Study on intraspecific diversity of Ralstonia solanacearum strains in West Malaysia using whole cell fatty acid analysis

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Study on intraspecific diversity of *Ralstonia solanacearum* strains in West Malaysia using whole cell fatty acid analysis

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Abstract

Surveys were conducted between the years of 2005 and 2006 at several locations in the northern, central and southern parts of West Malaysia to study the polymorphism of *Ralstonia solanacearum* strains. These sites included vegetables and farms with 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, in selected areas. The bacterium was isolated in all samples using semi-selective tetrazolium chloride medium (TZC). The bacteria strains were detected by using the BIOLOG identification system and were confirmed by nested-PCR. Fatty Acid Methyl Esters (FAME) profiling was performed to determine polymorphism among 58 bacterial isolates. The results showed that the fatty acid composition varied for all *R. solanacearum* isolates. Grouping of *R. solanacearum* isolates by fatty acid composition suggested that the existence of distinct groups that were significantly related to host of bacteria but low correlation between fatty acid profiles and biovar or sampling site was detected. A unique FAME profile was found among the strains that have been isolated from banana.

Keywords: *Ralstonia solanacearum*, bacterial wilt, FAME, diversity, West Malaysia

Introduction

*Ralstonia solanacearum*, previously known as *Pseudomonas solanacearum* (Smith, 1914), is an aerobic, Gram-negative, rod with a polar flagella tuft (Agrios 2005). This organism, which is responsible for bacterial wilt disease, can infect over 300 plant species belonging to over 30 botanical families (Hayward 1991). Major agricultural hosts include tomato, potato, eggplant, tobacco, chili and banana trees (Hooker 1990). The bacterium is a complex taxonomic unit in which the strains display an important diversity at different levels (physiological, serological, genetic characteristics, and host range) (Genin and Boucher 2004). In order to describe this intraspecific variability, several systems of classification have been proposed. Thus, the species is subdivided into five races according to its host range and into six biovars based on utilization of three disaccharides and three hexose alcohols (Schaad et al. 2001). Races and
biovars usually do not correspond and also there is considerable genetic variation among strains within each race or biovar (Hayward 1991; Genin and Boucher 2004). This genetic and pathogenic variation makes development of diagnostic, detection, and control measures of *R. solanacearum* more difficult. Moreover, individual strains in each biovar or race differ greatly in their ability to cause wilting in host plants and display varying degrees of aggressiveness against different host plants (Genin and Boucher 2004). To resolve this genetic plasticity, several methods have been developed to identify diversity and subdivide races or biovars of *R. solanacearum* in relation to their aggressiveness, geographic distribution and plant pathogenicity. Unfortunately grouping of *R. solanacearum* strains by recent molecular markers (e.g. RFLP, AFLP or rep-PCR) reveal limited relationships with traditional groups (biovar or race) and so far low relationship between genotype and pathogenic fitness (races) or biochemical groups (biovars) have been detected (Dookun et al. 2001; Horita and Tsuchiya 2001). Bacterial fatty acids (FA), unlike many phenotypic and some genotypic characteristics, are genetically highly conserved owing to their essential role in cell structure and function. Therefore, fatty acid can be used as a valuable chemotaxonomic tool for the classification of bacterial species and subspecies (Janse 1991; Schutter and Dick 2000; Stead 1992). The objectives of this study were to characterize *R. solanacearum* strains in West Malaysia by comparison of their fatty acid profiles and to determine the effects of biovar, host and sampling site on the fatty acid profiles.

**Materials and methods**

**Sample collection**

During the years of 2005–2006, several locations were selected within the northern, central and southern parts of West Malaysia to study the polymorphism among strains of *R. solanacearum*. Sampling sites were divided in to five different regions (Region 1, 2 . . . 5) based on their similarity in surface area, environmental conditions and host crops (Table 1). These sites included vegetable farms and other production areas planted with known hosts of the pathogen, such as banana and tobacco. Sampling was conducted by collecting wilted crop plants and weeds. Soil and water samples were randomly collected from selected areas. All field samples were stored at 8°C until ready for analysis.

*R. solanacearum* strains were isolated on semi-selective tetrazolium chloride medium (TZC) (Schaad et al. 2001). The cultures were incubated at 30°C for 36 hours. Typical *R. solanacearum* colonies were isolated and transferred to distilled water and kept at room temperature. The BIOLOG identification system was used to identify the bacterial isolates. A two-stage nested-PCR was performed to confirm the results of BIOLOG test by using the method of Pradhanang et al. (2000). The first stage PCR generated a 410 bp amplicon while the second stage PCR gave a 220 bp amplicon. PCR products were analyzed by electrophoresis on 1% agarose gels and visualized with UV light after ethidium bromide staining. Positive samples were selected and used for FAME test. Biovar determination was carried out for positive samples in accordance to the method described by Schaad et al. (2001).

**Fatty acid methyl esters (FAME) extraction**

*R. solanacearum* strains were grown on Tryptic Soy Broth Agar (TSBA) at 28°C for 24 hours. Extraction of FAMEs was conducted for each strain by MIDI protocol according to the method described by Schutter and Dick (2000). The extracts were analyzed using the Gas Chromatography (GC) System (Hewlett Packard, model 5898) equipped with a DB-5
column (25 m x 0.2 mm silica capillary column). The temperature program was ramped from 170°C to 250°C at 5°C per minute. Total running time was 34 minute. GC included a flame-ionization detector (FID) and Helium (He) as carrier gas. Each sample injection was repeated two times to decrease operative errors. A bacterial FAME standard (AccuStandard, FAMQ-005), containing representative compounds of common fatty acid groups in bacteria, was injected into the column prior to sample injection. Data analysis was performed using MiniTab 15 and EXCEL (Microsoft, 2007). Means and standard deviations for the individual fatty acid in FAME profile of each group of samples (Regions, Hosts and Biovars) were calculated (Table 1). Principle Component Analysis (PCA) was used to compare FAME profiles between different strains as well as relationships among different samples with multiple variables (fatty acids) from each sample.

Results

Among 101 bacterial strains that had a typical colony shape of R. solanacearum on TZC media, 69 strains were confirmed as R. solanacearum by the BIOLOG identification system. On the basis of results of nested-PCR, all the 69 strains had 410 bp amplicon for first PCR and a 220 bp ampiclon for second PCR (Figure 1); therefore all 69 strains were further confirmed as R. solanacearum. 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 (Schaad et al. 2001), 38 and 31 strains have been recognized as Biovar 3 and biovar 4, respectively. Only 58 bacterial strains grew under standard conditions (TSBA, 28°C for 24 h) as described by Schutter and Dick (2000). Eleven isolates were unable to grow on TSBA under the same conditions. The results of FAME on 58 samples showed that fatty acid composition varied for all R. solanacearum isolates. The mean percentage distributions of fatty acid in all R. solanacearum strains in each group (host, biovar and region) are shown in Table 1. Eight groups of fatty acid were identified and quantified among all positive strains. These included fatty acids with a carbon length of 14 to 20 carbons (Table 1). Four fatty acids (Myristoleic acid, Myristic acid, cis-9-Hexadecanoic acid and Palmitic acid were identified in all collected strains.

In addition, these four fatty acids included 65—80% of total fatty acid of all strains. Long chain fatty acids (cis-11, 14-Eicosadienoic, cis-11 Eicosenoic acid and cis-11-14-17 Eicosatrienoic acid) were found in a very low concentration just within strains that have

Figure 1. Results of nested-PCR; from right, fist three lines shown a 280 bp ampiclon that has been produced by primer JE2/OLI2, lines 4–6 shown a 340 bp ampiclon by primer Y2/OLI1, Line No. 7 is Marker (80, 100, 200 ... to 1000 bp) and line Nos. 8–12 are results of nested-PCR (220 and 410 bp).
Table I. Relative means amounts (%) of Fatty acids found in *R. solanacearum* strains.

<table>
<thead>
<tr>
<th>Fatty acid (FA)*</th>
<th>Common name (AUPAC)</th>
<th>Abbreviation</th>
<th>Biovar</th>
<th>Region**</th>
<th>Host***</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA 1</td>
<td>Myristoleic acid</td>
<td>14:1 3OH</td>
<td>7.17</td>
<td>7.46</td>
<td>6.13</td>
</tr>
<tr>
<td>FA 2</td>
<td>Myristic acid</td>
<td>14:0</td>
<td>8.2a</td>
<td>6.97b</td>
<td>6.91</td>
</tr>
<tr>
<td>FA 3</td>
<td>Methyl cis-9-Hexadecanoic acid</td>
<td>16:1</td>
<td>30.9</td>
<td>30.1</td>
<td>33.58</td>
</tr>
<tr>
<td>FA 4</td>
<td>Palmitic acid</td>
<td>16:0</td>
<td>34.82</td>
<td>32.87</td>
<td>32.82</td>
</tr>
<tr>
<td>FA 5</td>
<td>Heptadecanoic acid</td>
<td>17:0 cyclo</td>
<td>2.1a</td>
<td>4.7b</td>
<td>7.09a</td>
</tr>
<tr>
<td>FA 6</td>
<td>Linolelaidic, Linolenic &amp; Elaidic acid</td>
<td>Summed</td>
<td>12.88</td>
<td>14.27</td>
<td>12.42</td>
</tr>
<tr>
<td>FA 7</td>
<td>2-hydroxy oleic acid</td>
<td>18:1 2OH</td>
<td>2.18</td>
<td>2.55</td>
<td>2.7</td>
</tr>
<tr>
<td>FA 8</td>
<td>cis-11 Eicosenoic acid &amp; cis-11-14-17 Eicosatrienoic acid</td>
<td>20:1, 20:2</td>
<td>3.57a</td>
<td>2.58b</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*Fatty acids with mean of less than 2% not included in this table.

**Regions were shown by Region 1: Johor, Region 2: Kedah and Perak, Region 3: Kelantan, Region 4: Selangor and Region 5: Terengganu.

***Hosts were shown by Host 1: Banana, Host 2: Tomato, Host 3: Eggplant, Host 4: Chili and Host 5: Tobacco.

****Means of groups followed by the different letter(s) are significantly different (P < 0.01) by LSD. No letter means that no significant difference.
been isolated from banana. Meanwhile Heptadecanoic acid was found in all strains except some strains that have been isolated from banana.

Pearson’s correlation of individual fatty acids was used to measure the relationship between them and any two of these eight fatty acid groups. A significant correlation between all kinds of fatty acids (FA1–FA8) and a host of isolated bacteria has been observed while sampling site and biovar type were correlated with some groups of fatty acids. Low but significant correlation between Myristoleic acid (FA1) and two other fatty acids (Myristic acid (FA2) and cis-9-Hexadecanoic acid (FA3)) have been observed. In addition, a negative correlation between Palmitic acid (FA4) and group-eight (FA8) fatty acids (cis-11, 14-Eicosadienoic and cis-11 Eicosenoic acid and cis-11-14-17 Eicosatrienoic acid) has been observed.

Euclidean distances were calculated between fatty acid profiles of strains and clustered using the unweighted pair group method. The dendrogram based on fatty acid composition formed two major clusters (Figure 2). Cluster No. 1 had three subgroups, a, b, c. Subgroup

![Figure 2. Dendrogram by cluster analysis on R. solanacearum strains fatty acids.](image-url)
shown by ‘a’ was contained only strains that have been isolated from banana. One of the main clusters (cluster No. 2) comprised isolates only from tomato, eggplant and chili strains. Similar results could be drawn from principle component analysis. Grouping of \textit{R. solanacearum} strains using PCA suggested the existence of two main groups with eight subgroups namely group A, B . . . G and H (Figure 3). The first group (G1) included all banana strains. Only two chili strains were included in group one. This group was separated into six sub-groups (A to F). The second group was divided into two subgroups (G and H). Subgroup ‘G’ comprised most strains that have been isolated from chili, eggplants and tobacco while most strains that were isolated from tomato were found in subgroup ‘H’.

\section*{Discussion}

According to the results of BIOLOG and nested-PCR, the most important host of \textit{R. solanacearum} in West Malaysia was banana followed by chili and tomato. This is in agreement with the observations previously reported by Abdullah (1988). In addition, the results obtained from this study show that different biovars of \textit{R. solanacearum} are found randomly in West Malaysia and distributions of biovars of \textit{R. solanacearum} in West Malaysia are random. This also was in agreement with results obtained by many previous studies; unlike the distribution of races, for biovars of \textit{R. solanacearum} no regular geographical distribution has been report in the entire world (Hayward 1990).

Table 1 summarizes the whole fatty acid composition of different strains of \textit{R. solanacearum} in West Malaysia. Based on these results, fatty acid composition varied for all \textit{Ralstonia solanacearum} strains. This disparity in the fatty acid profile is consistent with previous studies (Stead 1992; Salete de Mole et al. 1999; Jaunet and Wang 1999). All of eight fatty acid groups significantly differed in \textit{R. solanacearum} strains from different hosts, indicating that FAME profiles of \textit{R. solanacearum} could be host specific. Therefore, it was observed that the type of...
host plant significantly influenced the fatty acid profiles of Ralstonia solanacearum while biovar type or sampling sites have a lower influence on the composition of fatty acid. This is in agreement with the observations made by Salete de Mole et al. (1999). Long chain fatty acids (cis-11, 14-Eicosadienoic, cis-11 Eicosenoic acid and cis-11-14-17 Eicosatrienoic acid) were found only in the banana strains (Table 1). There is no previous report on the existence of these kinds of fatty acids in strains of R. solanacearum (Jaunet and Wang 1999; Salete de Mole et al. 1999; Timms-Wilson et al. 2001). Although there was a report of a long chain unidentified fatty acid found in eucalyptus strains (Salete de Mole et al. 1999), this is the first report of the presence of these kinds of fatty acid in banana strains. The quantity of heptadecanoic acid in the strains that have been collected from Johor province (region 1) was significantly high. Few strains were collected from banana and as the results of this study have shown, some banana strains had no Heptadecanoic acid.

One notable observation in this study is the negative correlation between Palmitic acid (FA4) and fatty acids of group-eight (FA8). Palmitic acid is one of the major fatty acids in bacteria and is usually present in most bacteria including R. solanacearum (Stead 1992; Salete de Mole 1999; Varbanets et al. 2003). On the other hand, there are a few reports for the existence of long chain fatty acids (such as FA8) in different strains of R. solanacearum. Therefore, this relationship may be important for the pathogenicity and virulence of R. solanacearum on banana. Both PCA analysis and the dendrograms distinguish the banana isolates as a separate group. Both chili isolates that have been grouped in group one of PCA analysis have been isolated from the farms nearby banana fields so these farms could be contaminated by banana isolates. Therefore, from this study, it is clear that the strains that have been isolated from banana have different fatty acid composition. Tomato, chili, eggplant and tobacco strains are also different in quantity and quality of fatty acid compositions, but the FAME test is not a sufficient method for their differentiation.

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