

Comparative Diversity Analysis Of *Ralstonia solanacearum* Strains In Solanaceae Farms

Reza Khakvar¹, Kamaruzaman Sijam², Jeffery Jone³ and Kwai L. Thong⁴

1, Plant Protection Department, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
khakvar@tabrizu.ac.ir

2, Plant Protection Department, Faculty of Agriculture, University Putra Malaysia (UPM) Selangor, Malaysia
kama@agri.upm.edu.my

3. Plant Pathology Dept., University of Florida, IFAS, Gainesville, USA
jbjones@ifas.ufl.edu

4, Institute of Biological Sciences, Faculty of Science, University Malaya (UM), Selangor, Malaysia
thongkl@um.edu.my

Abstract—Pulsed Field Gel Electrophoresis (PFGE) and Fatty acid methyl ester (FAME) were comparatively used to determine the diversity of *Ralstonia solanacearum* strains collected from Solanaceae farms in West Malaysia. A total 47 strain isolated from different location was examined. The strains were a mix of pathogenic and non-pathogenic strains that have been collected from infected plant tissues and different depths of soil and water. PFGE results classified the strains into two main clusters highly related to their pathogenicity and five subgroups which were significantly related into geographical origin and could divide all strains into East and West coast strains. FAME was highly related into bacterial host origin but also was able to classify the strains into pathogenic and non-pathogenic groups which significantly correlated with PFGE genomic clusters among same nonpathogenic strains. The results confirmed that combined results of PFGE and FAME can be effectively used in diversity study of *R. solanacearum* strains especially in nonpathogenic strains.

Keywords Bacterial wilt; *Ralstonia solanacearum*; Genomic Diversity; PFGE; FAME

I. INTRODUCTION

Ralstonia solanacearum, the causal agent of bacterial wilt disease, results in serious world-wide economic losses particularly in the tropics (Agrios, 2005). Although it is difficult to estimate total economic losses that causes directly or indirectly by bacterial wilt, it ranks as one of the most important plant diseases in entire world (Gnanamanickam, 2006). The pathogen is soil-borne and has an extremely wide host range that can infect over 300 plant species, belonging to over 30 botanical families (Hayward, 1991). Members of the family of *Solanaceae* such as potato, tomato, eggplant, chili and tobacco are the major host crops for this pathogen (Jones et al., 1991). In many parts of Peninsular (West) Malaysia, bacterial wilt caused by *R. solanacearum* is one of the major constraints in agricultural production especially in solanaceae crops (Abdullah, 1988; Khakvar et al., 2008a).

R. solanacearum has complex taxonomic properties with a great degree of diversity at all levels, including physiological, phenotypic, genotypic, and host range (Genin and Boucher, 2004; Poussier et al., 1995). Traditionally the

bacterium has been subdivided into six biovars on the basis of carbohydrate catabolism and five races, designated by host range (Schaad et al., 2001). The determination of DNA–DNA homologies of isolates of *R. solanacearum* has shown that the relatedness between isolates of this species is often less than the limit of >70% which has been considered a threshold level within a species (Castillo and Greenberg, 2007).

Recently the bacterium has been classified into four phylotypes and 23 sequeres based on phylogenetic analysis of 16S-23S ITS but still there is no general consensus about sub-classification of *R. solanacearum* species (Fegan and Prior, 2005). Therefore, sub-specific classification studies, which categorize this polymorphism, are still valuable and needed to give sufficient information for prediction in the context of epidemiology and control of the bacterial wilt disease. In most parts of South-East Asia, the bacterial wilt pathogen has been isolated and characterized unambiguously and most of strains originated from this part of the world South-East Asia are placed in phylotype IV (Horita and Tsuchiya, 2001; Ito et al., 1996; Jaunet and Wang, 1999; Melanie et al., 2007; Thammakijawat et al., 2004). But still little is known about the genetic diversity of this pathogen in Malaysia.

Hence, the objective of the study was to analyze the genetic diversity of *R. solanacearum* isolated from different parts of West Malaysia using Pulsed Field gel Electrophoresis (PFGE) typing. PFGE is generally accepted as one of the most powerful tools in diversity studies of *R. solanacearum*. In the other hand, previous reports have indicated that fatty acid methyl ester (FAME) profiling corresponded well with DNA and rRNA homology, and accordingly FAME profiling is known to be the best phenotypic marker for *R. solanacearum* strains classification (Dawyndt et al., 2006; Varbanet et al., 2003; Weller et al., 2000). Therefore, the study also aimed to determine whether FAME profiling can be effectively used for classification of *R. solanacearum* strains in combination with PFGE.

II. MATERIAL AND METHODS

Bacterial strains: A total of 42 strains of *R. solanacearum* were collected from different parts of West Malaysia in years 2005-2007 and confirmed as *R. solanacearum* using biochemical tests, BIOLOG identification system and nested-PCR (Khakvar *et al.*, 2008 a&b). Five additional isolates from a 1984-1985 collection (Abdullah, 1988) were included for comparison. The isolates were recovered on semi-selective tetrazolium chloride (TZC) plates and were incubated at 28°C for 48 to 72h before analysis.

Pathogenicity Test: To determine pathogenicity and aggressiveness of collected strains, pathogenicity test was performed for all strains according to the method described by Klement *et al.* (1990). Tomato and chilli seedlings were used for this experiment. Bacterial isolates were streaked on TZC medium and incubated for 24h at 28°C before inoculation. The stem of test plants were wounded with a sharp scalpel and 2ml of the bacterial inoculum (10^8 cfu/mL in distilled water) were poured into cut surfaces. Control plants were similarly inoculated using sterilized distilled water in place of the bacterial inoculum. Inoculated test plants were monitored for two weeks.

PFGE Analysis: Bacterial DNA was prepared using the method described by Thong *et al.* (2003) with a few modifications. The slices of bacterial DNA plugs were digested with 20 units of restriction enzyme *Xba*I (VIVANTIS Labs) in accordance with the manufacturer's instructions for at least two hours at 37°C. The restricted DNA fragments in plugs were separated by pulsed field gel electrophoresis through 1% agarose type-I at 14°C in CHEF Mapper XA system (Bio-Rad, USA). After electrophoresis, the gels were stained with ethidium bromide and the banding patterns were analyzed using the GelCompar II Version 5 software (Applied Mathematics, Belgium).

Fatty Acid Methyl-Esters (FAME) profiling: Extraction of FAMES was conducted for each strain by using MIDI protocol combined with the method described by Schutter and Dick (2000). Bacterial cells were mixed with 3M NaOH in Methanol:H₂O (1:1), vortexed briefly and were saponified by heating in a boiling water bath. The tubes were cooled to room temperature, then methanolic HCl (Methanol:HCl (20%); 40:60 v/v) was added and the mixture was heated at 80°C for 10min and the tubes were quickly cooled inside ice-box. N-hexane/methyl-tert-butyl ether (1:1; v/v) was added and the phases were allowed to separate. The lower aqueous layer was carefully removed then NaOH (0.25M) was added and mixed with the remaining organic layer. Upper phase was removed, then the approximately two-thirds of the organic layer containing the fatty acid methyl esters (FAMES) was then transferred to a dark GC vial and were kept at -18°C. The extracts were analyzed with the Gas Chromatography (GC) System (Hewlett Packard, model 5898) equipped with a DB-5 column (25 m x 0.2 mm silica capillary column). GC included with a flame-ionization detector (FID) and Helium (He) as carrier gas. Pearson correlation was calculated between fatty acid profiles of

different isolates by using The MiniTab version-14 (Statics Software).

III. RESULTS

Based on pathogenicity test and symptom on inoculated seedling, 69% stains were determined as pathogenic stains. The reminders (13 stains) were not able to produce wilting in inoculated plants within two weeks therefore were consider as non-pathogenic or weakly pathogenic. All of these nonpathogenic strains had been isolated from soil or water samples.

All 47 (42new+5old isolates) strains of *R. solanacearum* displayed *Xba*I profiles and generated 22–28 fragments ranging in size from approx. 18 to 690 kb. The similarity of the patterns varied from 68 to 99% and the combination of the digestion profiles defined 68 different haplotypes. Based on the cluster analysis of PFGE profiles, all strains were divided into two main clusters at the 68% similarity level and eight main subgroups (G1-G8) at the 82% similarity level. Each of the main clusters consisted of pathogenic or non-pathogenic strains separately. Only few strains with nonpathogenic behavior were placed among pathogenic clusters. High similarity was observed among the strains from same geographical region (province) with same biovar. All subgroups also appeared to be correlated with two geographic origins (West or East coast). The strains isolated from South provinces (Johor) were separated into both groups. Strains of biovar 4 showed the highest similarity (80-99%) than strains of biovar 3 (62-90%) since there was no biovar 3 that similarity was 95 percentages (Fig. 1a)

The results of FAME analysis with *R. solanacearum* strains showed that fatty acid composition were very variable. The mean percent distributions of fatty acids in different groups of *R. solanacearum* strains are shown in Table 1. Nine types of fatty acids were identified and quantified among all 47 strains. Fatty acids 12:0 and 16:1 2OH were just found in the non-pathogenic strains in low quantities (always <2%). Concentrations of fatty acid (16:0) in the pathogenic isolates were always higher than non-pathogenic isolates. The dendrogram based on all fatty acids composition formed nine major clusters (Fig. 1b). All clusters were highly correlated with host and pathogenicity of bacterial strains but low correlation was found between biovar and FAME clusters. All 9 isolates that were clustered in groups A, B and C were nonpathogenic with two main characteristics; two types of fatty acids (12:0 and 16:1 2OH) and lower concentration of one of main fatty acid (16:0). Also there is a significant correlation between these FAME clusters and three non-pathogenic groups in PFGE clustering. The rest (D to I) were included with all pathogenic strains. Correlation between FAME clusters and biovars in nonpathogenic groups was higher than pathogenic groups. Pearson's correlation tests showed that there is highly significant correlation between pathogenicity and concentration of one of individual fatty acid (14:0) (table 3). Also low but significant correlation was found between biovar type and 14:0 and 17:0 cyclo.

IV. DISCUSSION

This study was conducted to determine the relatedness among *R. solanacearum* strains isolated from Solanaceae farms in West Malaysia. Two different markers, PFGE and FAME, have been used for differentiation of *R. solanacearum* strains. The results confirmed that *R. solanacearum* strains in West Malaysia were diverse. Except for two strains, all non-pathogenic strains were grouped in one cluster. Previously the capability of PFGE in differentiation of different groups of *R. solanacearum* (biovar, race, pathogenicity and geographical origin) has been demonstrated (Dawyndt et al., 2006; Grothues and Radolph, 2006; Smith et al., 1995 a&b). In the current study, except two strains, all bacterial strains were divided into pathogenic and nonpathogenic types based on their symptoms on the inoculated seedlings after two weeks. Therefore those two strains (Se-1-4-1w and J-5-3) could be placed into nonpathogenic group due to their very low pathogenicity. Also the analysis of PFGE patterns revealed the significant regional similarity among the strains. Based on PFGE results, the strains of *R. solanacearum* were genetically similar within same regions and they are divided into two main West and East groups. These results can be explained by attention to topographic map of Peninsular Malaysia (Fig 2). Peninsular Malaysia is divided into East and West Coast regions by the Titiwangsa Mountains Range and it acts as a natural geophysical barrier. There is no link between the rivers and streams on both sides of the Range, and therefore the strains of *R. solanacearum* on both sides of this natural barrier have diverged through the centuries of isolation. Similar observation by authors has been reported in the strains that isolated from banana farms in Peninsular Malaysia (Khakvar et al., 2008b). Total fatty acid profiles were clustered into 9 groups. Like PFGE, a highly significant correlation was found between total fatty acid clusters and pathogenicity and host of bacterial strains and could separate bacterial strains based on their pathogenicity and host origin. Clough et al, (1997) showed that palmitic acid (16:0) has great importance in physiology of *R. solanacearum* and it acts as a virulence factor for this bacterium. This is in agreement with the results of current study and there is a significant difference between concentration of this fatty acid in pathogenic and nonpathogenic strains. On the other hand, with the exception of nonpathogenic strains, low relationship was found between FAME clusters and geographical origin and biovars. Janse (1991), Salet de Mole, et al (1999), Stead 1992, Timms-Wilson et al (2001) and Varbanets et al (2003) showed similar grouping among pathogenic strains. Total fatty acid profiles have been used as a common phenotypic marker for identification of some other types of bacteria at the subspecies level (Cothier et al., 1992, Stead et al., 1992; Walcott et al, 2000). Previously it has been reported that the family of *Pseudomonaceae* species can be divided into eight FAME groups that parallels RNA homology grouping (Weller et al., 2000). In this FAME grouping some saturated fatty acids such as Myristic acid (14:0) and Palmitic acid (16:0) and unsaturated fatty acid such as Oleic acid have

been used for classification of many *Pseudomonas* at different levels (species and subspecies) but for *R. solanacearum* no reports for differentiation of *R. solanacearum* species at the subspecies levels (biovar or race) by total fatty acid clustering. All FAME experiments on the strains of *R. solanacearum* had been focused on the pathogenic strains and so far there was no report on FAME results on non-pathogenic strains of *R. solanacearum*. The results of current study demonstrated that PFGE and FAME combined profiling could be used in differentiation of pathogenic and nonpathogenic strains of *R. solanacearum*.

ACKNOWLEDGEMENT

This work was supported by grant KTP(BS)035/10/003 under Malaysian Technical Cooperation Program (MTCP) from Ministry of Higher Education of Malaysia. We also thank Dr. Siti Havana from Horticulture division in Institute of Malaysian Agricultural Research and Development (MARDI) for her kind cooperation during sample collection.

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TABLE I. MEAN AMOUNT (%) OF FATTY ACIDS FOUND IN DIFFERENT GROUPS OF *R. SOLANACEARUM*

Phenotypic groups		Fatty acid composition (% of total)								
		12:0	14:1 3OH	14:0	16:1	16:1 2OH	16:0	17:0 cyclo	SF8	18:1 2OH
Biovar	3	0.80	6.95	8.00	30.5	0.98	33.1	4.32	13.5	2.22
	4	0.75	7.54	7.13	30.0	1.17	32.5	5.82	13.9	2.70
Pathogenecity	Pathogenic	0.60	7.11	5.95	30.0	1.18	34.0	6.10	14.1	12.23
	Non-patho	0.95	7.25	7.98	30.2	1.11	30	4.90	13.8	2.20

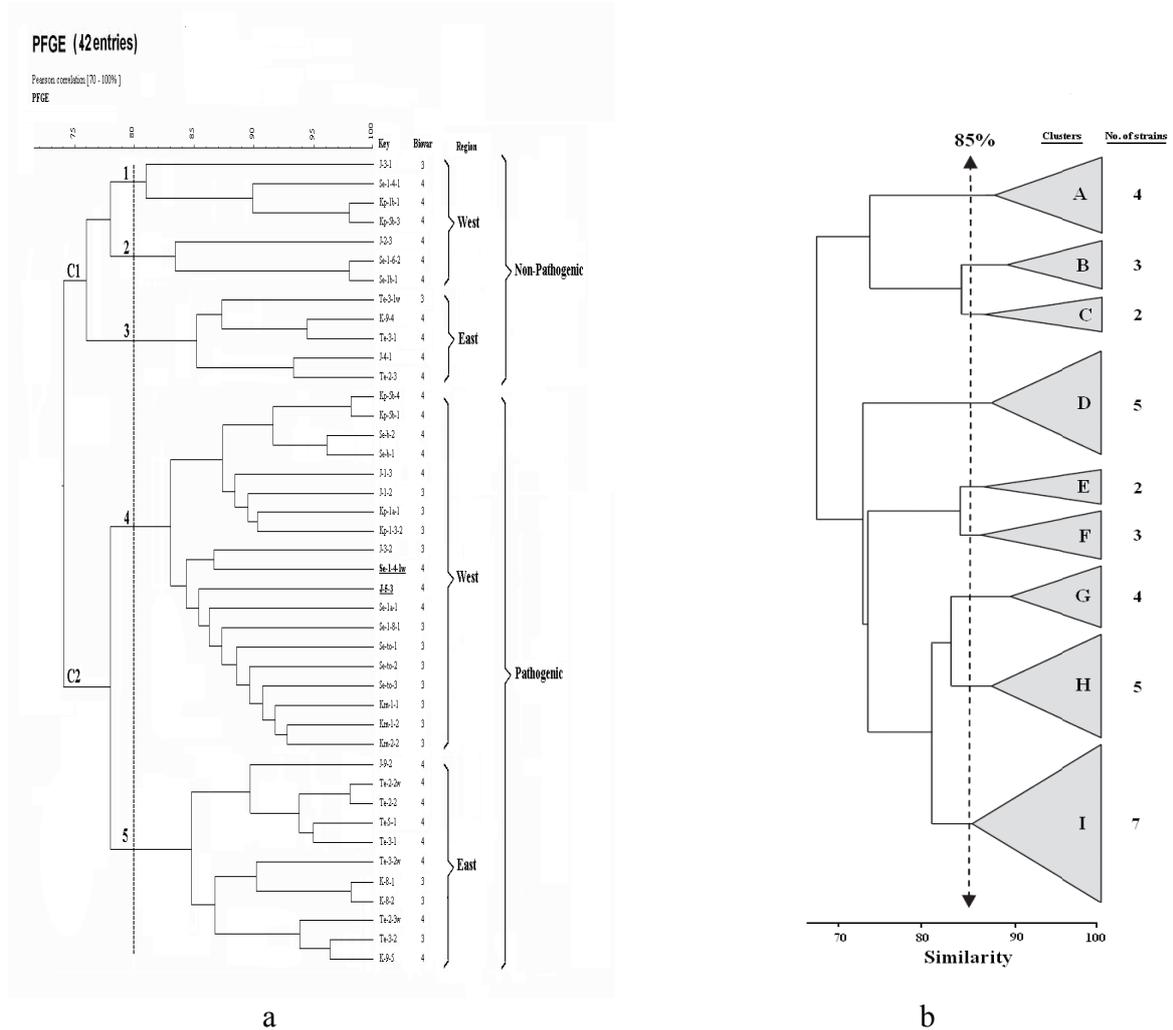


Figure 1. Results of PFGE (a) and FAME (b) clustering of *R. solanacearum* strains.

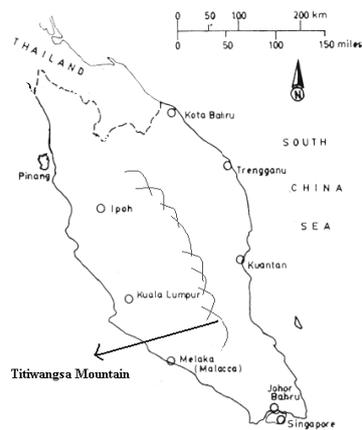


Figure 2. Geography of West Malaysia and Titiwangsa Mountain