

Original Research Article

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Isolation and Characterization of *Ralstonia solanacearum* Causing Bacterial Wilt of Solanaceae Crops

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is the most destructive disease of crop plants. Six isolates of *R. solanacearum* causing wilt disease in tomato (*Solanum lycopersicum*) and brinjal (*S. melongena*) were collected from different locations of Karnataka, India. All the six isolates were identified as *R. solanacearum* by colonies phenotype, simple staining, streaming and *R. solanacearum* species specific PCR. In this study, six isolates were differentiated into race on the basis of their ability to infect different host. The isolates-1, 2, 4 and 5 were established as race-3 and isolates-3 and 6 were established as race-1. Biovar characterization was performed by their ability to utilize disaccharides [Sucrose, lactose, and maltose (Himedia)] and sugar alcohols [mannitol, sorbitol and dulcitol (Himedia)]. According to Hayward's classification system, the isolates-1, 2, 4 and 5 were belongs to biovars-2 and isolates-3 and 6 were belongs to biovars-3. Molecular analysis, including multiplex PCR-based phylotyping and sequence analysis of 16s rDNA were used to determine the ITS sequences of six *R. solanacearum* strains. The results of Pmx-PCR for all six isolates revealed that all isolates are belonged to phylotype I. Therefore, it may be confirmed that *R. solanacearum* causing bacterial wilt of tomato and brinjal in Karnataka belong to Biovar-2, 3 and Race 1, 3 and phylotype I.

Keywords

Ralstonia solanacearum,
Bacterial wilt,
Tomato,
Phylotype, Biovar.

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Introduction

Bacterial wilt pathogen *Ralstonia solanacearum* (Smith, 1896; Yabuuchi *et al.*, 1995) is a gram negative, rod shaped, β -proteobacteria, nonsporulating, aerobic, soil-borne, motile bacterium with a polar tuft of flagellum. It causes wilt diseases in plants by invading through xylem vessels (Genin, 2010). It colonizes the root surface and then invades xylem vessels by degrading cell wall and produces ample quantities of exopolysaccharides which in turn blocks water flow causing chlorosis, wilting of plants

and eventually plant death. Its ability to persist in soil and infesting plant parts adds to the difficulty in elimination of inocula (Genin and Denny, 2012). It occurs widely in tropical, subtropical and warm temperate parts of the globe (Liu *et al.*, 2009) and causes catastrophic yield loss in major crops like tomato, eggplant, potato, tobacco, pepper, banana, peanut, ginger, etc. (Peeters *et al.*, 2013). It has a powerful and tissue specific tropism in host plants, specifically invading and rapidly multiplying in the xylem vessels.

It has an extensive host range of ~ 450 crop species (Swanson *et al.*, 2005) across 54 families (Wicker *et al.*, 2007). There is huge genetic diversity in *R. solanacearum* (formerly *Pseudomonas solanacearum*, Yabuuchi *et al.*, 1995) across the globe and thus it is termed as *R. solanacearum* species complex (RSSC). These marked differences in geographical distribution suggest separate evolutionary development. The variability in *R. solanacearum* species complex is classified by different workers on the basis of different criteria like host range, utilization of different carbon sources, phylogenetic relationship, etc. (OEPP 2004).

The race, biovar and phylotype classification has gained wide acceptance for subdividing *R. solanacearum* species complex. The racial pattern system groups the strains of *R. solanacearum* according to their ability to infect different host plants, viz., race 1 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 (Buddenhagen *et al.*, 1962; Aragaki and Quinon, 1965; He *et al.*, 1983; Begum, 2005; Rahman *et al.*, 2010; Chandrashekara *et al.*, 2012, Popoola *et al.*, 2015). Five races are also different in geographical distribution and ability to survive under different environmental conditions (French, 1986). *R. solanacearum* was grouped into four biovars on the basis of utilizing and/or oxidizing three hexoses (mannitol, dulcitol and sorbitol) and three disaccharides (lactose, maltose and cellobiose) (Hayward, 1954; He *et al.*, 1983; Begum, 2005; Rahman *et al.*, 2010; Chandrashekara *et al.*, 2012, Popoola *et al.*, 2015).

Fegan and Prior (2005) developed a new hierarchical classification scheme by analysis

of the internal transcribed spacer (ITS) region, the endoglucanase (*egl*) gene and the *hrpB* gene. A phylotype-specific multiplex PCR (Pmx-PCR) was employed in this scheme to distinguish four phylotypes (I, II, III, IV). A number of different phenotypic and genotypic methods are presently being employed for the identification and classification of bacteria, including plant pathogenic bacteria like *Ralstonia*. Each of these methods permits a certain level of phylogenetic classification from the genus, species, subspecies, biovar to the strain level. Moreover, each method has its advantages and disadvantages with regard to ease of application, reproducibility, requirement for equipment and level of resolution. Modern phylogenetic classification is based on 16S rRNA sequence analysis (Cook *et al.*, 1989; Gillings *et al.*, 1993; Seal *et al.*, 1992; Seal *et al.*, 1993; Poussier *et al.*, 2000; Popoola *et al.*, 2015)

There exists a 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. Understanding local pathogen genetic diversity is the first step in a successful breeding and integrated disease management programme. One of the purposes of the present investigation on six isolates of *R. solanacearum* causing wilt on tomato and brinjal isolated from different agro-climatic zones of Karnataka is to characterize them into races, biovars and phylotype.

Materials and Methods

Isolation and characterization of *Ralstonia solanacearum*

Collection of sample

Plant and soil samples were collected for isolation of *R. solanacearum* from diverse

sites around Karnataka, India (Table 1). The presence of pathogen was tested by placing longitudinal sections of the collar portion containing vascular tissues from diseased plants in a test tube containing clean water. The infected tissues shows fine milky white strands composed of masses of bacteria, which oozes out from the margin of the cut portion within few minutes.

Isolation of *Ralstonia solanacearum*

The bacterial wilt disease confirmed tissue was used for isolation. The outer parts of infected material were removed with a sterilized scalpel.

The small pieces were placed in distilled water for 10 to 15 minutes. The inoculation loop was dipped in the ooze and streaked on Triphenyltetrazolium Chloride (TZC) (Himedia) media. [TZC media was 10 g peptone, 1 g casein hydrolysate, 10 g dextrose, 18 g agar and 5 ml of 1 per cent triphenyltetrazolium chloride (TZC) for one litre of distilled water].

The streaked plates were incubated at $28\pm 1^{\circ}\text{C}$ for 24 to 48 hours. The virulent (pink color at the centre with fluidal in nature) colonies were isolated and then suspended in sterilized distilled water in screw capped vials and stored at room temperature. They were regularly renewed and checked for virulence by plating on TZC medium.

Identification of virulent and avirulent isolates

The virulent (colonies with pink or light red color or characteristic red center and whitish margin) and avirulent (smaller, brick red and non-fluidal colonies) strains of *R. solanacearum* were identified in TZC medium containing 0.005% TZC (Kelman, 1954).

Characterization of the pathogen

Different biochemical tests were performed to characterize the *R. solanacearum* are described below.

Gram staining

A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet (Himedia) solution (0.5%) was spread over the smear for 30 seconds and then gently washed with slow running tap water for one minute. It was then flooded with iodine for one minute, rinsed in tap water and decolorized with 95% ethanol until colorless runoff. After washing, the specimen was counter-stained with safranin (Himedia) for approximately 10 seconds, washed with water, dried and observed under microscope at 40X using immersion oil (Schaad, 1980).

Streaming test

A presumptive test was carried out on infected tomato plant to diagnose the presence of *R. solanacearum*. Stems of infected tomato plants were cut above the soil level and the cut surfaces were suspended in test tube containing clean water.

Host range studies and race determination

Races were assigned based on host range (Buddenhagen *et al.*, 1962; Schaad *et al.*, 2001). Studies on four species in the family Solanaceae (tomato-*Solanum lycopersicum*, chilli-*Capsicum annum*, brinjal-*Solanum melangena* and potato- *S. tuberosum*) and one in Moracea family (Mulberry-*Morus alba L.*) (Table 3) were used. The one month old seedlings were inoculated with *R. solanacearum* by trimming the roots and dipping in bacterial suspension for 30 minutes (Klement *et al.*, 1990). The inoculated plants

were then kept in net house until the symptoms were developed. *R. solanacearum* from the wilted plant was reisolated on TZC agar and colonies were compared with the original culture.

Determination of biovars

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides [Sucrose, lactose, and maltose (Himedia)] and sugar alcohols [mannitol, sorbitol and dulcitol (Himedia)] (Table 4) as described previously by Hayward (1954) and He *et al.*, (1983). The biovars were determined in the mineral medium (Himedia) (NH₄H₂PO₄ 1.0g, KCl 0.2g, MgSO₄.7H₂O 0.2g, Difcobao peptone 1.0g, Agar 3.0g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 200 µl of the melted medium is dispensed into the wells of microtitre plate. Inocula for each group of isolates was prepared by adding several loopful of bacteria from 24-48h old cultures to distilled water to make suspension containing about 10⁸ CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate and incubated at 28-32°C. The tubes were then examined at 3 days after inoculation for change in pH by color change (Schaad *et al.*, 2001).

Molecular characterization of *Ralstonia solanacearum*

Genomic DNA extraction and purification

Genomic DNA isolation was performed by Amnion bacterial g DNA isolation protocol.

***Ralstonia solanacearum* confirmed by Polymerase chain reaction (PCR) amplification**

All six 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 (Table 5). PCR products were resolved on 1.2% agarose gel.

Phylotype determination

A phylotype-specific multiplex PCR (Pmx-PCR) was performed to determine the phylotype affiliation of all strains (Fegan and Prior, 2005). A set of four phylotype-specific forward primers (Nmult:21:1F, Nmult:21:2F, Nmult:23:AF, Nmult:22:InF, and Nmult:22:RR) with a unique and conserved reverse primer (Nmult:22:RR) targeted in the 16S-23S intergenic spacer region (internal transcribed spacer) were used for the study (Table 6). The mixture contained 12.5µl of 2×PCR buffer, 0.6µl of each forward primer (10 µmol/l), 2.4 µl of reverse primer (Nmult:22:RR), 0.4 µl of 759R and 760F primers and 2µl template mix (about 50 ng/µl). The final volume was made up to 25 µl using PCR-grade water. The PCR reaction was prepared and subjected to thermocycling at the following temperatures: 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s and a final extension of 72°C for 10 min. PCR products were resolved using agarose 1.5% (wt/vol) gel electrophoresis. Amplicon sizes were estimated by comparison to a 100-bp DNA ladder. This Pmx-PCR produces the following phylotype specific PCR products: a 144-bp amplicon from phylotype I strains, a 372-bp amplicon from phylotype II strains, a 91-bp amplicon from phylotype III strains, and a 213-bp amplicon from phylotype IV strains.

Results and Discussion

Isolation and characterization of isolates of *R. solanacearum* from Karnataka

The six isolates were isolated from infected plant sample and characterized by morphological, biochemical and molecular methods.

The result of this objective described in the following subheading.

Confirmation for *R. solanacearum*

Six wilt causing bacterial isolates were identified as *R. solanacearum* based on their typical fluidal, irregular colony morphology with a characteristic spiral pink centre on TZC agar amended with 2,3,5-teiphenyl tetrazolium chloride (Fig. 1). The isolates represented geographically distinct locations such as Gandhi Krishi Vignyan Kendra, Indian Institute of Horticulture Research Hesaraghatta, Main Agricultural research station Hebbal and Sadahalli farm Devanahalli Bengaluru, Karnataka.

Assessment of cfu of isolates of *Ralstonia solanacearum* at different locations

The number of colony forming unit (cfu) were assessed at different dilutions of isolates of *R. solanacearum* (Table 2 and Fig. 3). In all isolates, the cfu were uncountable at zero dilution. At first dilution (10^{-1}) the cfu were uncountable in isolate-2, 4, 5 and 6, while cfu were recorded 120 and 180 in isolate-1 and 3, respectively. At second dilution (10^{-2}), the isolates⁵ and 6 were uncountable whereas isolate-1, 2, 3 and 4 recorded 41, 80, 90 and 150 respectively. The maximum cfu were recorded in isolate-2(75), isolate-6(61), isolate-6(45) and isolate-3(28) whereas minimum cfu was recorded in isolate-1 *i.e.* 29, 33 and 0 at 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions, respectively

Identification of virulent/avirulent strains of *R. solanacearum*

All isolates of *R. solanacearum* collected from different growing areas produced pink or light red coloured colonies with characteristic red centre and whitish margin on TZC medium (Fig. 2). It indicates that, all the isolates were belongs to virulent.

Host range studies and race determination

The races of *R. solanacearum* were identified by pathogenicity test on wide host ranges (tomato, chilli, potato, mulberry, brinjal and banana). The results of pathogenicity test revealed that, the isolate-1, isolate-2, isolate-4 and isolate-5 showed wilting symptoms only in solanaceous families (tomato, chilli, potato and brinjal) not in other crops, hence it was identified as race-3 whereas isolate-3 and isolate-6 showed wilting symptom in solanaceous families (tomato, chilli, potato and brinjal). The banana was also identified as race-1 (Table 3 and Fig. 5, 6, 7 and 8).

Streaming test

A presumptive test was carried out on infected tomato plant to diagnose the presence of *R. solanacearum*. Stems of infected tomato plants were cut above the soil level and the cut surfaces were suspended in beaker containing millipore water and it appeared as cloudiness streaming down (Fig. 8).

Biochemical tests

Biochemically *R. solanacearum* was confirmed by gram staining and Biovar differentiation test.

Gram staining

Bacterial staining reddish pink color indicates that these bacteria are gram-negative (G-ve), All six isolates exhibited reddish pink color indicated that these are gram negative (Fig. 4).

Biovar differentiation

The biovar of *R. solanacearum* isolates were identified by utilizaion of disaccharides and sugar alcohols. The result of the biovar test showed that, all six isolates of *R. solanacearum* oxidized disaccharides

(Sucrose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) within 7-10 days (Table 4). The oxidation reaction was indicated by the change of color. The isolates 3 and 6 changed the color to yellow from blue indicating the oxidation of sugars and alcohol and hence they belong to biovar-III. Further Isolate-1, Isolate-2 Isolate-4 and isolate-5 utilized only sugar but not sugar alcohol and hence they belonged to biovar-II (Table 4 and Fig. 10). On the other hand, all the control plates of different sugars and sugar alcohols remain unchanged.

Bacterial genomic DNA isolation and quantification

Genomic DNA was extracted from the six isolates of *R. solanacearum* and quantified (Fig. 11) by nanodrop. The maximum DNA amount was observed in isolate-2 (1455 ng/μl) followed by isolate-3 (1399 ng/μl), isolate-4 (1390 ng/μl), isolate-5 (1220 ng/μl), isolate-6(1219 ng/μl) and isolate-1 (1207 ng/μl). Purity of DNA was observed by A260/280 ratio. The A260/280 ratio was found to nearer to 1.8 in all isolates of *R. solanacearum*.

Molecular characterization of six isolates of *Ralstonia solanacearum*

Isolates of *R. solanacearum* were characterized by ten DNA Markers (Table 4 and 5). The genomic DNA was subjected to PCR, the PCR amplification resulted in a ~300 base pair (bp) product (Fig. 12) for (OLI1 and Y2) and 292 bp product (Fig. 13) for (Y1 and Y2) from all six isolates of *R. solanacearum*.

OLI1 and Y2 primer derived from 16S rDNA sequence of *R. solanacearum* and Y1 and Y2 derived from 16S rRNA. This result confirmed that all bacterial isolates were *R. solanacearum*.

Phylotype determination

A phylotype-specific multiplex PCR (Pmx-PCR) was performed to determine the phylotype affiliation of all strains. A set of four phylotype-specific forward primers (Nmult:21:1F: Nmult:21:2F: Nmult:22:InF: and Nmult:23:AF:), with a unique conserved reverse primer (Nmult:22:RR:) and *R. solanacearum* specific primers (759R and 760F) (Table 5), these primers targeted in the 16S-23S intergenic spacer region (internal transcribed spacer). The Pmx-PCR of six isolates of *R. solanacearum* yielded 144-bp amplicon and 280 bp amplicon (Fig. 14). It revealed that all isolates were *R. solanacearum* and belonged to phylotype I.

Isolation and characterization of six bacterial isolates

R. solanacearum was isolated from infected samples of six locations of Karnataka and culturing was achieved through streaking a bacterial suspension (in water) onto a selective medium, tetrazolium chloride (TZC) agar plates (Elphinstone *et al.*, 1998; Wenneker *et al.*, 1999; Chandrashekara *et al.*, 2012). It produced irregular-shaped white colonies with pink centers, resembling those of *R. solanacearum*. All the isolates grown on TZC medium had similar characteristics with minor variations which are depicted in (Table. 1). This result also supported by Kelman (1954), Klement (1990), Rahman *et al.*, (2010), Chandrashekara *et al.*, (2012), Popoola *et al.*, (2015). Kelman (1954): used triphenyltetrazolium chloride (TZC) medium to distinguish *R. solanacearum* among other bacteria during isolation. Also, when TZC medium was used with *R. solanacearum*, has showed the difference between avirulent colonies that look dark red from the fluidal virulent that are white with pink center, here also we found similar differences between virulent and avirulent colony appearance (Fig. 2).

Table.1 Collection of six bacterial isolates from Karnataka

Sl.No.	Isolates	Source	Crop	Morphology on TZC media
1	Isolates 1	IIHR, Bangalore	Tomato	Irregular with smooth margin, convex, creamish colony with light pink centre.
2	Isolates 2	IIHR, Bangalore	Brinjal	Spherical, convex, pink centered with cream border colonies
3	Isolates 3	Hebbal, Bangalore	Tomato	Spherical, dull white colonies with pink centre, convex
4	Isolates 4	GKVK, Farm, UAS, Bangalore	Tomato	Spherical, dull white colonies pink at centre
5	Isolates 5	Karavali, Karnataka	Tomato	Spherical pink colored colonies, convex
6	Isolates 6	GKVK, Farm, UAS, Bangalore	Brinjal	Irregular, smooth margin, pink centered with cream border colonies,

Table.2 Number of colony found at six dilution of six isolates of *R. solanacearum*

	10 ⁰		10 ⁻¹		10 ⁻²		10 ⁻³		10 ⁻⁴		10 ⁻⁵		10 ⁻⁶	
	OD	CFU	OD	CFU	OD	CFU	OD	CFU	OD	CFU	OD	CFU	OD	CFU
Isolate 1	1.76	Uncountable	0.82	120	0.64	41	0.51	29	0.43	3	0.36	3	0.10	0
Isolate 2	1.75	Uncountable	0.90	Uncountable	0.74	80	0.65	75	0.36	35	0.23	40	0.08	8
Isolate 3	1.05	Uncountable	0.90	180	0.77	90	0.52	73	0.25	59	0.13	30	0.06	28
Isolate 4	1.89	Uncountable	0.90	Uncountable	0.78	150	0.54	70	0.30	35	0.23	33	0.14	25
Isolate 5	2.23	Uncountable	0.99	Uncountable	0.96	Uncountable	0.75	70	0.61	47	0.34	27	0.27	21
Isolate 6	2.22	Uncountable	0.99	Uncountable	0.99	Uncountable	0.71	70	0.59	61	0.38	45	0.14	21

OD- optical density, CFU- Colony Forming Unit

Table.3 Race of *R. solanacearum* by host range

Sl.NO.	Race	Natural host	Geographical distribution	Biovars
1	1	Wide variety: Ginger, olive, chili pepper, peanut, <i>Solanum</i> spp., and tobacco	Asia, Africa, Australia, North America, and South America	1, 3, 4
2	2	<i>Musa</i> spp. (banana and plantain), peanut, <i>Heliconia</i> , and tomato	Caribbean, Asia, Central America, South America, and Hawaii	1
3	3	Solanaceous, and <i>Pelargonium</i> spp. (Geranium)	Worldwide (except Canada and United States)	2
4	4	Ginger	Australia, India, Asia, and Hawaii	3,4
5	5	<i>Morus</i> spp. (Mulberry)	china	5

Table.4 Differentiation of *R. solanacearum* strains into biovars based on the ability to utilize disaccharides and oxidize hexose alcohols producing acid when positive (+)

Biochemical test	Biovar				
	1	2	3	4	5
(Utilization of)					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
<i>Oxidation of</i>					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

Table.5 List of 16S Rrna primer name and sequence information

SL.N.	universal primers	Sequence of the primer	
1	OLI1	5'GGGGGTAGCTTGCTACCTGCC3'	~300 bp
2	Y2	5'CCCACTGCTGCCTCCCGTAGGAGT3')	
3	Y1	5'TGGCTCAGAACGAACGCGGCGGC3'	292 bp
4	Y2	5'CCCACTGCTGCCTCCCGTAGGAGT3')	

Table.6 List of primers used for multiplex PCR (determination of phylotype)

SL.N O.	Primer	Primer sequence	Expected band size	Remarks
1	Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144 bp	Phylotype I (Asiaticum)
2	Nmult:21:2F	5'-AAGTTA TGGACGGTGAAGTC-3'	372 bp	Phylotype II (Americanum)
3	Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3	213 bp	Phylotype IV (Tropical)
4	Nmult:23:AF	5'-ATTACGAGAGCAATC GAAAGATT-3'	91 bp	Phylotype II (African)
5	Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3		Amorce reverse unique
6	759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280 bp	Universal <i>R.solanacearum</i> specific primers
7	760F	5'-GTCGCCGTGAGCAATGCGGAATCG-3'		

Fig.1 Colonies morphology of *R. solanacearum* on TZC Media

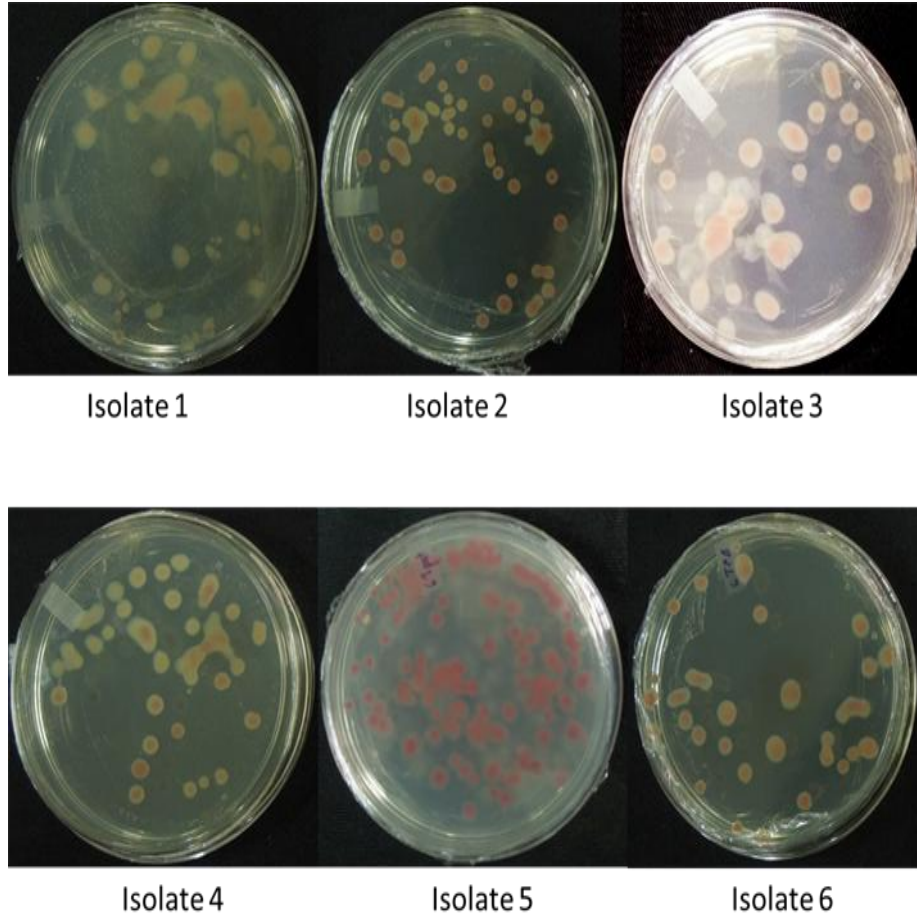


Fig.2 Plate showing virulent/nonvirulent strains of *R. solanacearum*

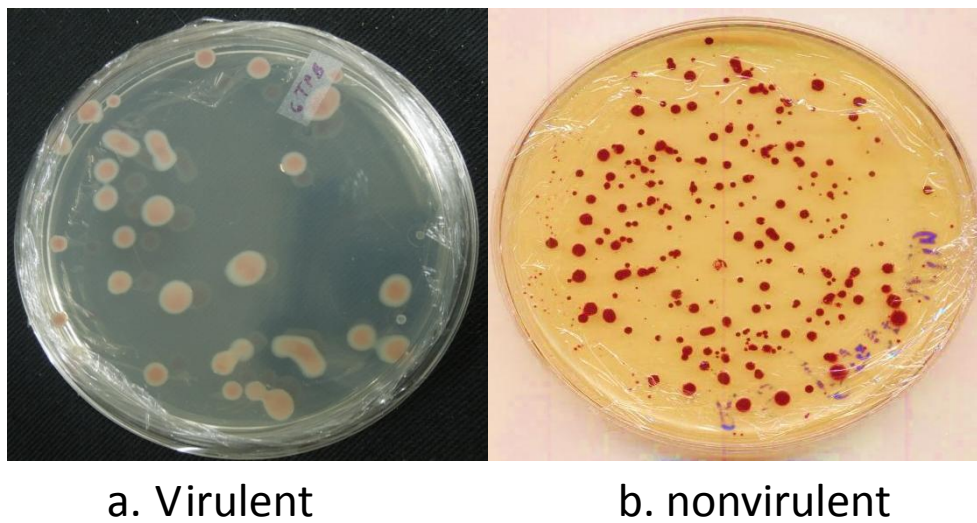


Fig.3 The CFU (colony forming units) count at different dilutions of isolates of *R. solanacearum*

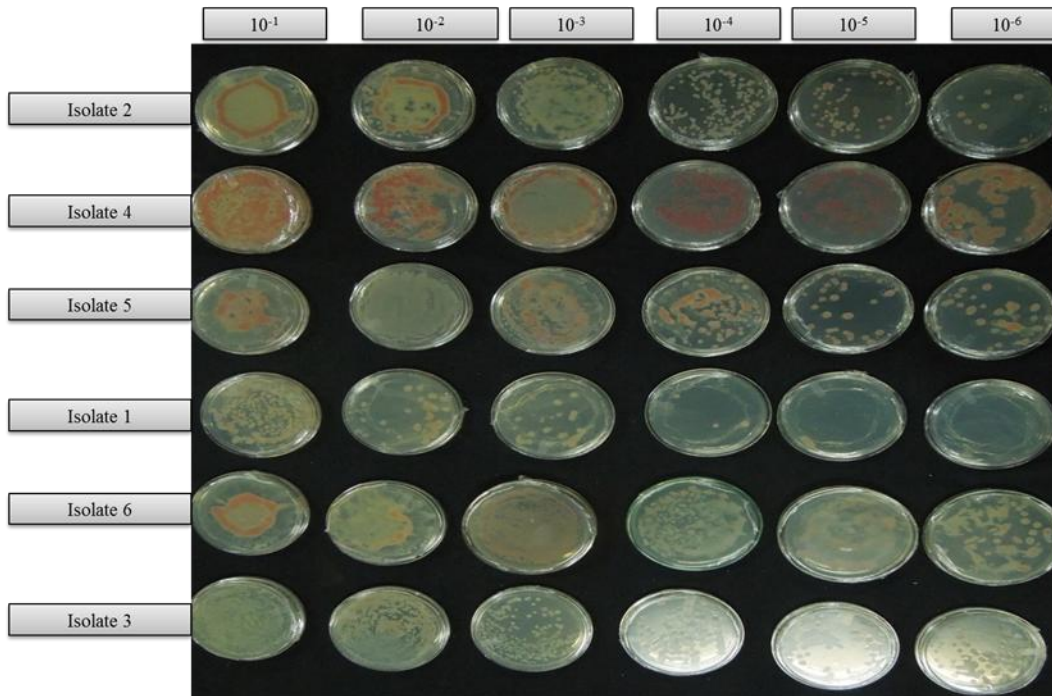


Fig.4 Gram's stain response of the six isolates of *R. solanacearum*

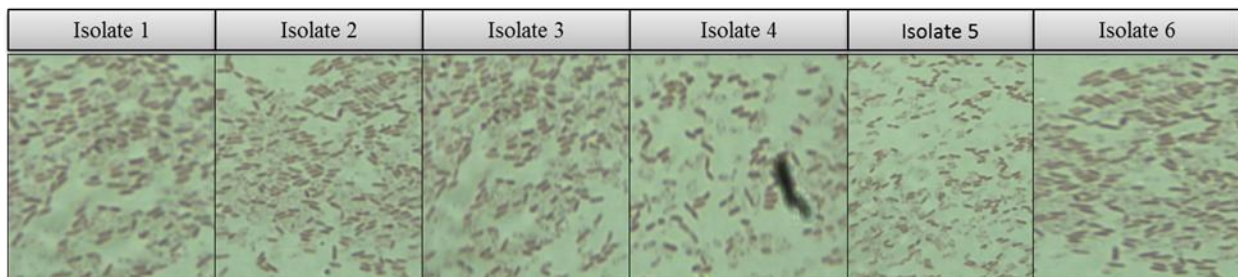


Fig.5 Bacterial wilt symptoms of six isolates in *Capsicum annum*



Fig.6 Bacterial wilt symptoms of six isolates in *Solanum melongena*



Fig.7 Bacterial wilt symptoms of six isolates in *Solanum lycopersicum*



Fig.8 Bacterial wilt symptoms of six isolates in *Morus alba*

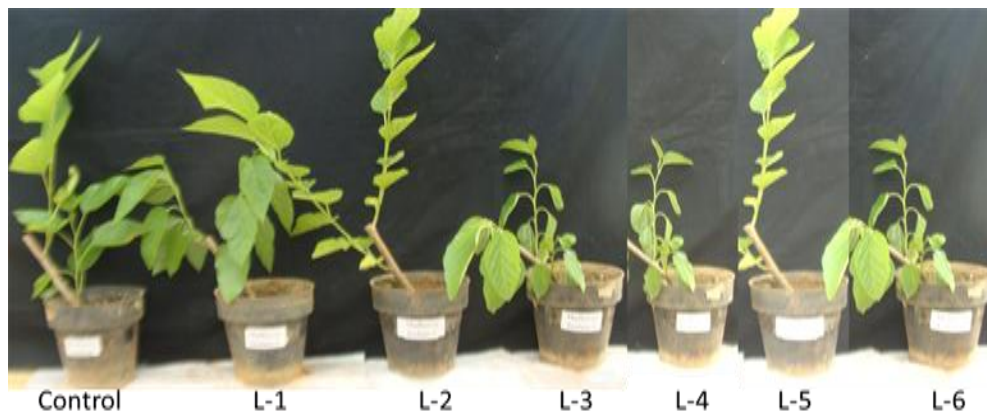


Fig.9 Bacterial streaming test

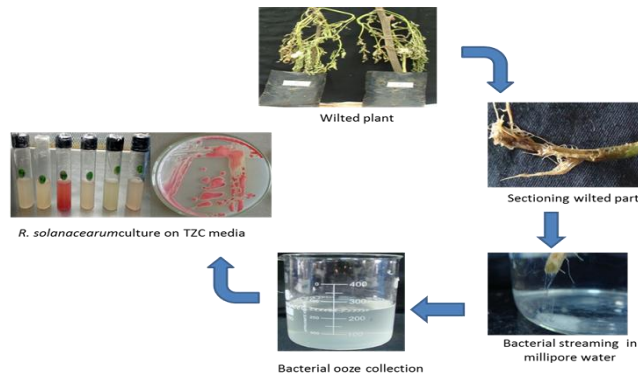
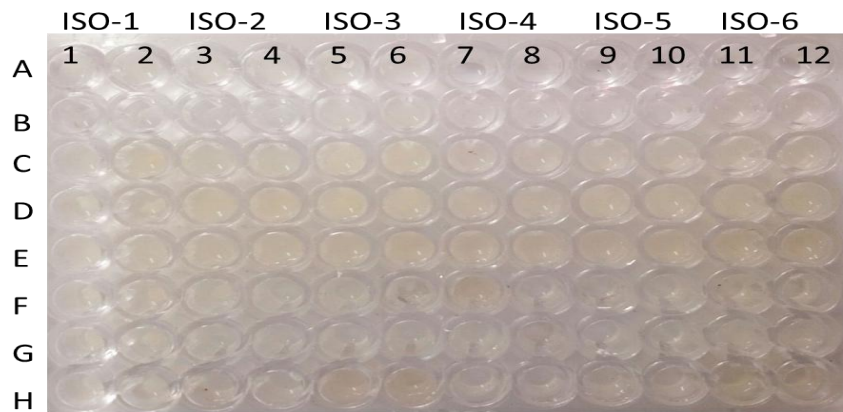
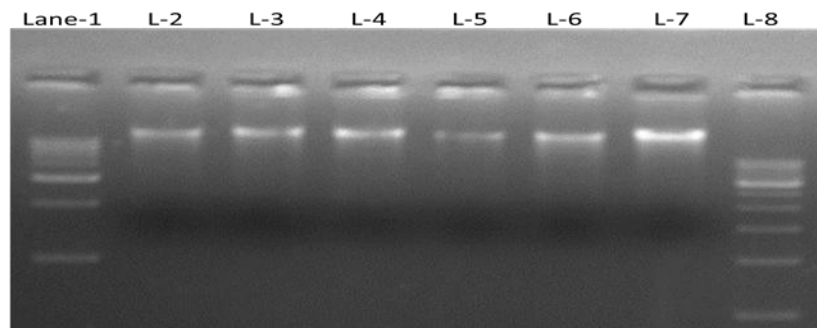


Fig.10 Biovar test showing positive (+ve) yellow color and negative (-ve) green color reaction indicating the utilization of sugar and alcohol by *Ralstonia solanacearum* isolates in microtitre plate



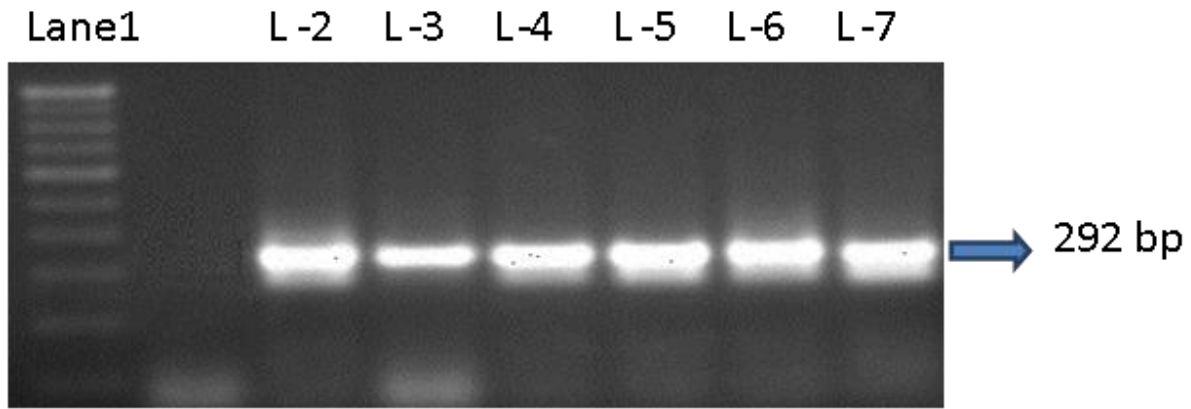
wells 1,2- Isolate(1) 3,4- Isolate(2) 5,6-Isolate-3 7,8-Isolate(4) 9,10-Isolate(5) 11,12-Isolate(6)
 A- without carbohydrate, B-without *R. solanacearum*, C-Lactose, D-Maltose, E-Cellobiose, F-Mannitol, G-Sorbitol, H-Dulcitol

Fig.11 Agarose gel profile of total genomic DNA of *Ralstonia solanacearum*



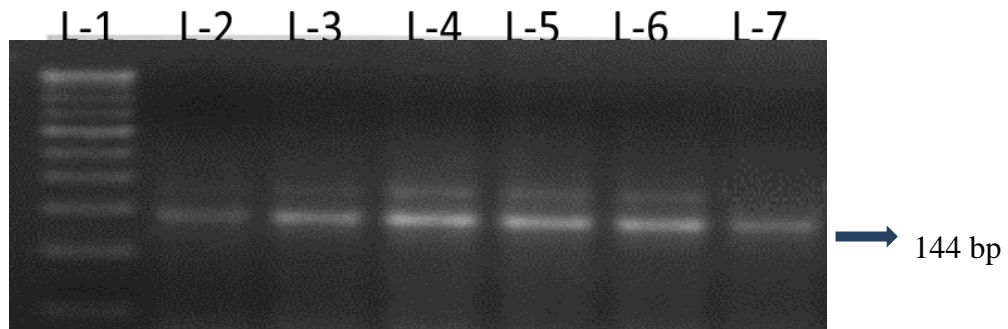
Lane1-Ladder 5kb, Lane8-Ladder1kb, Lane(2-7)- Isolates

Fig.12 Electrophoretic analysis of PCR amplified DNA from six isolates of *Ralstonia solanacearum* using the primer Y1 and Y2



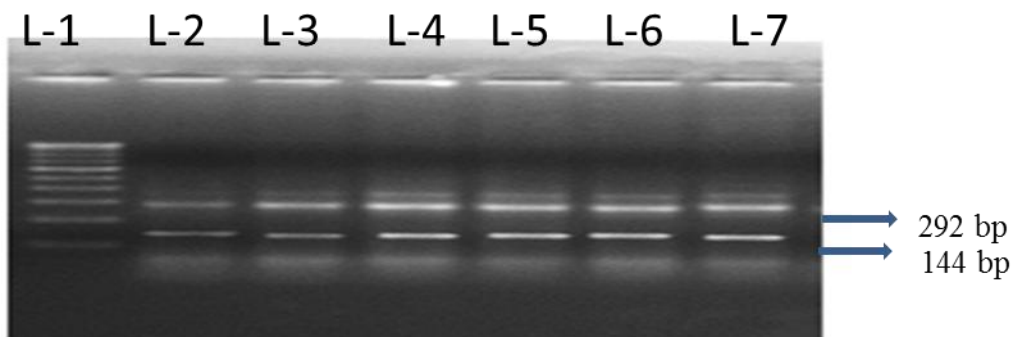
Lane1-1kb ladder, lane(2-7)-isolates

Fig.13 Electrophoretic analysis of PCR amplified DNA from six isolates of *Ralstonia solanacearum* using the primer OLII & Y



Lane1 -ladder, Lane(2-7)- isolates

Fig.14 PMX-PCR using a phylotype specific primer showing PCR product 280bp amplicon for all isolates and 144bp phylotype 1



Lane1- ladder 1kb, lane(2-7)- isolates

The number of colony forming unit (cfu) observed at six dilutions of all isolates of *Ralstonia solanacearum*. In all isolates the colony forming units were uncountable at zero dilution. This result in conformity with Adams *et al.*, (2014), Kwiatkowski *et al.*, (2015), Mensah and Twumasi (2016), Hendry *et al.*, (2014), even they could find 40 plates out of 1440 were having uncountable colonies due to spreading of the colonies. It was observed that the plates were having uncountable colonies at zero dilution because of more number of colonies and due to spreading. At sixth dilution, the maximum cfu were recorded in isolate-3(28), isolate-4(25), isolate-5(21), isolate-6(21), isolate-2(8) and no colony forming unit (cfu) observed in isolate-1(0).

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman Tetrazolium Chloride (TZC) agar test. Results of this test showed that all isolates of *R. solanacearum* collected from different growing areas produced pink or light red color colonies with characteristic red centre and whitish margin on TZC medium. This indicates that all *R. solanacearum* isolates were virulent. Kelman (1954) reported that avirulent colony types of *R. solanacearum* could be easily differentiated by the pigmentation from the wild virulent types. *R. solanacearum* developed two types of colonies on tetrazolium chloride (TZC) medium on which virulent colonies appear as white with pink centre and non-virulent colonies appear as small off-white colonies. On this medium, typical bacterial colonies appear fluidal, irregular in shape, and white with pink centres after 2 to 5 days incubation at 28°C as reported by Champoiseau (2008). *R. solanacearum* produced fluidal colonies with pink or light red color on TZC media after 24 hours of inoculation as reported previously by Rahman *et al.*, (2010), Ahmed *et al.*, (2013).

Denny and Hayward (2001) identified race of *R. solanacearum* by host specificity with infiltration/inoculation methodology used as simple and quick means of determining the race of any particular virulent isolate of this pathogen (Lozano and Sequiera, 1970; Lemessa and Zeller, 2007). In the present study, four isolates belonged to Race 3 while two belonged to Race 1. The findings of the present study are also supported by Buddenhagen *et al.*, (1962), Ahmed *et al.*, (2013), Popoola *et al.*, (2015). *R. solanacearum* Race 1 strains infect tobacco, tomato, and many other solanaceous crops, and certain diploid bananas. Race 3 causes wilting in potato, tomato and rarely other solanaceous plants. Race 2 is known to infect triploid banana (*Musa* spp.) and *Heliconia* spp., while Race 4 infects mulberry (He *et al.*, 1983; OEPP/EPPO 2004). He *et al.*, (1983) reported Race 5 on Mulberry from China. Therefore, five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions (French, 1986). Race 3 is highly virulent on potatoes, tomatoes and other *solanaceous* crops (Hudelson *et al.*, 2002; Janse *et al.*, 2004). Its temperature optimum is lower than that of other races and this might partly its preponderance in temperate regions of the World and places like Bangalore (Karnataka).

To diagnose the presence of *Ralstonia* Streaming test was performed. Popoola *et al.*, (2015) observed similar results. The Gram's staining reaction was performed using crystal violet as described by Schaad (1980). The microscopic results showed that all the isolates of *R. solanacearum* did not retain violet colour *i.e.* the isolates retained counter stain (pink colour). Therefore, all isolates of *R. solanacearum* representing each group are gram negative and straight or curved, rod shaped. These results are in conformity with the findings of Suslow *et al.*, (1982),

Chaudhry and Rashid (2011) and Ahmed *et al.*, (2013), Popoola *et al.*, (2015).

Biovar characterization showed that most of the *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol). The oxidation reaction was indicated by the change of color. In present study, isolate-3 and isolate-6 of *R. solanacearum* belong to biovar III whereas isolate-1, isolate-2, isolate-4 and isolate-5 belonged to biovar-2 (Table 3 and Fig. 10). The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported previously by Hayward (1964), He *et al.*, (1983), Kumar *et al.*, (1993), Chaudhry and Rashid (2011), Ahmed *et al.*, (2013) and Popoola *et al.*, (2015). They also observed that, biovar III oxidizes both disaccharides and hexose alcohols, biovar II oxidizes only disaccharides whereas Biovar I oxidizes hexose alcohols only, and biovar IV oxidizes only alcohols.

Genomic DNA was extracted from all six isolates of *R. solanacearum* and subjected to PCR by *R. solanacearum* specific primers. Partial sequences of 16S rDNA and 16S rRNA genes are excellent targets for identification of bacteria at the species level as they are species-specific and available in multiple copies in microbial genome. The corresponding specific rDNA and rRNA sequences have been used as targets for PCR amplification (Woese, 1987).

In this study, the PCR amplicon resulted in a ~300 base pair (bp) product for (OLI1 & Y2) and 292 bp products for (Y1 & Y2) from all isolates of *R. solanacearum*. This result confirmed that all the isolates are *R. solanacearum*. It was supported by earlier results of Seal, (1993), Chandrashekara (2012) and Chandrashekara (2012) for *R. solanacearum*.

For phylotype identification, a set of four phylotype-specific forward primers (Nmult:21:1F: Nmult:21:2F: Nmult:22:InF: and Nmult:23:AF:), with a unique and conserved reverse primer (Nmult:22:RR:) and set of *R. solanacearum* specific primers (759R and 760F) as described (Fegan and Prior 2005; Prior and Fegan 2005) was used. This primers sequence was targeted to the 16S-23S intergenic spacer region (internal transcribed spacer).

The results of Pmx-PCR of six isolates of *R. solanacearum* were 144-bp amplicon and 280 bp amplicon. It revealed that all isolates were *R. solanacearum* and belonged to phylotype I. These results were confirmed by Fegan and Prior 2005; Prior and Fegan 2005; Wang *et al.*, (2013); Popoola *et al.*, (2015) and Vanitha *et al.*, (2014).

In conclusion the goal of this research was to collect and characterize the isolates from bacterial prone areas of Karnataka and characterization. In this study, we collected six isolates were collected and characterized as Biovar-2, 3 on the basis of ability to utilize disaccharides (Sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol), Race 1, 3 were identified based on their ability to infect different hosts we could class these races as phylotype I based on the ITS sequences of six *R. solanacearum* strains.

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