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MORPHOLOGICAL, MOLECULAR AND PATHOLOGICAL VARIABILITY OF Phytophthora spp. FROM PERENNIAL CROPS IN MALAYSIA

LATIFAH MUSANIF

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By

LATIFAH BINTI MUSANIF

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MORPHOLOGICAL, MOLECULAR, AND PATHOLOGICAL VARIABILITY OF *Phytophthora* spp. FROM PERENNIAL CROPS IN MALAYSIA

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LATIFAH BINTI MUSANIF

December 2016

Chairman : Nusaibah Binti Syd Ali, PhD
Faculty : Agriculture

Phytophthora causes various types of devastating diseases in different types of plants including annual and perennial crops, ornamental and forest trees in the tropical, subtropical, and temperate climates. In the tropics, *Phytophthora* spp. is considered as one of the most destructive pathogens of cocoa, rubber, durian and other commodity crops. However, the potential of *Phytophthora* spp. to cross-infect susceptible non-host plants were not extensively studied. *Phytophthora* spp. cross-infection studies are particularly important in intercropping systems practiced in plantations. Hence, the purpose of this study was to isolate, characterize and identify *Phytophthora* spp. from major perennial crops in Malaysia. In addition, pathogenicity and cross-pathogenicity of *Phytophthora* spp. between hosts and non-hosts such as oil palm were also studied. Identification of *Phytophthora* spp. was done through morphological, cultural and molecular characterizations based on Internal Transcribed Spacer (ITS) regions of ribosomal deoxyribonucleic acid (rDNA). Pathogenicity tests were carried out to confirm its pathogenicity towards its native host. Cross-pathogenicity tests were conducted to determine the potential of *Phytophthora* spp. in infecting susceptible non-hosts. Morphological characterizations of the isolated *Phytophthora* spp. indicated that most isolates from cocoa and durian formed striate and stellate colonies while isolates from rubber exhibited fluffy colonies on cornmeal agar (CMA), carrot agar and vegetable juice agar (V8). Optimum mycelial growth for all isolates was 28 ± 1.5°C. In addition, sporangia from cocoa and durian were caducous with length to breadth ratios ranging from 1.51 to 1.69 and possessed short occluded pedicels. However, *Phytophthora* isolates from rubber displayed non-caducous sporangia with length to breadth ratios of 1.3 to 1.38. Nonetheless, all isolates were observed to produce abundant chlamydospores after two weeks of incubation. Therefore, in the present

study, 18 isolates of *Phytophthora* spp. were successfully isolated from cocoa, durian and rubber; 12 isolates from cocoa and durian were identified as *P. palmivora*, while six isolates were from rubber, *Phytophthora nicotianae* (synonym *P. parasitica*). Molecular identifications supported morphological and cultural characterizations of the isolated *Phytophthora* spp. *In-vitro* pathogenicity tests of 18 isolates on detached leaves and unripe cocoa pods exhibited the progressive development of lesions on its native host. Moreover, lesions were also developed in all wounded detached leaves and unripe cocoa pods regardless of hosts and isolates in cross-pathogenicity tests. However, there was a significant difference in the length of lesions developed, whereby longer lesions were detected on its native host compared to its non-native host. Additionally, inoculation of *Phytophthora* isolated from durian stem canker, cocoa pod rot, and rubber pod rot displayed infections on young immature oil palm leaflets by the development of discoloration. This discoloration developed after three days of incubation under moist conditions. Cross-inoculation studies also demonstrated that *Phytophthora* spp. was pathogenic to oil palm seedlings through the development of lesions on the buds. The pathogens were successfully re-isolated from the developed lesions of inoculated tissues, thus fulfilling Koch's postulates and this confirmed that *Phytophthora* spp. are pathogenic to oil palm. As conclusion, both conventional method (morphology and cultural studies), together with molecular identification confirmed that *Phytophthora* isolates from cocoa and durian were *P. palmivora*, whilst rubber isolates known as *P. nicotianae*. There was development of lesion on plant tissues regardless of host and non-host was noted, however more aggressiveness and length of lesion on own host in *in-vitro* test was observed. Both of *in-vitro* test and field trials also showed that *P. palmivora* and *P. nicotianae* in the present study were pathogenic to immature oil palm leaflets.

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MORFOLOGI, MOLEKULAR DAN KEPELBAGAIAN PATOLOGIKAL *Phytophthora* spp. PADA TANAMAN SAKA DI MALAYSIA

Oleh

LATIFAH BINTI MUSANIF

Disember 2016

Pengerusi : Nusaibah binti Syd Ali, PhD
Fakulti : Pertanian

Phytophthora menyebabkan pelbagai penyakit serius kepada banyak jenis tanaman sama ada tanaman saka, tanaman semusim, pokok-pokok hiasan serta pokok-pokok hutan di kawasan beriklim tropika, separa-tropika dan temperat. Di kawasan tropika, *Phytophthora* spp. dianggap sebagai salah satu patogen tumbuhan yang paling berbahaya pada pokok koko, getah, durian serta tanaman komoditi yang lain. Walaubagaimanapun, kajian mengenai potensi *Phytophthora* spp. untuk menjangkiti bukan perumah yang rentan masih tidak meluas. Kajian mengenai kebolehan *Phytophthora* spp. dalam jangkitan silang diantara perumah dan bukan-perumah adalah sangat penting dalam amalan pertanian campur yang diimplementasikan di ladang-ladang. Justeru itu, tujuan kajian ini dijalankan adalah untuk memencilkan, mencirikan serta mengenalpasti identiti *Phytophthora* spp. daripada tanaman saka yang utama di Malaysia. Tambahan pula, aras keagresifan serta potensi kepatogenan-silang *Phytophthora* spp. diantara perumah dan bukan perumah seperti pokok sawit juga menjadi salah satu objektif kajian ini. Pengecaman spesis *Phytophthora* telah dilakukan melalui ciri-ciri morfologi, kultura serta pencirian secara molekul berdasarkan kawasan "Internal Transcribed Spacer" (ITS) pada asid deoxyribonukleik di kawasan ribosom (rDNA). Ujian kepatogenan telah dijalankan bagi mengesahkan keagresifan pencilan *Phytophthora* terhadap perumahnya. Manakala ujian kepatogenan-silang dijalankan bagi menentukan kebolehan pencilan *Phytophthora* dalam menjangkiti bukan perumah yang rentan. Ciri-ciri morfologi pencilan daripada koko dan durian kebanyakannya mempunyai koloni "striate" dan "stellate", manakala pencilan daripada getah mempamerkan koloni yang gebu di atas media jagung (CMA), lobak, dan jus sayuran (V8). Suhu optimum pertumbuhan miselia bagi kesemua pencilan *Phytophthora* adalah 28 ± 1.5°C. Selain itu, sporangia daripada pencilan koko dan durian adalah "caducous" (sporangia terpisah daripada sporangiofor) dengan nisbah panjang kepada lebar daripada 1.51 hingga 1.69 dan mempunyai pedisel yang pendek dan "occluded". Sebaliknya, pencilan daripada getah mempamerkan sporangia yang "non-caducous" dengan nisbah panjang kepada lebar daripada 1.30 hingga 1.38. Selain itu, kesemua pencilan didapati menghasilkan klamidospora dalam

kuantiti yang banyak selepas diinkubasi selama dua minggu. Oleh itu, dalam kajian ini, sebanyak 18 pencilan *Phytophthora* spp. telah berjaya dipencilkan daripada koko, durian dan getah; dimana 12 pencilan daripada koko dan durian telah diidentifikasi sebagai *P. palmivora* manakala enam lagi pencilan daripada getah, adalah *P. nicotianae* (sinonim dengan *P. parasitica*). Pencirian yang dilakukan secara molekul menyokong kaedah pencirian secara morfologi dan kultura terhadap pencilan-pencilan *Phytophthora*. Ujian kepatogenan secara *in-vitro* yang dijalankan terhadap 18 pencilan pada buah koko dan daun menunjukkan pencilan-pencilan tersebut berupaya menyebabkan lesi pada perumah asal dan bukan perumah asal. Walaubagaimanapun, kesan lesi adalah lebih panjang dan kritikal pada perumah asal berbanding dengan bukan perumah. Lesi juga terhasil apabila pencilan-pencilan *Phytophthora* diinokulasi ke atas pucuk muda kelapa sawit secara *in-vitro*. Pencilan-pencilan *Phytophthora* spp. daripada kesemua perumah terbukti patogenik pada anak sawit apabila berjaya menghasilkan lesi pada bahagian pucuk muda setelah diinokulasi secara buatan. Perubahan warna diperhatikan pada pucuk muda anak sawit selepas tiga hari diinkubasi dalam persekitaran yang lembap. Kedua-dua spesies *Phytophthora* telah berjaya dipencilkan kembali daripada kesan lesi pada anak-anak sawit akibat jangkitan dan ini memenuhi 'Koch postulate' serta mengesahkan *Phytophthora* spp. yang dikaji adalah patogenik terhadap anak sawit. Kesimpulannya, kedua-dua kaedah pencirian secara konvensional (pengkajian morfologi dan kultura) serta molekul telah mengesahkan pencilan-pencilan *Phytophthora* daripada koko dan durian adalah *P. palmivora* manakala pencilan-pencilan daripada getah dikenali sebagai *P. nicotianae*. Terjadi perkembangan lesi pada tisu perumah dan juga bukan perumah, akan tetapi lesi tersebut lebih agresif dan panjang saiznya pada tisu perumah dalam ujian *in-vitro* setelah diperhatiakan. Kedua-dua, *P. palmivora* dan *P. nicotianae* dalam pengkajian ini adalah patogenik kepada tisu pucuk sawit belum matang berdasarkan pengkajian menerusi ujian *in-vitro* dan ujian lapangan.

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

Nusaibah Syed Ali, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Kamaruzaman Sijam, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Dr. Nusaibah Syed Ali

Signature of Member of Supervisory Committee: _____

Assoc. Prof. Dr. Kamaruzaman Sijam

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
CA	Carrot agar
CMA	Cornmeal agar
CRD	Complete Randomized Design
CTAB	N-Cetyl-N,N,N-Trimethyl-ammonium bromide
°C	Degree Celsius
ddH_2O	double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
g	Gram
ITS	Internal Transcribed Spacer
L	Liter
MEGA	Molecular Evolutionary Genetics Analysis
M	Molar
mL	Milliliter
mg	Milligram
min	Minute
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
PCNB	Pentachloronitrobenzene
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
%	Percent
rpm	rotation per minute
rDNA	ribosomal Deoxyribonucleic acid
TAE	Tris-acetate EDTA
Tris	Tris (hydroxymethyl) aminomethane
µg	Microgram
µL	Microliter
V8A	Vegetables Juice Agar
V	Voltage
w/w	weight per weight

CHAPTER 1

INTRODUCTION

Phytophthora means 'plant destroyer' in Greek; is a plant pathogen causing various devastating diseases in different types of plants including annual and perennial crops. Some *Phytophthora* spp. infect only one or two species of host plants though most cause diseases on many types of host plants (Agrios, 2005). *Phytophthora* spp. infection occurs in all life stages of forest trees including roots and crowns causing trunk cankers to foliar blights with a huge impact on forest ecosystems (Erwin and Ribeiro, 1996). It is a flexible and very effective pathogen due to its uncommon genetic architecture that enables *Phytophthora* to cause rapid evolution in pathogenicity (Jiang *et al*., 2008; Raffaele *et al*., 2010; Seidl *et al*., 2011).

The importance of *Phytophthora* genus in plant pathology eventually started when *P. infestans* destroyed Ireland's staple potato crops in 1845 and 1846, a time known as the Irish Potato Famine. The famine resulted in severe potato blights and it is estimated that Ireland lost one-fourth of its eight million inhabitants due to starvation and emigration (Bourke, 1991).

By the year 1996, there were 58 *Phytophthora* spp. that had been described by Erwin and Ribeiro (1996). Currently, the genus *Phytophthora* consists of more than 100 species and this continues to rise (Érsek and Ribeiro, 2010). Thus, there is no doubt that *Phytophthora* will be a continued global threat for decades to come.

To date, the threat still remains with a new wave of late blight epidemics in the 1990s due to the emergence of highly aggressive and fungicide-insensitive isolates in North America and Europe (Fry and Goodwin, 1997a, 1997b; Schiermeier, 2001; Smart and Fry, 2001). Although chemicals targeting the pathogen can provide some level of control, late blight is still a damaging disease particularly since the crops infected by *P. infestans* are grown in every state in the United States of America. As a result, crop losses and control measures are estimated to cost several billion dollars annually worldwide (Duncan, 1999; Schiermeier, 2001).

In another study, Rizzo *et al*. (2002) discovered *P. ramorum*, a new pathogenic species in oak trees in California and it was also found on several horticultural species, larch and Sitka spruce in Britain. Apart from that, *P. alni* was identified as the causal agent of lethal root and collar rot of the alder species in Europe (Brasier and Kirk, 2004).

Recently, Hee *et al*. (2013) reported that 'Jarrah dieback' caused by *P. cinnamomi* had caused the widespread decline of the dominant forest species of *Eucalyptus marginata* (jarrah). In Western Australia, *P. cinnamomi* is known as a biological bulldozer as 2284 of 5710 plant species are susceptible or highly susceptible (Shearer *et al*., 2004).

In Southeast Asia (Thailand, Malaysia, Indonesia, Vietnam and Philippines) the economic impact of *Phytophthora* on cocoa, durian, rubber, coconut, pepper, citrus and potato was estimated to be at least US$2.3 billion (Drenth and Sendall, 2004). Losses due to *Phytophthora* ranged from 5-10% for coconut and black pepper, to 15-25% for rubber, durian and cocoa (Drenth and Guest, 2004). Annual global losses due to *Phytophthora* spp. to the cocoa industry were estimated to be around 450 000 tones, valued at over US$1 billion (Drenth and Guest, 2004; IPARC, 2012).

Rubber cultivation remained as an important element in the Malaysian economy instead of Malaysian 'golden crops'- oil palm. Malaysia is currently the world's fifth largest producer of natural rubber (NR) after Thailand, Indonesia, Vietnam and China. NR production in Malaysia increased by 4.1% from 0.67 million tonnes in 2014 to 0.72 million tonnes in 2015. Meanwhile, the exports of NR recorded a decline of 6.5% from 1.2 million tonnes in 2014 to 1.1 million tonnes in 2015.Nonetheless, Malaysia is still a net exporter of NR since Malaysia provide high-quality raw rubber with SMR (Standard Malaysian Rubber) grades. Malaysia produces speciality rubber such as ENR (Epoxidized Natural Rubber), DPNR (Deproteinized Natural Rubber), TPENR (Thermoplastic epoxidized natural rubber) and latex concentrates including Low Protein Latex (MREPC, 2016). In addition, Malaysia is globally renowned for its high-quality rubber product which currently exported to more than 190 countries globally. The value of exports of rubber products from Malaysia surpassed RM15 billion in 2014 and reached nearly RM18 billion in 2015 (MREPC, 2016).

Durian is a potential fruit to be commercialised since it is well received for its sweet and creamy flavour. It was consumed locally in Malaysia though a significant amount of good quality durians were exported to other countries. In 1991, Malaysia exported USD16.3 million worth of fresh durian, with about 90% to Singapore (Graef and Klotzbach, 1995). Recently, the durians are gaining in popularity among Chinese causing demand of frozen durian pulp enter China market (FAMA, 2014). The export value of Malaysian frozen durians to China had reached RM1.2 million in August last year, adding that the export value of the whole of last year was expected to surpass the RM1.6 million figure achieved in 2013 (News Straits Times, 2015). This potential profit value will be loss if *Phytophthora* disease did not manage effectively since *Phytophthora* spp. could infect at parts and at all growth stages of durian tree.

Cocoa is known as among primary commodity crops that planted in Malaysia after oil palm, rubber, kenaf and pepper. According to Department of Statistic Malaysia of Malaysian Cocoa Board (2016), the export value of cocoa industry was reached the amount of RM3.25 billion with 2809 tonnes cocoa beans production in 2013. However, the amount of cocoa beans production (upstream sector) still can't fulfil the demand of downstream sector cocoa product in Malaysia nowadays. Hence, the effort to increase the cocoa production through pest and disease management was the very important approach in order to reduce the amount of imported cocoa beans which needed cost more than RM2 billion annually.

Diseases caused by *Phytophthora* spp. are common in countries having tropical climates with high annual rainfall such as Malaysia. One of the most common species in the

tropics is *P. palmivora* which causes black pod in cocoa, root rot and blight of citrus, bud rot in palms, black stripe in rubber, stem canker, and fruit rot in durians (Drenth and Guest, 2004). The favourable environment enables the pathogen to cause disease throughout the year to susceptible host.

Phytophthora palmivora was identified as the causal agent of bud rot disease in oil palm in Columbia and its neighboring countries. It destroyed at least 45 000 hectares of oil palm estate causing significant economic losses (Martinez, 2009a; Torres *et al*., 2010). The threat of the oil palm bud rot disease from South America hangs over the Malaysian oil palm industry as *P. palmivora* pathogenic to cocoa, durian, rubber and papaya are also present in Malaysia. However, reports of pathogenicity among hosts has not been extensively studied. In addition, no documented report of its pathogenicity on oil palm in Malaysia has been reported.

Thus, the specific objectives of this research were to isolate, characterize and identify *Phytophthora* spp. from major perennial crops in Malaysia, to determine its pathogenicity and cross-pathogenicity between hosts and to study its pathogenic potential on oil palm

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