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Geographical Distribution of Biovars of *Ralstonia solanacearum* in Brazil

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Fifty–seven bacterial wilt pathogens were isolated from infected tomato, eggplant, potato, cucumber, pepper, banana, *Solanum gilo* and eucalyptus plants collected at different regions in Brazil. They were identified as *Ralstonia solanacearum* by the routine identification tests and the TLC method. The isolates were divided into seven groups on the basis of pathogenicity tests to tomato, eggplant, potato and tobacco. Moreover, based on the results of cultural, physiological and biochemical tests, twenty–four isolates were grouped as biovar 1, seventeen as biovar 2 and sixteen as biovar 3. Biovar 1 isolates are common and widely distributed in Brazil. Biovar 2 isolates are predominant on potato and prevail in the central west, south–east and south areas. Biovar 3 isolates exist mainly in the northern and north–eastern parts of Brazil.

INTRODUCTION

Ralstonia (syn. *Burkholderia, Pseudomonas*) *solanacearum* (E. F. Smith) Yabuuchi *et al.*, the causal agent of bacterial wilt, is an important plant pathogenic bacterium in tropical, subtropical and warm temperate regions, causing great losses in production of many crops in worldwide including Brazil (Kelman, 1953, Hayward, 1991). Its isolates from different countries have so far been grouped into five races and five biovars (Hayward, 1964, French and Sequeira, 1970, He *et al.*, 1983).

The importance of bacterial wilt in Brazilian agriculture is confirmed by the number of papers published on this subject (Mariano *et al.*, 1998). The high destructive capacity of *R. solanacearum* coupled with its large host range has meant that this pathogen is still associated with heavy losses. Thus, there is a need for more in depth studies on host range, biovar distribution, survival, epidemiology and control of *R. solanacearum*.

The present study was undertaken to characterize the bacterial wilt pathogen and to clarify its biovar distribution in Brazil.

MATERIALS AND METHODS

Isolation and preservation of bacterial wilt pathogen

Pepper, potato, tomato, eggplant, cucumber, eucalyptus, banana and *Solanum gilo* which showed symptoms of bacterial wilt were collected from different locations in Brazil (Table 1). Bacteria were isolated from diseased stem tissues by cutting and soaking in

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Table 1. Brazilian isolates used in this experiment.

Bacterial isolate	Host	Place	Bacterial isolate	Host	Place
13,72,92,128,578, 613,799,933	potato	Brasilia	19,628,630,	tomato	Para
61,113,131	potato	Parana	31,33,35	tomato	Amapa
66	potato	Minas Gerais	855,1033	tomato	Brasilia
67	potato	Bahia	62,76,985	tomato	Pernambuco
98,106,964	potato	Santa Catarina	140,535	tomato	Amazonas
982,1005	potato	Rio de Janeiro	49,1102,1103, 1104	tomato	Maranhao
7	pepper	Amazonas	51	eggplant	Para
127	pepper	Brasilia	56	eggplant	Pernambuco
162	pepper	Maranhao	71,79,87	eggplant	Brasilia
582	pepper	Ceara	534	eggplant	Maranhao
20,629	pepper	Para	574,575,576,577, 579,603	eucalyptus	Para
129	cucumber	Brasilia	47	*Solanum gilo*	Amazonas
656	cucumber	Amazonas	73	banana	Sergipe

sterile distilled water. The resulting suspension was streaked onto tetrazolium chloride (TTC) medium, which is semi-selective for *Ralstonia solanacearum*. The plates were incubated at 30 °C for 3 days. Bacterial colonies displaying characteristics of *R. solanacearum* were selected and purified by re-streaking on TTC medium. Fifty-seven isolates listed in Table 1 were selected for further tests. A type strain (ATCC11696) and Japanese strain (C319) *of R. solanacearum* were used as positive reference cultures for all determinative tests. The individual virulent colonies were selected based on cultural characteristics and preserved in sterilized distilled water in screw capped test tubes at room temperature.

Pathogenicity test

Fifty-seven isolates of the suspect bacteria were tested for pathogenicity on tobacco (*Nicotiana tabacum* L. cv. White Burley), tomato (*Lycopersicon esculentum* mill. cv. Sekai ichi), eggplant (*Solanum melongena* cv. Chikuyou) and potato (*Solanum tuberosum* L. cv. Dejima).

Inocula were prepared as followed; each bacterial stock suspension in sterile distilled water was spread on TTC medium, and the plate was incubated at 30 °C for 48 hr for selecting virulent colonies. Typical virulent colonies were inoculated by the stem-puncture method (Winstead and Kelman, 1952). Sterile distilled water was used as a control. The inoculated plants were incubated at 30 °C for 24 hr in a moist chamber (RH 100%), then transferred to a greenhouse bench at 25–30 °C. The inoculated plants were observed daily to record wilt symptom for 30 days.

Identification and characterization of isolates

The following tests were carried out according to the method described in the references to characterize the pathogenic fifty-seven isolates: Gram reaction (Gredersen,

1978), levan production (Hildebrand, 1988), poly-β-hydroxybutyrate (PHB) accumulation (Hildebrand, 1988), oxidase reaction (Kovacs, 1956), catalase production (Digat, 1971), gelatin liquefaction (Frazier, 1926), indole production (Hayward, 1964), arginine dihydrolase (Thornley, 1960), starch hydrolysis (Sands *et al.*, 1980), lecithinase (egg yolk) (Mc Clung and Toabe, 1947), pigment production (Kelman, 1954). Nutritional studies were also performed. All carbon sources were filter-sterilized and added to the Ayer's basal medium (Pelczar, 1957) to give a final concentration of 0.5% (w/v). A suspension of bacterial cells grown on TTC agar medium was inoculated into broth of each test medium, incubated at 30°C, and the characteristics were evaluated periodically for 21 days.

Simple TLC

The simple TLC (Khan and Matsuyama, 1998) was conducted for the isolates including the type strain ATCC 11696 and C319 of *R. solanacearum*. Each isolate was grown on slant of King's B medium (Eiken Chem. Co., Tokyo) at 30°C for 3 days. One loopful of bacterial cells was suspended in 0.2 ml of chloroform-methanol (2:1, v/v) in a small glass vial and kept at least for 15 min at room temperature for lipid extraction. Ten μl of lipid extract was spotted on the origin of silica gel TLC plate and completely dried by a drier. The plate was developed with chloroform-methanol-0.2% calcium chloride solution (55:35:8, v/v/v) at 25°C for 1 hr. After development, aminolipid spots were detected by spraying ninhydrin followed by heating at 100°C in an oven for 10 min. The chromatograms were recorded by photocopy, photograph and/or computer (Adobe Photoshop 3.0J).

Biovar identification

To classify the isolates into biovars, carbohydrate oxidation test was conducted following standard procedures (Hayward, 1964, 1976, He *et al.*, 1983, Swanepoel and Young, 1988). In this test, three disaccharides (cellulose, lactose and maltose) and three hexose alcohols (dulcitol, mannitol and D-sorbitol) were used.

RESULTS AND DISCUSSION

Characterization of isolates

All isolates tested in the present study produced highly fluidal, creamy-white colonies with light pink or pinkish red center on TTC medium after 48 hr. The characteristics of the isolates revealed that all cultures were virulent isolates of *R. solanacearum* (Kelman, 1954; Hayward, 1964). They showed *R. solanacearum* type of chromatographic profile under direct colony TLC and simple TLC tests as described by Matsuyama *et al.* (1993a, 1993b) and Khan and Matsuyama (1998), respectively. All were pathogenic to at least one kind of plant tested and produced characteristic symptoms of bacterial wilt after inoculation. In particular, all isolates were pathogenic to tomato plant.

All isolates were gram-negative, and cells accumulated poly-β-hydroxybutyrate (PHB) as a carbon reserve material. They gave a negative reaction for arginine dihydrolase, indole production, starch hydrolysis, gelatin liquefaction, levan production and lecithinase. In the test for utilization of carbon source, all isolates gave positive

Table 2. Comparison of physiological and biochemical characteristics of Brazilian isolates with *Ralstonia solanacearum*.

Characteristic	Brazilian isolate (57)[a]	*R. solanacearum* ATCC11696[T]	*R. solanacearum*[b]
Pigment producution	d	+	d
Indole production	−	−	−
Levan formation from sucrose	−	−	−
Lecithinase	−	−	−
Catalase	+	+	+
Arginine dihydrolase	−	−	−
Oxidase	+	+	+
Hydrolysis of			
Starch	−	−	−
Gelatin	−	−	−
Poly-β-hydroxybutyrate accumulation	+	+	+
Utilization of			
glucose	+	+	+
α-ketoglutarate	+	+	+
m-hydroxybenzoate	−	−	−
α-amylamine	−	−	−
levulose	+	+	+
testosterone	−	−	−
L-histidine	+	+	+
adonitol	−	−	−
erythritol	−	−	−
saccharate	+	+	+
2,3 butylene glycol	−	−	−
geraniol	−	−	−
meso-inositol	+	+	d
β-alanine	d	+	d
D-ribose	d	+	d
levulinate	d	−	d
L(+)tartrate	d	+	d
meso-tartrate	d	+	d
azelate	d	−	d
L-serine	+	+	d
sarcosine	d	+	d
glycolate	d	−	d
p-hydroxybenzoate	−	−	d
trehalose	d	+	+
L-valine	d	−	−
D-xylose	d	−	−
L-rhamnose	d	−	−
citraconate	d	−	−
mesaconate	d	−	−
malonate	d	−	−
betaine	d	−	−
L-arabinose	d	−	−

Symbols: +=positive, −=negative, d =11–89% of strains are positive.

a) Fifty-seven isolates were tested.

b) Data from Bergy's Mannual of Systematic Bacteriology (Palleroni, 1984).

reactions for glucose, α-ketoglutarate, levulose, L-histidine, saccharate, *meso*-inositol and L-serine. They showed negative reactions in the test for utilization of m-hydroxybenzonate, α-amylamine, testosterone, adonitol, erythritol, 2,3-butylene glycol, geraniol and *p*-hydroxybenzoate. The above results of physiological and biochemical tests are the evidence of *R. solanacearum* (Table 2).

On the basis of pathogenicity tests, Brazilian isolates of *R. solanacearum* were divided into seven groups (Table 4). Considerable variations in Brazilian isolates were also observed in regard to utilization of trehalose, D-xylose, L-arabinose, L-rhamnose, L-valine, citraconate, mesaconate, malonate and betaine (Table 2).

Biovar identification and their distribution

Marked differences were observed in the ability of the isolates of *R. solanacearum* to

Table 3. Confirmatory test for determination of biovar of *Ralstonia solanacearum* isolates from Brazil.

Biovar	Results for carbohydrate oxidation test						Isolate
	Disaccharides			Hexose alcohols			
	cellobiose	lactose	maltose	dulcitol	mannitol	sorbitol	
1	−	−	−	−	−	−	13,19,31,72,73,76,127,128,129,140, 574,575,576,577,578,579,603,613, 629,656,982,985,1005,1033
2	+	+	+	−	−	−	7,61,66,67,71,79,87,92,98,106,113, 131,162,799,855,933,964
3	+	+	+	+	+	+	20,33,35,47,49,51,56,62,534,535, 582,628,630,1102,1103,1104
4	−	−	−	+	+	+	not isolated
5	+	+	+	−	−	−	not isolated

Table 4. Pathogenicity of Brazilian isolates of *Ralstonia solanacearum* to tomato, eggplant, potato and tobacco plants.

Tentative grouping	Pathogenicity				Brazilian isolate
	tomato	eggplant	potato	tobacco	
I	+	+	+	+	19,98,127,574,578,613,630
II	+	+	+	−	7,13,20,33,51,56,61,62,66, 79,131,534,535,579,582, 603,656,799,855,933,964, 1033,1102
III	+	+	−	+	628
IV	+	+	−	−	140,985
V	+	−	+	−	31,35,67,72,76,87,92,106, 113,128,162,575,576,577, 982,1005,1103
VI	+	−	−	+	629,1104
VII	+	−	−	−	47,49,71,73,129

oxidize three disaccharides and three hexose alcohols. Twenty–four isolates did not oxidize any disaccharides and hexose alcohols. They were classified as biovar 1. Seventeen isolates oxidized disaccharides but not any hexose alcohols, and classified as biovar 2. Sixteen isolates oxidized both groups of carbohydrates, and were classified as biovar 3. Any isolates belonging to biovar 4 or 5 were not isolated in Brazil (Table 3).

As can be seen in Fig. 1, the frequency of presence in the collection of biovars isolated in Brazil was plotted on a map with information about the host plants. Biovar 1 isolates are common and widely distributed in Brazil. Biovar 2 isolates are predominant on potato and prevail in the central west, south–east and south areas. Biovar 3 isolates exist mainly in the northern and north–eastern parts of Brazil. Thus, the geographical distribution of each biovar varied in Brazil.

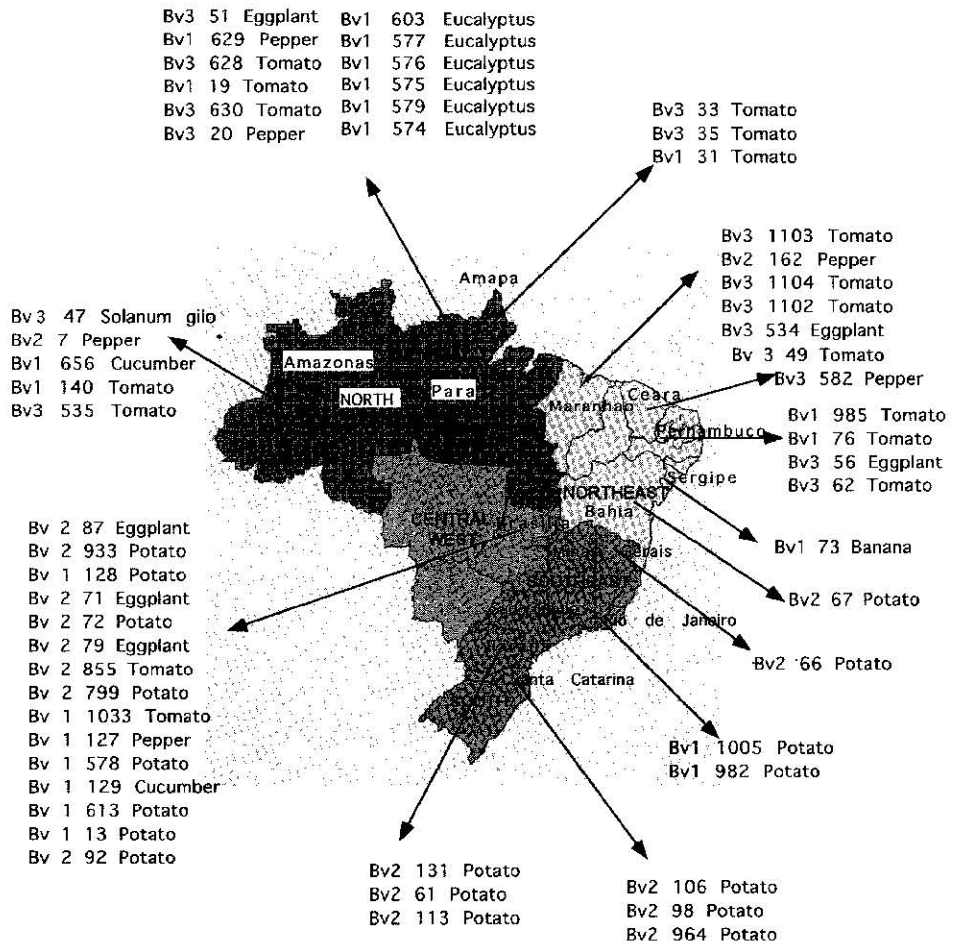

Fig. 1. Geographical distribution of biovars 1, 2 and 3 of *Ralstonia solanacearum* in Brazil.

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