



**MOLECULAR CHARACTERIZATION OF *Phytophthora palmivora* Butler
AND EVALUATION OF *Morinda citrifolia* L. LEAF EXTRACT
FOR CONTROLLING STEM CANKER ON DURIAN**

By

NOR DALILA BINTI NOR DANIAL

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Science**

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Chair : Siti Idera Ismail, PhD
Faculty : Agriculture

Commercial durian production in Malaysia has doubled in the last decade and durian growers are receiving premium price from their fruits from local and international consumers. Stem canker, caused by *Phytophthora palmivora* is a major problem for durian farmers in Malaysia, but limited studies addressed their genetic diversity using multiple genes. Therefore, this study was conducted to characterize *Phytophthora* species from main durian orchards infected by stem canker in Malaysia using morphological, sequence analyses of the internal transcribed spacer (ITS) regions of ribosomal deoxyribonucleic acid (rDNA) and cytochrome oxidase c (COX) regions. Fungicides containing metalaxyl and phosphonate have been long used for this disease management in durian fields. However, the repeated and extensive use of similar fungicide developed resistant *Phytophthora* isolates. Research into new approaches of managing such pathogens are of importance. *Morinda citrifolia*, a medicinally valued plant that are commonly found in Malaysia can be a beneficial complement to chemical fungicides. This study was also to identify the compounds of *M. citrifolia* leaf crude extracts extracted using methanol, acetone, ethyl acetate and hexane. In addition, this study was carried out to evaluate *in vitro* and *in vivo* efficacy of *M. citrifolia* leaf crude extracts against the mycelial growth of *P. palmivora*. A total of 21 *Phytophthora* isolates were obtained from water-soaked lesions of durian stem tissues collected from 4 different states of Malaysia in 2019, Selangor ($n=10$), Perak ($n=4$), Pahang ($n=4$) and Melaka ($n=3$). All isolates had morphological characteristics similar to that of *P. palmivora*. For molecular identification, the results of BLASTn using the ITS and COX sequences identified them as *P. palmivora* with 99-100% with *P. palmivora* culture (GenBank accession no. ON834450 for the ITS region and JF771543 for the Cox region). Results of phylogenetic analyses of the ITS rDNA and COX confirmed that all *Phytophthora* isolates belonged to *P. palmivora* and clustered into one clade with reference *P. palmivora* isolates, supported by high bootstrap values for the ITS (100%) and COX (76%) regions. Pathogenicity tests showed all isolates were

pathogenic on durian leaves, where PMM02 had significantly the lowest virulence among all isolates with a lesion diameter of 64.04 mm at day 8. The detached leaf assays using durian leaves of D10, D24 and Mousang King varieties showed there was significance among durian varieties, where D10 was proven to be more susceptible towards *P. palmivora*. GC-MS analysis of *M. citrifolia* leaves crude extract revealed the presence of squalene. *In vitro* evaluation of *M. citrifolia* crude extracts acetone and ethyl acetate showed an effective concentration 50% (EC_{50}) values of 36.12 mg/mL and 38.10 mg/mL, respectively. Plate treated with acetone solvent at 60 mg/mL altered the morphology and inhibit the mycelial growth of *P. palmivora* at microscopic observation using scanning electron microscope. *In vivo* bioassay evaluation, plants treated with *M. citrifolia* extract of ethyl acetate 76 mg/mL had a lesion growth of 30.24 mm on the stem. This treatment was not significantly difference to control positive treatment applied with Ridomil fungicide. *M. citrifolia* methanol, acetone and ethyl acetate contain antifungal compounds and can be used as biofungicides for stem canker disease management.

Keywords: antifungal activity, canker, *Durio zibethinus*, *Morinda citrifolia*, oomycete

SDG: GOAL 9: Industry, Innovation and Infrastructure

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

**PENCIRIAN MOLEKUL *Phytophthora palmivora* Butler DAN PENILAIAN
EKSTRAK DAUN *Morinda citrifolia* L. UNTUK MENGAWAL PENYAKIT
KANKER DURIAN**

Oleh

NOR DALILA BINTI NOR DANIAL

Februari 2023

Pengerusi : Siti Izera Ismail, PhD
Fakulti : Pertanian

Pengeluaran durian secara komersial di Malaysia telah meningkat dua kali ganda beberapa dekad yang lalu dan penanam durian menerima harga premium terhadap buah-buahan mereka daripada pengguna tempatan dan antarabangsa. Kanker batang yang disebabkan oleh *Phytophthora palmivora* adalah masalah utama bagi penanam durian di Malaysia, tetapi kajian berkenaan kepelbagaian genetik menggunakan pelbagai gen adalah terhad. Oleh itu, kajian ini dijalankan untuk mencirikan spesies *Phytophthora* daripada dusun durian utama yang dijangkiti kanker batang di Malaysia menggunakan morfologi dan analisis jujukan kawasan *internal transcribed spacer* (ITS) *ribosomal deoxyribonucleic acid* (rDNA) dan *cytochrome oxidase c* (COX). Racun kulat yang mengandungi metalaksil dan fosfonat telah lama digunakan untuk pengurusan penyakit tanaman durian. Walaubagaimanapun, penggunaan racun kulat yang sama dan secara berpanjangan telah menghasilkan populasi *Phytophthora* yang rintang terhadap racun tersebut. Penyelidikan berkaitan pendekatan baru untuk menguruskan patogen seperti ini adalah penting. *Morinda citrifolia*, tumbuhan perubatan bernilai tinggi biasa ditemui di Malaysia boleh menjadi pelengkap yang bermanfaat kepada racun kulat kimia. Kajian ini juga adalah untuk mengenalpasti sebatian ekstrak mentah daun *M. citrifolia* yang diekstrak menggunakan methanol, aseton, etil asetat dan heksana. Di samping itu, kajian ini dijalankan untuk menilai keberkesanan *in vitro* dan *in vivo* ekstrak mentah daun *M. citrifolia* terhadap pertumbuhan miselia *P. palmivora*. Sebanyak 21 isolat *Phytophthora* dipencil daripada simptomatik tisu batang durian yang disampel pada tahun 2019 dari 4 negeri di Malaysia, negeri Selangor ($n=10$), Perak ($n=4$), Pahang ($n=4$) dan Melaka ($n=3$). Semua isolat mempunyai ciri morfologi yang serupa dengan *P. palmivora*. Untuk pengenalpastian molekul, hasil BLASTn menggunakan jujukan ITS dan COX telah mengenalpasti isolat sebagai *P. palmivora* dengan 99-100% dengan kultur *ex-type P. palmivora* (GenBank ON834450 untuk primer ITS dan JF771543 untuk primer COX). Keputusan analisis filogenetik ITS rDNA dan COX mengesahkan bahawa semua isolat *Phytophthora* adalah *P. palmivora* dan

dikelompokkan ke dalam satu kluster dengan rujukan isolat *P. palmivora*, disokong oleh nilai *bootstrap* yang tinggi bagi primer ITS (100%) dan Cox (76%). Ujian patogenisiti menunjukkan semua pencilan adalah patogenik pada daun durian, di mana PMM02 mempunyai tahap virulen yang paling rendah di antara semua isolat dengan diameter lesi 64.04 mm pada hari ke 8. Ujian daun *detach* menggunakan daun durian varieti D10, D24 dan Mousang King menunjukkan D10 terbukti lebih rentan terhadap patogen *P. palmivora*. Analisis GC-MS terhadap ekstrak mentah daun *M. citrifolia* mendedahkan kehadiran *squalene*. Penilaian *in vitro* ekstrak mentah *M. citrifolia* aseton dan etil asetat memberikan kepekatan keberkesanan sebanyak 50% (EC₅₀) dengan nilai masing-masing, 36.12 mg/mL dan 38.10 mg/mL. Rawatan pelarut aseton dalam piring petri pada 60 mg/mL dilihat telah mengubah morfologi dan menghalang pertumbuhan miselium *P. palmivora* pada pemerhatian mikroskopik menggunakan *Scanning Electron Microscope*. Penilaian *in vivo* bioesei, pokok yang dirawat dengan ekstrak *M. citrifolia* etil asetat 76 mg/mL mempunyai pertumbuhan lesi 30.24 mm pada batang. Rawatan ini tidak mempunyai perbezaan yang signifikan dengan rawatan positif yang mengandungi racun kulat Ridomil. Ekstrak mentah *M. citrifolia* metanol, aseton dan etil asetat mengandungi sebatian antikulat dan boleh digunakan sebagai biofungisid dalam pengurusan penyakit kanker batang durian.

Kata Kunci: antikulat, *Durio zibethinus*, kanker, *Morinda citrifolia*, oomycete

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Siti Izera Ismail, PhD

Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Norhayu Asib, PhD

Senior Lecturer, TS.
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Tosiah Sadi, PhD

Principle Research Officer
Organic Farming Programme
Soil Science, Water and Fertilizer Research Centre
Malaysia Agriculture Research and Development Institute
(Member)

ZALILAH MOHD SHARIFF, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 June2024

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
ITS	Internal Transcribed Spacer
COXI	Cytochrome c oxidase subunit 1
COXII	Cytochrome c oxidase subunit 2
Ha	Hectare
°C	Degree Celsius
Mt	Metric Ton
BLAST	Basic Local Alignment Search Tool
RNA	Ribonucleic Acid
%	Percentage
CMA	Corn Meal Agar
g	Gram
L	Litre
mL	Millilitre
PCNB	Pentachloronitrobenzene
V8	Campbell's Vegetable 8 Juice
μL	Microliter
TBE	Tris borate EDTA
kb	Kilobase
V	Volts
PCR	Polymerase chain reaction
ANOVA	Analysis of Variance
PDA	Potato Dextrose Agar

L/B	Length/ breadth
bp	Base pair
rpm	Revolutions per minute
ppm	Parts per million
ATP	Adenosine triphosphate
EC ₅₀	Effective Concentration
mg	Miligram
SAS	Statistical Analysis System
PIRG	Percentage inhibition of radial growth

CHAPTER 1

INTRODUCTION

Durian (*Durio zibethinus*) is a sought-after fruit especially in Asian countries. In Malaysia specifically, this economically important crop valued at RM 7.49 billion (Department of Agriculture, 2020) is the largest cultivated crop making it a major source of income for both small scale and commercial farmers. Stem canker disease caused by *Phytophthora palmivora* is still a major issue for durian growers. *Phytophthora palmivora* is considered one of the most destructive organisms causing stem canker in almost all growth stages of durian. Despite several efforts conducted to mitigate the losses caused by this disease, the pathogen's diversity has been a significant element in disease management. Hence, analysis of the genetic diversity is essential in understanding the species' evolutionary process and the development of an effective management strategy (Mohamed Azni *et al.*, 2019). The preliminary step in identifying the *Phytophthora* species is based on morphological characteristics. As to complete the identification of the microorganism, molecular identification techniques are used as it is rapid, more accurate and sensitive (Capote *et al.*, 2012). Advances in molecular methods for detecting microorganisms have helped to a better understanding of the phylogeny and molecular diversity of organisms such as the *Phytophthora* genus (Scibetta *et al.*, 2012). Furthermore, molecular approaches have been widely employed to distinguish closely related species and strains within a species (Singh *et al.*, 2006).

It is stated in a phylogenetic study conducted by Baldauf *et al.* (2000), that true fungi and oomycetes are distantly related as they have different metabolic pathways. As a result, most fungicides used in the agriculture industry are ineffective in the treatment of plant disease caused by the oomycete pathogens. Thus, rapid pathogen identification is critical for disease control in terms of quarantine and disease transmission in planting materials. Several management measures have been used to control stem canker of durian, including phytosanitary measures, cultural approaches, chemical control, biological control and genetic resistance, but none have shown acceptable results or entirely control the disease so far (Guest, 2007). As a result, research into new approaches of managing such pathogens are of importance.

The *Morinda citrifolia* is a medicinally valued plant that is cultivated for its fruits, leaves and roots (Zin *et al.*, 2002). Not only are the leaves consumed as a vegetable (Zhang *et al.*, 2016), it has long been used for prevention of various chronic diseases (Lim *et al.*, 2016). Considering *M. citrifolia* leaves are commonly found in Malaysia, the use of this plant extract can be a beneficial complement to chemical fungicides.

There are currently no publications on the genetic diversity of *P. palmivora* of durian plantations in Malaysia. Moreover, identification and characterization of

this pathogen using the internal transcribed spacer (ITS) and Cytochrome c oxidase (COX) region. Also, the control of this disease is limited and there is no information on the use of *M. citrifolia* against this pathogen.

The main objectives of the study were:

- i. to identify and characterize *Phytophthora palmivora* isolates causing stem canker disease on *Durio zibenthinus* using morphological, molecular and pathogenic characteristics
- ii. to identify the compounds of *Morinda citrifolia* leaf crude extracts extracted using methanol, acetone, ethyl acetate and hexane
- iii. to evaluate *in vitro* and *in vivo* efficacy of *Morinda citrifolia* leaf crude extracts against the mycelial growth of *Phytophthora palmivora*

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