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PREVALENCE OF RACES AND BIOTYPES OF *RALSTONIA SOLANACEARUM* IN INDIA

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Abstract: Bacterial wilt caused by *Ralstonia solanacearum* is the most destructive disease of plants. Fifty-seven isolates of *R. solanacearum* causing wilt on different host plants *viz.,* tomato (*Solanum lycopersicum*), brinjal (*S. melongena*), potato (*S. tuberosum*), bird of paradise (*Strelitzia reginae*), ginger (Zingiber officinale), chili (*Capsicum annuum*), capsicum (*Capsicum annuum*), davana (*Artemisia pallens*) and coleus (*Coleus forskohlii*) were collected from the different agro climatic zones of Karnataka and other parts of India. In this study, 57 isolates were differentiated into race on the basis of their pathogenicity and their ability to infect different host. The isolates were established as race-1. None of the isolates infected mulberry and banana. Fifty-four isolates oxidized and utilized both the disaccharides and sugar alcohols. These isolates were positioned as biovars-3 according to Haywards classification system. Three isolates from Kerala, two ginger, and one tomato strain were not able to utilize dulcitol and lactose. Hence, they were categorized into a new taxo group within the system and designated as biovar-3B for the first time in India. There were 54 isolates which were confirmed as race-1, biovar-3, and 3 isolates were confirmed as race-1, biovar-3B by morphological, physiological, biochemical and pathogenicity studies. Two sets of primers (OLI1 & Y2 and Y1 & Y2) were used in this study to authenticate the organism. Furthermore, the identity of the isolates was confirmed by a serological diagnostic kit obtained from the International Potato Research Center, Lima, Peru, and single chain variable fragment antibody specific to *R. solanacearum.*

Key words: *Ralstonia solanacearum,* race, biovar, Kerala, taxo

INTRODUCTION

Ralstonia solanacearum (*Pseudomonas solanacearum*) (E.F. Smith) (Yabuuchi *et al.* 1995) as a species has an extremely wide host range. Different pathogenic varieties (races) within the species, though, may show more restricted host ranges. Several hundred species of tropical, subtropical and warm temperature plants are susceptible to one or more of the races of *R. solanacearum*. The results are heavy losses in many economically important crops (Hayward 1991; Chandrashekara and Prasannakumar 2010). *R. solanacearum* is highly heterogeneous and complex. It is a major constraint in the production of many important vegetables, fruit, and cash crops. This devastating, soil-borne plant pathogen has a global distribution and an unusually wide host range. *R. solanacearum* causes wilt in over 450 host species in 54 botanical families (Allen *et al.* 2005) which may give the pathogen an evolutionary advantage. The number of new species continues to increase (Hayward 1991).

The race and biovar classification has gained wide acceptance for subdividing *R. solanacearum*. The racial pattern system groups the strains of *R. solanacearum* according to their ability to infect different host plants, *viz.,* race 1 is comprised of many strains having a wide host range and pathogenic on different solanaceous plants and weed hosts, race 2 is restricted to triploid banana and *Heliconia*, race 3 (potato race) affects potato, race 4 infects ginger, and race 5 is pathogenic on mulberry (He *et al*. 1983). The biovar scheme divides the species into five groups on the ability of the strains to metabolise or oxidise specific hexose sugars and disaccharides (Hayward 1991). At present, there is lot of controversy regarding the prevalence of strains in the various parts of the world. In India however, scant information is available about the prevalence of biovars/races and strains in various parts of the country. Therefore, the present investigations on 57 isolates of *R. solanacearum* causing wilt on different host plants were carried out. The objectives were to find the races and bi-

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ovars prevailing in different agro climatic zones of Karnataka and other parts of India.

MATERIALS AND METHODS

Isolation of *R. solanacearum* strains from the field

Wilt affected host plants *viz*., tomato (*Solanum lycopersicum*), brinjal (*S. melongena*), potato (*S. tuberosum*), bird of paradise (*Strelitzia reginae*), ginger (*Zingiber officinale*), chili (*Capsicum annuum*), capsicum (*Capsicum annuum*), davana (*Artemisia pallens*) and coleus (*Coleus forskohlii*) showing typical symptoms of wilt were collected from different agro climatic zones of Karnataka and other parts of India (Table 1). An appropriate water streaming test was carried out as described by Danks and Barker (2000). Bacterium was isolated on Semi-selective media South Africa (SMSA) media (Elphinstone *et al*. 1998; Wenneker *et al*. 1999). The isolates were subjected to identification and confirmation based on morphological, physiological, cultural, biochemical and pathogencity studies (Kelman 1954). The isolates were differentiated into biovars according to the description of Schaad 1988 and Hayward 1991.

Pathogenicity test on different hosts

Fifty-seven isolates of *R. solanacearum* were multiplied in casein peptone glucose (Casamino acid 1 g/l, Peptone 10 g/l, Glucose 10 g/l pH 7.2) to inoculate on differential hosts under artificial conditions. The bacterial suspension was adjusted to a concentration of $5x10^8$ cfu/ml using spectrophotometer readings (Spectronic 20 D, M and R, USA) at a wave length equivalent to A600 nm = 0.8 to 1.0. Plants used as differential hosts viz., tomato (*Lycopersicon esculentum* Mill.) cv. Avinash-II, banana (*Musa accuminata*) cv. yallaki bale, ginger (*Z. officinale*), mulberry (*Morus alba*) cv. M-5 and potato (*S. tuberosum*) cv. kufri jyothi seedlings were grown in greenhouse under artificial conditions. Twenty-day-old seedlings of tomato were pulled out gently, washed free of soil and a few tertiary roots were clipped with sterilized scissors and dipped in the bacterial culture for 10 minutes. The inoculated seedlings were transplanted to plastic bags containing sterilized soil. *In situ* inoculation of potato, banana, mulberry and ginger plants was carried out by scrapping off the surface soil and pouring 20 ml of bacterial suspension around the roots separately with each isolate, and then all was covered with soil. Plants similarly inoculated with sterile water served as the control.

Sugar utilization test

The isolates were differentiated into biovars as described by Schaad 1988, using carbohydrate fermentation discs to confirm the obtained results. The bacterial suspension belonging to 57 isolates containing a population of $5x10^8$ cfu/ml, were seeded separately with 0.8 Optical Density (OD) in a spectrophotometer at 600 nm. The flask was mixed to get a uniform suspension. The seeded medium was poured onto a petri plate and was allowed to cool for 3 hrs. Then, fermentation discs *viz*., cellobiose, lactose, maltose and sugar alcohols such as dulcitol, mannitol and sorbitol were placed in marked positions in three locations. A total of nine discs were maintained and plates were incubated at 32°C for 48 hrs. Observations were made and changes in colour recorded. Observations were recorded once at 18–24 hrs and again at the end of 48 hrs. A hypersensitive test on tobacco was carried out to determine plant pathogen presence and its virulence (Granada and Sequeira 1975). All tests *viz*., starch hydrolysis, nitrate reduction, oxidase, indole production, esculin hydrolysis, arginine dihydrolase, and curdling of skimmed milk were carried out using the methods described in the Manual of Microbial methods (Anonymous 1957) and the Laboratory guide for identification of Plant Pathogenic Bacteria (Schaad 1988).

Genomic DNA extraction and purification

R. solanacearum culture purified on SMSA was used for inoculation and incubated overnight in 100 ml of casein peptone glucose (CPG) medium at room temperature. Bacterial culture was centrifuged, (7,000 rpm for 10 min) and pellets were frozen at –20°C for 2 hrs. Frozen bacterial pellets were thawed for 3 min at 37°C, suspended in 10 ml of lysis buffer (0.15 M NaCl, 0.05 M sodium citrate buffer) and further incubated with 200 µl of lysozyme (10 mg/ml) at 37°C for 60 min. The cells were lysed with 500 µl of 20% Sodium dodecyl sulfate (SDS) by gently shaking. Next, 15 ml of extraction buffer were added [2.5 ml of 5 M sodium per chlorate and 12.5 ml of chloroform: isoamylalcohol mixture (24:1)]. The contents were incubated at –20°C for 2 hrs and centrifuged at 5,000 rpm for 10 min. Supernatant was precipitated with an equal volume of isopropanol, washed with 70% ethanol, the DNA was air dried and dissolved in 1 ml $T_{10}E_1$ buffer. After quantification on a spectrophotometer, the DNA was analysed on agarose 0.7% gel electrophoresis.

Polymeraze chain reaction (PCR) amplification and serological detection

All 57 isolates were PCR amplified using two sets of primers corresponding to 16S rDNA (OLI1 and Y2) and 16S rRNA (Y1 and Y2), as described by Seal *et al.* 1993, using universal primers (OLI1- 5'GGGGGTAGCTTGCTACCTGCC3' & Y2-5'CCCACTGCTGCC TCCCGTAGGAGT3') and (Y1-5'TGGCTCAGAACGAACGCGGCGGC3' & Y2-5'CCCACTGCTGCCTCCCGTAGGAGT3'). PCR products were resolved on 1.2% agrose gel.

Serological confirmation was carried out by using CIP's post-enrichment Double Antibody Sandwich ELISA (DAS-ELISA) kit, International Potato Center, Lima, Peru, (Priou *et al*. 1999). Monoclonal scFv antibody specific to *R. solanacearum* race-1, biovar-3 (Chandrashekara *et al*. 2006) was used to confirm bacterial isolates. The microtitre plate was coated with 60 mg of *R. solanacearum* antigen per well with 200 ml of carbonate buffer, and pH 9.6 along with appropriate controls, then incubated overnight at room temperature. The wells were rinsed three times with 1X Phosphate buffered saline (PBS) and excess buffer was removed by flipping over the plate. The plate was blocked with 200 ml of 3% Bovine serum albumin (BSA) in 1X PBS for 90 minutes at room temperature and then rinsed three times with 1X PBS. Two hundred ml of

anti – *R. solanacearum* – Alkaline phosphatase (ALP) conjugate in 1:10 dilution was prepared in 3% BSA-1X PBS and added to each well except for the control well. The microtitre plate was incubated for 90 minutes at room temperature and washed three times with 1X PBS-Tween 20. An addition of 200 ml of pNPP substrate was added to all the wells, and they were incubated in the dark for 30 minutes. A reading was taken at 415 nm.

RESULTS

Isolation of *R. solanacearum* strains from the field

Isolation of the bacterium was done from tomato (*S. lycopersicum*), brinjal (*S. melongena*), potato (*S. tuberosum*), bird of paradise (*Strelitzia reginae*), ginger (*Z. officinale*), chili (*C. annuum*), capsicum (*C. annuum*), davana (*A. pallens*) and coleus (*C. forskohlii*) plants showing typical symptoms of bacterial wilt. Such signs were: lower leaves turning pale yellow, loss of leaf turgidity followed by drooping of leaves and sudden wilt of the plants. The vascular bundles of the infected plants showed brown discoloration. Plants with symptoms were collected from different agro climatic regions of Karnataka and various parts of India (Table 1). Tentative diagnosis of the diseased plants collected from different hosts and different places was done by ooze test in a test tube containing clear sterilized distilled water.

The colonies of all the 57 isolates were well separated, irregular with smooth margins, slimy dull white colonies with a pink to red center on SMSA medium. However some variations in colony characters were observed (Table 2). Based on the morphological studies it was found that all the isolates were gram-negative, rod shaped, non-capsulated and non-spore forming. The isolates were negative for indole and gelatin lignifications, positive for oxidase, levan production, nitrate reduction and did not produce fluorescent pigment on Kings B medium.

Pathogenicity test on different hosts

All the 57 isolates produced typical wilt symptoms on their respective hosts on being inoculated by root injury technique. Similar methods were followed by Wang and Berk (1997). Artificial inoculation of the pathogen plants expressed symptoms in 3–4 weeks time, while Bird of paradise (BOP), coleus and davana isolates took longer. Thus, on the basis of host range, and the ability to cause wilt in solanaceous and non-solanaceous plants the 57 isolates were designated as race 1. On the basis of various morphology and pathogenicity characteristics and on comparison of these characters with the characters reports for *R. solanacearum* from all 57 isolates from different geographical areas, *R. solanacearum* was indentified.

PCR amplification and serological detection

The genomic DNA was subjected PCR, the PCR amplification resulted in a ~300 base pair (bp) product for (Y2 & OLI1) and 292 bp product (Y1 & Y2) from all 57 isolates of *R. solanacearum*. This is in agreement with the earlier results (Seal *et al*. 1993) and the tested isolates were grouped into race 1.

Detection of *R. solanacearum* by Enzyme-Linked Immunosorbent Assay on Nitrocellulose membrane using enriched samples (NCM-ELISA), originally developed to detect latent infection in potato seed tubers, was adopted to authenticate the isolates (Priou *et al*. 1999). Furthermore, the scFv monoclonal antibody (anti-*Ralstonia solanacearum*-ALP conjugate) detected *R. solanacearum* (Chandrashekara *et al*. 2006). The ELISA results obtained were on par to positive control of *R. solanacearum* which was 5–8 times greater than the negative control value, which confirmed the isolates of *R. solanacearum* belong to race 1, biovars 3.

DISCUSSION

The bacterium was isolated on SMSA medium (Elphinstone *et al*. 1998; Wenneker *et al*. 1999) where it produced irregular-shaped white colonies with pink centers, resembling those of *R. solanacearum*. All the isolates grown on SMSA medium had similar characteristics with minor variations which are depicted in table. 2. The colonies were highly fluidal, white coloured with slight pink center and a bluish margin, and round to irregular in shape (Kelman 1954). Brinjal isolates produced copious slime and were highly fluidal. BOP isolate produced miniature colonies with dark red pigmentation in the center of the colony. The isolates did not differ much in cultural characters with the exception of the brinjal isolates.

All the isolates produced typical wilt symptoms in their respective hosts on artificial inoculation by the root injury technique (Wang and Berk 1997). Artificial inoculation of the pathogen plants expressed symptoms in 4–5 weeks time, while BOP isolate took longer. Two ginger isolates could infect all other solanaceous host plants, but failed to infect mulberry and Musa plants. Consequently, on the basis of host range and the ability to cause wilt in solanaceous and non-solanaceous plants, the 57 isolates were designated as race 1. Similar results were also reported in the past by several workers about prevalence of race in India as race 1 (Khan 1974).

The *R. solanacearum* isolates was classified into different biovars on the basis of their ability to utilize or oxidize three hexose alcohols (mannitol, sorbitol and dulcitol) and three disaccharides (lactose, maltose and cellobiose) (Hayward 1964). Among the 57 isolates, 54 isolates oxidized disaccharides, and hexahydric alcohols tested are presented in table 2. Shekhawat *et al*. (1978) recorded that strains causing brown rot of potato belonged to race 1 and biovar 3 and 4. In 1992, Shekhawat *et al*. reported that pathogen isolated from the plains area belonged to race 1, and that biovar 3, biovar 4 was encountered only among the isolates from eastern parts of India. Race 3 and biovars 2 were obtained only from a few places in the central plains and Deccan plateu. Bhattacharya *et al*. (2003) reported the prevalence of race 1 and biotype 3 infecting potato, tomato, aurbargine, chilli, jute and banana from West Bengal.

The three isolates from Kerala *viz*. Kerala Ralstonia solahacearum tomato (KERT-1), Kerala Ralstonia solahacearum ginger (KERG-1 and KERG-2) behaved dif-

Table 1. Designation of *R. solanacearum* isolates collected from different agro-climatic zones of Karnataka and other states of India

No.	Isolate code	Host	Place
1	SRP-1	potato	*Simla (GSC-26)
2	SRP-2	potato	*Simla (134)
3	SRP-3	potato	*Simla (JGS 3/118)
4	KRP-4	potato	Sulebele, Kolar
5	CRP-6	potato	Kolar
6	ChRP-7	potato	Chikkabalapur
7	KRP-8	potato	Chikamaglur
8	ChRP-6	potato	Bagepalli, Kolar
9	BRP-9	potato	Chikamaglur
10	HRP-10	potato	Bangalore
11	BRP-11	potato	Kandli, Hassan
12	KERT-1	tomato	Kerala
13	BRT-2	tomato	Bangalore
14	KRT-3	tomato	GKVK, Bangalore
15	HRT-4	tomato	Chintamani, Kolar
16	KRT-5	tomato	Hassan
17	BRT-6	tomato	Sreenivaspura, Kolar
18	BRT-7	tomato	Bangalore
19	KRT-8	tomato	Hebbal, Bangalore
20	KRT-9	tomato	Gouribidnur, Kolar
21	TRT-10	tomato	Chintamani, Kolar
22	BRT-11	tomato	Hossur, Tamil Nadu
23	APRT-12	tomato	Hoskote, Bangalore
24	KRT-13	tomato	Anantapur, Andhra Pradesh
25	BRT-14	tomato	Malur, Kolar
26	DRT-15	tomato	Anekal, Bangalore
27	KRT-16	tomato	Davangere
28	TuRT-17	tomato	Kaivara, Kolar
29	MRT-18	tomato	Tumkur
30	KRT-19	tomato	Mysore
31	ChiRT-20	tomato	Sreenivaspura
32	HRT-21	tomato	Chitradurga
33	HRT-22	tomato	Madenur, Hassan
34	HRT-23	tomato	Bhuvaneshwar, Orissa
35	HRT-24	tomato	Arkalgud, Hassan
36	HRT-25	tomato	Kandli, Hassan
37	TuRT-26	tomato	Hassan
38	HRT-27	tomato	Tumkur
39	BRT-28	tomato	Hassan
40	DhRT-29	tomato	Kanakpura
41	SiRT-30	tomato	Dharwad
42	BiRT-31	tomato	Sirsi
43	KRB-1	brinjal	Bijapur
44	HRB-2	brinjal	Kolar
45	ARB-3	brinjal	Kandli, Hassan
46	ARB-4	brinjal	Arabhavi
47	BRCa-1	capsicum	Arabhavi
48	CRD-1	davana	GKVK, Bangalore
49	BOP-1	bird of paradise	Chikkabalapur
50	ORT-1	tomato	Bangalore
51	ORT-2	tomato	Bhuvaneshwar, Orissa
52	ORB-1	brinjal	Bhuvaneshwar, Orissa
53	MRT-1	tomato	Nasik, Maharastra
54	MRT-2	tomato	Nasik, Maharastra
55	KERG-1	ginger	Trissur. Kerala
56	KERG-2	ginger	Trissur. Kerala
57	APRC-1	coleus	Tirupati (AP)

*indicates reference strains used in the study provided by the Central Potato Research Institute, Shimla, Himachal Pradesh, India

Table 2. Morphology of 57 isolates of bacterium on Semi-selective media South Africa (SMSA) media

Isolates	Morphology on SMSA media
KERT-1, BRT-2, KRT-3, HRT-4, KRT-5, BRT-6, BRT-7, KRT-8, KRT-9, TRT-10, BRT-11, APRT-12, KRT-13, BRT-14, DRT-15, KRT-16, TuRT-29, MRT-18,	irregular with smooth margin, slimy dull white colonies with pink to red center
KRT-19, ChiRT-20, HRT-21, ORT-22, HRT-23, HRT-24, HRT-25, TuRT-26, HRT-27, BRT-28, DhRT-29, SiRT-30, BiRT-31	moderately fluidal, regularly shaped, convex, red colored colonies
SRP-1, SRP-2, SRP-3, KRP-4, KRT-5, CRP-6, ChRP-7, KRP-8, ChRP-6, BRP-9, HRP-10, BRP-11, BRCa-1	moderately fluidal, irregularly shaped, convex, dull white colonies with pink colored center and bluish margin
KRB-1, HRB-2, ARB-3, ARB-4	dull white colonies with light pink colored center, and the colonies were highly fluidal and flowing producing copious slime
ORB-1	the colonies were well-separated, fluidal, white colonies with dark yellow coloring and the colonies were relatively rigid
BOP-1	fluidal, white colonies with dark red colored center and the colonies were relatively small rigid
CRD-1, ORT-1, ORT-2, MRT-1, MRT-2, KERG-1, KERG-2	highly fluidal, irregularly shaped, convex, dark reddish colonies with red colored center and whitish margin

ferently and were unable to utilize dulcitol and lactose. Hence, a new taxonomic group at the subspecies level within the biotype was created and these three isolates have been designated as biovar-3B. Variation in the ability of isolates to utilize sugars such as dulcitol were also reported by Mathew *et al.* 2002. They designated such strains as biovar 3A. Similarly, Sunaina *et al*. (1997) found differences among the strains infecting potato in India and the ability of the strains to utilize sugars. One strain could not utilize mannitol and maltose, which was designated as a typical strain.

Sequences of 16Sr DNA and 16Sr RNA genes are often species-specific and are present as multiple copies in microbial genomes, hence they make excellent targets for identification of bacteria at the species level. The corresponding specific rDNA and rRNA sequences have been used as targets for PCR amplification (Woese 1987). The PCR amplification resulted in a 300 bp and 292 bp product from all the 57 isolates of *R. solanacearum* respectively for (Y2 & OLI1) and (Y1 & Y2). The amplification conditions allowed the specific detection of *R. solanacearum* isolates. This confirmed that all 57 isolates tested were *R. solanacearum*. Similar results were obtained by Seal *et al*. (1993) and isolates tested were grouped in race 1. The amplification conditions allowed for the specific detection of the isolates of *R. solanacearum.* These primers can be used to diagnose *R. solanacearum* strains. All the isolates produced bright purple coloration at a concentration of 10^7 cfu/ml on the nitrocellulose membrane which was compared with the positive control strips provided in the kit (Priou *et al*. 1999). The scFv monoclonal (anti-*Ralstonia solanacearum*-ALP conjugate) antibody also confirmed the detected *R. solanacearum* isolates has race 1, biovar 3 (Chandrashekara *et al.* 2006).

CONCLUSIONS

R. solanacearum isolates of Karnataka and other states in India were characterized as race 1 and biovars 3. A new taxonomic group has been given as biovars 3B within the biovar system for the 3 isolates from Kerala, which did not oxidize dulcitol and lactose. Optimization of PCR conditions using two sets of universal primers can be used as diagnostic primers for detection and identification of *R. solanacearum.* Serological diagnostic kit International Potato Centre-Enzyme Linked Immunosorbent Assay on Nitrocellulose Membrane (CIP-NCM-ELISA) used in this study confirmed the scope of the kit for detection of the isolates from other host and sub species identification of *R. solanacearum* and scFv monoclonal (anti-*Ralstonia solanacearum*-ALP conjugate) to confirm causative organism as *R. solanacearum* race 1, biovar 3.

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