan terhadap nematod-bengkak akar. Sepuluh PL baka tempatan dan sejenis baka BT pembawa urutan gen Cry6 dan Cry14 telah dipencilkan sebagai langkah inisiatif untuk memerangi masalah penyakit ulat-bengkak akar. Dalam bioassai pada nematod betina di atas agar-agar berair, PL

menunjukkan pengkolonian yang bererti dan tinggi (> 90%, P < 0.01) pada nematod betina. Dalam ujian parasitisme telur, ampaian spora (10^5 spora /ml) PLA mempamerkan 78.8% pemparasitan pada telur. Sementara itu, kadar penetasan telur nematod yang dieram dalam ampaian spora PLA selama tujuh hari telah mengalami pengurangan yang ketara; 89% telur telah terhalang daripada menetas berbanding dengan ujian kawalan (26%). Ujian berpasu telah dijalankan untuk menilai keberkesanan PL dan BT dalam mengurangkan infestasi RKN pada keratan lada hitam di bawah pondok terbuka melalui rawatan tunggal (PL sahaja, BT sahaja) dan rawatan seiring (BT + PL). Semua jenis rawatan menunjukkan penurunan indeks bengkak akar yang bererti berbanding dengan ujian kawalan dalam rawatan selama 70 hari. Dalam rawatan selama 140 hari, fenamifos dan PL mencatat bilangan bengkak akar per sistem akar yang terendah (masing-masing 14.6 dan 71.9). Peratus peunuranan dalam faktor pembiakan nematod untuk kedua-dua rawatan ini adalah yang tertinggi masing-masing pada 99.8% dan 99.2%. BT dan rawatan seiring (PL+BT) menunjukkan perbezaan yang tidak bererti dalam aspek purata bengkak per gram akar berbanding dengan ujian kawalan bagi kedua-dua jenis rawatan selama 140 hari dan 180. Dalam eksperimen I dan II, PLA menunjukkanh penindasan RKN per sistem akar yang lebih baik daripada rawatan seiringan (BT + PLA). Bagi rawatan selama 180 hari, faktor pembiakan RKN untuk rawatan fenamifos, PLA, BT dan rawatan seiring (BT + PLA) adalah masing-masing 0, 47, 113 dan 108. Penyelidikan di atas telah membuka peluang untuk menilai keberkesanan PLA dalam percubaan ladang secara pengurusan bersepadu.

ACKNOWLEDGEMENTS

First of all, I wish to thank my supervisor Dr Stephen Leong for his academic support, patience and help. I also wish to extend my gratitude to my co-supervisors, Dr Wong Sing King and Dr Osumanu Haruna Ahmed for their guidance, advice and insight. Many thank to all members of my supervisory committee for their time, patience and input in reviewing and correcting my manuscript.

I also wish to extend my appreciation to Dr Lily Eng for her help and invaluable advice about some of the laboratory skills on nematology. I am grateful to her for teaching me the technique of preparing perineal pattern, sending me a photocopy of her literature and lending me her Bridge counting disks.

My profound gratitude to Prof Dato Dr Nik Muhammad Majid, black pepper research team leader, for his insight and constructive comment. I would also like to register my thanks for all lecturers and members in the black pepper research team for their input during monthly meeting and progress presentation. Gratitude is also expressed to the Dean and Deputy Dean of Faculty of Agriculture and Food Science, Prof. Dr. Japar Sidik Bin Bujang and Dr. Mohd. Hanafi Bin Idris, and all UPM lecturers who have contributed constructive idea to my studies. Many thanks for their friendship and moral support.

Also, my deep appreciation to Malaysia Pepper Board for funding this study and to UPM Graduate School for the scholarship that I received and for the financial support during my research and studies in UPM Bintulu. My gratitude to Bahagian

Tajaan of Kementerian Pelajaran Malaysia for granting me with full time unpaid study leave so that I could concentrate on research.

I would also like to thank our lab assistants, Mohammad Ezwandi and Maini Chee for their technical assistance on some of our laboratory equipments. To all my laboratory mates and coursemates, Sharon Wong, Amelia Tang, Hsien Loong, Chin Tsong, Latifah, Kona, Latip, thank you for your friendship, encouragement and assistance. Thank you also to Dr Yiu Pang Hong for allowing me to use your homogenizer in your chemistry laboratory. My deepest appreciation extended to English lecturer in IPG, Mr Tay Nguong Yong who carefully corrected the grammatical errors in my thesis.

Also, my heartfelt respect and gratitude to all my examiners, Prof. Dr Sepiah Muid, Assoc. Prof. Dr. Radziah Othman, Assoc. Prof. Dr. Kamaruzaman Sijam and chairperson of viva voce, Asso. Prof. Dr. Joseph Bong Choon Fah for their reviews, constructive comments and suggestions in improving the content of my thesis.

Lastly, thanks to all my siblings and relatives for their moral support and help during this course of study. No part of this research was conducted as a sole endeavor and you all have contributed at different levels in different ways. It is a pleasure for me to thank those who made this master thesis possible. Lastly, my best regards and blessings to all who have directly or indirectly supported me in any aspect during the completion of this thesis.

I certify that a Thesis Examination Committee has met on 13 August 2012 to conduct the final examination of PAU CHEN GUAN on his thesis entitled "Biological control of root-knot nematodes (*Meloidogyne incognita*) in black pepper (*Piper nigrum L.)* using *Paecilomyces lilacinus* and *Bacillus thuringiensis*" in accordance with Universities and University Colleges Act 1971 and the Constitution of the University Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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Date: 26 February 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

PAU CHEN GUAN

Date: 13 August 2012

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LIST OF ABBREVIATIONS

1	BT	*Bacillus thuringiensis*
2	CFUs	Colony Forming Units
3	CRD	Complete Randomized Design
4	Fig	Figure
5	J2	2nd stage Juveniles
6	MCAs	Microbial Control Agents
7	PCR	Polymerase Chain Reaction
8	MI	*Meloidogyne incognita*
9	PDA	Potato Dextrose Agar
10	PDA+	Potato Dextrose Agar amended with 0.01% (w/v) Chloramphenicol and 3% (w/v) Sodium Chloride
11	PDB	Potato Dextrose Broth
12	PGPR	Plant Growth Promoting Rhizobacteria
13	PL	*Paecilomyces lilacinus*
14	RCBD	Randomized Complete Block Design
15	R_f	Reproduction factor
16	RGI	Root Gall Index
17	RKN	Root Knot Nematode
18	RLCC	Relative Leaf Chlorophyll Content
19	SDS	Sodium Dedocylsulphate
20	Tab	Table
21	TAE	Tris Acetic acid EDTA
22	TE	Tris EDTA
23	VC	Vegetative Cell

CHAPTER 1

INTRODUCTION

Black pepper (*Piper nigrum L.*), the king of spices, is an important cash crops of Sarawak. Sarawak exported about 19,748 tonnes of pepper in 2004 and 22,218 tonnes in 2008, valued at RM 113.2 million and RM170.0 million respectively (Lim, 2009). About 6,125 tonnes of black pepper were exported from January to June 2010, valued at RM77.889 million (Anonymous, 2010). Today, pepper is one of the important cash crops supporting the livelihood of about 67,000 rural dwellers in upland areas of Sarawak (Lim, 2009).

However, most of the black pepper farms in Sarawak are being infested by root-knot nematodes, *Meloidogyne* spp. (Kueh, 1978,; Leong, 1986; Ramana and Eapen, 2000; Eng, 2001) which has always been a major constraint to the black pepper production. In a survey conducted by Eng (2001) on 43 black pepper farms in Sarawak, root-knot nematodes (RKN) were reported presence in all the farms. Badly infested vines, with yellowish speckle especially at the interveinal areas of the leaves and galls on their roots, are stunted and vulnerable to moisture or temperature pressure as well as other pathogenic infections such as *Phytophthora* (Winoto, 1972). Subsequently, the vines became unproductive and abandoned, resulting in substantial economic losses to farmers each year.

Currently, no RKN resistant black pepper cultivar is available (Eng, 2001). For the past decades, chemical nematicides have been used in managing nematodes infestation on crops but gradually are being reappraised in respect of health and environmental concern and limited availability in developing nations. On the other hand, microbial control agents (MCAs) are gaining popularity in integrated nematodes management due to their promising results in nematodes control and are safer than synthetic nematicides (Mukhtar & Pervaz, 2003; Dong & Zhang, 2006).

Paecilomyces lilacinus (PL), a saprophytic soil fungus, commonly known as natural facultative egg parasite of root-knot and cyst nematodes (Rumbos & Kiewnick, 2006) has drawn many research attentions for the past decade owing to its efficacy in parasitizing and controlling population of phytonematodes (Jatala, 1986; Dube & Smart, 1987; Hewlett *et al.*, 1988; Freitas *et al.*, 1995; Nagesh *et al.*, 1997; Khan *et al.*, 2006a; Kiewnick & Sikora, 2006a; Kiewnick & Sikora, 2006b; Brand *et al.*, 2010). It was reported with high frequency of occurrence in tropics and subtropic (Morgan *et al.*, 1984; Chen *et al.*, 1996) and can be found in most of agricultural soils (Brand *et al.*, 2010). According to the report of Eng (2001), 82.9% of the total forty one surveyed farms in Sarawak contained PL despite intensive application of fungicide in the farm. Cabanillas *et al.* (1989) observed maximum growth of PL at temperature ranged from 24ºC–30ºC. They claimed that PL was able to grow on a wide range of common organic substrates and remain competitive with other microbes in soil. Besides, it also tolerated broad range of soil pH and was able to grow well at 15°C–30°C. Since PL has high adaptability in its life strategy, it is competitive in a broad spectrum of range adaptability.

Meanwhile, *Bacillus thuringiensis* (BT), a well-known enthomopathogenic bacteria used for pest insects control since four decades ago (Brar *et al*., 2006) has also drawn intensive studies on its nematicidal effects against economically important phyto-parasitic nematodes (Deviddas and Rehberger, 1992; Siddiqui and Mahmood, 1994; Carneiro *et al*., 1998; Mozgovaya *et al.*, 2002; El-Nagdi and Youssef, 2004; El-Sherif *et al.*, 2007; Mohammed *et al.*, 2008; Khan *et al.*, 2010). Carneiro *et al.* (1998) claimed that culture of BT was efficacious in killing freshly hatched 2nd stage juvenile (J2) of *Meloidogyne javanica*. Mozgovaya *et al.* (2002) reported 80% mortality of nematodes after *in vitro* treatment with BT. El-Nagdi and Youssef (2004) commented that soaking faba beans with BT reduced the population density of *M. incognita* but increased the plant growth. According to El-Sherif *et al.* (2007), BT applied alone improved the growth parameters of egg plant and reduced nematode development. Mohammed *et al.* (2008) reported that the spore/crytal proteins of BT showed highest nematicidal activities against *Meloidogyne incognita.* However, no current report was found on the positive effect of BT in controlling RKN development in black pepper. Therefore, an effort is initiated to explore the use of BT and PL as alternatives to the chemical nematicides currently in use for the control of root-knot nematodes in black pepper. The objectives of this study were:

1. To isolate and evaluate the local isolates of *Paecilomyces lilacinus* and *Bacillus thuringiensis* for their nematicidal properties against *Meloidogyne incognita* in the laboratory bioassay.

2. To produce, formulate and evaluate the selected strains of *Paecilomyces lilacinus* and *Bacillus thuringiensis* for their biological control of *Meloidogyne incognita* on black pepper in pot trial.

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BIODATA OF STUDENT

Pau Chen Guan, born in 18 May 1974, originated from Sri Aman, Sarawak. He completed his primary school in Sekolah Rendah Kebangsaan Stampin in 1986, secondary school in SMK Gapor Stampin, Kuching in 1992 and high school in SMK Green Road, Kuching in 1994. He successfully obtained Bachelor of Science (Hons) in Microbiology with second class upper (CGPA 3.149) from Universiti Putra Malaysia, Serdang in 1999. After that he worked as a temporary teacher in SMB St Thomas, Kuching for a year and as an education officer in Sarawak Biodiversity Centre for six months under contract before he proceeded for diploma in education in Maktab Perguruan Keningau, Sabah for nine months. After completed his diploma in education, he was posted to SMK Simanggang, Sri Aman in Janauary 2002 as a form six biology teacher for three years before transferred back to Kuching. He taught SPM biology and chemistry in SMK Lumba Kuda for four and a half year. In July 2009, he was granted with two year unpaid study leave from Bahagian Tajaan, Kementerian Pelajaran Malaysia to pursue Master of Science (Plant Pathology) which he is undertaking now in Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus. In July 2011, he was posted back to his former school, SMK Green Road, Kuching to teach biology at STPM and SPM level while waiting for his viva voice and final thesis correction.

LIST OF PUBLICATIONS

1. Pau, C.G., C.T.S. Leong, S.K. Wong, L. Eng, M. Jiwan, F.R. Kundat, Z.F.B.A. Aziz, O.H. Ahmed and N.M. Majid, 2012. Isolation of indigenous strains of *Paecilomyces lilacinus* with antagonistic activity against *Meloidogyne incognita. Int. J. Agric. Biol.*14: 197–203 [Published]

2. Pau, C.G., C.T.S. Leong, S.K. Wong, L. Eng, M. Jiwan, F.R. Kundat, Z.F.B.A. Aziz, O.H. Ahmed and N.M. Majid. 2012. Evaluation of *Paecilomyces lilacinus* and *Bacillus thuringiensis* as microbial control agents for *Meloidogyne incognita* in black pepper (*Piper nigrum* L.) planted in pots under open house condition. *Biocontrol Science and Technology* (Under review).