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https://doi.org/10.5109/24306

出版情報：九州大学大学院農学研究院紀要. 44 (1/2), pp.59-65, 1999-11. Kyushu University

J. Fac. Agr., Kyushu Univ., 44 (1·2), 59–65 (1999)

Identification of *Ralstonia solanacearum* Isolated from Wilted Tobacco Plant by Fatty Acid Profiles and PCR–RFLP Analysis

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(*Received July 19, 1999 and accepted August 24, 1999*)

Identification of *Ralstonia solanacearum*, isolated from wilted tobacco plant was conducted by fatty acid profiles and PCR–RFLP analysis. The tobacco strain showed identical patterns of fatty acid composition with *Ralstonia solanacearum* and *R. pickettii*, and it was distinctly different from *Burkholderia gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli*. Palmitoleic acid (16:1 *cis* 9) content was much higher in tobacco strain, *R. solanacearum* and *R. pickettii*. On the other hand, an unidentified (unidentified–6) fatty acid was detected (9.55 to 11.98%) in *B. gladioli* pv. *gladioli*, *B. cepacia and B. caryophylli*, and it was absent in tobacco strain, *R. solanacearum* and *R. pickettii*. The tobacco strain showed identical PCR–RFLP profiles (*Msp*I digestion) with *R. solanacearum* and *R. pickettii*. The PCR–RFLP profiles with *Hae*III digestion further differentiated the tobacco strain and *R. solanacearum* from *R. pickettii*. Both fatty acid profiles and PCR–RFLP analysis suggested that tobacco strain is *R. solanacearum*. The physiological and biochemical tests also confirmed this conclusion.

INTRODUCTION

Bacterial wilt disease caused by *Ralstonia solanacearum* is a widely distributed and devastating disease of many economically important crops (Kelman, 1953; Hayward, 1991). Different physiological and biochemical tests under a determinative scheme and a host pathogenicity test are generally used to identify the bacterium. However, all these tests require about two months to perform. Therefore, rapid identification has been emphasized. Recently, thin layer chromatography (Matsuyama *et al.*, 1993; Matsuyama, 1995; Khan and Matsuyama, 1998; Rahman *et al.*, 1998), fatty acid profiles (Janse, 1991; Stead, 1992) protein profiles (Dristing and Dianese, 1990; Li and Hayward, 1994) and PCR amplified RFLP (Cook *et al.*, 1989) were used for characterization and identification of *R. solanacearum*.

In this paper, we describe the identification of *R. solanacearum*, isolated from wilted tobacco plant by fatty acid profiles and PCR–RFLP analysis of amplified ribosomal DNA (rDNA). Simultaneously, the conventional physiological and biochemical tests were also conducted to verify the results of fatty acid profiles and PCR–RFLP analysis of amplified rDNA.

MATERIALS AND METHODS

Bacterial strains

A tobacco wilting pathogen isolated from Matsuyama City in 1997 was included in this experiment. The type strains of *Ralstonia solanacearum* ATCC 11696^T, *R. pickettii*

ATCC 27511^T, *Burkholderia gladioli* pv. *gladioli* ATCC 10248^T, *B. cepacia* ATCC 25416^T, *B. caryophylli* ATCC 25418^T and *R. solanacearum* C–319, a Japanese strain, were used as reference strains.

GLC analysis

Whole cellular fatty acid analysis was conducted followed by Khan *et al.* (1999). Bacteria were grown in 523 broth (Kado and Heskett, 1970) at 30°C for 48 hr in shake culture. Five milligrams of the lyophilized cells was methylated with 0.5 ml of 5% HCl–methanol at 100°C for 3 hr in a sealed glass tube. The content was cooled at room temperature and transferred to a new Eppendorf tube. The fatty acid methyl ester (FAME) derivatives were added with 0.5 ml of distilled water and petroleum ether and centrifuged at 5000 rpm for 5 min. The solvent phase was collected and washed with 0.5 ml distilled water and dehydrated by mixing with 0.5 g anhydrous sodium sulfate. The organic phase was concentrated by nitrogen gas blowing and subjected to GLC (Shimadzu C–R–7A plus; column: HR–SS–10). The column and injection–port temperatures were maintained at 180°C and 250°C, respectively, and the flow rate of nitrogen gas was 50 ml/min. Fatty acids were identified by the comparison of retention times with the standard and average values of fatty acid composition were used to differentiate the strains. Relative similarities among the strains based on fatty acid composition were assessed with average linkage cluster analysis procedure using the statistics package software SYSTAT.

PCR–RFLP analysis

About 5 mg bacterial cells was homogeneously suspended in 500 μl lysis buffer (50 mM Tris–HCl, pH 7.2, 50 mM EDTA, 1% sodium *N*–lauroyl sarcosinate and 1% 2–mercaptoethanol) and incubated at 65°C for 1.5 hr. The homogenate was thoroughly shaken using 500 μl of chloroform – phenol – isoamyl alcohol (25:24:1, v/v/v) mixture. After centrifugation for 15 min at 15000 rpm, the water phase was collected and 400 μl of chloroform – isoamyl alcohol (24:1) was added, shaken thoroughly and centrifuged at 15000 rpm for 15 min. The water phase was collected and 200 μl of ammonium acetate (7.5 M) was added. DNA was precipitated with 95% ethanol and collected by centrifugation at 15000 rpm for 20 min and then washed with 80% aqueous ethanol solution, dried *in vacuo*, and dissolved in 200 μl of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Two primers ITS 1A (5'–CTGGATCACCTCCTTT–3') and ITS 2D (5'–CGCTTGACC-CTATAACG–3') were used for amplification of ITS region of rDNA.

PCR was carried out in a 100 μl reaction volume; contained 77.5 μl deionized water, 10×PCR buffer 10 μl, 5 mM DNTPs 5 μl, DNA solution 5 μl, each primer 1 μl and *Tth* DNA polymerase 0.5 μl, and it was overlaid with 50 μl of mineral oil. The thermal cycles were conducted for 30 times, with parameters of 94°C for 1 min, 50°C for 0.45 min and 72°C for 1 min. After amplification, DNA fragments were examined by horizontal electrophoresis in 1% agarose gel.

RFLP was conducted for ITS 1A/ITS 2D primed PCR amplified rDNA. Each PCR product was digested with two restriction enzymes, *Msp*I and *Hae*III, and then subjected to electrophoresis in 1.5% agarose gel in TBE buffer.

Physiological and biochemical tests

To verify the results of fatty acid profiles and PCR–RFLP analyses, physiological and biochemical tests were conducted. Gram stain, oxidase, catalase, argininc dihydrolase, growth at 41 °C and gelatin liquefaction tests were performed followed by standard procedures (Kovacs, 1956; Thornley, 1960; Suslow *et al.*, 1982; Schaad, 1988). Carbon source utilization tests were also conducted by adding each carbon source (0.1% wt/vol) to the mineral base medium of Ayers *et al.* (1919). Carbon stock solutions were sterilized by autoclaving except for the solutions heat labile, which were sterilized by filtration. Bacterial cells suspensions were streaked onto each test medium, incubated at 30 °C and evaluated periodically at 7, 14 and 21 days. The results on the minimal medium without any addition of carbon source were used as control.

RESULTS AND DISCUSSION

Bacterial strains used in this experiment contained eight major (constitutes more than 5% of total composition) fatty acids and six minor (constitutes less than 5% of total composition) fatty acids. Among the major fatty acids, myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1 *cis* 9), oleic acid (18:1 *cis* 9), *cis*–vaccenic acid (18:1*cis*11) and one unidentified fatty acid (unidentified–1) were common in all the strains. Unidentified–3 was recorded in the tobacco strain, *R. solanacearum*, *R. pickettii* and *B. caryophylli*, and unidentified–6 was detected in *B. gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli*. The tobacco strain, *R. solanacearum* and *R. pickettii* predominantly contained palmitoleic acid (16:1 *cis* 9), which constituted 34.21 to 40.19% singly; while it was detected 7.55 to 10.25% in *B. gladioli* pv. *gladioli*, *B. cepacia*, and *B. caryophylli*. Unidentified–1 and unidentified–6 were recorded 7.92 to 13.75% in *B. gladioli* pv.

Table 1. Percentage composition of whole cellular fatty acids in tobacco strain and reference strains.

Bacterial strain	Fatty acid composition													
	14:0	16:0	16:1 *cis* 9	18:0	18:1 *cis* 9	Un–1	18:1 *cis* 11	18:3 *cis* 6,9,12	Un–2	16:0 2–OH	Un–3	Un–4	Un–5	Un–6
Tobacco strain	11.08	15.95	36.37	0.99	6.18	2.65	18.79	0.81	0	0.34	6.51	0	0.29	0
R. solanacearum C 319	11.04	13.02	34.21	0.94	8.53	1.29	21.25	0.53	0	0.67	7.71	0	0.76	0
R. solanacearum ATCC 11696T	15.99	16.48	38.35	2.18	6.46	3.45	9.71	0	0	1.08	0.79	0	5.47	0
R. pickettii ATCC 27511T	11.15	21.60	40.19	2.56	9.69	1.80	9.93	0.66	0	0	0.98	0	1.36	0
B. gl. pv. *gladioli* ATCC 10248T	8.44	31.60	8.04	2.22	11.96	11.59	8.94	0	1.54	2.30	0	3.78	0	9.55
B. cepacia ATCC 25416T	7.69	28.82	7.55	2.74	7.50	13.75	12.69	0	0.58	2.21	0	4.44	0	11.98
B. caryophylli ATCC 25418T	4.86	21.69	10.25	2.95	14.29	7.92	17.42	0	0	3.99	2.07	0	3.37	11.13

Un–, unidentified

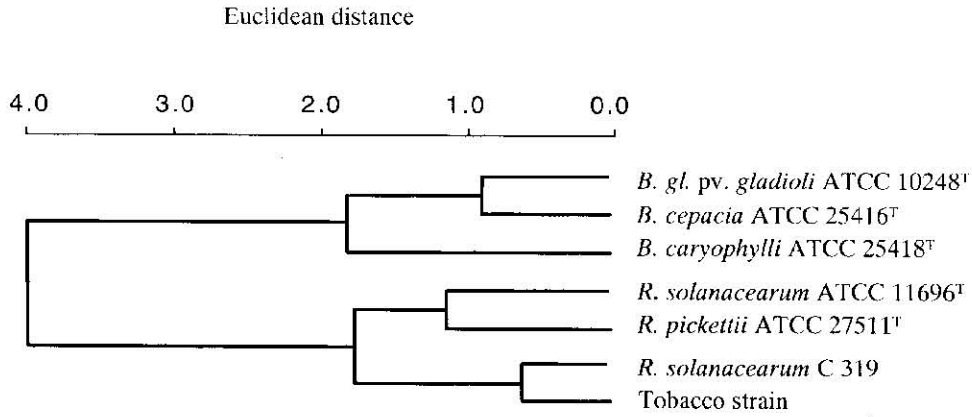

Fig. 1. Dendrogram by cluster analysis on tobacco strain and reference strains.

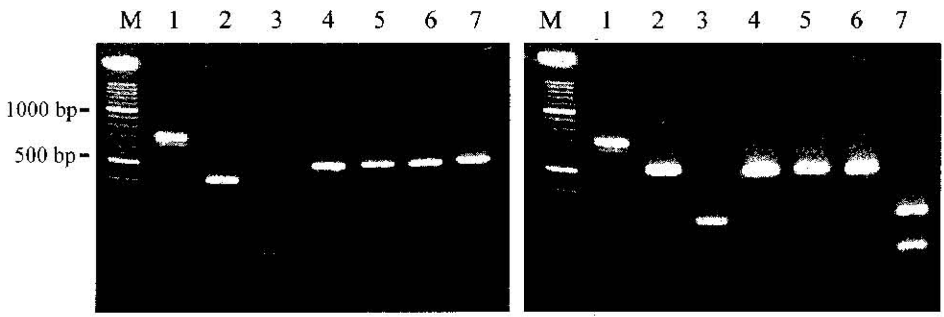

Fig. 2. Electrophosetic patterns of PCR-amplified ITS region of rDNA digested with *Msp*I (left) and *Hae*III (right).
Lanes M: Molecular size marker (100-bp ladder); 1: *B. caryophylli* ATCC 25418T; 2: *B. gl.* pv. *gladioli* ATCC 10248T; 3: *B. cepacia* ATCC 25416T; 4: *R. solanacearum* ATCC 11696T; 5: *R. solanacearum* C 319; 6: Tobacco isolate; 7: *R. pickettii* ATCC 27511T.

gladioli, *B. cepacia* and *B. caryophylli*; while, in the case of tobacco strain, *R. solanacearum* and *R. pickettii*, unidentified-1 was detected 1.29 to 3.45% and unidentified-6 was absent (Table-1). These results showed that *R. solanacearum* and *R. picketti* will be clearly differentiated from *B. gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli* by their fatty acid compositions.

The dendrogram of cluster analysis based on fatty acid composition formed two major clusters (Fig. 1). One cluster was formed by tobacco strain, *R. solanacearum* and *R. pickettii*, and other consisted with *B. gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli*. Previous studies showed that *R. solanacearum* and *R. pickettii* were very similar in fatty acid pattern and easily discriminated from *B. gladioli* pv. *gladioli*, *B.*

cepacia and *B. caryophylli* by the differences in percentages of the fatty acids (Janse, 1991; Stead, 1992). The results of our present experiment also agreed with the previous findings. The results also suggested that tobacco strain was closely related with *R. solanacearum* and *R. picketti*. However, *R. pickettii* is a human clinical bacterium. Hence, the tobacco strain will be *R. solanacearum*.

The ITS rDNA product, a single fragment was detected by agarose gel electrophoresis following amplification with PCR (data not shown). RFLP profiles obtained after digestion of PCR amplified product of rDNA represented specific fragment patterns by

Table 2. Physiological and biochemical tests for tobacco strain and reference strains.

Tests	Strain						
	R. solanacearum ATCC 11696^T	*R. solanacearum* C 319	Tobacco strain	*R. pickettii* ATCC 27511^T	*B. gl.* pv. *gladioli* ATCC 10248^T	*B. cepacia* ATCC 25416^T	*B. caryophylli* ATCC 25418^T
Gram stain	−	−	−	−	−	−	−
Oxidase	+	+	+	+	W	+	+
Catalase	+	+	+	+	+	+	−
Arginine dihydrolase	−	−	−	−	−	−	−
Denitrification	+	+	+	+	−	−	+
Gelatin liquefaction	−	−	−	−	+	+	−
Growth at 41 °C	−	−	−	+	W	+	+
Utilization of :							
D–Xylose	−	−	−	+	+	+	+
Trehalose	−	+	+	−	+	+	+
D–Ribose	−	−	+	+	+	+	+
D–Arabinose	−	−	−	−	+	+	+
Saccharose	+	+	+	−	−	+	+
L-Rhamnose	−	−	−	−	−	−	+
Levulose	+	+	+	+	+	+	+
Maltose	−	−	−	−	−	−	−
Cellobiose	−	−	−	−	+	+	+
Lactose	−	−	−	−	+	+	−
Sorbitol	−	+	+	−	+	+	+
Mannitol	−	+	+	−	+	+	+
Dulcitol	−	+	+	−	+	+	−
Adonitol	−	−	−	−	+	+	−
Inositol	+	+	−	−	+	+	+
Sarcosine	−	−	+	−	+	+	−
Salicin	−	−	−	−	+	+	+
Citraconate	−	−	−	+	+	+	−
2–Ketoglutarate	+	+	+	+	+	+	+
D–Tartrate	−	−	−	−	+	−	−
Benzoate	−	−	−	−	+	+	−
Malonate	−	−	−	+	+	+	−
Mesaconate	−	−	−	−	+	−	−
L–Aspartate	+	+	+	+	+	+	+

+, positive; −, negative; w, weakly positive electrophoresis (Fig. 2). Two restriction enzymes *Msp*I and *Hae*III were used to differentiate the strains. The RFLP patterns with *Msp*I digestion showed that tobacco strain was identical with *R. solanacearum* and *R. pickettii*, and distinctly different from *B. gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli*. The RFLP patterns with *Hae*III digestion discriminated *R. pickettii* from tobacco strain and *R. solanacearum* as well as *B. gladioli* pv. *gladioli*, *B. cepacia* and *B. caryophylli* (Fig. 2). These results indicate that tobacco strain is *R. solanacearum*. Furthermore, this method would involve the possibility of genetic diagnosis and can be used for identification of *B. gladioli* pv. *gladioli*, *B. cepacia*, *B. caryophylli*, *R. solanacearum* and *R. pickettii*.

Physiological and biochemical tests were also conducted to verify the results of fatty acid analysis and PCR–RFLP analysis. Tobacco strain was positive in oxidase, catalase and denitrification tests, and negative in gram stain, arginine dihydrolase, growth at 41 °C and gelatin liquefaction tests. Furthermore, other characteristics summarized in Table 2 confirmed that tobacco strain is *R. solanacearum*.

Identification of *R. solanacearum* by fatty acid profiles and PCR–RFLP analysis in combination with some selected physiological and biochemical tests is more convenient and a practical approach.

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