UNIVERSITI PUTRA MALAYSIA

OIL-BASED NANOEMULSION OF Metarhizium anisopliae (Metschn) SOROKIN TO CONTROL RED PALM WEEVIL Rhynchophorus ferrugineus (Olivier)

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By

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Chairman : Professor Dzolkhifli Omar, PhD
Faculty : Agriculture

Oil based emulsion formulations of *Metarhizium anisopliae* were prepared, characterised and evaluated for their effectiveness against the larvae and adults of *Rhynchophorus ferrugineus*. Infested *R. ferrugineus* adults were collected from Terengganu,and*M. anisopliae* was isolated by cadavers of red palm weevil adults. Four strains were obtained and identified via morphology and molecular technique as *M. anisopliae*. The virulence of these strains was evaluated against the adult and larvae of *R. ferrugineus* by time exposure mortality bioassay. The strain coded as D1 gave the lowest LT_{50} values of 7.2 and 5.2 days at the conidia concentration of 10^6 and 10^7 spores/mL, respectively against the larvae. While against the adult, the D1 strain also gave the lowest LT_{50} values of 6 and 5 days at same conidia concentrations. Oil emulsion formulations of the most virulence isolate conidia of *M. anisopliae* were prepared through ternary phase diagram consisting of 20% (w/w) surfactant, 40% (w/w) oil and 40% (w/w) water containing 10^7spore/mL. The surfactants and oil were first evaluated for their compatibility with conidia by using direct plating. The effect of the surfactants on conidia germination was evaluatedby counting the germination rate of the conidia using a microscope. Agnique PG9116 at 1% concentration gave 87.5 % germination while at 5% surfactant Emereen1604 and EW70 gave 70% conidia germination. In a study of the effect of oils on the conidia germination, glycerin oil gave highest conidia germination rate. Sunflower and glycerin showed less inhibition at 1% concentration with 49.13 and 44.13% growth rate respectively.Palm oil at 5% concentration was the best with 56.88% growth rate. At 10% concentration of oils, soybean and glycerin gave 48.63 and 45.38% growth rate respectively. Eight ternary phase diagram systems were then constructed. The selected systems showed large isotropic regions. They were Agnique PG9116/ glycerin/ water, Emereen1604/ glycerin/ water, Tensiofix 96 DB08/ glycerin/ water, Tensiofix 96 DB10/ glycerin/ water, Tensiofix EW 70/ glycerin/ water, Termul 1284/ glycerin/ water, Tween20/ glycerin/ water and Tween80/ glycerin/ water. Eight oil emulsion formulations were

derived and characterised. All the formulations were stable under centrifuge, storage at 26 ± 1 °C, 60 ± 5 % RH and under high temperature (54± 1 °C) for two weeks. On the particle size, seven formulations were in the range below <100 nm sizes indicating that the formulations were in the category of the nanoemulsion. The zeta potential of the formulations ranged between -7.22 to -39.06 mV, the pH ranged from 4 to 6.34, the surface tension ranged from 32.03 to 41.83 mN/m, and the viscosity ranged from 2.40 to 28.8 mPas. In the study on the toxicity of the oil nanoemulsion formulations of *M. anisopliae* conidia against the larvae, the formulation coded as E1604 gave the LT_{50} of 4.90 days while the conidia water suspension gave LT_{50} of 6 days. On adults, the LT_{50} was 2.20 days while the conidia water suspension was 5 days. Effect of oil nanoformulations on the conidia germination on the cuticle of *R. ferrugineus* was also observed, and after 20 hrs., the E1604 showed 55% germination compared to conidia water suspension of 49.8%. The formulation E1604 showed the longest germ tube of 41.34 µm and full penetration while the conidia water suspension gave 5.28 µm length of a germ tube. The E1604 recorded 100% cumulative mortality after 6 and 4 days on larvae and adults respectively. The oil nanoemulsion of *M. anisopliae* conidia shows good potential for the sustainable control of both adults and larvae of *R. ferrugineus*.

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

NANOEMULSI BERASASKAN MINYAK *Metarhizium anisopliae* (Metschn) SOROKIN UNTUK MENGAWAL KUMBANG PALMA MERAH *Rhynchophorus ferrugineus* (Olivier)

Oleh

ALI ZACHI ABDULQADER

Januari 2018

Pengerusi : Profesor Dzolkhifli Omar, PhD
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Formulasi emulsi berasaskan minyak *Metarhizium anisopliae* disediakan, dicirikan dan dinilai untuk keberkesanannya terhadap larva dan peringkat dewasa *Rhynchophorus ferrugineus*. Peringkat dewasa *R. ferrugineus* dikumpulkan dari Terengganu dan *M. anisopliae* telah diasingkan oleh peringkat dewasa *R. ferrugineus* yang telah mati. Empat strain diperoleh dan dikenal pasti melalui morfologi dan teknik molekul sebagai *M. anisopliae*. Keberkesanan strain ini dinilai terhadap peringkat dewasa dan larva *R. ferrugineus* oleh bioessei mortaliti pendedahan terhadap masa. Strain yang dikodkan sebagai D1 memberikan nilai LT50 terendah sebanyak 7.2 dan 5.2 hari pada kepekatan konidia 106 dan 107 spora / mL masing-masing terhadap larva. Manakala terhadap peringkat dewasa, D1 juga memberikan nilai LT50 paling rendah sebanyak 6 dan 5 hari kepekatan conidia yang sama. Formulasi emulsi minyak yang paling berkesan ialah daripada conidia *M. anisopliae* telah disediakan dengan pembinaan diagram fasa ternari dengan surfaktan 20% (w / w), minyak 40% (w / w) dan air 40% (w / w) yang mengandungi spora 107 / mL. Surfaktan dan minyak mula-mula ditentukan untuk kesesuaian dengan conidia dengan menggunakan secara langsung. Kesan surfaktan pada percambahan konidia ditentukan dengan menggunakan mikroskop untuk menghitung kadar percambahan conidia. Agnique PG9116 pada kepekatan 1% memberikan percambahan 87.5% manakala pada 5% surfactant Emeere 1604 dan EW70 memberikan percambahan conidia 70%. Dalam kajian mengenai kesan minyak pada percambahan konidia, minyak gliserin menunjukkan kadar percambahan konidia tertinggi. Lapan sistem diagram fasa ternari kemudian dibina dan sistem yang dipilih menunjukkan kawasan isotropik yang besar. Ia adalah Agnique PG9116 / gliserin / air, Emereen 1604 / gliserin / air, Tensiofix 96 DB08 / gliserin / air, Tensiofix 96 DB10 / gliserin / air, Tensiofix EW 70 / gliserin / / air dan Tween80 / gliserin / air. Lapan formulasi emulsi minyak diperoleh dan dicirikan. Semua formulasi adalah stabil di bawah emparan, penyimpanan pada 26 ±

1 ° C, 60 ± 5% RH dan di bawah suhu tinggi (54 ± 1 ° C) selama dua minggu. Pada saiz zarah, tujuh formulasi adalah dalam julat di bawah saiz <100 nm yang menunjukkan bahawa formulasi berada dalam kategori nanoemulsi. Potensi zeta dari formulasi antara - 7.22 hingga -39.06 mV, pH antara 4 hingga 6.34, ketegangan permukaan antara 32.03 hingga 41.83 mN / m dan kelikatannya antara 2.40 hingga 28.8 mPas. Dalam kajian mengenai ketoksikan formula nanoemulsion minyak *M. anisopliae* conidia terhadap larva, rumusan itu dikodkan sebagai E1604 memberikan LT50 sebanyak 4.90 hari manakala suspensi air conidia memberikan LT50 dari 6 hari. Pada peringkat dewasa, LT50 adalah 2.20 hari manakala suspensi air conidia adalah 5 hari. Kesan formula nano minyak pada percambahan konidia pada kutikel *R. ferrugineus* juga diperhatikan dan selepas 20 jam, E1604 menunjukkan percambahan 55% berbanding dengan penggantungan air konidia sebanyak 49.8%. Perumusan E1604 menunjukkan tiub germanium paling panjang 41.34 μm dan penembusan penuh manakala penggantungan air konidia memberikan panjang 5.28 μm. E1604 mencatatkan kematian kumulatif 100% selepas 6 dan 4 hari pada larva dan dewasa. Emulsi nano minyak *M. anisopliae* conidia menunjukkan potensi yang baik untuk kawalan mampan kedua-dua peringkat dewasa dan larva *R. ferrugineus*.

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I certify that a Thesis Examination Committee has met on 17 January 2018 to conduct the final examination of Ali Zachi Abdulqader on his thesis entitled "Oil-Based Nanoemulsion of *Metarhizium anisopliae* (Metschn) Sorokin to Control Red Palm Weevil *Rhynchophorus ferrugineus* (Olivier)" 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 Doctor of Philosophy.

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

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ITS	Internal transcribed spacer
L	liter
G	gram (s)
Mg	milligram(s)
mL	milliliter (s)
cm	centimeter(s)
mm	millimeter (s)
h	hour(s)
%	percent
°	degree
°C	degree(s) Celsius
CRD	Complete Randomized Design
DAT	Day after treatment
S.E	Standard Error
Sec.	second
UPM	Universiti Putra Malaysia
a.i	active ingredient
w/w	weight over weight
rpm	revolutions per minute
no.	number
&	and
i.e	*exempli gratia,* for example
et al.,	*et alii,* and others
RPW	Red Palm Weevil
Dtx	destruxin
♀	male
♂	female
µL	microliter
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
v/v	volume to volume
LT_{50}	median lethal time
mN/m	milli newton per meter
Mv	millivolt
SEM	scanning electron microscope
UV	ultraviolet
EPF	entomopathogenic fungi
ULV	ultra low volume

CHAPTER 1

INTRODUCTION

The red palm weevil *R. ferrugineus* (Coleoptera: Rhynchophoridae)is a phytophagous insect which feeds explicitly on palm trees. It is a global concern due to its harmful feeding habits within palm trees and further threats to the ornamental and other date palm species. RPW is a severe pest of coconut palm *Cocos nucifera* and was reported from 15% of the coconut growing countries worldwide (Faleiro, 2006).The RPW rapidly spread to date palm-growing countries during the past two decades In Malaysia; studies show red palm weevil as a pest of palm trees especially the economically important coconut, *C. nucifera*, and the sago, *Metroxy lonsagu*(Idris *et al.*, 2014).Plantation of palm oil plays a significant role in the economy of the country as country's GDP relies on it with a maximum percentage. Therefore, red palm weevil is a high threat toitsdirect effects on people's livelihood in many countries such as Malaysia.

An imperative role of chemical pesticides has been recorded in crop protection programs to serve humankind significantly. An estimated quantity of about 2.5 million tons of pesticideshas been used each year at the cost of $20 billion worldwide. However, the unselective use of these pesticides has resulted in resistance, resurgence and outbreaks of new insect pest species. Furthermore, ground-breaking public exposure of the risk to the environment including benefits organism and public health posed by the frequent use of these chemical threatening the sustainability of ecosystems (Chagnon *et al.*, 2015; Aktar*et al.*,2009). There has been a continuous on going endeavour to reduce harmful effects of these pesticides, either by the development of more targeted compounds that exhibit less side effects, abandon of highly hazardous chemicals or by the development of non-chemical methods of pest management (Gilland Garg 2014).

The functional utilisation of biological agents as pesticides have efficiently delivered thesatisfactorily results in reducing the incidences of insect pests, weeds and diseases for many years. There isa number of entomopathogenic agents that have been documented(Gindin *et al.*, 2006).*M. anisopliae* is one of the worthiest examples in this regard that has been considered as an essential biological agent in reducing the pest population of different insect orders (Zimmermann,2007).

The fungus was initially isolated from Coleopteran insects (*Anisopliae austriaca*)in 1878 by Leland (2001). It possesses ahigh rate of germination and responsible for theproduction of various enzymes that may result in insect toxins (Schrank and Vainstein, 2010) but not infectious or toxic to mammals. Although, entomopathogenic plays a vitalpart in pest management,butthey are still facing many challenges with regard toimproving the efficiency of these insect-microbial pesticides formulation, shelf life and compatible with theenvironment. Therefore, improving the conidial

formulation of *M. anisopliae* is anindispensable step to optimiseits biological control strategy.

It has been well documented that the oil in water emulsion enhances the efficacy of entomopathogens against various insects in controlled and uncontrolled conditions (Polar *et al.*,2005).The oil formulation of theCitoweetoil enhanced the fungal virulence on *Eurygaster integriceps* (Sedighi*et al.*, 2013).Also, the oil formulations are useful in strengtheningthe transfer of conidia to the areas of thinner cuticle (Ibrahim *et al.*, 1999). Similarly, these bio fungicides ensure the greater ability of adherence to the host body and the combination of oil and conidia further assists in protecting the fungus against fast dehydration in low-humidity environments (Bateman *et al.*, 1993), high temperature (McClatchie *et al.*, 1994) and ultraviolet radiation (Moore *et al.*,1996; Alves*et al.*, 1998; Bateman and Alves, 2002).The preference thus has been given to the use oil based formulations fungi that havebeen shown to beeffectivein controllingvarious arthropods in laboratory and field conditions (Bateman *et al.*, 1993; Batta, 2003).

In arecent decade, red palm weevil (RPW) *R. ferrugineus*has becomea noxious pest of coconut and date palm trees in most Asian countries. An introduction of *M.anisopliae* has displayed a great ability to control this pest and was found to be the main pathogen against it through using proper formulation and application method (Francardi *et al.*, 2016).It is also suggested that the fungi could spread infecting healthy insects of *R. ferrugineus* by horizontal transmission as the insect is highly promiscuous and live in aggregation (Francardi *et al.*, 2013 and Llácer *et al.*,2013). The oil-based formulation of *Beauveria bassiana*has reported thelittle effect on RPW(Abdel-Samad *et al.*,2011) in comparison with *M. anisopliae* that shows the highest efficacy against RPW larvae and adults (Francardi *et al.*, 2012 and 2013).The oil-based formulations of *M. anisopliae* weremore effective than aqueous suspensions against eggs, larvae and engorged females of *Rhipicephalus microplus* (Camargo *et al.*, 2012).

Themycopesticide containing *M. anisopliae*has been developed worldwide to control numerous insect pests including *R. ferrugineus*. However, there is lack of information of the *M. anisopliae* formulated as oil emulsion for the control of RPW(Francardi *et al.*, 2016).Therefore, this study was conducted to expand the knowledge on a specific entomopathogenic fungus *M. anisopliae* and to examine a novel approach in preparingoil nanoemulsions formulations as a microbial biopesticide with following objectives.

1) Toisolate and identify of *M. anisopliae*.
2) Prepare and characterizethe oil based emulsion formulation of *M. anisopliae.*
3) To evaluate the biological effectiveness of oil based formulation against RPW.
4) To investigate the effect of the oil emulsion formulation on the penetration of germ tube through RPW cuticle.

It is expected that this study will contribute towards an improved understanding of the fundamental aspects of oil based nano-emulsion of biopesticide. Accordingly, the information obtained can be further exploited for their proper application that will contribute towards the proper pest management of palm oil in Malaysia and other palm oil producing countries.

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