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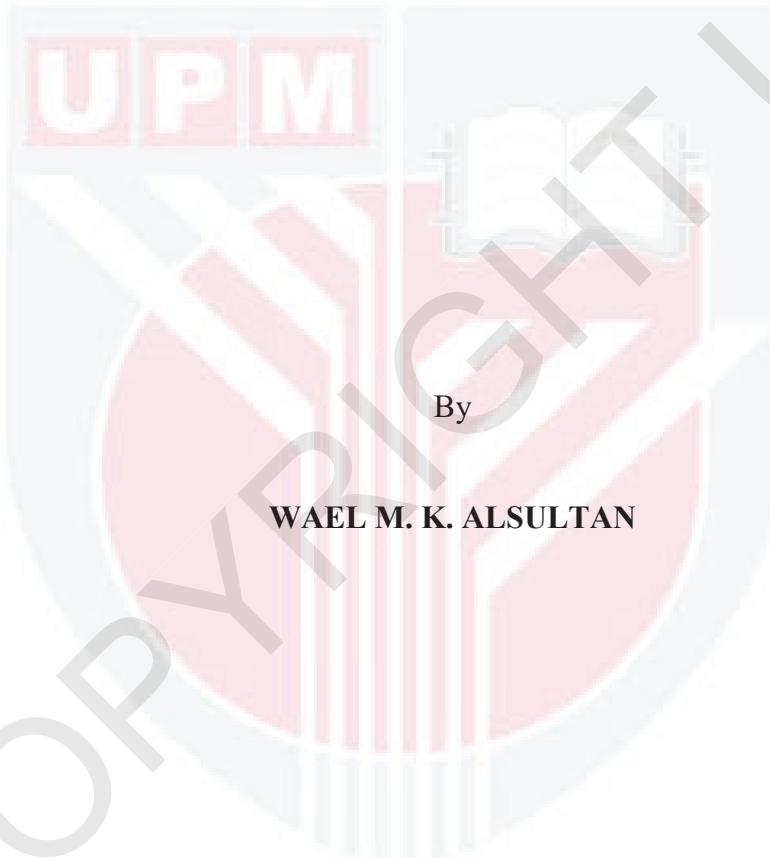
CHARACTERIZATION AND GENETIC DIVERSITY OF
Phytophthora palmivora FROM COCOA IN MALAYSIA AND ITS
BIOLOGICAL CONTROL USING ENDOPHYTIC BACTERIA

WAEEL M. K. ALSULTAN

FP 2019 56



**CHARACTERIZATION AND GENETIC DIVERSITY OF
Phytophthora palmivora FROM COCOA IN MALAYSIA AND ITS
BIOLOGICAL CONTROL USING ENDOPHYTIC BACTERIA**



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

April 2019

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DEDICATION

This Thesis is dedicated to

The most precious people in my life; my Beloved Parents

Mahmoud and Fatimah

My Beloved Brothers

My Lovely sisters

For their unconditional everlasting love

They began my education

They motivated me to continue it

They will always contribute to it



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

**CHARACTERIZATION AND GENETIC DIVERSITY OF
Phytophthora palmivora FROM COCOA IN MALAYSIA AND ITS
BIOLOGICAL CONTROL USING ENDOPHYTIC BACTERIA**

By

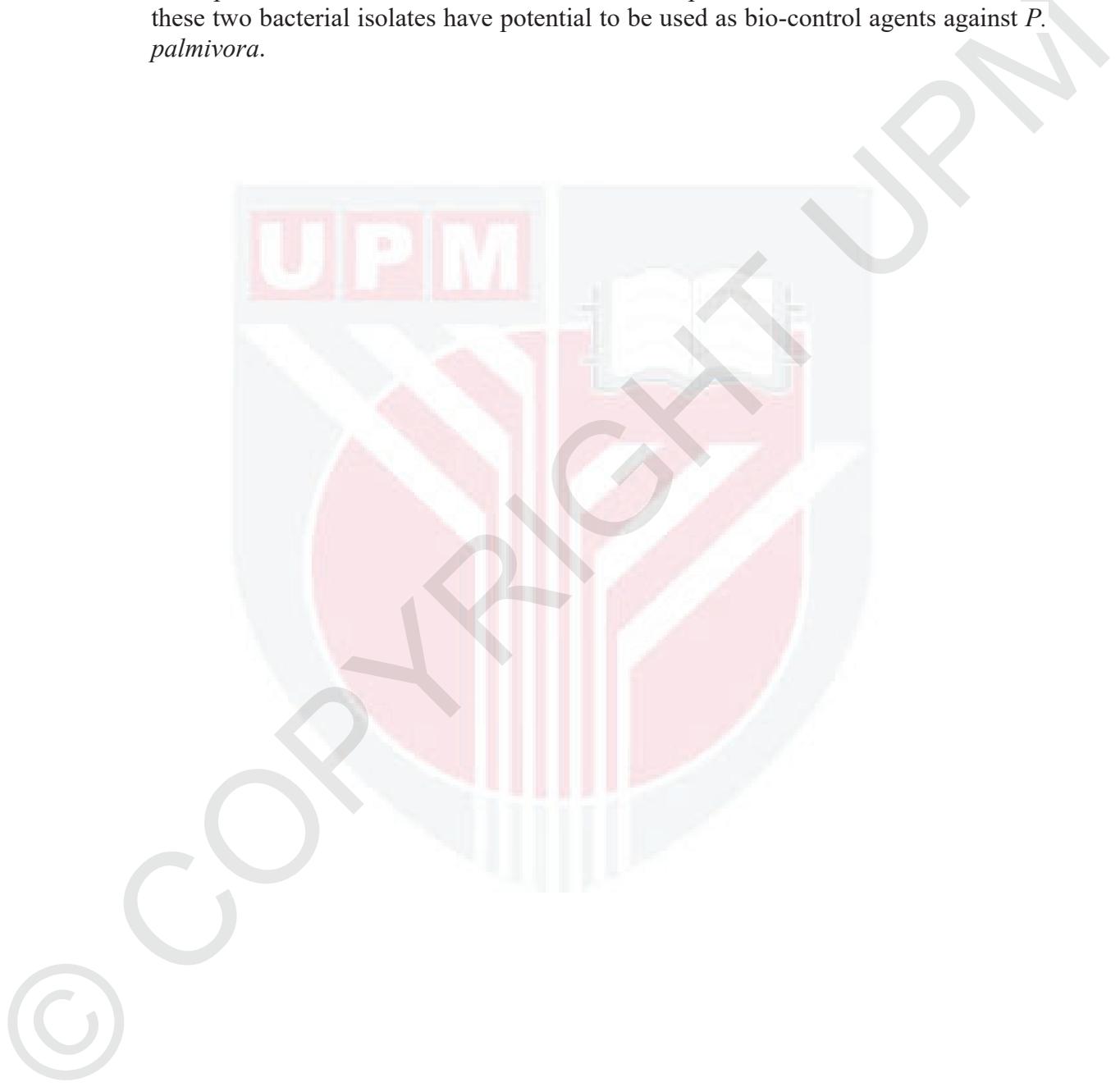
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April 2019

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Black pod disease of cocoa, caused by *Phytophthora palmivora*, is a serious problem to cocoa growers in Malaysia and worldwide. There are a few research efforts have been conducted to study the causal agent of black pod disease of cocoa in Malaysia. At present, there are no reports available on the determination of genetic diversity among *P. palmivora* populations in cocoa growing areas of Malaysia. Therefore, this study was initiated to isolate and identify *Phytophthora* species from main cocoa plantations infected by black pod disease in Malaysia using sequence analyses of the ITS rDNA, cytochrome c oxidase subunit 1, and translation elongation factor 1 α regions. This study was also examined the genetic diversity within 56 *P. palmivora* isolates obtained from the different host, geographical regions and years in Malaysia using Inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. In addition, this study was carried out to isolate, characterize and screen bacterial endophytes from cocoa plants for their biological control activities. Their mechanisms of action as well as the ability to reduce black pod rot disease were also investigated. A total of 36 *Phytophthora* isolates were obtained from different infected cocoa plantations from five states of Malaysia. Six *Phytophthora* isolates obtained from durian and available at the Department of Plant Protection, Universiti Putra Malaysia were also used in this study. Results of phylogenetic analyses of individual and combined dataset of the ITS rDNA, COX I and EF-1 α confirmed that all *Phytophthora* isolates belonged to *P. palmivora*. The results also confirmed that the three examined regions could differentiate *P. palmivora* at the species level. *P. palmivora* isolates obtained from cocoa and durian were clustered into different subclades based on the three regions examined. The results of both markers indicated relatively high diversity among *P. palmivora* isolates. The complete separation was based on host and year of isolation. In total, 100 endophytic bacterial isolates were obtained from healthy cocoa tissues (leaves, branches and fruits) from seven states of Malaysia. The isolated bacteria were screened by dual culture assay and culture filtrate

test for their antagonistic properties towards *P. palmivora* *in vitro*. The best two isolates *Pseudomonas aeruginosa* (AS1) and *Chryseobacterium proteolyticum* (AS2) with more than 80% inhibition of radial growth (PIRG) were selected for subsequent experiments. Bioactive volatile compounds from ethyl acetate crude extract were identified using gas chromatography-mass spectrometry (GC). The effectiveness of *Pseudomonas aeruginosa* and *Chryseobacterium proteolyticum* isolates in reducing black pod lesion was confirmed on detached cocoa pods. These results confirmed that these two bacterial isolates have potential to be used as bio-control agents against *P. palmivora*.



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

**PENCIRIAN DAN KEPELBAGAIAN GENETIK *Phytophthora palmivora*
DARIPADA KOKO DI MALAYSIA DAN KAWALAN BIOLOGINYA
MENGGUNAKAN ENDOFITIK BAKTERIA**

Oleh

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Penyakit buah hitam koko yang disebabkan oleh *Phytophthora palmivora* adalah masalah serius bagi penanam koko di Malaysia dan seluruh dunia. Terdapat beberapa kajian telah dijalankan untuk mengetahui agen penyebab penyakit buah hitam koko di Malaysia. Buat masa sekarang tidak terdapat sebarang laporan untuk menentukan kepelbagaian genetik antara populasi *Phytophthora palmivora* di kawasan penanaman koko di Malaysia. Oleh itu, kajian telah dimulakan untuk memencarkan dan mengenalpasti spesis utama *Phytophthora* di ladang utama penanaman koko yang dijangkiti penyakit buah hitam di Malaysia menggunakan analisa bagi penjurukan ITS rDNA, sitokrom c oksidase bahagian (subunit) 1 dan bahagian faktor pemanjangan translasi 1 α (*EF-1 α*). Kajian juga dijalankan untuk memeriksa kepelbagaian genetic dalam 56 pencilan *P. palmivora* yang diperolehi daripada pelbagai perumah, kawasan geografi dan tahun yang berbeza di Malaysia menggunakan *Inter Simple Sequence Repeat (ISSR)* dan penanda Pengaruan Rawak DNA Polimorfik (RAPD). Disamping itu, kajian dijalankan untuk memencarkan, menciri dan menyaring bakteria endofit daripada pokok koko untuk aktiviti kawalan secara biologinya. Mekanisma tindakbalas dan kemampuannya untuk megurangkan penyakit reput buah hitam juga telah disiasat. Sejumlah 36 pencilan *Pytophthora* telah diperoleh daripada pelbagai ladang koko yang dijangkiti daripada lima negeri di Malaysia. Enam pencilan *Phytophthora* yang diperoleh daripada durian yang terdapat di Jabatan Perlindungan Tumbuhan di Universiti Putra Malaysia juga telah digunakan dalam kajian ini. Keputusan daripada analisa filogenetik secara individu atau gabungan bagi set data ITS rDNA, COX1, dan *EF1- α* telah pasti hingga ke peringkat spesis bahawa semua pencilan *Phytophthora* adalah *P. palmivora*. Pencilan *P. palmivora* yang diperoleh daripada durian dan koko telah dikelompokkan kepada subklad berbeza berdasarkan tiga kawasan yang diperiksa. Keputusan daripada kedua-dua penanda menunjukkan relatif kepelbagaian yang tinggi diantara pencilan *P. palmivora*. Perpisahan yang lengkap adalah berdasarkan perumah dan tahun pemencilan. Kesemuanya 100

pencilan bakteria endofit telah diperoleh daripada tisu koko yang sihat (daun, batang, buah) daripada 7 buah negeri di Malaysia. Bakteria yang telah dipencarkan telah disaringkan menggunakan eseai kultur berkembar dan ujian tapisan kultur untuk sifat antagonis kepada *P. palmivora* secara in vitro. Dua pencilan terbaik *Pseudomonas aeruginosa* (AS1) dan *Chryseobacterium proteolyticum* (AS2) dengan lebih daripada 80% halangan pertumbuhan jejari (PIRG) telah dipilih untuk eksperimen seterusnya. Kompaun bioaktif yang meruap daripada ekstrak etil asetat mentah telah dikenalpasti menggunakan kromatografi gas- spektrometri jisim (GC). Keberkesanan pencilan *Pseudomonas aeruginosa* dan *Chryseobacterium proteolyticum* dalam mengurangkan luka penyakit buah hitam telah dipastikan pada buah koko yang dicabut. Keputusanya telah mengesahkan bahawa kedua-dua pencilan bakteria ini mempunyai potensi untuk digunakan sebagai agen kawalan bio melawan *P. palmivora*.



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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. The members of the Supervisory Committee were as follows:

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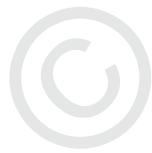
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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
FAO	Food and Agriculture Organization of the United Nations
LSD	Least Significant Difference
VJA	Vegetable Juice Agar
pH	Potential Hydrogen
SAS	Statistical Analysis System
UPM	Universiti Putra Malaysia
BLAST	Basic Local Alignment Search Tool
USD	United States Dollar
PCR	Polymerase chain reaction
ITS	Internal Transcribed Spacer
COX I	Cytochrome c oxidase subunit 1
<i>EF-1α</i>	Translation elongation factor 1 α
DNA	Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic Acid
EDTA	Disodium ethylene diamine tetraacetate
Min	Minute
Sec	Second
CTAB	Hexadecyltrimethyl-Ammonium Bromide
TBE	Tris borate EDTA
UV	Ultra violet
V	Volts
Bp	Base pair
Tris	Tris (hydroxymethyl) aminomethane
Kb	Kilo base
DAI	Days after inoculation
ISSR	Inter-simple sequence repeat
RAPD	Random amplified polymorphic DNA
rpm	Revolutions per minute

CHAPTER 1

INTRODUCTION

Cocoa (*Theobroma cacao* L.) from the family Malvaceae, is an economically important tree in many tropical countries where it is grown as well as for many chocolate manufacturing countries that process the beans into a range of confectionery products. Cocoa is produced in countries in a belt between 10°N and 10°S of the Equator, where the climate is appropriate for growing cocoa trees. The largest producing countries are Côte d'Ivoire, Ghana and Indonesia. (ICCO, 2013). The global economic importance of cocoa cannot be underestimated, as it plays key roles in producing and non-producing countries. Ivory Coast, Ghana, Nigeria and Cameron are the major producer of cocoa beans in the African continent. While Indonesia, Malaysia and Papua New Guinea are the top major producing countries in Asia and Oceania. The largest cocoa producing countries in Americans are Brazil, Ecuador and Colombia. Almost 90% of world cocoa production comes from small farmers of under 5 hectares (ICCO, 2013).

In many countries, plant diseases are the limiting factor for cocoa production. Cocoa crop is affected by many diseases caused by fungi, viruses and bacteria, besides nutritional disorders with estimated losses ranged between 30 to 40% of global production (ICCO, 2015). Rapid identification of pathogens is significant for integrated disease management. *Phytophthora* species are a serious threat to the cocoa industry worldwide. Accurate identification of *Phytophthora* species based on morphological characteristics is very essential to support the preliminary step of identification. However, molecular identification methods are faster, more specific and more sensitive than morphological identification of *Phytophthora* species (Capote et al., 2012). With the development of molecular techniques together with the recent comprehensive multigene phylogenetic analysis of the genus (Blair et al., 2008), sequence databases are available to simplify identification of unknown species.

Black pod disease can be managed primarily using resistant cultivars (Adejumo TO, 2005). This is the destructive cosmopolitan genus of Oomycete and it is of primary interest due to many *Phytophthora* species are plant pathogens of significant economic importance. Phylogenetic analyses have assisted in clarifying equivocation in the traditional classification system of *Phytophthora*. Development in molecular biology has brought new influential tools to fungal taxonomists such as the means to clarify the relationship among fungal species and the possibility for rapid identification of isolates. Moreover, molecular methods have been extensively used to differentiate between closely related species and to distinguish strains within a species (Singh et al., 2006). Several management strategies are used to control black pod of cocoa such as phytosanitary, chemical control, cultural methods, biological control and genetic resistance, but none has been able to generate acceptable results and completely controlled the disease so far (Guest, 2007).

Currently, there is no report available on the determination of genetic diversity among *Phytophthora palmivora* populations in cocoa plantations of Malaysia. Also, little is known about the use of endophytes to control black pod of cocoa in Malaysia. Biological control research of black pod disease conducted in Malaysia revealed that certain bacteria isolated from the surfaces of infected cocoa pods are antagonistic to *P. palmivora* such as *Burkholderia* sp., *Pseudomonas putida* biotype A and *Bacillus* sp. (Ahmad Kamil, 2004). Moreover, there are a few research efforts have been conducted to identify the causal agent of black pod disease of cocoa in Malaysia using sequence analysis of the internal transcribed spacer (ITS) region only (Mohamed et al., 2017). Thus, the objective of the present research was to identify *Phytophthora* isolates causing black pod disease in the infected cocoa plantations in Malaysia, determine the genetic diversity within the isolates and determine its biological control using endophytic bacteria for effective management strategies.

The specific objectives of this study were as follow:

- (i) To isolate, characterize and identify *Phytophthora* isolates from cocoa plantations in Malaysia using sequence analyses of the ITS, COX I and *EF-1 α* .
- (ii) To examine the genetic diversity within *P. palmivora* isolates using ISSR and RAPD markers.
- (iii) To isolate, characterize and screen bacterial endophytes from cocoa plants for their biological control activities and to determine the mechanisms of antagonistic endophytes to control the disease.
- (iv) To investigate the efficacy of endophytic bacteria in *P. palmivora* suppression on Detached pod.

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LIST OF PUBLICATIONS

Wael Alsultan, Jugah Kadir, Ganesan Vadmalai, Halimi Mohd Saud & Ahmed Kamil M.J (2016). Potential of Endophytic Bacteria for Control Black Pod Disease of Cocoa. Kuching City, Sarawak: Proceedings from the 9th International Conference on Plant Protection in the Tropics (9th ICPPT), Aug.3-5, Malaysia.

Wael Alsultan, Ganesan Vadmalai, Khairulmazmi Ahmad & Osamah Rashed (2017). Detection and Identification of Cacao Black Pod Causal Agent by Polymerase Chain Reaction (PCR), International Conference on Big Data Applications in Agriculture (ICBAA2017), Malaysia.

Wael Alsultan, Ganesan Vadmalai, Ahmad Khairulmazmi, Halimi Mohd Saud, Abdullah M. Al-Sadi, Osamah Rashed, Ahmad Kamil Mohd Jaaffarand & Abbas Nasehi (2019). Isolation, identification and characterization of endophytic bacteria antagonistic to *Phytophthora palmivora* causing black pod of cocoa in Malaysia. *European Journal of Plant Pathology*. (Accepted with minor correction).



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