



UNIVERSITI PUTRA MALAYSIA

**EPIDEMIOLOGICAL AND MOLECULAR CHARACTERISATION OF
CITRUS HUANGLONGBING (HLB) DISEASE IN MALAYSIA**

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By

KHAIRULMAZMI AHMAD

**Thesis Submitted to the School of Graduate Studies,
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DEDICATION

Special dedication to:

My dearest father, Ahmad b. Abdullah, mother, Zaleha bt. Mohammad, brother, Khairul Naim and sisters; Nor Muzalni, Nor Asmizan and Nor Hazalzhiah for their endless and boundless love, understanding and encouragement throughout my study.



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

EPIDEMIOLOGICAL AND MOLECULAR CHARACTERISATION OF CITRUS HUANGLONGBING (HLB) DISEASE IN MALAYSIA

By

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Citrus huanglongbing (HLB) disease is considered as one of the most destructive diseases of citrus in Asian, African and American countries. It is caused by *Candidatus Liberibacter* species. In Asian countries, the causal agent is *Candidatus Liberibacter asiaticus*. Surveys of the HLB disease on infected citrus trees were carried out in the major citrus growing areas in Peninsular Malaysia namely Selangor, Pahang, Kelantan and Pahang. The occurrence of HLB disease was confirmed by polymerase chain reaction (PCR) and transmission electron microscope (TEM) tests. *Candidatus Liberibacter asiaticus* was detected positive in samples collected from honey mandarin (*Citrus reticulata*), pummelo (*C. grandis*), Mexican lime (*C. aurantifolia*), mandarin (*C. suhueinsis*), calamondin (*C. madurensis*), Cleopatra (*C. reticulata*), Troyer citrange (*Poncirus trifolia* X *C. sinensis*) and citrimelo (*P. trifolia* X *C. paradise*). Field-infected citrus trees showed typical symptoms of HLB disease such as interveinal chlorosis, green vein, dieback of twigs, lopsided fruit shape, small fruit size and premature



fruit drop easily. All the major citrus growing areas in Peninsular Malaysia were found infected with HLB disease. The percentage mean estimate disease incidence ranged from 28.3 - 53.8% and 0.0 - 46.03% depending on localities and citrus species, respectively. The seriousness of the disease justified further studies on the epidemiology and molecular characterization of the causal agent, *Candidatus Liberibacter asiaticus* in Malaysia. HLB vector, *Diaphorina citri* were more abundant in the lowland areas such as Selangor and Terengganu. It was absence in Cameron Highland, Pahang but moderate level was recorded in Lojing Highland, Kelantan. Further study on characterization and strain differentiation of Malaysian *Candidatus Liberibacter asiaticus* isolates was carried out. Based on their biological (pathological) properties, Malaysian isolates exhibited six types of HLB symptoms such as olive green of leaves, green vein, vein yellowing, mottling, stunted and dieback of twigs. In terms of disease incidence and disease severity reactions, Malaysian isolates showed sigmoid pattern of disease progress curves and caused polycyclic type of disease. Based on their aggressiveness, Malaysian isolates could be classified into three groups i.e. severe, moderate and mild. In terms of molecular properties, all isolates produced intense accumulation of starch granule inside the tissue of infected leaves. TEM study revealed that Malaysian isolates were pleomorphic and consisted of two types of bodies i.e. elongated and spherical forms. The body lengths ranged from 100-1200 nm depending on their body shape and the isolates. Differentiation of their modal length and composition ratio revealed that the Malaysian isolates could be classified into two groups i.e. group 1 comprises



of GFB-T and GFB-S and group 2 comprises of GFB-PK. Characterization of their 16S rDNA gene sequences revealed that Malaysian isolates produced about 1156-1167b.p of nucleotide sequences. Gene sequences between Malaysian isolates showed high percentage of nucleotide similarity that ranged from 96-99%. Similar trends were observed on their genetic distances. Analysis of outer membrane protein (OMP) gene also showed differences between Malaysian isolates tested, namely GFB-Mandarin and GFB-Pummelo. A study on host preference of *D. citri* and susceptibility of citrus species against GFB-T isolate indicated that *D. citri* most preferred to colonize and feed on jasmine orange (*Murraya paniculata*) followed by sour orange (*C. aurantium*), pummelo and honey mandarin. Host susceptibility study revealed that jasmine orange and pummelo were resistant to HLB infection while sour orange was found to be tolerant. Calamondin was susceptible and honey mandarin was very susceptible to HLB infection. The effect of calcium, zinc and copper application on plants' recovery and severity following infection of HLB disease showed slight protection against the HLB bacterium but was inconsistent in some cases. Citrus trees treated with combination of calcium and zinc at 600 ppm, and 10 ppm respectively resulted to significant increase ($P \leq 0.05$) in terms of fruit production and total soluble solid (TSS) content. This treatment also resulted in reduction of AUDPC value of disease severity and also improved mean leaf length, mean leaf width and mean leaf area. To date, there is no successful treatment available throughout the world including Malaysia to control HLB disease in the orchards. Perhaps the combination of present treatment together with good agriculture

practices could improve the efficiency of HLB management in the orchards by enhancing tree immunity, delaying disease onset to prolong lifespan of citrus trees.



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**EPIDEMIOLOGI DAN PENCIRIAN MOLEKUL PENYAKIT HUANGLONGBING
(HLB) LIMAU DI MALAYSIA**

Oleh

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APRIL 2008

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Penyakit huanglongbing (HLB) limau, adalah dianggap sebagai salah satu daripada penyakit limau yang paling merbahaya di negara-negara Asia, Afrika dan Amerika. Ianya disebabkan oleh spesis *Candidatus Liberibacter*. Di negara-negara Asia, agen penyebab adalah *Candidatus Liberibacter asiaticus*. Tinjauan penyakit HLB terhadap tanaman limau telah dijalankan di kawasan-kawasan penanaman limau utama seperti Selangor, Pahang, Kelantan dan Terengganu. Kejadian penyakit ini telah disahkan menggunakan kaedah transmisi elektron mikroskop (TEM) dan reaksi berangkai polimerase (PCR). *Candidatus Liberibacter asiaticus* telah dikesan positif pada sampel-sampel yang diambil daripada honey mandarin (*Citrus reticulata*), pummelo (*C. grandis*), Mexican lime (*C. aurantifolia*), mandarin (*C. suhuiensis*), calamondin (*C. madurensis*), Cleopatra (*C. reticulata*), Troyer citrange (*Poncirus trifolia* X *C. sinensis*) dan citrimelo (*P. trifolia* X *C. paradisi*). Pokok limau terjangkit menunjukkan simptom-



simptom jangkitan HLB yang tipikal seperti klorosis di antara urat daun, urat daun menghidu, mati rosot pada reranting, bentuk buah yang tidak simetri, buah kecil dan mudah gugur. Semua kawasan penanaman limau utama di Semenanjung Malaysia didapati telah dijangkiti oleh penyakit HLB. Peratusan purata jangkakan kejadian penyakit adalah di antara 28.3-53.8% and 0.0-46.03% bergantung kepada kawasan dan jenis limau. Keterukan kejadian penyakit ini menjastifikasikan kajian epidemiologi dan pencirian molekul terhadap agen penyebab, *Candidatus Liberibacter asiaticus* di Malaysia. Vektor penyakit HLB, *Diaphorina citri* banyak ditemui di kawasan rendah seperti Selangor dan Terengganu. Ianya tidak dapat dikesan di kawasan tinggi seperti Tanah Tinggi Cameron, Pahang tetapi ditemui pada peringkat sederhana di Tanah Tinggi Lojing, Kelantan. Kajian lanjutan mengenai pencirian dan perbezaan strain *Candidatus Liberibacter asiaticus* Malaysia telah dijalankan. Berdasarkan pencirian biologi (patologi) isolat-isolat Malaysia menunjukkan enam jenis simptom jangkitan HLB seperti daun hijau zaiton, urat daun kehijauan, urat daun kekuningan, daun bercapuk, kerencatan dan mati rosot pada reranting. Berdasarkan reaksi kejadian penyakit dan keterukan penyakit, isolat-isolat Malaysia menunjukkan corak 'sigmoid' pada keluk perkembangan penyakit dan menyebabkan jangkitan jenis 'polycyclic'. Berdasarkan kepada peringkat kegarangan, isolat-isolat Malaysia dapat dibahagikan kepada tiga kumpulan iaitu parah, sederhana dan ringan. Berdasarkan pencirian diperingkat molekul pula, semula isolat mengasikkan pengumpulan kanji yang banyak di dalam tisu daun terjangkit. Kajian TEM, menunjukkan semua isolat Malaysia adalah berbentuk



'pleomorphic' dan mempunyai dua jenis jasad iaitu memanjang dan bulat. Panjang jasad adalah di antara 100-1200nm bergantung kepada jenis jasad dan isolat. Pengasingan isolat berdasarkan panjang modal jasad dan nisbah komposisi jasad menunjukkan ianya terbahagi kepada dua kumpulan iaitu kumpulan 1 mengandungi GFB-S dan GFB-T manakala kumpulan 2 mengandungi GFB-PK. Pencirian terhadap jujukan gen 16S rDNA, semua isolate Malaysia menghasilkan jujukan nucleotida di antara 1156-1167bp. Jujukan gen pada isolat Malaysia menunjukkan peratusan kesamaan nukleotida yang tinggi, 96-99%. Corak yang sama dilihat pada jarak genetik mereka. Analisis gen outer membrane protein (OMP) juga menunjukkan perbezaan di antara isolat-isolat Malaysia yang dikaji iaitu GFB-Mandarin dan GFB-Pummelo. Kajian kegemaran perumah oleh *D. citri* dan kerintangan spesis limau terhadap GFB-T menunjukkan bahawa *D. citri* sangat gemar untuk mengkolonisasi dan menghisap sap daun jasmine orange (*Murraya paniculata*) diikuti sour orange (*C. aurantium*), pummelo dan honey mandarin. Kajian kerintangan perumah menunjukkan jasmine orange dan pummelo sangat rintang terhadap jangkitan PGL. Sour orange pula sangat toleran. Calamondin adalah rentang dan honey mandarin adalah sangat rentang terhadap jangkitan HLB. Kesan penggunaan kalsium, zink and kuprum terhadap pemulihan dan keparahan pokok terjangkit oleh penyakit HLB telah dijalankan. Rawatan menunjukkan terdapat sedikit perlindungan terhadap bakteria HLB tetapi keputusannya tidak stabil pada sesetengah kes. Dalam kajian lapangan, pokok limau yang dirawat menggunakan kombinasi kalsium dan zink pada kepekatan 600 dan 10ppm telah



memberi kesan peningkatan yang bererti ($P \leq 0.05$) terhadap penghasilan buah dan jumlah pepejal terlarut (TSS). Rawatan ini juga mengurangkan nilai AUDPC bagi keterukan penyakit dan menambah purata panjang daun, purata lebar daun dan purata luas permukaan daun. Buat masa ini, tiada rawatan berkesan di dunia mahupun di Malaysia untuk mengawal penyakit HLB di ladang. Semoga dengan kombinasi rawatan ini bersama dengan amalan pertanian yang baik boleh meningkatkan imuniti pokok, melambat jangkitan penyakit seterusnya melanjutkan hayat tanaman.



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I certify that an Examination Committee has met on 28 April 2008 to conduct the final examination of Khairulmazmi bin Ahmad on his Doctor of Philosophy thesis entitled "Epidemiological and Molecular Characterisation of Citrus Huanglongbing Disease in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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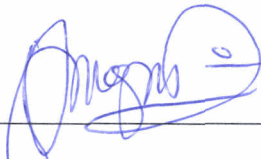
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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



KHAIRULMAZMI B. AHMAD

Date: 30/5/2008

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