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Genomic Diversity of *Ralstonia solanacearum* Strains Isolated from Banana Farms in West Malaysia

[1]R. Khakvar, [1]K. Sijam, [1]M.Y. Wong, [2]S. Radu, [3]J. Jones and [4]K.L. Thong
[1]Department of Plant Protection, Faculty of Agriculture, University Putra Malaysia (UPM), Malaysia
[2]Department of Food Science, Faculty of Food Science and Technology, UPM, Malaysia
[3]Department of Plant Pathology, University of Florida, IFAS, Gainesville, USA
[4]Institute of Biological Sciences, Faculty of Science, University Malaya (UM), Malaysia

Abstract: Pulse-Field Gel Electrophoresis (PFGE) and BOX-PCR patterns were used to determine the phylogenetic relationships among 32 strains of *Ralstonia solanacearum* collected from banana farms in West Malaysia. Eighty two percent (25 stains) were biovar 3 and the remainders were biovar 4. Cluster analysis based on PFGE and BOX-PCR fingerprinting showed that there was significant genetic variation among all strains within this species in West Malaysia. Patterns of DNA fingerprinting in both methods were significantly correlated with sampling sites and revealed the regional similarity among the strains. The PFGE patterns varied from 78 to 99% while BOX-PCR patterns showed a high degree of variation among *R. solanacearum* strains. Genomic fingerprinting by PFGE also revealed one that was significantly correlated to biovar type while BOX-PCR pattern analysis was unable to differentiate various biovars. This study clearly showed that *R. solanacearum* strains were phylogenetically similar within a region but diverse between regions despite biovar designation.

Key words: *Ralstonia solanacearum*, genomic diversity, banana diseases

INTRODUCTION

Ralstonia solanacearum, the causal agent of bacterial wilt disease, is a devastating soil-borne plant pathogen that is a member of the Pseudomonaceae. It causes disease on over 200 plant species in over 50 families (Agrios, 2005; Jones *et al*., 1991). The optimum growth temperature of this bacterium is 25 to 35°C, consequently it is a plant pathogen of tropical, subtropical regions (Hayward, 1991). It is also considered one of the dominant plant pathogenic microorganisms in soils of many countries of temperate zone (Elphinstone, 2005). Strains of *R. solanacearum* represent a heterogeneous group that has been sub-divided traditionally into five races based on host range and 6 biovars based on utilization of three disaccharides and three hexose alcohols (Schaad *et al*., 2001). No general correlation between races or biovars has been reported (Genin and Boucher, 2004). Recently Fegan and Prior (2005) proposed a hierarchical classification for *R. solanacearum*, based on phylogenetic analysis of 16S-23S ITS and they proposed to dividing *R. solanacearum* species into four phylotypes. Of course, in recent years, greater understanding of the infraspecific grouping within *R. solanacearum* has been gained through the use of a number of molecular techniques and perhaps as a result of information gained from these study there may be sufficient information to split this complex species into two or more subspecies or species (Timothy, 2006).

In many parts of Malaysia, bacterial wilt of caused by *R. solanacearum* is one of the major potential constraints in production of banana (*Musa acuminate*). Earlier studies have confirmed the existence of biovars 3 and 4 of this bacterium in Malaysia and the ability of both biovars in infection of banana in Malaysia (Abdullah, 1988; Khakvar *et al*., 2008), but still little is known about the genetic and phylogenetic relationships among strains of *R. solanacearum* in Malaysia. The aim of this study was to assess phylogenetic relationships within local populations of *Ralstonia solanacearum* isolated from banana farms in West Malaysia (or Peninsular Malaysia).

MATERIALS AND METHODS

Bacterial strains and culture conditions: The strains used in this study, isolation location relating to region

Corresponding Author: Reza Khakvar, Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Iran Tel: 0098-411-3356006 Fax: 0098-411-3345332

Table 1: Characteristics of *Ralstonia solanacearum* strains used in this study

No.	Isolate	Isolation part	Province(s)	Biovar
1	Se-1a-2	Root	Selangor	3
2	Se-1a-3	Root	Selangor	3
3	Se-1a-4	Rhizosphere	Selangor	3
4	Se-1b-2	Rhizosphere	Selangor	3
5	Se-1b-3w	Irrigation water	Selangor	3
6	Se-1b-5	Rhizosphere	Selangor	3
7	Se-1-7-2	Stem	Selangor	3
8	Se-1-8-5	Rhizosphere	Selangor	3
9	K-8-4	Rhizosphere	Kelantan	4
10	K-9-3	Rhizosphere	Kelantan	4
11	K-10-1	Root	Kelantan	3
12	Kp-1a-3	Rhizosphere	Kedah	3
13	Kp-1b-2	Rhizosphere	Kedah	3
14	Kp-1b-3w	Irrigation water	Kedah	3
15	Kp-1b-4	Rhizosphere	Perak	3
16	Kp-1b-5	Root	Perak	3
17	J-1-1	Root	Johor	3
18	J-3-2	Rhizosphere	Johor	3
19	J-3-3	Root	Johor	3
20	J-8-1	Rhizosphere	Johor	3
21	J-8-4	Rhizosphere	Johor	3
22	Te-1-1	Root	Terengganu	4
23	Te-1-2	Rhizosphere	Terengganu	4
24	Te-2-1	Rhizosphere	Terengganu	3
25	Te-2-1w	Irrigation water	Terengganu	3
26	Te-2-4	Rhizosphere	Terengganu	3
27	Te-3-3	Rhizosphere	Terengganu	3
28	Te-3-4	Rhizosphere	Terengganu	3
29	Te-3-4w	Irrigation water	Terengganu	3
30	Te-5a-4	Rhizosphere	Terengganu	4
31	Te-5b-1	Rhizosphere	Terengganu	4
32	Te-5b-2	Rhizosphere	Terengganu	4

and biovar types are shown in Table 1. These strains were already collected from different parts of West Malaysia in year 2005-2006 and confirmed as *R. solanacearum* by Khakvar *et al*. (2008). Seven out of 32 strains were biovar 4 and the remaining 25 strains were biovar 3. All bacterial strains were maintained as suspensions in sterile water and were retrieved by streaking on CPG medium (5.0 g L^{-1} glucose, 1.0 g L^{-1} casamino acids, 10.0 g L^{-1} peptone, 17.0 g L^{-1} agar-agar). Following recovery, all *R. solanacearum* strains were grown at 28°C on tetrazolium chloride medium (CPG medium amended with 0.05% (wt./vol) tetrazolium chloride) (Schaad *et al*., 2001).

PFGE analysis: For PFGE analysis, agarose gel plugs containing total cellular DNA were prepared by the method described by Thong *et al*. (2003) with a few modifications. Briefly, strains of *R. solanacearum* were grown in CPG medium at 28°C for 48 h. Bacterial cells were removed from agar and suspended in cell suspension buffer (10 mM Tris, 1 mM EDTA in deionized water, pH 8). The cell density of the suspensions was adjusted to optical density of 1.3-1.4 at OD_{610}. The standardized cells suspensions were mixed with an equal volume of molten Incert-agarose (1.6%) and immediately dispensed into labeled plug molds. After the plugs solidified, they were transferred into tubes containing cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8, 1% N-lauroylsarcosine and 1 mg mL^{-1} proteinase K) and then incubated at 54°C for at least 4 h. After washing the plugs with preheated (50°C) sterile distilled water, slices (1 mm) from plugs were digested with 20 Units of restriction enzyme *Xba*I in accordance with the manufacturer's instructions for at least 2 h at 37°C. The DNA restriction fragments in plugs were separated by electrophoresis through 1% Sigma agarose in solution of Tris-Borate-EDTA buffer at 14°C in CHEF Mapper XA system (Bio-Rad, USA). The running condition was: 2 to 50 sec at 6 volt cm^{-1} (110-130 mAmper) for 20 h. Bacteriophage lambda PFGE marker and *Xba*I-digested Salmonella Branderup were used as DNA markers. Gels were stained with ethidium bromide (0.5 μg mL^{-1}) and photographed under UV transillumination. The results were analyzed using the GelCompar II Version 5 software (Applied Mathematics, Belgium). Using this software, the analysis of the restriction fragments and determination of similarity coefficients for all combinations was performed using Pearson correlation.

BOX-PCR analysis: Total genomic DNA of *R. solanacearum* strains was prepared using the procedure described by Chen and Kuo (1993). After DNA extraction, the quality of DNA was analyzed under UV translluminator after staining with ethidium bromide. Two microliter of these extracted DNA (appr. 10 ng) was used in PCR in a total volume of 25 μL. The BOX primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') was synthesized by Research Bio-Labs Co. (Singapore). DNA amplification was performed in a thermocycler (BioRad, USA) with an initial denaturation step of 95°C for 7 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min and 65°C for 8 min with a final extension step of 65°C for 15 min (Horita and Tsuchiya, 2001). The results were analyzed using the GelCompar II software.

RESULTS

PFGE: All 32 strains of *R. solanacearum* were typeable by PFGE and produced discernable banding patterns consisting of band ranging from 15 to 740 kb (Fig. 1). In total, only 18 different bands were used in gels analysis and the rest were omitted due to low clarity and non-reproducibility. The similarity of the patterns varied from 78 to 99%. High similarity was observed among the strains from the same geographic regions. The strains that were collected from Terengganu Province showed the highest similarity (95-99%). Based on the PFGE analysis, strains of biovar 4 showed little variation in fingerprinting pattern

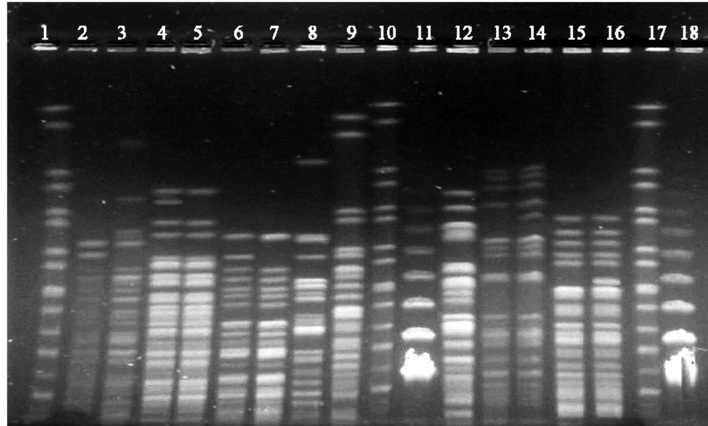

Fig. 1: Representative PFGE profiles of *R. solanacearum* strains. Lanes 1, 10 and 17 are standard marker strain, *S. branderup* restricted by *Xba*I; Lanes 11 and 18 are Lambda DNA concatemer marker

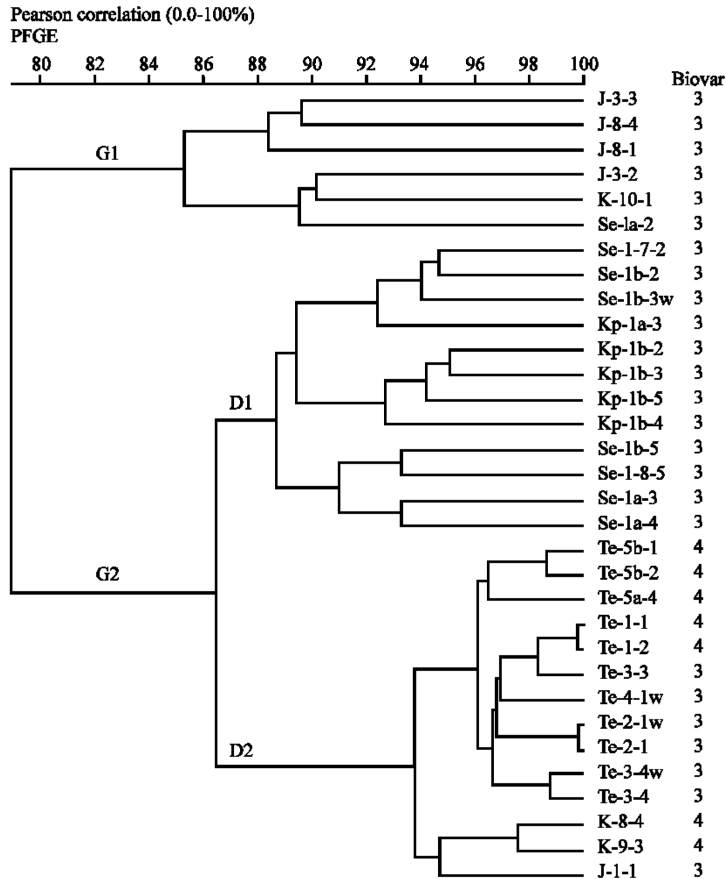

Fig. 2: Dendrogram constructed by using Pearson correlation showing correlations between PFGE profiles of 32 strains of *R. solanacearum*

(similarity from 94-98%) than strains of biovar 3 (similarity from 78-99%); even similarity between strains of biovar 4 from different origins (provinces) was higher than most strains of biovar 3.

Based of cluster analysis of PFGE pattern, all strains were differentiated into two main groups G1 and G2 (Fig. 2) at 85% similarity. Group 1 contained two subdivisions (shown by D1, D2); one subdivision

Fig. 3: BOX-PCR patterns of *R. solanacearum* strains; the lanes No. 5, 12 and 20 are DNA ladder

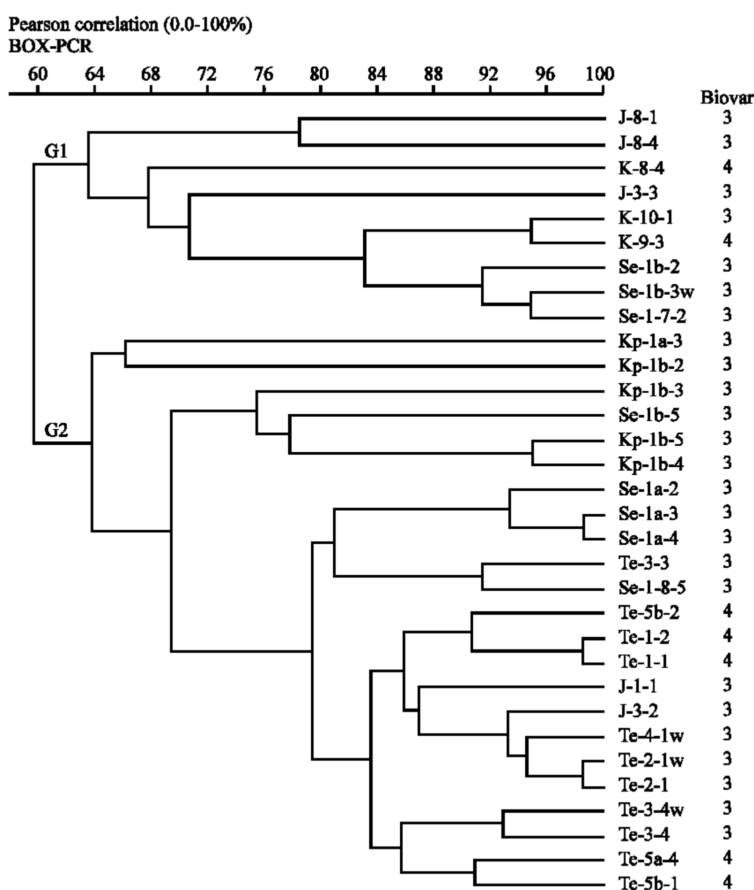

Fig. 4: Dendrogram constructed by using Pearson correlation showing correlations between BOX-PCR profiles of 32 strains of *R. solanacearum*

(D2) contained all 7 strains of biovar 4 plus 9 biovar 3 strains. This division (D2) consisted of all strains that originated in Terengganu Province. Other subdivision (D1) contained strains that were collected from Selangor, Kedah and Perak Provinces. Group 2 was a mix from strains, were collected from Selangor, Johor and Kelantan Provinces.

BOX-PCR: All of the strains were also typeable BOX-PCR fingerprinting method and produced varied banding patterns (Fig. 3). All fingerprints were determined twice for each strain 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 (Fig. 4) revealed same

similarity with PFGE among all strains, but not as many identical groups as obtained with PFGEs. A close relationship was found for all strains that have been collected from same origins but just one distinct group (shown by G1 in Fig. 4) has been observed; consisted from some strains collected from different province (Fig. 4). No distinct group has been identified for different biovars or strains from different provinces.

DISCUSSION

We identified phylogenic relationships among strains of *R. solanacearum* isolated from banana farms of West Malaysia. Previously it was demonstrated that PFGE is a very powerful tool for detecting genomic diversity of *R. solanacearum* and for understanding the ecology and phylogeny of this microorganism (Ito *et al*., 1996; Smith *et al*., 1995). Also in many earlier reports, BOX-PCR analysis has been used for differentiation of *R. solanacearum* species (Horita and Tsuchiya, 2001; Thwaites *et al*., 1999). In this study, both PFGE and BOX-PCR analysis were used for phylogenic clustering of *R. solanacearum* strains but PFGE revealed more distinct groups at the infrasubspecific level and it was able to distinguish DNA fingerprinting of strains based on location of isolation of the strains were and which biovar type they were; while BOX-PCR only was able to differentiating bacterial strains by geographical origins.

Based on PFGE analysis, the strains collected from Terengganu Province (in East part of Peninsular Malaysia) were closely related to each other genetically (94-98% similarity) and composed a very distinct genetic group. This group was similar to other strains collected from Johor Province followed by strains collected from Kelantan. The strains of Selangor, Perak and Kedah Provinces had low similarity as compared to strains of Terengganu. These results can be explained by attention to topographic map of West Malaysia. Titiwangsa Mountains in West Malaysia is a mountain range that acts as a natural barrier, dividing West Malaysia into East and West Coast regions. Although the climate and weather condition in both regions are very similar there is no link between the rivers and streams of both sides. Therefore through the centuries of isolation, the strains of *R. solanacearum* on both sides of this natural barrier have diverged. On the other hand, in PFGE analysis, strains of biovar 4 collected from different areas showed very close DNA fingerprinting profile while the variation within all strains of biovar 3 was higher than strains of biovar 4 except those that have been collected from Terengganu Province. This observation is unique result and show that genetic diversity among strains of biovar 4 is less than strains of biovar 3. Recent phylogenetic studies indicate that biovar 3 and 4 are part of a separate genetic lineage distinct from the other biovars (biovar 1, 2 and 6) (Timothy, 2006), the results of this study is not far from this theory because in this study only strains from biovars 3 and 4 have been studied and in the meantime the similarity between strains of biovar 4 and most of strains biovar 3 was higher than 90%.

Based on BOX-PCR analysis, there was significant similarity among all strains of *R. solanacearum* that have been collected from same origin in West Malaysia. The capability of BOX-PCR in differentiation of *R. solanacearum* strains from different sources already has been confirmed by many reports [10, 16]. However, BOX-PCR was not able to differentiate DNA fingerprinting of different strains of *R. solanacearum* into very distinct groups, presumably because of the conserved nature of the repetitive target sequences in *R. solanacearum* species.

In summary, this study clearly shows that the strains of *R. solanacearum* originating from different geographic regions of West Malaysia are phylogenetically diverse. DNA sequencing of these bacterial strains can release more information about the relationship of these genomic groups with other phylotypes of *R. solanacearum* in the world.

Considering this diversity and existence of different genomic groups of *R. solanacearum* in different parts of West Malaysia, probably local quarantines can be effective in preventing of distribution of different types of this bacterium. *R. solanacearum* is known as dominant plant pathogenic bacteria in all parts of West Malaysia (Abdullah, 1988; Khakvar *et al*., 2008) but still this bacterium is not an epidemic problem in this country. Most of commercial banana varieties in this part of country are still tolerant to this bacterium and it is known as an opportunistic pathogen, therefore without proper and internal quarantines for bacterium, more pathogenic and aggressive strains can be produced by genomic transformation between different groups of *R. solanacearum* strains in West Malaysia.

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