



UNIVERSITI PUTRA MALAYSIA

***ISOLATION, CHARACTERIZATION AND HISTOPATHOLOGICAL STUDY
OF PATHOGENIC BACTERIA ASSOCIATED WITH CROWN ROT OF
PAPAYA (*Carica papaya* L.) IN PENINSULAR MALAYSIA***

BIBI NAZIHAH BINTI MOHD DIN

FP 2014 57



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By

BIBI NAZIHAH BINTI MOHD DIN

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

July 2014

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

ISOLATION, CHARACTERIZATION AND HISTOPATHOLOGICAL STUDY OF PATHOGENIC BACTERIA ASSOCIATED WITH CROWN ROT OF PAPAYA (*Carica papaya* L.) IN PENINSULAR MALAYSIA

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July 2014

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Papaya, *Carica papaya* L. is a fast-growing, semi-woody tropical herb and is one of the major fruit crops worldwide that is primarily consumed as fresh fruit. The fruits consist mostly of water and carbohydrate, and are low in calories and rich in natural vitamins and minerals. It is an economically important fruit crop grown in Malaysia with an export value of about RM100-120 million per year. In 2006, a dieback disease was detected in the west coast states of peninsular Malaysia, affecting 800 hectares with significant yield losses and resulting in the destruction of 1 million trees. Affected plants initially show greasy and water-soaked lesions on young stems, petiole axis and buds (crown parts). A total of 40 bacterial isolates were isolated from diseased crowns but only one bacterium (KD33) fulfilled Koch's Postulate. The bacterium was successfully identified as *Chryseobacterium indologenes* based on morphological characteristics and biochemical tests as described in the literature. The pathogenicity of the bacterium (*C. indologenes*) to its host (papaya) was confirmed by Koch's postulate through repeated trials in glasshouse experiments. As the disease develops, necrotic lesions spread downward into the internal tissues resulting in shrinking and drying up of seedlings, and leading to dieback and death of trees, while no symptoms were observed in control plants. The Biolog identification system was used to identify the bacterium as *C. indologenes/gluem* with a similarity (SIM) index value of between 0.5 and 0.74 at 24 hours of incubation. A high infection probability was recorded. Molecular genotypic identification was used to confirm that the pathogenic bacterium was truly *C. indologenes* with a 100% sequence similarity and no nucleotide difference between the isolate and reference strain (*C. indologenes* strain LMG 8337; GenBank Accession Number: NR_042507.1). Neighbour-joining

phylogenetic tree analysis revealed that the bacterium belonged to the genus *Chryseobacterium* and this was supported by a high bootstrap value (99%). The disease progress rate on the petiole axis was the highest ($r_L = 0.58$ unit/day), followed by buds ($r_L = 0.51$ unit/day) and young stems ($r_L = 0.50$ unit/day), while the AUDPC was highest for petiole axis (680 unit²), followed by young stems (600 unit²) and buds (580 unit²). The histological study using light and electron microscopy confirmed the localization and distribution of the isolate bacterium in the infected host, thus demonstrating the high pathogenicity of the *C. indologenes* bacterium towards papaya. This is a first report of *C. indologenes* as the pathogen of papaya crown rot in Peninsular Malaysia.



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

PENGASINGAN, PENCIRIAN DAN KAJIAN HISTOPATOLOGI PADA BAKTERIA BERPATOGEN BERKAITAN DENGAN PENYAKIT REPUT SILARA PADA POKOK BETIK (*Carica papaya* L.) DI SEMENANJUNG MALAYSIA

Oleh

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Betik, *Carica papaya* L. adalah sejenis tumbuhan yang membesar dengan cepat, herba tropika separa-kayu, dan salah satu daripada tumbuhan di dunia yang biasanya digunakan sebagai buah-buahan segar. Kandungan buahnya terdiri daripada air dan karbohidrat, kalori rendah dan kaya dengan vitamin semula jadi dan mineral. Ia merupakan tanaman ekonomi yang penting di Malaysia dengan nilai eksport kira-kira RM100-120 juta setahun. Pada tahun 2006, penyakit mati rosot dikesan di negeri pantai barat semenanjung Malaysia, yang menjejaskan 800 hektar dengan hasil kerugian yang ketara dan mengakibatkan kemusnahan 1 juta pokok. Pokok yang terjejas pada mulanya menunjukkan gejala lesi berair dengan kilauan gris pada bahagian batang muda, paksi daun dan pucuk (bahagian silara). Sebanyak 40 isolat bakteria telah diasingkan daripada bahagian silara yang berpenyakit tetapi hanya satu bakteria sahaja (KD33) yang memenuhi dalil Koch. Bakteria ini telah berjaya dikenalpasti sebagai *Chryseobacterium indologenes* berdasarkan kepada ciri morfologi dan ujian biokimia seperti yang dinyatakan dalam sorotan kajian. Kepatogenan bakteria (*C. indologenes*) terhadap perumahannya (betik) telah disahkan oleh dalil Koch menerusi percubaan berulang dalam eksperimen rumah kaca. Sebaik sahaja penyakit ini berkembang, lesi nekrotik merebak ke bawah ke dalam tisu dalaman mengakibatkan pengecutan dan pengeringan anak benih, yang membawa kepada mati rosot dan kematian pokok manakala tiada gejala yang diperhatikan pada pokok kawalan. Sistem pengenalan Biolog digunakan untuk mengenal pasti bakteria ini sebagai *C. indologenes/gluem* dengan persamaan (SIM) nilai indeks antara 0.5 dan 0.74 pada tempoh 24 jam inkubasi. Kebarangkalian jangkitan yang tinggi telah

direkodkan. Pengenalan genotip molekul telah digunakan untuk mengesahkan bahawa bakteria patogenik ini adalah benar *C. indologenes* dengan 100% persamaan jujukan dan tiada perbezaan nukleotid antara isolat dan strain rujukan (*C. indologenes* strain LMG 8337; GenBank Accession Number: NR_042507.1). Analisis 'neighbor-joining' filogenetik mendedahkan bahawa bakteria ini tergolong dalam genus *Chryseobacterium* dan ini disokong oleh nilai bootstrap yang tinggi (99%). Kadar perkembangan penyakit pada paksi daun adalah yang tertinggi ($r_L = 0.58$ unit/hari), diikuti oleh pucuk ($r_L = 0.51$ unit/hari) dan batang muda ($r_L = 0.50$ unit/hari), manakala AUDPC adalah tertinggi pada paksi daun (680 unit²), diikuti oleh batang muda (600 unit²) dan pucuk (580 unit²). Kajian histologi menggunakan mikroskop cahaya dan elektron mengesahkan penyetempatan dan penyebaran isolat bakteria di dalam perumah yang dijangkiti, seterusnya menunjukkan kepatogenan yang tinggi oleh bakteria *C. indologenes* terhadap betik. Ini adalah laporan pertama *C. indologenes* sebagai patogen kepada reput silira betik di Semenanjung Malaysia.

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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:

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LIST OF ABBREVIATIONS

g	Gram
mg	Milligram
%	Percentage
IU	International unit
cm	Centimeter
mm	Millimeter
°C	Degree Celsius
mg/L	Milligram/liter
μm	Micrometer
Kcal	Kilocalorie
Mt	Metric ton
kg	Kilogram
Ha	Hectare
cm ²	Centimeter square
cm ³	Centimeter cubic
rpm	Rotation per minute
ml	Milliliter
μl	Micro liter
ng	Nanogram
μm	Micrometer
v/v	Volume/volume
cfu	Colony forming unit
OD	Optical density
RH	Relative humidity
cc/ml	Centimeters cubic /milliliters

NA	Nutrient Agar
NB	Nutrient Broth
r_L	Apparent infection rate values obtained from epidemic rate by transforming disease severity data using logistic model
DI	Disease incidence
DS	Disease severity
SE	Standard Error
R^2	Coefficient of determination
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
sp	Species
nm	Nanometer
M	Molar
mM	Milimolar
bp	Base pair
V	Volt
min	Minute
h	Hour
sec	Seconds
SIM	Similarity
EDTA	Ethylenediaminetetraacetic acid
CO ₂	Carbon dioxide
ANOVA	Analysis of Variance
CRD	Completely Randomized Design
AUDPC	Area Under Disease Progress Curve
LM	Light Microscopy
SEM	Scanning Electron Microscopy



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CHAPTER 1

INTRODUCTION

Papaya, as its scientific name *Carica papaya* L., is a fast-growing, semi-woody tropical herb (Chan and Theo, 2000) and is one of the most important global fruit crops that are primarily consumed as fresh fruit. The fruits consist mostly of water and carbohydrate, have small amount of calories and rich in natural vitamins and minerals (Chan and Tang, 1979). It is consumed as jams, pickles, and desserts. Papaya plants are also grown commercially for papain and chymopapain, two industrially important proteolytic enzymes found in the milky white latex exuded by unripe fruits (Madrigal *et al.*, 1980). It is an economically important fruit crop grown in Malaysia with an export value of about RM100-120 million per year (Rabu and Mat, 2005). However, it has been affected by a very serious disease which is crown rot that leads to dieback disease. It was first reported in Batu Pahat by the Johor State Department of Agriculture in late 2003 and another incidence was later reported in Bidor, Perak, in October 2004 (Noriha *et al.*, 2011). By the end of 2006 it had spread to five states on the west coast of Peninsular Malaysia, affecting 800 ha with significant yield losses and resulting in the destruction of one million trees (Maktar *et al.*, 2008b).

A previous study had reported that the causal agent of papaya dieback in Malaysia was *Erwinia papayae* (Maktar *et al.*, 2008a, 2008b), but the latest study by Noriha *et al.* (2011) showed that *E. mallotivora* was the causal pathogen of papaya dieback in peninsular Malaysia. However, no significant biochemical tests were performed to distinguish between *E. papayae* and *E. mallotivora*, which are closely related species. *Erwinia papayae* was also first reported by Garden *et al.* (2004) as the causal organism of papaya bacterial canker in the Caribbean region, while *E. mallotivora* was reported to cause leaf spot in *Mallotus japonicus* (Japanese common name: Akamegashiwa) (Masao, 1976). The early symptoms of the disease include yellowing and necrosis (formation of brown spots) along the leaf edges and mid-ribs (Anonymous, 2009) followed by greasy and water-soaked lesions on the bases of leaf stalks (Anonymous, 2009) and crowns (Maktar *et al.*, 2008b). Besides, the symptoms on fruits include the appearance of dark spots on the skin (Anonymous, 2009) and water-soaked lesions on the flesh (DOA, Malaysia). Later, necrotic and water-soaked areas developed on stems and spread into internal tissues. In advanced stages, the bending of water-soaked leaf stalks occur leading to dieback and death of trees.

Since there were two bacterial species that have been reported as the causal pathogen of papaya dieback in Malaysia, the objectives of the present research were to re-confirm the causal agent of the disease through phenotypic and genotypic information, and conduct a host-pathogen interaction study to confirm the presence of bacteria in the host plant. Isolation was carried out on the crown parts of papaya plants that showed typical symptoms of rotting of young stems, petiole axis and buds.

The specific objectives of this study were to:

1. Isolate, identify and characterize the pathogenic bacteria associated with papaya crown rot and confirm its pathogenicity through Koch's Postulate.
2. Further confirm the identity of the causal bacterium through a combination of phenotypic and genotypic information.
3. Conduct histopathological study on the ability of the bacterial isolate to penetrate, colonize and establish in the infected host plant.



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