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SYNERGISTIS EFFECT OF Metarhizium anisopliae AND FIPRONIL ON Coptotermes curvignathus

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By

YII JING EE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

July 2016

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

SYNERGISTIC EFFECT OF *Metarhizium anisopliae* AND FIPRONIL ON *Coptotermes curvignathus*

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July 2016

Chairman: Associate Professor Joseph Bong Choon Fah, PhD

Faculty: Agriculture and Food Sciences, Bintulu

Termites are severe economic threats to agriculture and urban structures in the Indo-Malaysia region. As a consequence of rapid expansion of the oil palm industry on peat area, termite infestation on monoculture oil palm becomes serious and *Coptotermes curvignathus* is identified as the main culprit that caused palm damage and loss. In current study, an enhancement of the infectivity and application methods against termites by combining entomopathogenic fungi with sublethal dosages of pesticides as stressor to weaken the pests towards fungal infection using termite baits was introduced as management tool. In order to choose the most compatible isolate for bait formulation, the germination, vegetative growth and spore production of *Metarhizium anisopliae* local isolates (TA, LR2 and MG) were examined in compatibility test with several sublethal doses of fipronil. Fipronil was found compatible and relatively less detrimental to fungal growth of isolate TA. Rubber sawdust bait supplemented with glucose was developed based on feeding preference of *C. curvignathus* through food size and nutrient test. The formulated bait incorporated with fipronil, *Metarhizium*, and glucose had shown no repellence to termites. The effective shelf life of formulated bait was determined through termite virulence test and HPLC method. In the present formulation studied, the formulated bait was shown to be effective against termites up to five months of storage. The synergism in efficacy of fungus-insecticide combinations in formulated bait was determined against *C. curvignathus* and the results showed synergies against subterranean termite mortalities at fipronil-*Metarhizium* combinations of 0.05 mg a.i. L^{-1} fipronil with either 10^7 or 10^8 conidia g^{-1} in bait at 8 DPT. Moreover, with the addition of glucose in bait, synergistic effect was manisfested at 10^8 conidia g^{-1} *M. anisopliae* alone bait and also 0.001 mg a.i. L^{-1} fipronil with 10^8 conidia g^{-1} in bait. The synergy between *M. anisopliae* and fipronil in termites has not been reported. The insecticidal stress caused by sublethal fipronil in formulated bait may weaken the termites and reduce their defense mechanism, which facilitates fungus infection on termites. Hence, termite defensive behavioral response such as grooming, cellular immunity response, tunneling, and spatial distribution of termite cadavers after treated with formulated bait were evaluated. As the first line of termite's defensive mechanisms, the total frequency of

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termite allogrooming within 72 hours post treatment was lowered down in the presence of fipronil in formulated bait which accelerated the probability of fungus infection against termites compared to the single treatment of fungus bait. Inhibition of grooming had been identified as the key factor to the enhanced infection rates. In histopathology study, *C. curvignathus* was proved to possess cellular immune response against fungus infection using Periodic Acid Schiff (PAS) staining. The occurrence of cellular encapsulation and melanization for termites that consumed formulated bait was lower than *Metarhizium* alone bait which was 45 and 65 times, respectively. The result suggested a possibility that a decrease of the cellular immune responses of termites was due to the addition of fipronil in formulated bait that weakening the immune system of termites and thereby increasing the susceptibility of termites to *M. anisopliae* infection. This finding can be improved in future work with more quantitative biochemical data to further prove the statement. Besides, slow action of formulated bait (fipronil 0.001 mg a.i. L^{-1} + *Metarhizium* 10^8 conidia g^{-1} + 1% glucose) was shown in infected termites which were still as active as the healthy termites in control treatment after bait feeding for three days with average tunneling speed 0.011 cm^2 $hour^{-1}$ $termite^{-1}$. This increased the chance to create epizootic in subterranean termite colony when the disease was able to be delivered to nest by the active foragers. Another impact of fungus-insecticide combination was manifested in the spatial distribution of termite cadavers in laboratory test, whereby the infected cadavers were distributed in all primary and secondary harborages but significantly fewer in bait area. This indicated the diseases are able to disseminate to further area, presumably termite nest or satellite nests, and infect more colony members. The formulated bait was further evaluated on termite infested oil palm trees through above and underground baiting station for seven months. Rubber wood stakes consumption was used as an essential parameter to estimate the suppression of termites in the colony in underground station. The consumption of rubber wood by termites was significantly reduced 58.70% at first month post treatment of fungus-insecticide-glucose bait combination (fipronil 0.001 mg a.i. L^{-1} + *Metarhizium* 10^8 conidia g^{-1} + 1% glucose). It implied that the number of foraging termites was reduced, perhaps as a result of weakened colony. The observed synergism treatment in laboratory and field trial showed the potential for integrated fungus-insecticide control method for subterranean termite management in oil palm industry.

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Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN SINERGI BAGI *Metarhizium anisopliae* DAN FIPRONIL TERHADAP *Coptotermes curvignathus*

Oleh

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Julai 2016

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Anai-anai merupakan suatu ancaman yang membimbangkan terhadap pembangunan ekonomi bagi industri pertanian serta kawasan bandar di rantau Indo-Malaysia. Akibat daripada perkembangan pesat industri kelapa sawit di atas tanah gambut, serangan anai-anai pada kelapa sawit monokultur menjadi serius dan *Coptotermes curvignathus* dikenal pasti sebagai punca utama yang menyebabkan kerosakan dan kerugian kelapa sawit. Dalam kajian semasa, peningkatan jangkitan dan aplikasi kaedah terhadap anai-anai dengan menggabungkan kulat entomopatogenik dengan dos sublethal racun perosak sebagai penekan untuk melemahkan perosak terhadap jangkitan kulat dalam umpan anai-anai telah diperkenalkan sebagai alat pengurusan. Dalam usaha untuk memilih pencilan yang paling serasi untuk perumusan umpan, percambahan, pertumbuhan vegetatif dan spora pengeluaran *Metarhizium anisopliae* pencilan tempatan (TA, LR2 dan MG) telah diperiksa dalam ujian keserasian dengan beberapa dos sub maut fipronil. Fipronil ditemui sesuai dan agak kurang menjejaskan pertumbuhan kulat pencilan TA. Rumusan umpan gabungan fipronil, *Metarhizium*, dan glukosa telah diformulasikan berdasarkan makanan keutamaan *C. curvignathus* melalui ujian saiz dan nutrien umpan. Umpan yang diformulasikan ini menunjukkan tidak menghalau anai-anai. Efektif angka hayat umpan yang dirumuskan juga disiasat melalui ujian virulen anai-anai dan kaedah HPLC. Dalam formulasi yang dikaji, bahan aktif fipronil dan spora *Metarhizium* dalam umpan telah terbukti berkesan terhadap anai-anai selama lima bulan penyimpanan. Sinergi dalam keberkesanan kombinasi kulat dan racun serangga dalam umpan telah disiasat terhadap *C. curvignathus* dan keputusan menunjukkan sinergi terhadap mortaliti anai-anai bawah tanah atas gabungan 0.05 mg a.i. L^{-1} fipronil sama ada dengan 10^7 atau 10^8 konidia g^{-1} *M. anisopliae* dalam umpan pada 8 DPT. Selain itu, penambahan glukosa dalam umpan telah menunjukkan kesan sinergi pada 10^8 konidia g^{-1} *M. anisopliae* dan juga 0.001 mg a.i. L^{-1} fipronil dengan 10^8 konidia g^{-1} dalam umpan. Sinergi di antara *M. anisopliae* dan fipronil tidak pernah dilaporkan pada anai-anai. Tekanan yang dihasilkan oleh sub-maut fipronil dalam umpan yang dirumuskan boleh melemahkan anai-anai dan mengurangkan mekanisme pertahanan mereka agar memudahkan jangkitan kulat pada anai-anai. Oleh itu, tindak balas tingkah laku pertahanan anai-anai

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seperti dandanan, maklum balas imuniti selular, membina terowong, dan pengedaran ruang mayat anai-anai selepas dirawat dengan umpan yang difomulasikan telah dinilai. Sebagai barisan pertama mekanisme pertahanan anai-anai, jumlah kekerapan dandanan sesama anai-anai dalam masa 72 jam selepas rawatan telah diturunkan dalam rumusan umpan yang mengandungi fipronil. Ini telah memudahkan jangkitan kulat entomopatogenik terhadap anai-anai berbanding dengan umpan kulat sahaja. Perencatan dandanan adalah faktor utama kepada peningkatan kadar jangkitan. Dalam kajian histopatologi, *C. curvignathus* telah terbukti memiliki tindak balas imun selular atas jangkitan kulat dengan menggunakan Periodic Asid Schiff (PAS). Pengkapsulan selular dan proses melanisasi telah diturunkan dalam anai-anai yang makan rumusan umpan yang mengandungi fipronil berbanding dengan *Metarhizium* umpan sahaja, iaitu 45 dan 65 kali. Ini berkemungkinan disebabkan gabungan racun serangga fipronil dalam kaedah kawalan biologi berkesan dalam melemahkan sistem imun selular anai-anai dengan mengurangkan respons imunnya, untuk meningkatkan jangkitan anai-anai terhadap *M. anisopliae*. Penemuan ini boleh diperbaiki dengan meningkatkan data kuantitatif biokimia untuk membuktikan kenyataan itu. Selain itu, umpan yang bertindak perlahan ini (fipronil 0.001 mg a.i. L^{-1} + *Metarhizium* 10^8 konidia g^{-1} + 1% glukosa) telah dibuktikan dalam anai-anai yang dijangkiti tetapi keaktifan sama dengan rawatan kawalan dengan purata kelajuan pembinaan terowong 0.011 cm^2 jam^{-1} $anai^{-1}$ selepas makan umpan selama tiga hari. Ini telah meningkatkan peluang untuk mewujudkan kesan epizootik kepada koloni anai-anai di bawah tanah apabila penyakit dapat disampaikan ke sarang oleh pekerja anai-anai. Salah satu kesan gabungan kulat dan racum serangga telah dimanifestasikan dalam pengagihan ruang mayat anai-anai dalam ujian makmal, di mana mayat yang dijangkiti telah diedarkan dalam semua pangkalan utama dan kecil tetapi ketara kurang di kawasan umpan. Ini menunjukkan penyakit dapat disebarkan ke kawasan yang lebih jauh, mungkin ke sarang atau sarang satelit anai-anai, dan menjangkiti lebih ramai ahli koloni. Umpan dirumuskan telah dinilai dalam lapangan pada pokok-pokok kelapa sawit yang diserangi oleh anai-anai melalui atas dan bawah tanah stesen umpan selama tujuh bulan. Pengambilan kayu getah oleh anai-anai telah digunakan sebagai satu parameter penting untuk menganggarkan penindasan anai-anai di stesen bawah tanah. Pengambilan kayu getah oleh anai-anai telah dikurangkan 58.70% pada bulan pertama selepas rawatan kombinasi umpan kulat, racun serangga dan glukosa (fipronil 0.001 mg a.i. L^{-1} + *Metarhizium* 10^8 konidia g^{-1} + 1% glukosa). Ia menunjukkan bahawa bilangan anai-anai pencari makanan telah dikurangkan, berkemungkinan akibat daripada kelemahan koloni. Rawatan sinergi yang diperhatikan dalam kerja makmal dan lapangan menunjukkan kaedah kawalan kulat dan racun serangga bersepadu berpotensi dalam pengurusan anai-anai bawah tanah dalam industri kelapa sawit.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. Members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

a.i.	Active ingredient
ANOVA	Analysis of Variance
BI	Biological index
CL	Confidence limit
CPO	Crude Palm Oil
CRD	Completely Randomized Design
DNMRT	Duncan's New Multiple Range Test
DPT	Day post treatment
GABA	Gamma-aminobutyric acid
HPLC	High Performance Liquid Chromatography
i.e.	That is
IPM	Integrated Pest Management
LC	Liquid Chromatography
LC_{50}	Median Lethal Concentration
LT_{50}	Median Lethal Time
M_E	Expected mortality
mo	Month
PP	Polypropylene
PTFE	Polyetrafluoroethylene
PVC	Polyvinyl chloride
RCBD	Randomized Complete Block Design
RH	Relative Humidity
RP	Reversed-Phase

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SDAY	Sabouraud dextrose agar + yeast
SDB	Sabouraud dextrose broth
SE	Standard error
SOP Sdn. Bhd.	Sarawak Oil Palm Sendirian Berhad (incorporated)
Tukey's HSD test	Tukey's honest significant difference test
UPM	Universiti Putra Malaysia
UV	Ultra Violet
UV/vis	Ultraviolet-visible
v/v	Volume per volume
Var.	Variety
w/v	Weight per volume
w/w	Weight per weight
χ^2	Chi-square test

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CHAPTER 1

INTRODUCTION

1.1 Background and problem statement

Termite damage is a serious predicament worldwide, especially in agriculture of the Indo-Malaysia region. As the rapid development of oil palm industry moves onto peat land in the country, termite problems arises unceasingly against this monoculture crops in every stages of palm growth. The main culprit of the termite pest in oil palm plantation is identified as *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). This pestiferous termite species is a living tissue consumer, which feeds on the apical meristematic tissue of young oil palm and built the nest inside the trunks of mature palms (Cheng *et al.*, 2008). Infested palms can be recognized by the moist dark brown mud sheet on the trunk from the palm base upwards to the shoot. Severely infested palms often topple over as inner part of the tree is hollowed up which weaken the palm structure. More than 3% of the standing palms are dead as a consequence of termite damage each year (Zulkefli *et al*., 2012). This influences the yield production of oil palms dramatically with total loss of RM 217.8 (US$ 58) million yr^{-1} in fresh fruit bunches (Zulkefli *et al*., 2012). In the long term, termite infestation will inevitably cause a severe economic loss and menace to the industry if no action is being taken.

Termite control is perhaps the most important and difficult pest management work of oil palm plantation on peat. In Malaysia, enormous cost of RM 30 - 37.5 (US$8 - 10) million is spent for battling against termite infestation every year (Verma *et al*., 2009). Concurrent with the increased damage from this insect pest, several ways have been applied against these natural calamities in the oil palm plantation, such as the use of chemical and biocontrol agents (Sudharto *et al*., 1991; Lim and Silek, 2001). However, there are pro and cons in both methods. The use of chemicals to control termites leads to rapid killing of the termites but heavy usage of chemical insecticides brings hazardous effect to the beneficial insects and environment. The most common chemical pesticide used by plantation owners is fipronil, a phenylpyrazoles insecticide, which acts on nervous system and used for control of many soil and foliar insects through contact and stomach poison activity (Yu, 2014). However, it is just a short term termite control method, whereby the plantations are still constantly suffering from termite infestations. Fipronil is applied regularly to prevent reinfestation of termite and this will inevitably increase the cost of termiticides usage. For biocontrol agents, the control effect of using biocontrol agent is slow and rather unstable, especially in natural environment if compared to chemical control. However, they are more persistence, cost effective, target specific and less potential for damage to environment or non-target organisms (Lacey *et al*., 2001, 2015; Milner *et al*., 2003; Srivastava *et al*., 2009). With the growing of environmental awareness, it is an urge to figure out an alternative that is effective, economic, and environmental friendly termite management tool.

The use of entomopathogenic fungus, *Metarhizium anisopliae* as biocontrol agents has been envisaged as a promising method and widely used in other pests control

management. However, it turns into a problematic issue when it comes to the subterranean termites *C. curvignathus* due to termites' natural cryptic living habits and an array of defensive behavior response against entomopathogens. For instance, triggering alarm behavior (Rosengaus *et al*., 1999; Myles, 2002a), aggregation around spore-treated individuals or avoidance of infective cadavers (Milner *et al*., 1998; Baverstock *et al*., 2010), grooming (Su *et al*., 1982; Yanagawa and Shimizu, 2007; Chouvenc *et al*., 2008; Yanagawa *et al*., 2008, 2010; Zhukovskaya *et al*., 2013), necrophagy or cannibalism (Myles, 2002b; Chouvenc and Su, 2012), necrophoresis or burial of infected cadavers (Jones *et al*., 1996; Yanagawa *et al*., 2011; Chouvenc *et al*., 2012b; Ulyshen and Shelton, 2012), cellular and humoral immunity (Chouvenc *et al*., 2009a; Avulova and Rosengaus, 2011), and antifungal gut activity (Chouvenc *et al*., 2009b, 2010). Termite defensive response against fungus infection induces a great barrier and often result in unsuccessful epizootic in subterranean termite colony in the last 50 years of research works (Chouvenc *et al*., 2011b). Therefore, the aim of termite control or elimination in the field is exceptionally difficult with the current circumstances.

To alleviate the shortcomings, alternative termite control methods should be developed such as the combination of both biocontrol agent and sublethal doses of chemical insecticide for the pest control, which has been promoted in the Integrated Pest Management (IPM) strategy. The integrated pest control methods are getting more appealing in the worldwide research of pest control. Numerous research studies have reported on the potential use of entomopathogenic fungi incorporated with agrochemicals in a whole range of pests. Several studies have documented that some insecticides have the capacity to induce a broad range of sublethal effects such as behavioral or physiological alterations in insect pests, for instance, abnormal movement, reduction in feeding and foraging activities, and reproductive impairment when used in sublethal doses (Serrao *et al*., 2000; Desneux *et al*., 2007; Zhu *et al*., 2013; Pisa *et al*., 2015), which might facilitate pathogen infection against the insect pests. In addition, baiting is the most recent method of termite pest control which sounds environmentally and had been successful implemented to control some termite species (Verma *et al*., 2009). Therefore, IPM strategies on termite baiting is suggested as an alternative termite control method in this study, whereby entomopathogenic fungus and chemical insecticide are incorporated in bait formulation for suppressing the cryptic underground nesting species, *C. curvignathus* in oil palm plantation. The hypothesis of the study is that the combination of fungus and insecticide is able to act synergistically by enhancing the infectivity, weakening the pests, and reducing their defense mechanism, in order to cause rapid decline in the *C. curvignathus* infestation.

1.2 Objectives

The objectives of this research are to:

i. Formulate a bait incorporating *Metarhizium anisopliae* and Fipronil to control *Coptotermes curvignathus*
ii. Evaluate the effect of formulated bait on defensive behavioral response of *Coptotermes curvignathus*
iii. Determine the effect of formulated bait against *Coptotermes curvignathus* infestation in oil palm plantation

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The student was born in Sibu Sarawak, Malaysia on 12th May 1986. She did her primary education at the Sekolah Rendah Bantuan Methodist, Sibu, and continued her secondary schooling at Sekolah Menengah Kebangsaan Deshon, Sibu where she obtained both her Penilaian Menengah Rendah (PMR) and Sijil Perperiksaan Malaysia (SPM). After that, she continued her pre-university program which formally known as Sijil Tinggi Persekolahan Malaysia (STPM) at Sekolah Menengah Kebangsaan Chung Hua, Sibu. She started her tertiary education at Universiti Putra Malaysia (UPM) Bintulu Sarawak Campus in 2006 and graduated with first class honor degree in Bachelor of Science Bioindustry in 2010. At the same year, she further pursues her postgraduate study at UPM in Doctor of Philosophy in Entomology with interest in the research of termite control. During the period of postgraduate study, she received Graduate Research Fellowship (GRF) from UPM and had assisted in teaching of diploma course lab work for 1 year. After that, she continues her study under sponsorship of The Ministry of Higher Education (MOHE) Malaysia in MyPhD program.

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LIST OF PUBLICATIONS

Yii, J.E., Bong, C.F.J., King, J.H.P. and Kadir, J. 2016. Synergism of entomopathogenic fungus, *Metarhizium anisopliae* incorporated with fipronil against oil palm pest subterranean termite, *Coptotermes curvignathus*. *Plant Protection Science* 52: 35-44.

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