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BIOLOGICAL CONTROL OF FUSARIUM WILT OF ROCK MELON USING EFFECTIVE MICROBES

SALHA IBNOUF ELMAHDI AHMED

FP 2015 43
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By

SALHA IBNOUF ELMAHDI AHMED

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

August 2015
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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.
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

By

SALHA IBBNOUF ELMAHDI AHMED

August 2015

Chairperson: Associate Professor Jugah Kadir, PhD
Faculty: Agriculture

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 acet ic 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 Alcaligenes faecalis as single or in combination respectively; and epidemic rate at 0.007 units day⁻¹. Histological observations revealed that B. amyloliquefaciens and Alcaligenes 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.
Penggunaan mikrob efektif diperolehi daripada rizosfera untuk menyerang patogen tumbuhan telah menerima perhatian dalam beberapa dekad kebelakangan ini sebagai alternatif kepada racun kimia dalam. Kajian ini dijalankan untuk mengetahui kesan pemakaian mikrob efektif (EMS) daripada 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 padatembikai wangi. Pencilan MKB04 telah dikenali pasti berdasarkan sistem Biolog sebagai *Bacillus amyloliquefaciens*; manakala pencilan MKB10 tidak berjaya dikenali pasti menggunakan sistem biolog berkemungkinan disebabkan kedalahan yang digunakan. Walau bagaimanapun, penjgujan 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 GenBank. Dua pencilan terbukti berkesan merendahkan Fom di dalam eseii *in vitro* dimana kedua pencilan ini menghasilkan bahan antibiotik yang menghambat pertumbuhan kulat masing-masing sehingga 92.05 dan 93.18% untuk MKB04 dan MKB10 berbanding dengan kontroldan percambahan spora pada 100%. Bahan antibiotik telah dihasilkan dalam bentuk merup dan juga metabolit tidak merup. 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 kompound mampu dianuikan yang diikatkan 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 pemendapan 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 Alcaligensis 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.
ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to all who made it possible for me to accomplish this work. First and foremost my grateful to the Almighty ALLAH the most merciful who gave me the strength and patience to complete this study.

Really, I am greatly indebted to my supervisor Prof. Dr. Jugah Kadir for his keen supervision, unlimited support, advice, constructive criticisms, guidance and fruitful discussion. Moreover, I would like to acknowledge his understanding and working group for the friendly working atmosphere. Special gratitude extends to my supervisory committee members, Prof. Dr. Mahmud Tengku Muda Mohamed for his close supervision, valuable advices for rendering all possible guidance in carrying out the research work, also great thanks, to Associate Professor Dr. Ganesan Vadamalai, I sincerely thank Prof. Dr. Sariah Meon my former supervisory committee member for her support, and guidance.

Thanks are also extended to all the staff members in the Biocontrol, Plant Pathology, Microbiology Laboratories; Mr. Nazri, Zawawi, Johery, Ms Asmalina, Junina and Mr. Suhimi from Botany laboratory for their kind and technical assistance.

Virtually I am greatly indebted to my friends, colleagues at Bahri University- Sudan; for their valuable assistance, continual encouragement and moral support. Special thanks, to my friends and colleagues (local and international) in Faculty of Agriculture UPM, particularly in biocontrol laboratory for their kindness cooperation and help throughout my study. I feel especially thankful to my Sudanese friends in Malaysia for their continual encouragement, valuable advices, and moral support.

Appreciably thanks also is extended to the key of success of this study, to my beloved sisters, brothers for their pray, blessings, endless love, sacrifice and encouragement during the study period.
I certify that a Thesis Examination Committee has met on 6 August 2015 to conduct the final examination of Salha Ibnoof Elmahdi Ahmed on her thesis entitled "Biological Control of Fusarium Wilt of Rock Melon Using Effective Microbes" 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.

Members of the Thesis Examination Committee were as follows:

Lau Wei Hong, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Kamaruzaman bin Sijam, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Phebe Ding, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Robert Chester Kemermait, Jr., PhD
Professor
University of Georgia
United States
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 September 2015
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. The members of the Supervisory Committee were as follows:

**Jugah Kadir, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Mahmud Tengku Muda Mohamed, PhD**  
Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

**Ganesan Vadamanai, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

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Name of Chairman of Supervisory Committee: Jugah Kadir, PhD

Name of Member of Supervisory Committee: Ganesan Vadmalai, PhD

Name of Member of Supervisory Committee: Mahmud Tengku Muda Mohamed, PhD

Signature: __________________________

Signature: __________________________
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5.4: Granular bio formulation: MKB04 (B. amyloliquefaciens) and MKB10 (Alcaligenes faecalis)
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>AIA</td>
<td>Actinomycetes Isolation Agar</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AUDPC</td>
<td>Area Under Disease Progress Curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Biological Control Agent</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CRD</td>
<td>Completely Randomized Design</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleoside Triphosphates</td>
</tr>
<tr>
<td>DI</td>
<td>Disease Incidence</td>
</tr>
<tr>
<td>DR</td>
<td>Disease Reduction</td>
</tr>
<tr>
<td>DS</td>
<td>Disease Severity</td>
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<tr>
<td>DMRT</td>
<td>Duncan’s Multiple Range Test</td>
</tr>
<tr>
<td>EMs</td>
<td>Effective Microbes</td>
</tr>
<tr>
<td>FAA</td>
<td>Formalin Acetic Acid</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>Fom</td>
<td><em>Fusarium oxysporum</em> f.sp.<em>melonis</em></td>
</tr>
<tr>
<td>IR</td>
<td>Induced Resistance</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GN</td>
<td>Gram negative</td>
</tr>
<tr>
<td>GP</td>
<td>Gram positive</td>
</tr>
<tr>
<td>hr</td>
<td>hours</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LCB</td>
<td>Lacto phenol Cotton Blue</td>
</tr>
<tr>
<td>LM</td>
<td>Light Microscope</td>
</tr>
<tr>
<td>LTGA</td>
<td>Lignolithoglycolic Acid</td>
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<tr>
<td>M</td>
<td>Monit</td>
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<tr>
<td>m</td>
<td>Meter</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MKB</td>
<td>Makmal Kawalan Biologi</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MPB</td>
<td>Malaysian Pepper Board</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient Agar</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient Broth</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>P</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato Dextrose Agar</td>
</tr>
<tr>
<td>PDB</td>
<td>Potato Dextrose Broth</td>
</tr>
<tr>
<td>PIRG</td>
<td>Percentage Inhibition of Radial Growth</td>
</tr>
<tr>
<td>PO</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PPO</td>
<td>Polyphenoloxidase</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyphenyl Pyrrolidone</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile Distil Water</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>V/V</td>
<td>volume / volume</td>
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<tr>
<td>vol</td>
<td>Volume</td>
</tr>
<tr>
<td>wt</td>
<td>Weight</td>
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</table>

The table lists common abbreviations and their full forms, such as 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 variety (known locally as ‘Golden Langkawi’) with the striking golden yellow colour 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 rank first in exported vegetables and second to mango in Sudan’s total horticultural 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 difficult to control partly due to the pathogen’s ability to 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* the causal 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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