



UNIVERSITI PUTRA MALAYSIA

***BIOLOGICAL CONTROL OF FUSARIUM WILT OF ROCK MELON
USING EFFECTIVE MICROBES***

SALHA IBNOUF ELMAHDI AHMED

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By

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirement for Degree of the Doctor of Philosophy**

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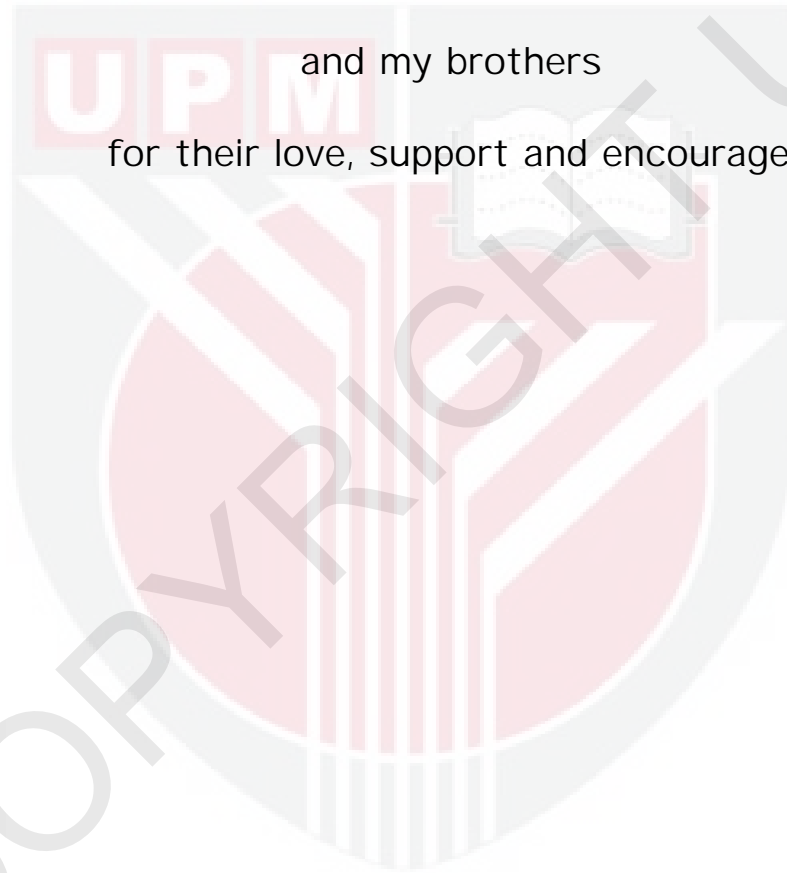
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DEDICATION

Special dedicate to:

My loving late father, Ibnouf Elmahdi
late mother, Amna Said, late brother Omer,
my affectionate sisters, Amal and Najat
and my brothers
for their love, support and encouragement.



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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

BIOLOGICAL CONTROL OF FUSARIUM WILT OF ROCK MELON USING EFFECTIVE MICROBES

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August 2015

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The use of effective microbes obtained from the rhizosphere to suppress soil borne plant pathogens has received greater attention in recent decades as an alternative to chemical fungicides. This study was conducted to explore the effects of effective microbes (EMs) from the rhizosphere of rock melon as bio- control agents (BCAs) against Fusarium wilt caused by *Fusarium oxysporum* f. sp. *melonis* (Fom). Two EMs namely MKB04 and KB10 were screened from 72 effective bacteria isolated from the rhizosphere soil of rock melon. The isolate MKB04 was identified based on the Biolog system as *Bacillus amyloliquefaciens*; though the isolate MKB10 was unsuccessfully identified using biolog system due probably to the limitation of the methods used. However, 16S rDNA sequencing for MKB04 and MKB10 isolates was confirmed at 100% of sequence similarity to *B. amyloliquefaciens* and *Alcaligenes faecalis*, respectively compared with related bacteria in the GeneBank. The two isolates were proved to be effectious towards Fom in *in vitro* biocontrol assay and showed different mechanisms of action, as they produced antibiotic substances which prevent the fungal growth up to 92.05 and 93.18% for MKB04 and MKB10 respectively compared with control and spores germination by 100%. Antibiotic substances were produced in the form of volatile as well as non volatile metabolites. Pyrrolopyrazine alkaloid compound was detected on GC/MS for both isolates which displayed significant biological activities against tested fungal pathogens. Furthermore, the two isolates produced hydrolytic enzymes that degrade the fungal cell components; and responded positively *invitro* for siderophore and HCN, indole acetic acid (IAA) production, and phosphate solubilisation. *B. amyloliquefaciens* and *Alcaligenes faecalis* bioformulations act as elicitors in the production of inducible compound associated with induced resistance (Peroxidase (PO), polyphenoloxidase (PPO), total phenol and lignin content); that subsequently enhance tolerance of the rock melon to Fusarium wilt based on parameters such as delay the symptoms onset, reduce disease incidence by (25, 33.33 and 33.33%), disease severity (18.83, 20.71 and 22.45%) for *B. amyloliquefaciens* and *Alcaligenensis faecalis* as single or in combination respectively; and epidemic rate at 0.007 units day⁻¹. Histological observations revealed that *B. amyloliquefaciens* and *Alcaligenensis faecalis* were able to colonise and produce massive deposition of new structures and products in the tissues of the rock melon, which were used as a defense mechanism against infection by Fom. Furthermore, the two bioformulations enhanced the

vegetative growth as observed by the increased chlorophyll content, dry weight of the root and shoot, shoot height and root length. Fruit fresh weight, firmness, total soluble solid, titratable acidity and ascorbic acid obtained in this study are within the range of quality standard and customer acceptance. Under storage conditions 4 and 25 C, the bioformulations performed retaining their viability over a longer period. *In vitro* and *in vivo* activities of the bioformulations of *B. amyloliquefaciens* and *Alcaligenes faecalis* as single or in combination against Fom of rock melon suggested that the bacterium has the potential to be a promising eco-friendly bio-control agent for *F. oxysporum* f. sp. *melonis* as well as plant growth promoters.



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

KAWALAN BIOLOGI LAYU FUSARIUM PADA TEMBIKAI WANGI MENGUNAKAN MIKROB EFEKTIF

Oleh

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Penggunaan mikrob efektif diperolehi daripada rizosfera untuk menyekat patogen tumbuhan telah menerima perhatian dalam beberapa dekad kebelakangan ini sebagai alternatif kepada racun kimiakulat. Kajian ini dijalankan untuk meneroka kesan mikrob efektif (EMS) dari rizosfera tembikai wangi sebagai agen biokontrol (BCAs) terhadap layu Fusarium disebabkan oleh *Fusarium oxysporum* f. sp. *melonis* (Fom). Dua EMS iaitu MKB04 dan KB10 telah disaring daripada 72 pencilan bakteria efektif yang berasal daripada rizosfera tanah padat tembikai wangi. Pencilan MKB04 telah dikenal pasti berdasarkan sistem Biolog sebagai *Bacillus amyloliquefaciens*; manakala pencilan MKB10 tidak berjaya dikenal pasti menggunakan sistem biolog berkemungkinan disebabkan had kaedah yang digunakan. Walaubagaimanapun, penjujukan 16S rDNA untuk pencilan MKB04 dan MKB10 telah disahkan masing-masing pada 100% urutan persamaan kepada *B. amyloliquefaciens* dan *Alcaligenes faecalis*, dibandingkan dengan bakteria berkaitan dalam GeneBank. Dua pencilan terbukti berkesan mereneat Fom di dalam esei *in vitro* dimana kedua pencilan ini menghasilkan bahan antibiotik yang menghalang pertumbuhan kulat masing-masing sehingga 92.05 dan 93.18% untuk MKB04 dan MKB10 berbanding dengan kontrol dan percambahan spora pada 100%. Bahan antibiotik telah dihasilkan dalam bentuk meruap dan juga metabolit tidak meruap. Sebatian alkaloid pyrrolopyrazine telah dikesan pada GC/MS untuk kedua-dua pencilan yang mempamerkan aktiviti biologi ketara ke atas patogen kulat yang diuji. Tambahan pula, kedua-dua pencilantelah menghasilkan enzim hydrolitik yang menghancurkan komponen sel kulat; dan bertindak balas positif di dalam *in vitro* untuk siderophore dan HCN, penghasilan indol asid asetik (IAA), dan solubilisasi fosfat. Bioformulasi *B. Amyloliquefaciens* dan *Alcaligenes faecalis* bertindak sebagai elisitor dalam penghasilan kompaun mampu diaruhkan yang dikaitkan dengan rintangan aruhan (peroksidase (PO), polyphenoloxidase (PPO), jumlah fenol dan kandungan lignin); yang seterusnya meningkatkan toleransi tembikai wangi kepadalayu Fusarium berdasarkan parameter seperti melambatkan simptom awal, mengurangkan insiden penyakit masing-masing pada (25, 33.33 dan 33.33%), keparahan penyakit (18.83, 20.71 dan 22.45%) untuk *B. amyloliquefaciens*, *Alcaligenes faecalis* dan kombinasi masing-masing; dan kadar wabak pada 0.007 unit hari⁻¹. Pemerhatian histologi mendedahkan bahawa *B. amyloliquefaciens* dan *Alcaligenes faecalis* mampu menghasilkan struktur pembedapan dan produk baru dalam tisu tembikai wangi,

yang mana telah digunakan sebagai mekanisme pertahanan terhadap jangkitan oleh Fom. Tambahan pula, kedua-dua bioformulasi meningkatkan pertumbuhan vegetatif seperti yang diperhatikan dengan peningkatan kandungan klorofil, berat kering akar dan pucuk, ketinggian pucuk dan panjang akar. Berat buah segar, keanjalan, jumlah pepejal larut, keasidan tertitrat dan asid askorbik yang diperolehi dalam kajian ini adalah dalam lingkungan piawai mutu dan penerimaan pelanggan. Di bawah keadaan penyimpanan 4 dan 25 C, bioformulasi menunjukkan pengekalan viabiliti mereka dalam tempoh yang lebih lama. Aktiviti *in vitro* dan *in vivo* daripada bioformulasi *B. amyloliquefaciens* dan *Alcaligenes faecalis* secara individual atau kombinasi ke atas Fom daripada tembikai wangi menunjukkan bahawa bakteria ini berpotensi untuk menjadi agen bio-kontrol mesra alam ke atas *F. oxysporum* f. sp. *melonis* serta penggalak pertumbuhan tanaman.



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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy.

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LIST OF ABBREVIATIONS

%	Percent
μ	Micro
AIA	Actinomycetes Isolation Agar
ANOVA	Analysis of Variance
AUDPC	Area Under Disease Progress Curve
BCA	Biological Control Agent
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cfu	Colony forming units
cm	Centimeter
CRD	Completely Randomized Design
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside Triphosphates
DI	Disease Incidence
DR	Disease Reduction
DS	Disease Severity
DMRT	M
EMs	Effective Microbes
FAA	Formalin Acetic Acid
FAO	Food and Agriculture Organization
Fom	<i>Fusarium oxysporum</i> f.sp. <i>melonis</i>
IR	Induced Resistance
g	Gram
GN	Gram negative
GP	Gram positive
hr	hours
K	Potassium
kb	Kilo base pair
L	Liter
LCB	Lacto phenol Cotton Blue
LM	Light Microscope
LTGA	Lignolithoglycolic Acid
M	Monit
m	Meter
mg	Milligram
min	Minute
MKB	Makmal Kawalan Biologi
ml	milliliter
mm	Millimeter
mM	Millimolar
MPB	Malaysian Pepper Board
N	Nitrogen
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
nm	Nanometer
OD	Optical Density

P	Phosphorus
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PIRG	Percentage Inhibition of Radial Growth
PO	Peroxidase
PPO	Polyphenoloxidase
PVP	Polyphenyl Pyrrolidone
rpm	Revolutions per minute
s	Seconds
SDW	Sterile Distil Water
SEM	Scanning Electron Microscope
TE	Tris-EDTA
U	Unit
UV	Ultraviolet
V	Volts
V/V	volume / volume
vol	Volume
wt	Weight

CHAPTER 1

INTRODUCTION

1.1 General

Melon (*Cucumis melo* L.) is among the most important cultivated cucurbits. It is mainly cultivated for its fruit, which has a typically sweet pulp and pleasantly fragrant flavour (Villanueva *et al.*, 2004). Melon is important also for its richness in vitamin A and C content (Wehner and Maynar, 2003). The global production of melon has doubled within the last two decades to 26 million tons in 2007 (FAOSTAT 2007). In Malaysia, rock melon is commonly cultivated under the rain-sheltered and open planting structure using the drip fertigation method. It is a popular fruit among locals compared to cereals or vegetable crops and Glamour is the most favourite choice among the commercial growers (UK assay). Rockmelon is an economically important crop that has become an export commodity (Alang *et al.*, 1990; Cantliffe *et al.*, 2001; Shaw *et al.*, 2000) for South East Asia, Central China and East Africa (Norlia, 1986; Jelaska, 1986; Dong *et al.*, 1990). Muskmelons exports in term of revenue (Abbas, 2004; AOAD 2008).

Rock melon is vulnerable to Fusarium wilt that is caused by (*Fusarium oxysporum* f. sp. *melonis* abbreviated as Fom) a seed and soil born fungus that is specific to melon. Fusarium wilt is a destructive vascular disease that leads to substantial economic losses especially when crops are planted in the same field without rotation (Martyn and Amador, 1987; Champaco *et al.*, 1993; Soriano-Martin *et al.*, 2006)). As the natural conditions in Malaysia and Sudan favour fungal growth, Fusarium wilt has been reported to cause severe destruction of commercial melon crops in Sudan, which can account for more than 40% loss (Mohamed *et al.*, 1994, 1995; AOAD, 2008).

Many management strategies have been tried to control Fusarium wilt of rock melon, but none has been able to generate acceptable results. However, resistant cultivars are promising and have been the primary choice of researchers and growers but time and cost factors limit its feasibility which is uncertain. Fungicides have been proposed to contain this pathogen but they are not perceived as the ideal long-term solution for the problem; this is because resistance development in pathogen may overwhelm the effect of these synthetic fungicides over time (McGrath, 2001 and Fernández *et al.*, 2006). On the other hand, the soil-borne pathogens are very durable and survive for several years as chlamydospores in soil or as mycelium in the plant vascular system which makes it urgent for alternative control strategies to be identified.

Recently biological control of Fusarium wilt has become a popular alternative for disease management fuelled by public and environmental concerns as chemicals and pesticides have been banned (Alabouvette, 1993; Conway *et al.*, 2004). There have

been many reports of the successful use of biological control of *Fusarium* which may eventually play a crucial role in improving overall crop productivity.

The idea of using effective microbes (EM) in Malaysia and Sudan is enticing, especially as the natural condition favour microbial growth; and due to the fact that pesticides technology resulted in a number of health and environmental hazards and socioeconomic problems. In addition to the increase in market demand for non-chemically treated rock melon plants; that free from pathogens as well as chemical residues and any other contaminants impairing its superior fruit quality, high yield as export cash crop. Moreover, no systemic and extensive works on *Fusarium* wilt of rockmelon plant have been done in Malaysia; also there is a lake of information on the use of EM in controlling *Fusarium* wilt of rock melon plant and as bio fertilizer as well (Zulkarami *et al.*, 2010). However, as to our knowledge no attempts in Sudan have been made for the management of *Fusarium* wilt by using the EM.

Therefore, this study was undertaken to establish the effect of effective microbe as BCAs of *Fusarium* wilt and the mechanism of action of the disease suppression directly through antagonism (antibiotics or inhibitory compounds production) towards *Fom*, and induces of plant resistance as well, or either indirectly through increased or promotion of plant growth yield and fruit quality.

1.2 Objectives of the Study

The main objective of this study was focused on the biological control of *Fusarium* wilt pathogens on rock melon and this can be achieved through the following specific objectives:-

1. Isolation and Identification of promising effective microbes involved in biocontrol of *Fusarium oxysporum f. sp melonis*;
2. Studying the mechanisms of action of disease control by antagonistic microbiota;
3. Investigating the impact of bioformulations of effective microbes against *Fusarium oxysporum f.sp.melonis* on rock melon under glasshouse condition.

1.3 Hypothesis

Hypothesis of this study are:

- < *Fusarium oxysporum f. sp. melonis* thecausal agent of *Fusarium* wilt of melon can be controlled by using effective microbiota.
- < Effective microbial communities in the rhizosphere of melon can be manipulated by mixing them with non- treated growth medium for improving the plant growth and bio control of the disease.

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