UNIVERSITI PUTRA MALAYSIA

INTERACTION BETWEEN AN ENTOMOPATHOGENIC FUNGUS Metarhizium anisopliae AND ITS HOST SUBTERRANEAN TERMITES Coptotermes curvignathus DURING INFECTION PROCESS

AHMAD SYAZWAN BIN SAMSUDDIN

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By

AHMAD SYAZWAN BIN SAMSUDDIN

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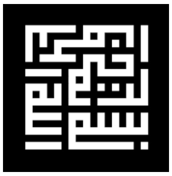
DEDICATION



This thesis was dedicated to my Ayah and Ibu, Samsuddin Mohamed and Zaharina Sharif for love, courage and support given.

Ahmad Syazwan Samsuddin

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

INTERACTION BETWEEN AN ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* AND ITS HOST SUBTERRANEAN TERMITES *Coptotermes curvignathus* DURING INFECTION PROCESS

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April 2017

Chairman : Professor Ahmad Said Bin Sajap, PhD
Faculty : Forestry

The subterranean termites, *Coptotermes curvignathus* is a destructive pest of tree plantation in Malaysia. Controlling termite population by chemical is not a good option since it may increase the resistance of pest towards the pesticide and cause several implications towards biodiversity and environment. *Metarhizium anisopliae*, an entomopathogenic fungus is a good option by controlling the termite biologically. In this study, ten *M. anisopliae* isolates were screened for their pathogenic effect on termites. The best isolate, PR1 which originated from Pantai Remis, Selangor is the most prudently isolates by yielded the highest mortality (96.97%) and the shortest median lethal time (LT_{50}= 1.47 days). This isolate was utilized in downstream study by conducting the histopathology and protein expression study; for each stages of pathogenesis up to 144 h of post-inoculation. Results demonstrated that it requires two days to kill infected host starting by germ tube penetration through the termite cuticle observed at 6 h post-inoculation. Injury caused by *M. anisopliae*'s appressorium in a way to penetrate the first layer of host defense mechanism, the cuticle, one of the factors that weakening the infected host. Dissemination and of fungal and hyphal bodies observed on cellular level is a proof that *M. anisopliae* invaded the host starting from the cellular level. Then, inoculated termites were mummified and the sporulation of *M. anisopliae* on host's carcasses observed at 96 h and 144 h of post-inoculation, respectively. Meanwhile in protein expression study, four and five significance expressed proteins have been identified from *C. curvignathus* and *M. anisopliae*, respectively. Expressed proteins in termite shows evidence that being involve in cell regulation and immune response towards infection by this entomopathogenic fungus. Meanwhile, the expressed proteins extracted from *M. anisopliae* were discovered to be involved in the protein transportation and also related with fungus virulencity during infection process. By knowing the interaction between this host-pathogen relationship, the achieved knowledge could be utilized in future development of biopesticide by using this fungus to control termite population effectively.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

INTERAKSI DI ANTARA KULAT PEMBUNUH SERANGGA *Metarhizium anisopliae* DAN ANAI-ANAI BAWAH TANAH *Coptotermes curvignathus* SEMASA PROSES JANGKITAN

Oleh

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Coptotermes curvignathus adalah salah satu spesies anai-anai merupakan perosak yang merbahaya terhadap dirian pokok di Malaysia. Pengawalan populasi anai-anai dengan menggunakan bahan kimia adalah salah satu pilihan yang kurang tepat memandangkan ianya mampu meningkatkan kerintangan serangga tersebut terhadap racun kimia tersebut, malahan mampu memberi impak negatif terhadap biodiversiti dan alam sekitar. Penggunaan kulat pembunuh serangga, *Metarhizium anisopliae* adalah salah satu alternatif terbaik dalam mengawal populasi serangga perosak ini secara biologi. Sepuluh pencilan kulat *M. anisopliae* telah disaring bagi menentukan kesan patogeniknya terhadap anai-anai. Kajian mendapati pencilan PR1, pencilan yang dipencil dari Pantai Remis, Selangor adalah pencilan yang terbaik. Ia mampu membunuh 96.97% dari populasi anai anai dan menyebabkan masa membunuh median, LT_{50} sebanyak 1.47 hari terhadap anai-anai yang terjangkit. Pencilan ini seterusnya digunakan di dalam kajian berikutnya iaitu kajian histopatologi dan ekspresi protein pada setiap peringkat patogenesis sehingga 144 h pasca-inokulasi. Keputusan menunjukkan ianya memerlukan dua hari untuk membunuh hos terjangkit bermula dengan penembusan tiub germa melalui kutikel pada 6 h pasca-inokulasi. Kecederaan yang disebabkan oleh appressorium kulat untuk menembusi lapisan pertama mekanisme pertahanan, iaitu kutikel serangga itu sendiri adalah merupakan faktor utama yang melemahkan hos terjangkit. Penyebaran kulat dan badan hifa dilihat di peringkat selular membuktikan penaklukan *M. anisopliae* bermula dari peringkat selular. Anai-anai terbalut dengan miselium dan sporulasi oleh kulat ini diperhatikan pada 96 h dan 144 h pasca-inokulasi. Manakala di dalam kajian ekspresi protein, penemuan mendapati empat dan lima protein yang signifikan dari kedua-dua organisma, *C. curvignathus* dan *M. anisopliae* dikenalpasti terlibat di dalam proses jangkitan. Protein yang diekspres oleh anai-anai menunjukkan bukti penglibatan protein di dalam regulasi sel dan tindakbalas imun apabila terjangkit oleh *M. anisopliae*. Selain itu, protein yang diekspres oleh kulat ini mempunyai peranan yang penting didalam pengangkutan protein dan virulensiti kulat ini sendiri ketika proses jangkitan. Berdasarkan interaksi yang dilihat dan dikenalpasti di dalam hubungan perumah-patogen ini, informasi yang didapati mungkin boleh

digunakan di dalam pembangunan racun serangga biologi dengan menggunakan kulat pembunuh serangga ini dengan lebih efektif dalam mengawal populasi anai-anai perosak ini di masa akan datang.

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I certify that a Thesis Examination Committee has met on 14 April 2017 to conduct the final examination of Ahmad Syazwan bin Samsuddin on his thesis entitled "Interaction Between an Entomopathogenic Fungus *Metarhizium anisopliae* and its Host Subterranean Termites *Coptotermes curvignathus* during Infection Process" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis 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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This is to confirm that:

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GLOSSARY OF TERM

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
cm	centimetre
Da	Dalton
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E	East
g	gram
G	Gamma
h	hour
IPG	Immobilized ph Gradient
ITS	Internal Transcribe Region
L	litre
LT_{50}	median lethal time
m	metre
M	molarity
mg	milligram
mg/ml	milligram per mililitre
min	minute
ml	millilitre
ml^{-1}	permillilitre
mm	millimetre
MS	Mass Spectra
MS/MS	Tandem Mass Spectrometry
N	North
ng/ml	nanogram per millimetre
OD	Absorbance
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
pI	Isoelectric point
ppm	Part per million
RH	Relative humidity
RNA	Ribonucleic acid
RPM	Rotation per minute
sec	second
SEM	Scanning electron microscope
TEM	Transmission electron microscope
US$	United States Dollar
UV	Ultra violet
V	Volt
Vh	Volt x hour
2-D	2-dimension
%	Percentage
^{o}C	Degree Celcius
µl	microlitre
µm	micrometre

CHAPTER 1

INTRODUCTION

1.1 *Coptotermes curvignathus* as pest

Coptotermes curvignathus is a species of termites commonly found in infested tree orchards in Malaysia. It is a severe pest of tree plantations such as rubber (Sajap et al., 2009) and oil palm (Bakti, 2004). This termite is widespread in subtropical and tropical countries such as Malaysia and other South East Asia countries (Tho, 1992; Klangkaew et al., 2002) as well as countries like Brazil (Costa-Leonardo et al. 2008) and the United States of America (Lenz et al., 2012). The colony performance of this termite is based on the various classes of caste in the colony, like the typical social insects (Deligne et al., 1981), makes it a serious pest of timber-based products and tree plantations.

Chemical-based pesticide generally used in controlling termite population is considered as an inefficient practice that could cause several implications toward the environment and pest resistances (Hokkanen and Lynch, 1995). As alternative application of a biological control such as *Metarhizium anisopliae* would be a potential approach to overcome these problems. *Metarhizium anisopliae* is an entomopathogenic fungus that does not give a negative effect on other organisms and the environment. Furthermore, it would give a long-lasting effect on the pests (Howarth, 1991). When compared to other entomopathogenic fungi such as *Beauveria bassiana*, *M. anisopliae* appeared to sporulated faster than *B. bassiana* (Sun et al., 2002). This criterion is important because it is the first in fungal pathogenicity to infect the host by accumulating its conidia on the host's body.

1.2 Study of *Metarhizium anisopliae* infection on *Coptotermes curvignathus*

The infection of *M. anisopliae* starts with the penetration of the germ tube through the insect's cuticle after germination and through several tissues before the hyphae colonizes the hemocoel and turns the termite into the moribund state (Sajap and Kaur, 1991). The first level of the insect immune system, which is the skin is made up of cuticle. This is the main weakness of the termites when they encounter entomopathogenic fungi.

Several hydrolytic enzymes such as chitinase and protease have been described as proteins that regulate the pathogenicity of *M. anisopliae* (St. Leger et al., 1992). The regulation of a series of chitinase genes such as *chi*, *chi1*, *chi2* and *chi3* shows the different patterns of expression in infected *Spodoptera litura* and *Helicoverpa armigera*. Different structure and composition of the insect's skin may cause different expression of these hydrolytic enzymes when under attack (Prakash et al., 2012). Therefore, hydrolytic enzymes and other pathogen-related protein expressions may be different in termites when attacked by *M. anisopliae*.

The immunity of individual termite plays a major role to encounter pathogen attack since the possibility of subterranean termites to get infected is high because they forage underground (Chouvenc et al., 2009). Grooming behavior between termites as a social insect when the pathogen infects them is not enough to repel the pathogen due to the slow cannibalism activity by termites in eliminating the infected member in the colony (Yanagawa and Shimizu, 2007).

1.3 Problem statement

Previous studies have shown that the application of *M. anisopliae* is commonly successful in the laboratory but not in the field. This problem arises when because there is lack of understanding in the interaction between the termites and *M. anisopliae* during the pathogenesis process (Chouvenc et al., 2011).

The interaction between *C. curvignathus* and several other *Coptotermes* spp. termites with *M. anisopliae* have been studied before (Hoe et al., 2009; Maketon et al., 2007). However, knowledge on the mechanisms of pathogenesis at different stages of infection at cellular and molecular level are still lacking.

1.4 Objectives of study

This study was to screen for highly pathogenic isolates from ten isolates of *M. anisopliae* for use in pathogenicity study on infected subterranean termites, *C. curvignathus*. The early and late stages of infection were observed using scanning electron microscopy (SEM) technique. Furthermore, the late stage of pathogenesis was observed by using transmission electron microscopy (TEM). The proteins involved during pathogenesis were separated using 2-dimensional gel electrophoresis. Protein gel images were analyzed and spots of the protein of interest were excised for protein expression profile and determination of protein of interest involved respectively, using software and mass spectrometry. Specific objectives were:

a) To identify highly virulent *M. anisopliae* isolates against *C. curvignathus*
b) To monitor the pathogenesis process in *C. curvignathus* using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)
c) To profile and identify proteins expressed during the interaction between *C. curvignathus* and *M. anisopliae* over a time course using a 2-dimensional gel electrophoresis and mass spectrometry, respectively.

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