UNIVERSITI PUTRA MALAYSIA

DETECTION OF CITRUS GREENING ORGANISM (LIBEROBACTER ASIATICUM) BY POLYMERASE CHAIN REACTION

MUH. ASAAD

FP 2001 8

DETECTION OF CITRUS GREENING ORGANISM (*LIBEROBACTER ASIATICUM*) BY POLYMERASE CHAIN REACTION

MUH. ASAAD

MASTER OF AGRICULTURAL SCIENCE
UNIVERSITI PUTRA MALAYSIA

2001

DETECTION OF CITRUS GREENING ORGANISM (*LIBEROBACTER ASIATICUM*) BY POLYMERASE CHAIN REACTION

By

MUH. ASAAD

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Agricultural Science in the Faculty of Agriculture Universiti Putra Malaysia

September 2001

DEDICATION

To

My parents, Mahmud Sara and Sitti Nurhayati

My wife, Warda Mustafa

My brothers and sisters

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

DETECTION OF CITRUS GREENING ORGANISM (*LIBEROBACTER ASIATICUM*) BY POLYMERASE CHAIN REACTION

By

MUH. ASAAD

September 2001

Chairman: Associate Professor Kamaruzaman Sijam, Ph.D.

Faculty: Agriculture

Citrus greening disease caused by greening organism (GO; *Liberobacter asiaticum*) is one of the most destructive diseases of citrus in Malaysia and Indonesia. To detect the GO in infected plant tissues, Polymerase Chain Reaction (PCR), an accurate, rapid and reliable detection method was applied to detect the 16S rDNA fragments of the GO in leaves showing one of several typical symptoms of greening collected from GO-infected mandarin trees in Malaysia and Indonesia.

In GO-infected mandarin trees, four typical symptoms of greening on leaves were observed, namely mottling (type I), mild chlorosis with green veins (type II), severe chlorosis with green veins (type III) and vein yellowing (type IV). Types II and III symptoms were mostly found in GO-infected mandarin trees in the field, followed by type I symptom, while type IV symptom was rare.

Before PCR was used for the detection of GO in infected plant tissues, several experiments relating to the optimization of the PCR condition were conducted. Results indicated that the best sample of citrus tissues for DNA extraction was the midrib plus the petiole. This can be shown by more intense band observed after agarose gel electrophoresis. A positive amplification was still visible when the reaction mixture contained 10 ng of total DNA was used. Results of the optimization of the PCR condition indicated that the optimal PCR buffer for amplification of GO's DNA was the standard buffer containing 78 mM Tris-HCl (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol and 200 μg of Bovine Serum Albumin (BSA). The optimal concentrations of $MgCl_2$, dNTP, primer and *Taq* DNA polymerase to be used in reaction mixture were 1.5 mM, 0.2 mM, 0.4 μM, and 1 Unit, respectively. The optimal annealing temperature and number of cycles of PCR condition were 55°C and 40 cycles, respectively.

The 16S rDNA fragments of the GO in expected size of 1160 bp were detected in each typical symptoms. These fragments were amplified from DNA extracted from mandarin cultivars infected with the GO and were not amplified from DNA extracted from healthy trees. These fragments were also detected in insect vector (*Diaphorina citri*) collected from GO-infected mandarin trees and were not amplified from DNA extracted from healthy vector collected from *Murayya paniculata* using cetyl trimethyl ammonium bromide (CTAB) method for DNA extraction.

Restriction enzyme analysis of the representative samples of six citrus growing areas in Malaysia and Indonesia indicated that the 16S rDNA fragments of GO were each digested into two fragments of the expected size of 640 bp and 520 bp using restriction enzyme ***Xba*I**. Therefore, it was confirmed that GO from infected mandarin trees in Malaysia and Indonesia was *L. asiaticum*.

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

PENGESANAN ORGANISMA PENYAKIT GREENING (*LIBEROBACTER ASIATICUM*) PADA LIMAU MELALUI TINDAKBALAS RANTAIAN POLIMERASE

Oleh

MUH. ASAAD

September 2001

Pengerusi: Profesor Madya Kamaruzaman Sijam, Ph.D.

Fakulti: Pertanian

Penyakit 'citrus greening' yang disebabkan organisma 'greening' (GO; *Liberobacter asiaticum*) merupakan salah satu penyakit yang paling merosakkan pokok limau di Malaysia dan Indonesia. Untuk mengesan kehadiran GO dalam tisu tanaman berpenyakit, tindakbalas rantaian polimerase (PCR), satu teknik pengesanan yang tepat, cepat dan boleh dipercayai telah digunakan untuk mengesan serpihan 16S rDNA GO pada daun-daun limau yang menunjukkan beberapa simptom tipikal penyakit 'greening'. Daun-daun ini diperolehi daripada pokok limau mandarin yang dijangkiti GO di Malaysia dan Indonesia.

Pada pokok limau mandarin yang dijangkiti GO, empat simptom tipikal penyakit 'greening' pada daun-daun telah diperhatikan iaitu 'mottling' (jenis I), klorosis yang tidak ketara (mild chlorosis with green vein, jenis II), klorosis yang ketara

(severe chlorosis with green vein, jenis III) dan urat daun menguning (vein yellowing, jenis IV). Simptom jenis II dan III adalah paling banyak ditemui pada pokok limau mandarin yang dijangkiti GO di lapangan, diikuti oleh simptom jenis I, manakala simptom jenis IV adalah simptom yang jarang ditemui.

Sebelum tindakbalas rantaian polimerase (PCR) digunakan untuk mengesan kehadira GO dalam tisu tanaman berpenyakit, beberapa kajian berhubung dengan pengoptimuman keadaan PCR dijalankan. Keputusan menunjukkan bahawa tisu limau yang terbaik digunakan untuk pengekstrakan DNA adalah 'midrib' dan 'petiole'. Ini dapat dilihat daripada produk PCR yang lebih terang diperhatikan setelah elektroforesis gel agarose. Amplifikasi yang positif masih boleh dilihat apabila campuran PCR mengandungi 10 ng DNA yang telah ditulinkan digunakan. Keputusan pengoptimuman keadaan PCR menunjukkan bahawa penimbal PCR yang optimum untuk amplifikasi DNA GO adalah penimbal yang lazim digunakan (standard) yang mengandungi 78 mM Tris-HCl, 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol dan 200 μg Bovine Serum Albumin (BSA). Kepekatan yang optimum untuk $MgCl_2$, dNTP, primer dan *Taq* DNA polimerase yang digunakan dalam campuran PCR adalah masing-masing 1.5 mM, 0.2 mM, 0.4 μM dan 1 unit. Suhu pelekatan primer dan bilangan kitaran untuk keadaan PCR adalah masing-masing 55°C dan 40 kitaran.

Serpihan 16S rDNA GO pada saiz 1160 bp yang dijangkakan dikesan pada setiap simptom tipikal penyakit 'greening'. Serpihan ini diamplifikasi daripada DNA yang diekstrak daripada limau mandarin yang dijangkiti oleh GO dan tidak diamplifikasi daripada DNA yang diekstrak daripada pokok limau yang sihat. Serpihan ini juga boleh dikesan pada vektor serangga (*Diaphorina citri*) yang diperolehi daripada pokok limau mandarin yang dijangkiti oleh GO dan tidak dikesan daripada DNA yang diekstrak daripada vektor sihat.

Keputusan analisis endonuclease pembatas untuk sampel-sampel daripada enam kawasan penanaman limau di Malaysia dan Indonesia menunjukkan bahawa serpihan-serpihan 16S rDNA GO masing-masing dipotong menjadi dua serpihan yang dijangkakan iaitu 640 bp dan 520 bp menggunakan enzim endonuclease pembatas X*ba*I. Oleh yang demikian, hasil kajian ini mengesahkan bahawa organisma 'greening' yang terdapat pada pokok limau mandarin di Malaysia dan Indonesia adalah *L. asiaticum*.

ACKNOWLEDGEMENTS

Praises to almighty Allah, whose blessing have enabled the author to complete this project.

The author expresses his sincere appreciation to Assoc. Prof. Dr. Kamaruzaman Sijam, the chairman of the supervisory committee for his active guidance, valuable advice, and generous help during the research work and preparation of this thesis. Gratitudes are also due to Dr. Suhaimi Napis, and Assoc. Prof. Dr. Mohd. Idris Zainal Abidin, the members of the supervisory committee for their fruitful suggestions and effective corrections in order to improve the quality of manuscript.

The author gratefully acknowledges the ARMP-II Project for the financial support for the study under Program of the Master of Agricultural Science. A very special appreciation is due to Dr. Ir. Endang Setyawati T., MSc. (ARMP Project Leader) who is in charge of this project, for her generosity and help in overcoming living problems in Malaysia. Thanks are also extended to UPM's technical and support staff notably Encik Yusuf, Encik Zawawi, Cik Junaina, Mr. Ong, and many others for providing the author with moral encouragement and homely atmosphere. Special thanks are due to all the staff members of the Faculty of Agriculture of UPM for their help in one way or other.

The author is indebted to Agency for Agricultural Research and Development (AARD) for approving a full-time deputation and for encouraging the author to continue his postgraduate study abroad.

Last but not least, the author is extremely grateful to his mother Sitti Nurhayati, his father Mahmud Sara, and his wife Warda Mustafa, for their sacrifice, patience and endless prayers without which this project could never have been completed.

I certify that an Examination Committee met on 27th September 2001 to conduct the final examination of Muh. Asaad on his Master of Agricultural Science thesis entitled "Detection of Citrus Greening Organism (*Liberobacter asiaticum)* by Polymerase Chain Reaction" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination committee are as follows:

Sariah Meon, Ph.D.
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairperson)

Kamaruzaman Sijam, Ph.D.
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Mohd. Idris Zainal Abidin, Ph.D.
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Suhaimi Napis, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

MOHD. GHAZALI MOHAYIDIN, Ph.D.
Professor/Deputy Dean of Graduate School
Universiti Putra Malaysia

Date: 9 OCT 2001

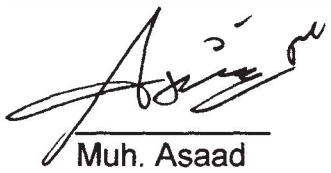
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Agricultural Science.

AINI IDERIS, Ph.D.
Professor
Dean of Graduate School
Universiti Putra Malaysia

Date: 13 DEC 2001

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.



Muh. Asaad

Date: 8 oktober 2001

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Chemicals / Units

CTAB	Cetyl trimethyl ammonium bromide
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
$MgCl_2$	Magnesium chloride
rDNA	ribosomal DNA
TBE	Tris-Borate- EDTA
TE	Tris-EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
bp	base pair
hr	hour
kb	kilobase
mg	milligram
min	minute
mM	millimolar
ng	nanogram
OD	optical density
pmol	picomole
sec	second
µL	microliter
µM	micromolar

CHAPTER 1

INTRODUCTION

1.1. Background of the Study

Citrus (*Citrus* spp.) is one of the major world trade commodities both as fresh or processed fruits. Citrus fruits comprising oranges, mandarins, lemons, limes, grapefruits, and pomelos are widely grown in all tropical and subtropical areas of the world. In Indonesia and Malaysia particularly, mandarin cultivar (*Citrus reticulata* Blanco) is widely cultivated by farmers because of its early vigour, well-adapted in the lowland, and offering a good income (Aubert, 1992; Ko, 1992; Sastijati, 1992; Sugiyanto, 1992).

In the world, the main citrus production countries are Brazil, USA, China, Spain, Mexico, Iran and Italy (FAO, 2001). Whereas, in South East Asia are Thailand, Indonesia and Philippines (Xu et al., 1991; FAO, 2001). During the period of1990 to 1998, the total harvested area and production of citrus in Indonesia averaged at 44,433 ha and 508,780 t, respectively (Ministry of Agriculture, Republic of Indonesia, 2001).

Production of citrus in Asian countries, particularly China and South East Asian countries decreased sharply since 1980's because of the presence of several major diseases. One of the most destructive diseases of citrus is citrus greening disease (CGD) that caused crop and tree loss in

many parts of Asia and Africa. Before it was identified as one disease, it was known by various other names such as yellow shoot (huanglungbin) in China (Lin and Lin, 1990), likubin (decline) in Taiwan (Su and Huang, 1990), dieback in India, leaf mottle in Philippines (Gonzales, 1987), citrus vein phloem degeneration (CVPD) in Indonesia, and yellow branch or greening in South Africa (da Graca, 1991). As it became clearer that all these were similar disease, the name "greening" was widely adopted.

Losses from greening to the citrus industry in detail have not been much published, but some data of the severity of the disease in several citrus-growing areas have been published. For example, in China the citrus production in Yangcun farm (one of the largest citrus farms) dropped to 5,000 t in 1982 from 450,000 t in 1977 because of huanglungbin (greening) disease (Ke, 1987), In Indonesia, estimated more than 8 million trees were infected and caused an annual loss of US$ 22,000 (Nurhadi et al., 1992) because of greening disease.

In Malaysia, evidence of the occurrence of greening disease was known after efforts on greening disease research were intensified by Malaysian government with FAO/UNDP since 1988 (Lim et al., 1990). Based on the typical greening symptoms, the presence of the vector (*Diaphorina citri*), and the positive results of transmission tests, greening disease was found in Kuala Terla (Cameron Highland), Jelebu, Kuala Jerangau, Serdang

and Klang (Lim et al., 1990; Aubert, 1992; Osman and Lim, 1992). In Indonesia the greening disease, known by the name of Citrus Vein Phloem Degeneration (CVPD), has apparently penetrated the Indonesian archipelago in the 1940s (Aubert et al., 1985) and seriously affected citrus production in Indonesia since 1950 (Setyobudi et al., 1992).

Greening disease is caused by a nonculturable, phloem-limited bacterium, which was proposed by Jaqoueix et al. (1994) the name *Liberobacter asiaticum* in Asia and *L. africanum* in Africa, after the nucleotide sequences of the 16S rDNA of the Indian and African isolates of the GO have been determined. According to Jagoueix et al. (1996), Xbal restriction enzyme should hydrolyze the 16S rDNA of *L. asiaticum* into two fragments (640 bp and 520 bp) and that of *L. africanum* into three fragments (520 bp, 506 bp and 130 bp). Greening disease was initially considered that the causal agent of greening disease was a mycoplasma-like organism (MLO). However, this organism was soon found to be enclosed by a 25 nm thick envelope, which was much thicker than the unit membrane envelope characteristic of MLOs (7 to 10 nm thickness). By analogy with MLO, this organism was called Bacterium-like Organism (BLO) or greening organism (Nakashima et al., 1996). This organism is transmitted by two insect vectors, the psyllid *Diaphorina citri* in Asia and *Trioza erytreae* in Africa (Martinez and Wallace, 1969; McClean et al., 1969).