UNIVERSITI PUTRA MALAYSIA

DIVERSITY AND CHARACTERIZATION OF *RALSTONIA SOLANACEARUM* STRAINS IN PENINSULAR MALAYSIA

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By

REZA KHAKVAR

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

May 2009

DEDICATION

WITH LOVE AND APPRECIATION TO:

My Parents: Rasool Khakvar and Masoomeh Fadavi

My Beloved Wife: Nasrin Sabour Moghaddam

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

DIVERSITY AND CHARACTERIZATION OF *RALSTONIA SOLANACEARUM* STRAINS IN PENINSULAR MALAYSIA

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REZA KHAKVAR

May 2009

Chairman: Associate Professor Kamaruzaman Sijam, PhD

Faculty : Agriculture

Surveys were conducted between the years of 2005 and 2007 at several locations in the northern, central and southern parts of West Malaysia to study the molecular characteristics of *Ralstonia solanacearum* strains. These sampling sites included vegetable farms and known hosts of the pathogen, such as banana, tomato, eggplant, chili and tobacco. Samples were collected from the suspected wilted plants and weeds, including soil and water samples, at the same areas. The bacterium was isolated from all samples using semi-selective medium and identified using BIOLOG identification system.

A two-stage nested-PCR was performed to confirm the results of BIOLOG test. The first PCR run produced a 410 bp amplicon and the second run produced 220 bp for all positive samples. Therefore all the strains were further confirmed

as *R. solanacearum*. A total of 69 strains were confirmed as *R. solanacearum* by BIOLOG identification system and nested-PCR. Thirty-two or 46.3% of positive samples were isolated from banana followed by chili (15.9%), tomato (13%), eggplant (7.2%) and tobacco (1.45%). Using routine biochemical tests, 38 and 31 strains have been recognized as Biovar 3 and biovar 4 respectively. Pulsed Field Gel Electrophoresis (PFGE), BOX-PCR and Fatty acid methyl ester (FAME) were used to determine the relatedness among these strains. All strains of *R. solanacearum* were typeable by PFGE and produced discernable banding patterns consisting of bands ranging from 15 to 800 kb. High similarity was observed among the strains from the same geographic regions. The strains that were collected from Terengganu (in northeast of West Malaysia) showed the highest similarity. Based on the PFGE analysis, strains of biovar 4 showed little variation in fingerprinting than strains of biovar 3. All strains except banana strains were divided into two main clusters in which each cluster consisted of pathogenic or non-pathogenic strains separately. All of the strains were also typeable by BOX-PCR fingerprinting method and in total 19 reproducible bands, ranging from 480 to 3200 bp, were scored and used for analysis. The similarity of patterns varied from 60 to 95%. Cluster analysis of the BOX-PCR patterns revealed same similarity with PFGE among all strains. The results of FAME analysis showed that fatty acid composition were very variable. Nine types of fatty acids were identified and quantified among all strains. Seven out of these nine fatty acids were identified as dominant fatty acids which consisted 98% of total fatty acids. Euclidean distances were calculated and the results showed that all FAME clusters were highly correlated with host and pathogenicity of

bacterial strains but low correlation was found between biovar and FAME clusters. A unique FAME profile was found among the strains that have been isolated from banana. This study clearly showed that *R. solanacearum* strains were phylogenetically similar within a region but diverse between regions despite biovar designation. Furthermore, fingerprinting by PFGE, BOX-PCR and FAME revealed a unique genomic and phenotypic distance within pathogenic and non-pathogenic strains of bacterium which previously was unrealized.

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

KEPELBAGAIAN DAN PENCIRIAN STRAIN *RALSTONIA SOLANACEARUM* DARIPADA MALAYSIA BARAT

REZA KHAKVAR

Mei 2009

Pengerusi: Profesor Madya Kamaruzaman Sijam, PhD

Fakulti : Pertanian

Survei telah dijalankan di antara tahun 2005-2007 di beberapa lokasi di utara, tengah dan selatan Malaysia Barat untuk mengkaji ciri molekul strain *Ralstonia solanacearum*. Kawasan persampelan termasuklah kawasan kebun sayur dan perumah patogen seperti pisang, tomato, terong, cili dan tembakau. Sampel-sampel telah dikutip daripada pokok-pokok yang disyaki layu, rumpai termasuk sampel tanah dan air daripada kawasan yang sama. Bakteria telah dipencilkan daripada semua sampel menggunakan media separa pilih dan telah dikenalpasti menggunakan sistem pengenalan BIOLOG.

Kaedah 'nested-PCR' dua peringkat telah digunakan untuk mengesahkan keputusan ujian BIOLOG dan keputusan menunjukkan kesemua 69 strain menghasilkan ’amplicon’ 410 bp pada peringkat pertama PCR dan ’amplicon’

220 bp pada peringkat kedua PCR. Oleh itu kesemua 69 strain telah disahkan sebagai *R. solanacearum* oleh ujian sistem pengenalan BIOLOG dan nested-PCR. Tiga puluh dua atau 46.3% sampel yang positif adalah dipencilkan dari pada pisang diikuti dengan cili (15.9%), tomato (13%), terong (7.2%) dan tembakau (1.45%). Ujian biokimia mengenalpasti 38 strain daripada biovar 3 dan 31 strain daripada biovar 4. Tiga kedah berbeza iaitu 'Pulsed Field Gel Electrophoresis (PFGE)' , 'BOX-PCR' dan "Fatty Acid Methyl Ester (FAME)' telah digunakan untuk menentukan keberkaitan di kalangan strain *R. solanacearum* yang telah dikumpulkan. Kesemua 69 strain *R. solanacearum* telah berjaya dikelaskan oleh PFGE dan menghasilkan corak-corak 'band' yang dapat dicerap mengandungi 'band' di antara 15-800 bp. Tahap kesamaan yang tinggi telah dilihat di kalangan strain daripada kawasan geografi yang sama. Strain yang dikutip daripada negeri Terengganu menunjukkan tahap kesamaan yang tinggi. Berdasarkan analisis PFGE, strain biovar 4 menunjukkan sedikit perbezaan berbanding dengan strain biovar 3. Kesemua strain (kecuali strain pisang) telah dikelaskan kepada dua kluster utama dan setiap kluster utama ini mengandungi strain patogenik dan bukan patogenik secara terpisah. Kesemua strain telah berjaya dikelaskan menggunakan kaedah 'BOX-PCR fingerprinting' dan kesemua 19 'band' boleh hasil semula, bersaiz diantara 480-3200 bp, telah dikenalpasti dan dianalisis. Kesamaan corak-corak adalah berbeza daripada 60-95%. Analisis kluster pada corak 'BOX-PCR' mendedahkan bahawa terdapat kesamaan strain seperti pada analisis 'PFGE'. Keputusan analisis 'FAME' menunjukkan komposisi asid lemak adalah sangat berbeza. Sembilan jenis asid lemak telah dikenalpasti dan dijumlahkan di kalangan strain. Tujuh daripada

sembilan asid lemak telah dikenalpasti sebagai asid lemak dominan yang mengandungi 98% daripada jumlah keseluruhan asid lemak. Pengiraan jarak 'Euclidean' telah dibuat dan keputusan menunjukkan bahawa kesemua kluster 'FAME' adalah sangat berkorelasi dengan perumah dan sifat kepatogenan strain bakteria tetapi dilihat kurang berkorelasi di antara biovar dan kluster 'FAME'. Kajian ini jelas menunjukkan bahawa strain *R. solanacearum* mempunyai kesamaan ciri filogenetik dalam kawasan yang sama tetapi berpelbagai antara kawasan meskipun biovar yang sama. Tambahan lagi kaedah 'fingerprinting PFGE, BOX-PCR' dan 'FAME' mendedahkan terdapat genomik yang unik dan jarak kecirian fizikal di kalangan strain bakteria patogenik dan bukan patogenik yang sebelum ini tidak disedari.

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I certify that a Thesis Examination Committee has met on 19th May 2009 to conduct the final examination of Reza Khakvar on his Doctor of Philosophy thesis entitled "Characterization and Diversity of *Ralstonia solanacearum* Strains in West Malaysia" in accordance with Universities and University Colleges Act 1971 and the Constituation of Universiti Putra Malaysia [P.U.(A)106] 15 March 1998. The Committee recommends that the student be awarded the PhD degree.

Members of the Thesis Examination Committee were as follows:

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

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Date : 9 July 2009

DECLARATION

I declared that the thesis is 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 Universiti Putra Malaysia or at any other institutions.

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REZA KHAKVAR

Date : 12 January 2009

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LIST OF ABBREVIATIONS

%	Percentage
µL	Microlitre
^{0}C	Centigrade
bp	Base pairs
CFU	Colony Forming Unite
cm	Centimeter
ELISA	Enzyme linked Immunosorbent Assay
FAME	Fatty Acid Methyl Ester
g	Gram
GC	Gas Chromatography
h	hours
HPLC	High Pressure Liquid Chromatography
Kg	Kilogram
L	Litre
mL	Millilitre
M	Molar
mM	millimolar
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
Rep-PCR	Repetitive-Element Sequence-Based PCR
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBE	Tris-Boric acid-EDTA
UV	Ultraviolet
V	Volt

CHAPTER 1
INTRODUCTION

Ralstonia solanacearum, previously known as *Pseudomonas solanacearum* (Smith, 1896) Smith 1914, is a plant pathogenic bacterium commonly found in the soils of tropical and subtropical countries where it devastates the cultures of many crop plants (Agrios, 2005). The bacterium was identified as straight or slightly curved rod (0.5 – 1.0 x 1.5 –5 µm), motile with one to several polar flagella, gram negative, nonspore-forming, noncapsulate and obligate aerobic. The bacterium multiplies readily in its hosts, but it is slow growing *in vitro* than some other bacterial pathogens and many plant and soil saprophytic organisms (Lelliott and Stead, 1987).

This organism, responsible for bacterial wilt, can infect over 300 plant species belonging to over 50 botanical families. Major agricultural hosts include tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicum esculentum* L.), potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.) and chilli (*Capsicum* spp.) and banana trees (*Musa* spp.) (Hooker, 1990). Weed hosts appear as an alternative host for the pathogen to survive in the absence of its susceptible host plants (Hayward, 1991).

Field symptoms of this bacterium are rapid and irreversible wilt under favourable conditions, stunting and yellowing of foliage (Agrois, 2005). Different symptoms may develop depending on the plant species, cultivars, growth stage and

environmental conditions but major symptom appeared as a rapid and irreversible wilt. If stems of wilted plants are dissected the vascular system can be seen to be discoloured and milky ooze exudes from cut surface (Lolliott and stead, 1987). The tropical and subtropical condition (such as Malaysia) was conductive for the growth of the pathogen and the development of the disease (Hayward, 1991).

Although most troublesome in the tropic and subtropics, *R. solanacearum* is a continuing threat in temperate climates (Denny, 2006). Meanwhile the bacterial wilt caused by this bacterium can occur in many types of mineral and organic soils, consequently it is also considered one of the dominant plant pathogenic microorganisms in all types of soils of many countries (Hayward, 1991)

This pathogen can lie dormant in water or soil until a host plant grows, then it enters the roots and colonizes water-conducting vessels, from where it spreads throughout the plant and multiplies to a high population density (Agrios, 2005). On the other hand, reportedly it has been found as one of common dominant microorganism in all kinds of soil in many countries that can easily live in soil without infecting plants for many years (Gnanamanickam, 2006).

Statement of Problems

According to Ivan Buddenhagen, there are many bacterial wilts and there are many '*Pseudomonas solanacearum*' (Danny, 2006). In order to describe this intra specific variability, several systems of classification have been proposed. Thus, the species was subdivided into five races according to its host range and pathogenicity and into five (recently 6) biovars based on the utilization of three disaccharides and three hexose alcohols (Schaad *et al*, 2001). As an sample, the majority of the Malaysian isolates were Biovar 3 followed by Biovar 4 and 2; also determination of race has showed that Malaysian isolated were in race 1 and 3; race 1 was the predominant race and was widespread, while race 3 was confined to the highland (Abdullah, 1988).

This genetic and pathogenic variations make the development of diagnostic, detection, and control measures of *R. solanacearum* more difficult and complex (Hayward, 1991). Therefore, sub-specific classification, which categorizes this polymorphism, is valuable and needed to give sufficient information for prediction in the context of epidemiology and control of the bacterial wilt disease.

In many parts of Malaysia, bacterial wilt caused by *R. solanacearum* is one of the major constraints in production of many agricultural crops. Previous studies have confirmed the existence of different biovars and races of this bacterium in Malaysia (Abdullah, 1988), but most of the previous reports have been done by traditional and phenotypic markers; therefore still little is known about the

molecular characteristics of the different populations of *R. solanacearum* in Malaysia.

In the other hand, most diversity studies on *R. solanacearum* have been done on strains isolated from very diverse geographical location such as different countries or different continents. In contrast, information on molecular variation among *R. solanacearum* within a restricted region such as West Malaysia is limited.

This study was conducted to characterize *Ralstonia solanacearum* strains in West Malaysia (Peninsular) at the molecular level. The results of the study could be used to distinguish populations of *R. solanacearum* collected from West Malaysia and identify the impact of location, geographical separation and host on pathogen population composition.