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CHARACTERIZATION OF Ralstonia solanacearum RACE 2 BIOVAR 1 ASSOCIATED WITH MOKO DISEASE OF BANANA IN PENINSULAR MALAYSIA

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By

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Moko disease caused by *Ralstonia solanacearum* race 2 biovar 1 (*R. solanacearum* R2Bv1) is a major disease affecting banana (*Musa* spp.) production. Although local reports suggested that this disease is widespreading in Malaysia, characterization of *R. solanacearum* strains associated with Moko disease in this country has not been done. This study was conducted to isolate, identify and characterize *R. solanacearum* R2Bv1 of Moko-causing strains in Peninsular Malaysia. During March 2011 to June 2012, 170 banana plants associated with Moko disease and adjacent soil samples were collected in 12 different locations of five outbreak states in Peninsular Malaysia comprising Kedah, Selangor, Pahang, Negeri Sembilan and Johor with disease incidence exceeding 80 % in some severely affected plantations. All 197 isolates produced fluidal colonies that were white to pink coloration after incubation at 24 to 48 hours at 29 °C on Kelman's TZC agar medium and were divided into two defined colony type, the B and SFR types. These isolates appeared as Gram-negative rods after Gram-stain, and positive for potassium hydroxide (KOH), Kovacs oxidase, catalase and lipase activity on Tween 80 solution tests. In biovar determination, only 30 isolates displayed characteristics of biovar 1 *R. solanacearum*, which was negative for utilization of disaccharides and hexose alcohols. Tobacco hypersensitivity assay revealed all isolates elicited hypersensitive response (HR) at 12 h after infiltration, suggesting that they were of race 2. In preliminary pathogenicity study, all 30 isolates were virulence towards three Moko most affected local banana cultivars namely *Musa paradisiaca* cv. Nipah, *Musa paradisiaca* cv. Tanduk and *Musa acuminata* cv. Berangan cultivars with diverse degrees of virulence; highly virulent, moderately virulent and weakly virulent with isolate NS-N1 as the most virulent, while isolates Ked-KN4 and Ked-KN5 were classified as weakly virulent. *Musa paradisiaca* cv. Nipah (ABB triploid) significantly exhibited the highest degree of severity to *R. solanacearum*, followed by *Musa paradisiaca* cv. Tanduk (AAB triploid) and *Musa acuminata* cv. Berangan (AAA triploid). Moreover, statistical results revealed

there were relationships between geographical origins of isolates and their severity, with the most and the lowest severity was related to isolates from Johor and Negeri Sembilan. This study represents the first evidence on the introduction of *R. solanacearum* biovar 1 associated with Moko disease of banana in Peninsular Malaysia. Partial 16S rDNA sequence analyses disclosed that all 30 isolates of *R. solanacearum* biovar 1 were clustered to the published *R. solanacearum* biovar 1 related to Moko-causing strains from the Philippines (MOD5 and R633) with 91 % Bayesian posterior probability support and completely different from *Ralstonia syzygii* (*R. syzygii*, S444E), blood disease bacterium (T520) and the outgroup strain, *Xanthomonas* spp. (55485). Meanwhile, phylogenetic analyses further demonstrated that all strains were grouped with 100 % posterior probability support to the published *R. solanacearum* race 2 insertion sequence gene, IS*Rso19* (AF450275). Phylotype-specific multiplex PCR (Pmx-PCR) showed all strains belonged to phylotype II displaying a 372 bp amplicon. Phylogenetic analyses of endoglucanase (*egl*) sequences clustered all 30 strains into phylotype II/4, together with the reference sequences strains from Peru (UW129, UW162 and UW163) and Colombia (UW070). Bioinformatics analysis of pooled rep-PCR fingerprinting method defined two major groups; cluster 1 (sub-group A and B) and cluster 2 (sub-group C), with 35 % average similarity coefficient within these two clusters. The sub-groups in cluster 1 were represented by strains from Kedah, Selangor, Negeri Sembilan and Johor; while cluster 2 sub-group was represented exclusively by strains of Pahang. This is indeed the first time that genetic diversity of *R. solanacearum* R2Bv1 has been characterized in this country, where rep-PCR technique clearly distinguished clonal lineages of Moko-causing strains in Peninsular Malaysia. These findings provide constructive documentations on *R. solanacearum* R2Bv1 in Malaysia, since banana has been identified as the second most important commercial fruit crop with a high economic value in this country.

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

PENCIRIAN KE ATAS *Ralstonia solanacearum* RAS 2 BIOVAR 1 DIKAITKAN DENGAN PENYAKIT MOKO PISANG DI SEMENANJUNG MALAYSIA

Oleh

DZARIFAH MOHAMED ZULPERI

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Penyakit Moko yang disebabkan oleh bakterium *Ralstonia solanacearum* ras 2 biovar 1 (*R. solanacearum* R2Bv1) adalah penyakit yang memberi kesan utama ke atas pengeluaran pisang (*Musa* spp.) di dunia. Walaupun terdapat laporan menyatakan penyakit ini semakin menular di Malaysia, pencirian ke atas strain *R. solanacearum* yang dikaitkan dengan penyakit Moko di negara ini belum pernah dijalankan. Oleh itu, kajian ini dijalankan untuk memencil, mengenalpasti dan mencirikan strain *R. solanacearum* R2Bv1 daripada penyakit Moko pokok pisang di Semenanjung Malaysia. Pada bulan Mac 2011 hingga Jun 2012, sebanyak 170 pokok pisang dengan simptom penyakit Moko dan sampel tanah berdekatan telah disampel secara rawak di 12 lokasi berbeza di lima negeri di Semenanjung Malaysia yang terdiri dari Kedah, Selangor, Pahang, Negeri Sembilan dan Johor, dengan simptom penyakit melebihi 80 % di ladang-ladang yang terjejas. Keseluruhan 197 isolat menghasilkan koloni berfluidal berwarna putih ke merah jambu selepas inkubasi selama 24 hingga 48 jam pada 29 ° C di atas agar TZC Kelman yang dibahagikan kepada dua jenis koloni iaitu B dan SFR. Kesemua isolat adalah Gram-negatif rod serta positif bagi ujian biokimia berikut; kalium hidroksida (KOH), Kovacs oxidase, catalase dan aktiviti lipase di dalam Tween 80. Dalam penentuan biovar, hanya 30 isolat *R. solanacearum* memaparkan ciri-ciri biovar 1, iaitu negatif terhadap penggunaan disakarida dan hexose alkohol. Ujian hipersensitiviti tembakau mendedahkan bahawa kesemua strain menghasilkan tindakbalas hipersensitif (HR) pada 12 jam selepas inokulasi. Penyaringan patogenisiti oleh keseluruhan 30 isolat ke atas tiga jenis kultivar pisang tempatan yang paling terjejas akibat penyakit Moko iaitu *Musa paradisiaca* cv. Nipah, *Musa paradisiaca* cv. Tanduk dan *Musa acuminata* cv. Berangan menghasilkan darjah virulen berbeza iaitu; sangat virulen, sederhana virulen dan kurang virulen dengan isolat NS-N1 sebagai yang paling virulen, manakala isolat Ked-KN4 dan Ked-KN5 dikelaskan sebagai paling kurang virulen. *Musa paradisiaca* cv. Nipah (ABB triploid) secara signifikan menghasilkan tahap kerentanan tertinggi terhadap penyakit Moko, diikuti oleh *Musa paradisiaca* cv. Tanduk (AAB triploid) dan *Musa acuminata* cv. Berangan

(AAA triploid) sebagai yang paling resistan terhadap penyakit Moko. Analisis statistik juga menunjukkan terdapat hubungan antara kedudukan geografi isolat dan tahap virulen, iaitu yang paling virulen adalah strain dari Johor dan paling kurang virulen adalah isolat dari Negeri Sembilan. Kajian ini adalah bukti kemasukan *R. solanacearum* biovar 1 yang dikaitkan dengan penyakit Moko pisang di Semenanjung Malaysia. Analisis jujukan 16S rDNA separa menunjukkan bahawa kesemua 30 isolat *R. solanacearum* biovar 1 menyamai strain-strain *R. solanacearum* biovar 1 rujukan penyakit Moko dari Filipina (MOD5 and R633) dengan 91 % sokongan kebarangkalian posterior Bayesian. Analisis filogenetik membuktikan kesemua strain telah dikelompokkan bersama gen rujukan bagi *R. solanacearum* ras 2, IS*Rso19* dengan 100 % sokongan kebarangkalian posterior. Multipleks PCR berfilotip khusus (Pmx-PCR) pula menghasilkan 372 bp amplikon yang menunjukkan kesemua strain adalah dalam kumpulan filotip II. Analisis filogenetik ke atas jujukan-jujukan endoglucanase (*egl*) membuktikan kesemua 30 strain berada dalam kumpulan filotip II sequevar 4, bersamaan dengan strain rujukan dari Peru (UW129, UW162 and UW163) dan Colombia (UW070). Analisis bioinformatik data PCR-berkelompok menghasilkan dua kumpulan utama; kelompok 1 (sub-kumpulan A dan B) dan kelompok 2 (sub-kumpulan C), dengan 35 % nilai koefisien. Sub-kumpulan kelompok 1 diwakili strain dari Kedah, Selangor, Negeri Sembilan dan Johor; manakala sub-kumpulan kelompok 2 diwakili hanya strain dari Pahang. Ini merupakan kali pertama pencirian kepelbagaian genetik itu *R. solanacearum* R2Bv1 dilaporkan di Semenanjung Malaysia. Oleh kerana pisang telah dikenal pasti sebagai tanaman buah-buahan komersial kedua terpenting di Malaysia, penemuan daripada kajian ini dapat menyediakan dokumentasi konstruktif ke atas *R. solanacearum* R2Bv1 di negara ini.

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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 were as follows:

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LIST OF ABBREVIATIONS

%	percent
°C	degree celcius
bp	base pair
CABI	Commonwealth Agricultural Bureaux International
DNA	deoxyribonucleic acid
DOA	Department of Agriculture
EDTA	ethylene-diamine-tetraacetic acid
FAO	Food and Agriculture Organization
g	gram
h	hour
kb	kilobase pair
L	liter
M	molar
Mb	megabase pair
min	minutes
ml	milliliter
mm	milimeter
mM	milimolar
ng	nanogram
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
rpm	rotation per minute
sec	seconds
TAE	tris-acetic EDTA
Taq	*Thermus aquaticus*
T_M	melting temperature
U	unit
UV	ultra-violet
V	voltan/volt
v/v	volume per volume
w/v	weight per volume
x g	gravity force
µg	microgram
µg/ml	microgram per mililiter
µl	microliter
µM	micromolar
µm	micronmeter

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Ralstonia solanacearum, the causal agent of bacterial wilt, is a soil-borne plant pathogen with a worldwide distribution that afflicts economically important crops and ornamentals (Lin *et al*., 2014; Denny, 2006; Agrios, 2005). This aerobic, Gram-negative organism, formerly known as *Pseudomonas solanacearum* is commonly encountered in tropical and subtropical areas. Its immense phenotypic and genotypic diversity contributes to its status as a major plant pathogen (Fegan and Prior, 2006; Agrios, 2005). *R. solanacearum* infects more than 200 plant species in 50 botanical families; among its hosts are tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and banana (*Musa* spp.) (Alvarez *et al.*, 2010; Hayward, 1994). Bacterial wilts of banana, known commonly as Moko, Bugtok and blood disease, are incited by distinct subgroups of the *R. solanacearum* species complex (RSSC) and pose a major threat to dessert and cooking banana production (Fegan and Prior, 2006).

Moko disease has been acknowledged as one of the remarkable major diseases threatening banana cultivation worldwide. After its first outbreak in Trinidad in the late 1890s, this disease caused by *Ralstonia solanacearum* (*R. solanacearum*) race 2 became endemic in several regions of Central and South America (Jones, 2002). In Jamaica as an example, Moko disease has become a devastating disease attacking banana plantains with estimated annual loss of about USD 5.8 million. This disease has been a major concern for banana growers in the Amazon region of Brazil where it has been the major production constraint for banana yields (Netto and Nutter, 2005).

The presence of Moko disease pathogen in Asia was first detected in the Philippines of Mindanao region (Eyres and Hammond, 2001). To date, the emergence and widespread of Moko disease has been identified in several countries in Asia, Africa, North America, Central America, South America, parts of the Caribbean Islands and Australia continents (EPPO, 2013).

1.2 Statement of the Problem

In Malaysia, the suspected outbreak of Moko disease was primary recognized in Muar, Johor in 2007 (Mokhtarud-din and William, 2011). Earlier findings revealed that tropical condition with a temperate climate like Malaysia was even more conducive for the growth of *R. solanacearum* and development of this disease in the infected region (Denny, 2006; Hayward, 1991). This vital situation on the epidemic of Moko disease has further diminished little enthusiasm of farmers on banana industry since the disease is amongst the most serious fruit diseases in the country where it widespread rapidly, retards banana plant growth, causes critical yield losses and can rigorously impact the banana growth sector. As banana has been recognized as one of the fruit types for special attention under the implemented Economic Transfer Programme (ETP) by Malaysian government, constant occurrences of this disease have been the most important and major constraint to the production of bananas, resulting to loss of yield and areas that are gradually becoming unsuitable for the production of the crops (Mokhtarud-din and William, 2011; Tengku Abdul Malik *et al*., 2011; Nik Hassan, 2003).

1.3 Significance of the Study

As banana (*Musa* spp.) remains the second most important economic-driven fruit crops in Malaysia for both local and export markets, scrutinizing records on the current status of Moko disease is of significant importance. Up to this point, none of the disease occurrences have been well documented in Malaysia since the first suspected outbreak in 2007. The results of our study will be an important pioneer documentation of Moko disease of banana in Malaysia. Taking this matter into serious account, our study would be a major platform on generating details documentation of Moko disease and its causal pathogen *R. solanacearum* race 2 biovar 1 in banana fruit crops in Malaysia by using combination of phenotypic characterization and molecular phylogenomics approaches.

1.4 Objective of the Study

Our study was carried out with the following objectives:

1. To isolate, identify and characterize *R. solanacearum* of Moko-causing strains in Peninsular Malaysia by using phenotypic characteristics.
2. To investigate genetic relationships and diversity of *R. solanacearum* race 2 biovar 1 strains of Moko disease via molecular characterization and phylogenetic analyses.

The output from this research perhaps may improve and increase efficiency in the development of accurate molecular diagnostic tests for detection and identification of *R. solanacearum* race 2 biovar 1. Indeed, the data obtained will be useful for quarantine purposes and suppression of Moko disease spread, thus bettering the banana industry in Malaysia.

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