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MORPHOLOGICAL AND MOLECULAR CHARACTERISATIONS OF PHYTOPHTHORA PALMIVORA INFECTING COCOA IN PAHANG AND PERAK, MALAYSIA

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By

WAEL M. K. ALSULTAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

This Thesis is dedicated to

The most precious people in my life; my dad and mum

Mahmoud and Fatemah

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 fulfillment of the requirement for the degree of Master of Science

MORPHOLOGICAL AND MOLECULAR CHARACTERISATIONS OF *PHYTOPHTHORA PALMIVORA* INFECTING COCOA IN PAHANG AND PERAK, MALAYSIA

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Chairman: Associate Professor Zainal Abidin Mior Ahmad, PhD
Faculty: Agriculture

Phytophthora spp. are one of the most destructive plant pathogens worldwide. Many economically important crop species such as cocoa, rubber, jackfruit, durian, taro, papaya, pepper, coconut, potato, citrus and forest trees are susceptible to various diseases caused by *Phytophthora* spp. Agronomists and plant pathologists have been aware of the economic importance of diseases caused by *Phytophthora* in Southeast Asia. However, there is a lack of published information on *Phytophthora* spp. in the tropics. Although many studies have been conducted over the past few decades to overcome diseases caused by *Phytophthora* spp., the general information is often not outlined with details while specific information of occurrence and economic impact are dispersed in many different publications in various languages. Black pod disease of cocoa caused by *Phytophthora palmivora* is a serious problem of economic importance to cocoa growers in Malaysia. It is also known to cause stem canker disease where their control in infected cocoa plantations is very difficult. This research was undertaken to study the cultural characteristics, reproduction, and pathogenicity as well as molecular characteristics of fourteen isolates of *Phytophthora* spp. randomly obtained from infected cocoa pods in two different states, Pahang and Perak in Peninsular Malaysia. The cultural characteristics of these isolates were assessed on four types of agar medium namely PDA , CA, VJA and CMA incubated at 25 ± 2°C showed various textures of colonies as fluffy with irregular margin on PDA, cottony with uniform margin on CA, slightly cottony with regular margin on VGA and flattish with regular margin on CMA. It did not show specific colony pattern on all four types of medium and each medium demonstrated its own morphological appearance. Growth rates of colonies were measured after three days of incubation on four types of agar media for one week and demonstrated that isolates grew significantly faster on CMA with 13.3mm/day, followed equally by VJA and CA with 10.0 and 10.2mm/day respectively, and the poorest growth was on PDA with 6.1mm/day. Dimensions of sporangia on CMA medium were measured averaging 43.48µm in length, 29.37 µm in width, 3.53 µm in pedicel length and 5.31 µm in size of exit pore. On culture media, the sporangia were of different shapes.

Sporangia of isolates were all caducous. Zoospores were induced on four types of medium CA, VJA and CMA. Isolates on CA medium produced the largest number of zoospores than on VJA and CMA medium. Chlamydospores were produced on VJ broth ranging between 25.21 to 21.55 µm in diameter. Molecular identification using ITS region of rDNA confirmed that all isolates were *Phytophthora palmivora*. Eight isolates were selected randomly for pathogenicity tests on green healthy pods of KKM22 cocoa cultivar. The test results confirmed that isolates RB03 and RB04 from Pahang significantly more virulent with size of lesion 129mm and 117.75mm correspondingly compared to other isolates after five days of inoculation. Hence, complete brown to black lesions covered all inoculated after nine days.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagi memenuhi keperluan untuk ijazah Master Sains Pertanian

PENCIRIAN MORFOLOGI DAN MOLIKUL *PHYTOPHTHORA PALMIVORA* PADA LADANG KOKO DI PAHANG DAN PERAK, MALAYSIA

Oleh

WAEL M. K. ALSULTAN

Jun 2015

Pengerusi: Profesor Madya Zainal Abidin Mior Ahmed, PhD
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Phytophthora spp. adalah salah satu pathogen tumbuhan yang paling memusnahkan diseluruh dunia. Banyak tanaman yang berkepentingan ekonomi seperti koko, getah, nangka, durian, ubi, betik, lada hitam, kelapa dan pokok hutan adalah rentan kepada pelbagai penyakit disebabkan oleh *Phytophthora* spesis. Ahli agronomi dan patologi tumbuhan telah mempunyai kesedaran tentang kepentingan ekonomi penyakit yang disebabkan oleh *Phytophthora* di Asia Tenggara. Walaubagaimanapun, terdapat kekurangan maklumat penerbitan mengenai *Phytophthora* spp. di kawasan tropika. Walaupun banyak kajian telah dijalankan dalam tempoh beberapa dekad yang lepas untuk mengatsi penyakit yang disebabkan oleh *Phytophthora* spp. Maklumat umum sering tidak di jelaskan dengan terperinci manakala maklumat khusus kejadian dan impak ekonomi adalah tersebar di dalam banyak penerbitan yang berbeza dalam pelbagai bahasa. Penyakit buah hitam koko disebabkan oleh *Phytophthora palmivora* adalah masalah serius berkepentingan ekonomi kepada penanam di Malaysia. Ia juga diketahui menyebabkan penyakit kanker batang dimana kawalan di ladang koko adalah amat sukar. Penyelidikan ini telah di jalankan untuk mengkaji ciri kultur, pembiakan dan kepatogenan serta ciri molikul sebanyak empat belas pencilan *Phytophthora* spp. yang diperolehi secara rawak dari buah koko berpenyakit daripada dua negeri yang berbeza, Pahang dan Perak di Semenanjung Malaysia. Ciri kultur kesemua pencilan telah dinilai pada empat jenis medium agar PDA, CA, VJA dan CMA dieram pada 25 ± 2°C menunjukkan pelbagai tekstur koloni seperti ‘fluffy’dengan margin tidak sekata pada PDA, berkapas dengan margin sekata pada CA, sedikit berkapas dan margin sekata pada VJA dan berleper serta margin sekata pada CMA. *Phytophthora* spp. daripada koko tidak menunjukkan bentuk koloni yang sekata pada semua media ujian, tetapi menunjukkan morfologi yang tersendiri bagi setiap satunya. Kadar pertumbuhan koloni diukur selepas tiga hari pada empat agar medium berkenaan selama satu minggu dan telah menunjukkan asingan tumbuh dengan kadar lebih pantas pada CMA dengan 13.3 mm/hari, diikuti kadar pertumbuhan yang sama pada VJA dan CA dengan 10.0 dan 10.2 mm/hari, dan kadar pertumbuhan terendah adalah pada PDA dengan 6.1 mm/hari. Ukuran besar sporangium pada medium CMA berpurata 43.48 µm panjang, 29.37 µm lebar, 3.53 µm panjang pedisel dan 5.31 µm untuk saiz lubang keluar. Pada medium kultur, sporangium

mempunyai bentuk yang berbeza. Sporangium pencilan kesemuanya adalah ‘caducous’. Zoospora telah dirangsang pada empat jenis media iaitu CA, VJA, dan CMA medium.. Pencilan pada medium CA menghasilkan bilangan zoospore paling banyak daripada VJA medium. Klamidospora di hasilkan pada cairan VJA dalam julat antara 25.21 ke 21.55 µm garis pusat. Pengenalpastian molikul menggunakan kawasan ITS rDNA mengesahkan kesemua pencilan adalah *Phytophthora palmivora*. Lapan pencilan dipilih secara rawak untuk ujian kepatogenan pada buah koko sihat kultivar KKM22. Keputusan ujian mengesahkan pencilan RB03 dan RB04 daripada Pahang adalah lebih virulen dengan saiz luka masing masing, 129 mm dan 117.75 mm dibandingkan dengan pencilan lain selepas lima hari inokulasi. Selepas Sembilan hari, seluruh buak koko menjadi warna perang kepada kehitaman.

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I certify that an Examination Committee met on 1st June 2015 to conduct the final examination of WAEL M K ALSULTAN on his Master of Science thesis entitled " Morphological and Molecular Characterisations of *Phytophthora palmivora* infecting Cocoa in Pahang and Perak, Peninsular Malaysia" in accordance with Universities and University college Act 1971 and the constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 Mach 1998. The Committee recommends that the student be awarded the Master of Science.

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Declaration by Graduate Student

I hereby confirm that:

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This to confirm that:

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- supervision responsibilities as stated in the Rule 41 in Rules 2003 (Revision 2012-2013) were adhered to.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percent
ANOVA	Analysis Of Variance
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BRD	Bud rot disease
CA	Carrot Agar
cm	Centimeter
CMA	Corn Meal Agar
CTAB	Hexadecyltrimethyl-Ammonium Bromide
DAI	Days after inoculation
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside Triphosphates
EDTA	Disodium ethylene diamine tetraacetate
FAO	Food and Agriculture Organization of the United Nations
g	gram
ha	hectare
ITS	Internal Transcribed Spacer
Kb	Kilo base
L	liter
LSD	Least Significant Difference
M	Molarity
Min	Minute
mm	millimeter
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
Nm	nanometer
ºC	Degree
P. megakarya	*Phytophthora megakarya*
P. palmivora	*Phytophthora palmivora*
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
pH	potential Hydrogen
PVP	Pimaricin-Vancomycin-Pentachloronitrobenzene
rRNA	Ribosomal Ribonucleic Acid
SAS	Statistical Analysis System
Sec	Second
TBE	Tris borate EDTA
Tris	Tris (hydroxymethyl) aminomethane
UPM	Universiti Putra Malaysia
USD	United States Dollar
UV	Ultra violet
V	Volts
VJA	Vegetable Juice Agar

CHAPTER 1

INTRODUCTION

Cocoa or *Theobroma cacao* is a small evergreen tree and native to the central and western Amazon region of South America (Pereira, 1992). Its seeds are the main ingredients for making cocoa powder and chocolate. Cocoa is a major beverage crop after coffee and tea in the world and is grown in about 45 countries.

A cocoa plantation has a critical social implication in the world as people are dependent on the cocoa industry. The generic name *Theobroma* literally means "Food of the Gods." Ivory Coast, Ghana, Indonesia, Malaysia, Brazil and Papua New Guinea are the major producers of the cocoa crop (Drenth and Guest, 2004). The projected world consumption of cocoa by 2010/2011 was estimated to be around 3.9 million tonnes while production and consumption in 2012 was estimated at four million tonnes (ICCO, 2012).

In Malaysia, cocoa is known as the third plantation crop after oil palm and rubber. It was introduced to the country for commercial cultivation as early as 1950s in the state of Sabah (MPIC, 2007). However, the crop failed to grow well due to dieback disease. Haddon (1960) reported that dieback problems were the main reason for the failure in experimental plantings of cocoa using Trinitario and imported Amelonado. During the 60s, Upper Amazon cocoa was grown successfully as a trial crop in Malaysia, and that was the first step for expansion in cocoa plantations. The cocoa plantation reached its peak in 1989 with the plantation area covering 414,236 hectares. Cocoa production in Malaysia had been reduced significantly from producing 9% of world production in the years of 1990-1991 to only below 2% of world production (Drenth and Guest, 2004). This decline was attributed to various factors including lower cocoa prices and the destruction of cocoa by the cocoa pod borer, which spurred farmers to grow other crops such as oil palm (Drenth and Guest, 2004). Cocoa is now planted in few states in Malaysia including Pahang, Perak, Selangor and Johor in the west, and Sabah and Sarawak in the east.

The highest record of cocoa bean production was 247, 000 tonnes in 1980, but this started to decline onwards as the plantation area was declining too. Nevertheless, in 2007 the cocoa bean production had increased even though the plantation area was decreasing. The production had increased to 35, 180 tonnes compared to 31, 937 tonnes in 2006 (MCB, 1992; 2008a; 2012). Cocoa is affected by various diseases caused by fungi, and one of the most destructive diseases of cocoa is black pod. The main symptom of black pod disease of cocoa is a brown or black spot on the pod that quickly grows to cover the whole pod (Smith et al., 1992). The causal pathogen of black pod disease was identified as *Phytophthora palmivora*. It is currently the most significant cocoa pathogen in South Asia and Papua New Guinea.

The genus *Phytophthora* is responsible for some of the most destructive diseases of cocoa including *Phytophthora* pod rot or black pod, stem canker, leaf and seedling blight, chupon wilt and flower cushion infections. Pod rot disease causes 20– 30% annual losses in cocoa beans production globally, and much higher losses in specific locations during particularly wet and humid conditions (Erwin and Ribeiro, 1996). In addition, *Phytophthora* attacks the roots, trunk, green stem, leaves and pods of the cocoa plant. High humidity provides the suitable environment for lesions on pods to spread and developed (Smith et al., 1992). Unquestionably, characterization of *Phytophthora* spp. are very essential to crop growers, environmentalists, legislators, policy makers and implementers to know the best way to managing and controlling diseases caused.

Phytophthora spp. are destructive fungal organism causing several diseases on crops in the tropics and it is one of the most-studied genera of plant pathogens. There is limited information in international herbaria and culture collections on the accession numbers used for the types of species. Many of the diseases caused on cocoa are not well studied in Malaysia, and there is a serious lack of locally published information.

Thus, this research was done to examine the variability and virulence of *Phytophthora* spp. present in cocoa plantations of two states in West Malaysia.

The two main objectives of the study were as follows:

(1) To conduct a detailed morphological and molecular characterization of *Phytophthora palmivora* isolated from cocoa plantations in the states of Pahang and Perak in peninsular Malaysia.

(2) To examine the pathogenicity of *Phytophthora palmivora* isolated from these cocoa plantations using artificial inoculation.

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