# DETECTION, ISOLATION AND CHARACTERIZATION OF TWO EGYPTIAN ISOLATES OF *SPIROPLASMA CITRI* FROM *CITRUS SINENSIS* TREES

**By**

**AYMAN FAISAL ABDOU OMAR**

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

**May 2006**

**DEDICATION**

To the soul of my beloved Prof Dr. Roshdy Abd- El-Bahi Omar in the heaven (Rahmatullah alieh), who regretfully did not live to see this work, my parents, brother, sisters, my fiancée Asmaa Mahmoud Emara, my father-in-law, my mother-in-law, my Prof. Dr. Shawky abd El-Rauf El-Kewey and Prof. Dr. Samir Armia Sidaros

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

**DETECTION, ISOLATION AND CHARACTERIZATION OF TWO EGYPTIAN ISOLATES OF *SPIROPLASMA CITRI* FROM *CITRUS SINENSIS* TREES**

By

**AYMAN FAISAL ABDOU OMAR**

**May 2006**

**Chairman: Associate Professor Kamaruzaman Sijam, PhD**

**Faculty: Agriculture**

Citrus stubborn disease is considered one of the most destructive diseases of citrus in the Mediterranean. It caused by *Spiroplasma citri* (Mollicutes, Order Entomoplasmatales, Family Spiroplasmataceae). Preliminary detection of *S. citri* of infected plants from Kafr El-Sheikh and El Qualubia governorates, Egypt was carried out using Dienes' stain of stem sections and electron microscope of ultrathin sections of leaf midribs. *Spiroplasma citri* was isolated from common orange (*Citrus sinensis*) trees showing typical symptoms of citrus stubborn disease and previously detected by Dienes' stain and electron microscope, one each from Kafr El-Sheikh and El-Qualubia. They were referred to as Fewa

and Qualubia isolates, respectively. The organisms were isolated from young leaves. Fried-egg shape colonies were obtained when *S. citri* was subcultured on C-3G solid medium. *S. citri* was confirmed using darkfield microscope, electron microscope and ELISA from *S. citri* cultures. Small helical filamentous and small round bodies were observed by dark field microscope, whereas electron microscope inspection detected helical structure of the filamentous organism and vesicular blebs on the filaments. The isolated organisms were confirmed by a positive reaction in ELISA test. Differences between the two *S. citri* isolates were detected by crossed immunoelectrophoresis (CIE) with intermediate gel and polyacrylamide gel electrophoresis. In the homologous reactions of Fewa isolate, eleven precipitin peaks were detected using CIE. Identical homologous reaction of Qualubia isolate was produced. One antigen (a) was specific for the Fewa isolate and one antigen (b) was specific for the Qualubia isolate when CIE with intermediate gel was used. One-dimensional electrophoresis analysis demonstrates very similar patterns of protein with two different minor protein bands between the two isolates of *S. citri.* Two Egyptian isolates of *S. citri* (Fewa and Qualubia) were studied based on nucleotide sequences of their spiralin and16S rDNA genes and 16S/23S rDNA intergenic spacer region. The 16S rDNA of the two isolates comprised 1529 nucleotides with a similarity

between them at 99.80% where only three nucleotide differences were observed. Both have more than 99.67% similarity to three published strains of *S. citri*, the R8A2HP, Car4 and Car6. Their 16S/23S rDNA intergenic spacer region has 305 nucleotides, with a similarity of about 99.76 %, where only one nucleotide difference was observed. The 16S/23S nucleotide sequence of Fewa isolate was identical to that of strains 169, Maroc and ATCC 27556. Five nucleotide differences were observed in spiralin gene sequences of the two isolates, which have 99.30 % similarity. The two spiralins comprised 241 amino acids with only three amino acids differences were observed between them, and their amino acids similarity was about 98.75%. Our findings indicated that both Fewa and Qualubia isolates have high similarity in the nucleotide sequences of their 16S rDNA, 16S/23S rDNA intergenic spacer region and the nucleotides and amino acids of spiralin gene. However, they were not identical and could be differentiated by using restriction enzyme *Bfa*I that digested at the recognition site CTAG within their spiralin gene sequence. Therefore, based on the above studies the two Egyptian isolates of *S. citri* (Fewa and Qualubia) are not identical.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENGESANAN, PENGASINGAN DAN PENCIRIAN DUA ISOLAT SPIROPLASMA CITRI DARIPADA LIMAU DI MESIR**

Oleh

**AYMAN FAISAL ABDOU OMAR**

**Mei 2006**

**Pengerusi: Profesor Madya Kamaruzaman Sijam, PhD**

**Fakulti: Pertanian**

Penyakit 'citrus stubborn' telah dianggap sebagai penyakit yang membawa kerosakan kepada limau di Mediterranean. Ia disebabkan oleh *Spiroplasma citri* (Mollicutes, order Entomoplasmateles, Famili Spiroplasmataceae). Pengesan awal pokok citrus yang dijangkiti oleh *S. citri* dari wilayah Kafr El-sheikh dan El-Qualubia di Mesir dengan menggunakan pewarnaan Dienes' pada bahagian batang dan mikroskop elektron terhadap potongan halus urat daun . *Spiroplasma citri* telah diisolat daripada pokok limau mandarin (*Citrus sinensis*) yang menunjukkan simptom-simptom tipikal penyakit 'citrus stubborn' yang sebelum dikesan melalui pewarnaan Dienes' dan mikroskop elektron. Setiap isolat merujuk kepada Fewa dan Qualubia.

Organisma-organisma ini telah diisolat daripada daun-daun muda. Koloni-koloni berbentuk telur goreng telah diperolehi apabila disub-kultur diatas media C-3G. *S. citri* telah dikenal pasti menggunakan mikroskop 'dark-field', elektron mikroskop dan ELISA daripada kultur *Spiroplasma citri* . Bebenang helikal kecil dan jasad berbulat kecil telah dilihat melalui mikroskop 'dark-field' manakala penelitian melalui mikroskop elektron, organisma bebenang berbentuk struktur helikal dan 'vesicular belb' pada filamen-filamen telah dikesan. Isolat-isolat organisma tersebut telah disahkan melalui reaksi positif dengan ujian ELISA. Perbezaan-perbezaan di antara kedua-dua isolat *S. citri* ini telah dikesan melalui ujian 'Immunoelectrophoresis' bersilang (CIE) dengan menggunakan gel 'intermediate' dan 'polyacrylamide gel electrophoresis'. Reaksi homologus pada isolat Fewa menghasilkan sebelas puncak-puncak 'precipitin' yang dikesan menggunakan ujian CIE. Reaksi homologus yang serupa pada isolat Qualibia juga telah diperolehi. Satu antigen (a) spesifik kepada isolat Fewa dan satu antigen (b) spesifik pada isolat Qualibia telah dikesan apabila menggunakan ujian CIE dengan gel 'intermediate'. Analisis 'electrophoresis' satu dimensi menunjukkan corak-corak protein yang sangat serupa, dengan dua jalur protein yang berbeza di antara dua isolat tersebut. Kedua-dua isolat *S. citri* daripada Mesir (Fewa dan Qualibia) telah dikaji berdasarkan jujukan nukleotida

gen-gen spiralin, 16S rDNA dan jujukan 'intergenic spacer region' 16S/23S rDNA. 16S rDNA bagi kedua-dua isolat mempunyai 1529 nukleotida dengan 99.80% keserupaan di antara mereka dengan hanya dua perbezaan nukleotida sahaja yang dapat dikesan. Kedua-dua isolat mempunyai lebih 99.67% keserupaan dengan tiga strain *S. citri* yang berdaftar iaitu R8A2HP, Car 4 dan Car 6. 16S/23S 'Intergenic spacer region' mereka mempunyai nukleotida-nukleotida dengan 99.76% keserupaan dengan dua nukleotida berbeza dilihat. Jujukan 16S/23S pada isolat Fewa adalah sama dengan strain 169, Maroc dan ATCC 27556. Lima perbezaan nukleotida dilihat pada jujukan gen spiralin bagi kedua-dua isolat dengan 99.30% keserupaan. Kedua-dua gen spiralin mengandungi 241 asid amino dan hanya tiga asid amino berbeza dilihat diantara mereka dengan keserupaan amino asid pada tahap 98.75%. Penemuan kami menunjukkan bahawa kedua-dua isolat Fewa dan Qualubia mempunyi tahap keserupaan yang tinggi pada jujukan nukleotida 16S rDNA, 16S/23S rDNA 'intergenic spacer region" dan nukleotida dan asid amino pada gen spiralin. Walaubagaimanapun mereka adalah tidak sama dan dapat dibezakan dengan menggunakan enzim penyekat *Bfa*I yang mencerna pada CTAG dalam jujukan gen spiralin. Berdasarkan kajian yang telah dijalankan didapati kedua-dua isolat dari Mesir ini adalah isolat yang berbeza.

**ACKNOWLEDGMENTS**

Praises to almighty Allah, the cherisher and sustainer of the world, whose blessings have enabled me to complete this study.

I would like to express my most sincere gratitude and deep appreciation to Associate Professor Dr. Kamaruzaman Sijam, Head of Plant Protection Department, Faculty of Agriculture and the chairman of the supervisory committee, for his invaluable guidance, encouragement, constructive comments and generous help during the research work and preparation of this thesis.

I am deeply indebted to my I wish to thank my previous co-supervisor Dr. Inon Sulaiman and co-supervisor Associate Professor Dr. Zainal Abidin Mior Ahmad, Department of Plant Protection, Faculty of Agriculture for their constant encouragement, and unfailing help during the research work. My sincere appreciation and gratitude to my co-supervisor Dr. Habibuddin Hashim, Biotechnology Centre, Malaysian Agricultural Research and Development Institute (MARDI) for his support, help, critical comments and suggestions, patience, tolerance, and fruitful advice.

I would like to express my deepest gratitude to my co-supervisor Prof. Dr. Om-Hashem M. El-Banaa, Department of Plant Pathology, Faculty of Agriculture, Cairo University for her guidance, understanding, and support. Her invaluable advice and comments are highly appreciated.

I gratefully acknowledge the "Malaysian Technical Co-operation Programme (MTCP) for giving me this opportunity and for their financial support for my phD programme. I wish to thank Dr. Inon Sulaiman, Department of Plant Protection, Faculty of Agriculture for her assistance throughout the course of the study.

I am certainly grateful to Associate Professor Gail E. Gasparich, Department of Biological Sciences, Towson University, USA for her critical discussion and comments.

I would like to express my gratitude to the staff members of Microbiology and Pathology laboratories, Mrs. Junaina, Mr. Abdul Khir, Mr. Mohamad Zawawi, Mr. Razali, Mr. Mohamad Yousf, Mr. Shamsudin, Mr. Johari, Mr. Mohamad Nazri and Mrs. Asmalina and also to the officers of the Plant Protection Department, Mr. Shahudin, Mrs. Norah Abdullah, Sabariah Buang, and Miss.

Norhumairah Shariff for their technical assistance and convenience.

It is worth expressing my special thanks to Mrs. Siti Maryam Othman, research assistant, Biotechnology Centre, MARDI for always being so willing to render assistance throughout the course of the study. Also, I wish to send my appreciation to all the members of Biotechnology Centre, MARDI, Dr. Tan Chon Seng, Dr. Lam Peng Fatt, Mrs. Jameah Baharom, Miss. Noor Suhana Adzaha, Miss. Sew Yun Shin, Miss. Tan Poh Hwa and Miss. Hayati Ahmad .

My profound gratitude goes to Associate Professor Dr. Mohammed Abd El-Ghany Gaber, and Prof. Dr. El Sayed Mosbah El Kady, Department of Plant Pathology, Faculty of Agriculture, Kafr El-Sheikh, Tanta University for his cooperation, encouragement and making facilities available. I thank Prof. Dr. Abd El-Salam Ebeid Draz, Deputy Director of Rice Research and Training Centre, Kafr El-Sheikh, Egypt for his assistance and use of the facilities of the centre.

Last but not least, very special thanks to my parents, brothers, sisters, father-in-law, mother-in-law and my fiancée, Asmaa

Mahmoud Emara for their sacrifices, patience, understanding, help and encouragement throughout the study. And also to all my friends especially Mohammed El-Bashir and Essam Natsheh.

I certify that an Examination Committee has met on 8 May 2006 to conduct the final examination of Ayman Faisal Abdou Omar on his Doctor of Philosophy thesis entitled "Detection, Isolation and Characterization of Two Egyptian Isolates of *Spiroplasma citri* from *Citrus sinensis* trees" 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:

**Jugah Kadir, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

**Sariah Meon, PhD**
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Wong Mui Yun, PhD**
Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Chung Jan Chang, PhD**
Professor
College of Agricultural and Environmental Sciences
University of Georgia, Griffin Campus
Griffin
(External Examiner)

---

**HASANAH MOHD. GHAZALI, PhD**
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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

**Zainal Abidin Mior Ahmad, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

**Habibuddin Hashim, PhD**
Biotechnology Centre
Malaysian Agricultural Research and Development Institute (MARDI)
(Member)

**Om-Hashem M. El-Banaa, PhD**
Associate Professor
Faculty of Agriculture
Cairo University
(Member)

---

**AINI IDERIS, PhD**
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

**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.

---

**AYMAN FAISAL ABDOU OMAR**

Date:

**TABLE OF CONTENTS**

**Page**