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# Isolation, identification and molecular characterization of *Ralstonia solanacerum* isolates collected from Southern Karnataka

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**Abstract**
Bacterial wilt caused by *Ralstonia solanacearum*, is the major threat to tomato cultivation in all tomato growing areas of Karnataka. *R. solanacearum* was isolated from the infected host plants collected from different locations of southern Karnataka. The identity of the isolates was established using morphological, biochemical, and molecular analysis using species specific PCR primers. The race and biovar specificity of pathogen was determined through pathogenicity test on different host plants and the ability of isolates to use carbohydrates, respectively. Phylotype classification was done by phylotype specific multiplex PCR using phylotype specific primers. All the bacterial isolates showed the characteristic creamy white fluidal growth with pink centre on the Tetrazolium chloride medium. Further, the isolates amplified at 280 bp, which confirmed the identity of pathogen as *Ralstonia solanacearum*. Our results showed that all isolates belonged to Race 1 of the pathogen. Among different isolates obtained, four isolates each were identified to be Biovar III and Biovar IIIA, repectively, while two isolates were identified as Biovar IIIB. All the ten isolates were affiliated to Phylotype I of *Ralstonia solanaceraum* species complex. These findings may help in devising the management practices for bacterial wilt of tomato in southern Karnataka.

**Keywords**: Bacterial wilt, Biovar, Phylotype, Race, *Ralstonia solanacearum*

## INTRODUCTION

*Ralstonia solanacearum* (Smith, 1896; Yabuuchi *et al*., 1995), is a soil borne, aerobic, gram-negative, rod shaped, non-spore forming bacterial plant pathogen and a serious threat to production of many crop plants around the world (Agrios, 1997). The bacterium invades vascular tissues and causes wilt disease in plants. It is the causal agent of moko disease of banana, brown rot of potato, bacterial wilt or southern wilt of tomato, tobacco, eggplant and some ornamental plant species (Stevenson et al., 2001). Bacterial wilt is a common bacterial plant disease in tropical, subtropical, and some temperate regions of the world (Fegan and Prior, 2005). The species, *R. solanacearum* has a very broad host range, but variability exists among the species with respect to their race specificity and host preference. Generally, the pathogen infects hundreds of plant species encompassing 44 families (Hayward, 1991), including economically-important plant species of Solanaceae or nightshade family (Stevenson, et al., 2001).

The soil-borne nature of the bacterium makes it difficult to control the pathogen due to the interaction of environmental factors and high pathogen diversity. Crop cultivars resistant to this pathogen were available in few plant species, however, their resistance is limited to only few strains of *R. solanaceraum* (Buddenhagen *et al*., 1962; Hayward, 1964, 1994). The pathogen is highly diverse, represented by hundreds of genetically distinct isolates and hence, *R. solanacearum* is considered as a species complex; a heterogeneous group of related strains (Fegan and Prior, 2005). The species is currently classified into five races based on its host specificity and grouped into six biovars with respect to carbohydrate utilization (Hayward, 1964). Race 1 of this pathogen is the most abun-

dant and widely distributed throughtout the world, which affects solanaceous crops and other plants species from several botanical families. Race 2 is mainly confined to tropical areas and causes the Moko disease of banana in South America, Philippines, Hawaii and Asia. This race also infects *Heliconia* sp. and other ornamental plant species of the family Musaceae. Race 3 usually occurs in tropical areas with higher altitude and temperate climate and affects mainly potato, tomato, and to a lesser extent, other hosts such as solanaceous weeds and geranium. Race 4 was reported on ginger in Philippines, while Race 5 was reported on mulberry in China (Buddenhagen *et al*., 1962).

Several studies were earlier conducted to isolate this pathogen from different host species and characterize the diversity within species in terms of race and biovar specificity using ERIC-PCR and BOX-PCR (Gilson *et al*., 2012). Phylogenetic analysis of sequences of part of *Egl* and *MutS* genes revealed predominance of phylotype II in Brazil. Seventy seven bacterial strains isolated from wilted bell pepper plants from north eastern Brazil were identified as *Ralstonia solanacearum* using multiplex PCR. The strains were further analysed to characterize their biochemical, physiological, and molecular diversities (Garcia *et al*., 2013).

A phylogenetic classification scheme based on the study of partial sequences of the spacer region, 16S-23S rDNA (ITS) divides the species complex into four phylotypes (Poussier *et al.,* 2000) corresponding to the four genetic clusters associated with their respective geographical origin. Phylotype I includes strains originated in Asia; phylotype II strains were originated from America; strains included under phylotype III were originated in Africa; and phylotype IV includes strains native to Indonesia (Fegan and Prior, 2005). The present study was conducted to isolate *R. solanacearum* from different host plants collected from different locations of southern Karnataka and characterize the race, biovar and phylotype specificity within the species to study the taxonomic diversity of the species in the region.

## MATERIALS AND METHODS

**Collection and isolation of *R. solanacearum* from wilt affected Solanaceae plants:** Infected host plants *viz.* tomato, brinjal, chilli, capsicum, and potato with typical symptoms of bacterial wilt were collected from different parts of southern Karnataka (Table 1). The disease in infected plants was diagnosed using ooze test (Sujeet Kumar *et al*., 2017). The disease confirmed vascular tissues of infected plants were processed for isolation of pathogen on Triphenyltetrazolium Chloride (TZC) medium (Peptone-10g; Casein hydrolylate-1g; Dextrose-5g; Agar-15g; 5 ml of 1% TZC ; Distilled water-1000 ml) using serial dilution method. Discoloured vascular tissues were cut into small pieces measuring 4-5 mm length and surface sterilized using 0.1% sodium hypochlorite (NaOCl) solution for 30 sec. Subsequently, the tissue bits were repeatedly washed with sterile double distilled water to remove traces of sodium hypochlorite, if any. The surface sterilized bits were suspended in a test tube containing 10 ml of sterile distilled water for 10 min. After the bacterial cells oozed out from the cut ends of tissue bits, the bacterial suspension in test tube was serially diluted in 9 ml of sterile water blanks. About 100 µl of bacterial suspension from the serial dilution was uniformly streaked onto the culture plates containing solidified TZC medium. The inoculated culture plates were incubated at 32°C for 48 hours.

**Characterization of *R. solanacearum* isolates:** The virulent isolates (colonies with characteristic red or pink centre and whitish margin) obtained from the infected tomato, brinjal, chilli, capsicum and potato plants were subjected to morphological, physiological, cultural, biochemical and pathogenicity studies as established by previous reports (Kelman, 1954; Buddenhagen *et al.,* 1962; Schaad 1992).

**Morphological and cultural characterization of *R. solanacearum:*** The pathogen was initially identified based on the colony morphology *viz.* shape, size, pigmentation and mucoidness. The morphological characteristics such as cell shape, gram reaction and capsule staining were done by following the descriptions given by Anonymous (1957), Kelman (1954), Bradbury (1970) and Schaad (1992).

**Gram staining:** A loop full of bacterial culture was smeared on to clean glass slide, air dried and heat fixed. Crystal violet was flooded for 1 min, the slide was washed with distilled water and flooded with iodine solution for 1 min. The iodine solution was washed with 95% (v/v) ethyl alcohol and subsequently with distilled water, drained and counter stained with safranin 1 min. The slide was washed with distilled water, air dried and examined under microscope using oil immersion objective lens.

**Physiological and biochemical characterization of *R. solanacearum:*** Physiological and biochemical tests *viz.* starch hydrolysis, nitrate reduction, oxidase and KOH solubility test were carried out as per the methods described in the Manual of Microbial methods (Anonymous, 1957) and laboratory guide for identification of Plant Pathogenic Bacteria (Schaad, 1992).

**Catalase test:** A loop full of bacterial culture was smeared on a clean glass slide and added with few drops of hydrogen peroxide (20 volumes). Production of gas bubbles indicates a positive reaction, which is related tot the presence of aerobic and facultative anaerobic bacteria.

**Oxidase test:** Fresh bacterial cultures grown on nutrient agar media with 1% (w/v) glucose were

patched onto a filter paper moistened with a fresh oxidase reagent (1% w/v aqueous solution of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) using a wooden stick. A purple reaction in 30 sec confirms the test as positive. This test is used to detect the presence of cytochrome C oxidase (oxidase positive).

**Nitrate agar test:** The nitrate agar test for the ten isolates was conducted as per the previous established procedure (http://www.asmscience.org/content/education/protocol/protocol.3660). In brief, this test confirms the ability of the bacterium to reduce nitrates into nitrites.

**KOH solubility test:** A loop full of the bacterial culture was smeared on a clean glass slide and 2-3 drops of 3% (w/v) KOH was added. The colony of the test strain was stirred in to the solution with clean loop for 5 to 10 sec. When the solution was viscous enough and sticks to the loop causing a thin strand of slime or thread, then the test is rendered positive (KOH soluble).

**Pathogenicity test for *R. solanacearum:*** Tomato, brinjal, chilli, capsicum, and potato seedlings were raised in polythene bags containing sterilized soil mixed with essential plant nutrients. The roots of 20 days old seedlings of tomato, brinjal, chilli, and capsicum were washed in water and few tertiary roots were sheared with sterilized scissors. The injured roots of the seedlings were dipped in the bacterial suspension **(**OD at $A_{480}$ηm**=**1.0) separately for each isolate of *R. solanacearum* for 10 min and the treated seedlings were transplanted to plastic bags containing sterilized soil. For potato seedlings, the bacterial suspension was drenched around the root zone.

**Race determination:** The race specificity of *R. solanacearum* isolates was determined through artificial inoculation of pathogen to the test plants. All the ten isolates of *R. solanacearum* were multiplied overnight in TZC broth and subsequently inoculated onto tomato, brinjal, potato, ginger, banana, and mulberry plants. The bacterial suspension was adjusted to cell concentration of $5x10^5$cfu/ml using spectrophotometer at wave length of 480nm, with an OD value equivalent to $A_{480}$ηm=0.8 to 1.0

**Determination of isolates into biovars:** Biovars of *R. solanacearum* are primarily differentiated according to their ability to oxidize disaccharides *viz.* cellobiose, lactose, and maltose and utilize sugar alcohols such as dulcitol, mannitol, and sorbitol (Hayward, 1964).

The three sugars *viz.* cellobiose, lactose, and maltose and three sugar alcohols *viz.* dulcitol, mannitol, and sorbitol were supplemented separately to a basal medium ($NH_4H_2PO_4$ -1.2 g; $MgSO_4$.7 $H_2O$-0.24 g; KCl-0.24 g; Peptone-1to 2 g; KOH-3% (w/v); Distilled water-1000 ml) at the rate of 0.03 g/litre. The pH of the basal medium was adjusted to 7.2 using 40% (w/v) NaOH solution until olivaceous green colour was observed in the basal medium. Bromophenol blue was added to the basal medium as an indicator for oxidation reaction (colour change).

The basal medium with bromophenol blue was autoclaved at 121$^o$C for 15 min and cooled to 45° C. Subsequently, the six sugar solutions were added to each flask separately and mixed thoroughly to get uniform distribution of sugar solution in the basal medium. Aliquots of 5 ml from each sugar supplemented basal medium was separately dispensed into 10 sterile Durham's tubes (6 inch) under aseptic conditions. The tubes containing the aliquots were seperately inoculated with 20 µl of bacterial suspension of ten isolates. The inoculated tubes were incubated at 30°C and the culture media were examined at 3, 7, 14 days for change in pH from basic to acidic producing yellow color from top downward.

**Molecular characterization of *Ralstonia solanacearum***

**Genomic DNA extraction:** The genomic DNA from the bacterial cells was isolated using AMpurE Bacterial gDNA Mini Spin kit (#AMRK016, Amnion Biosciences). The bacterial cells were collected from 24 hour nutrient broth cultures by centrifugation for 2 min at 12000 rpm. The pelleted cells were processed as per manufacturer's instruction for isolation of genomic DNA from bacterial cells. The extracted DNA was quantified using 0.8% (w/v) agarose gel.

**PCR for confirmation of *R. solanacearum:*** The 16S rDNA of all isolates were amplified by using universal specific primer set of 759/760 as described by Sagar *et al.* (2014). Each PCR reaction consisted of 50 µl mixture containing primers (20 pmols of 759/760 each), PCR master mix consisting of $MgCl_2$ (2mM), dNTP's (200 µM), Taq polymerase (2.5 U), template DNA (50 ng), and molecular grade water. The reaction mixture was amplified in Thermal cycler (Eppendorf) for 35 cycles after an initial denaturation at 96°C for 5 min. Each cycle consisted of 15 sec at 94°C, 30 sec at 59°C, 72°C for 30 sec and final extension for 10 min at 72°C. All the PCR products were electrophoresed using 2% (w/v) agarose gel for confirmation of template amplified.

**Molecular analysis of 16S rDNA:** The 16S rDNA fragments of bacterial DNA were amplified using Fd1 forward (GAGTTTGATCCTGGTCA) and Rp2 reverse (ACGGCTACCTTGTTACGACTT) primers. PCR reaction mixture (50 µl) contained 20 pmol of each primer, 50 ng of genomic DNA, 1X *Taq* DNA polymerase buffer, 1 U of *Taq*DNA polymerase, 0.2 mM of each dNTPs and 1.5 mM of $MgCl_2$. The thermal cycling program consisted of initial denaturation at 94 ºC for 3 min followed by 35 cycles with denaturation at 94 ºC for 1 min, annealing at 46 ºC for 30 sec and extension at 72 ºC for 4 min, with a final extension at 72 ºC for 10 min. Amplifi-

cation was performed in a DNA thermal cycler (Eppendorf). The purified PCR products were subjected to Sanger dideoxy sequencing at Amnion Biotech Pvt. Ltd. (Bangalore, India). The DNA sequences were compared with NCBI database using BLASt tool and the isolates were identified based on the nearest homology search results. The phylogenetic tree was constructed for bacterial isolates based on the closest relative organisms obtained in NCBI query using Mega 6 software.

**Phylotype identification:** Phylotype identification (Prior and Fegan, 2005) was done using Phylotype specific multiplex PCR (Pmx-PCR). The reaction mixture consisted of 25 ul final volume, containing 2X Master mix (PCR buffer, 1.5 mM $MgCl_2$, 200 uM of each dNTP, 50 mM KCI, 10 mM Tris-HCI and 1.25 U of Taq DNA polymerase), 6 pmoles of the primers, Nmult: 21:1F, Nmult: 21:2F, Nmult:22:lnF, 18 pmoles of the primer Nmult:23:AF and 4 pmoles of the primers 759 and 760 (Opine *et al.,* 1997) Table.2. The following cycling programme was used in a thermal cycler (Eppendorf): 96°C for 5 minute and then cycled through 30 cycles of 94°C for 15 s, 59°C for 30 s and 72°C for 30 s, followed by a final extension period of 10 minute at 72°C. The amplified PCR products were electrophoresed on 2% (w/v) agarose gel and visualized on a UV-trans illuminator.

## RESULTS AND DISCUSSION

**Collection, isolation and characterization of the *R. solanacearum* isolates:** The laterally dissected infected plants showed brownish discolouration of roots and stem portion just above the collar region. The disease was confirmed through ooze test (Plate.1) of infected tissues of host plants. Milky white bacterial ooze was observed from the cut ends of infected tissues placed in sterile distilled water (Vanitha *et al.*, 2009). The bacterial colonies of all the 10 isolates on TZC medium were well separated, irregular with smooth and dull white margin, and characteristic pink to red center. TZC medium is commonly used to differentiate the virulent and avirulent strains of *R. solanacearum* based on the morphological characteristics. All the isolates in the present study exhibited the typical characteristics of virulent strains (Plate.2) i.e. highly fluidal, round to irregular colonies with white margin with pink centre. Gram reaction identified that all the isolates were gram negative (reddish pink) with straight and slightly curved rod shaped cells, which is the characteristic feature of the pathogen (Smith, 1896).

**Physiological and biochemical tests:** The results of physiological and biochemical tests on the ten isolates of *R. solanacearum* were presented in Table.3. The results obtained in the present study were concurrent with the previous reports on the pathogen. The present study showed that all isolates were positive for oxidase test (Ahmed *et al*., 2013), which produced purple colour when the bacterial mass was rubbed on filter paper impregnated with oxidase reagent and this proved the aerobic nature of the pathogen. All the ten isolates of *R. solanacearum* obtained in the present study were found positive for nitrate test. Makhlouf and Hamedo (2013) also reported positive results for nitrate test with six isolates of *R. solanacearum* obtained from infected tomato plants. Positive reaction for nitrate test indicated the ability of bacteria to convert nitrates into nitrites. Similarly, all isolates in the present study were identified positive for the KOH solubility test as they formed slime threads when the bacterial cultures were mixed with 3% KOH solution. KOH test is a rapid and easy technique to distinguish the gram negative and gram positive bacteria as compared to the conventional Gram staining (Suslow *et al.,* 1982). Shahbaz *et al*. (2015) isolated *R. solanacearum* from infected chilly plants and identified the isolates positive for catalase test.

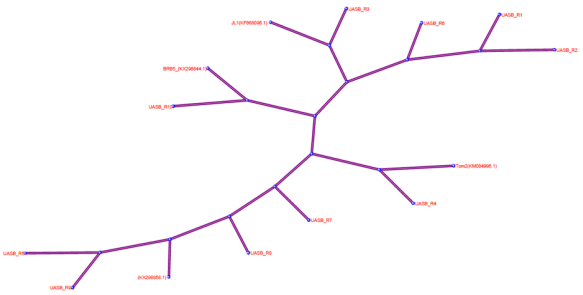

**Fig.1.** *Phylogenetic tree of ten isolates of R. solanacearum.*

**Pathogenicity test:** *R. solanacearum* is a soil borne pathogen and has both virulent and avirulent strains. It is essential to determine pathogenicity of isolates in order to confirm their virulence. The pathogenicity test for all the test isolates in the present study using root dip inoculation method (Avinash and Umesha, 2013) produced typical wilting symptoms in tomato plants within three weeks after treatment. The treated plants exhibited loss of turgidity, drooping of leaves and sudden wilting symptoms. Re-isolation of pathogen from the wilted plants yielded bacterial colonies that were similar to the original colonies used for inoculation. This confirmed the virulence of all isolates of *R. solanacearum* obtained in the present study. Thus on the basis of results obtained from morphological, cultural, biochemical, and pathogenicity tests, all the ten isolates obtained from different host plants collected from southern Karnataka were identified as *R. solanacearum*.

**Determination of race specificity:** The ten isolates of *R. solanacearum* were cross infected to different hosts and all the test isolates produced highly virulent reaction on tomato, brinjal, and po-

**Table 1.** Infected host plants collected from different parts of Southern Karnataka.

| Primers | Sequence (5′-3′) | Size (bp) | Specificity |
|---|---|---|---|
| Nmult:21:1F | CGTTGATGAGGCGCGCAATTT | 144 | Phylotype I |
| Nmult:21:2F | AAGTTATGGACGGTGGAAGTC | 372 | Phylotype II |
| Nmult:22:InF | ATTGCCAAGACGAGAGAAGTA | 91 | Phylotype III |
| Nmult:23:AF | ATTACSAGAGCAATCGAA | 213 | Phylotype IV |
| Nmult:22:RR | TCGCTTGACCCTATAACGAGTA | NA | All phylotypes |

**Table. 2.** Primers used for phylotype characterization.

| S. N. | Host | Location | Isolate designation |
|---|---|---|---|
| 1 | Tomato | GKVK, Bengaluru | UASBR1 |
| 2 | Brinjal | GKVK, Bengaluru | UASBR2 |
| 3 | Brinjal | Mandya | UASBR3 |
| 4 | Tomato | Devanahalli (Sadhalli) | UASBR4 |
| 5 | Brinjal | Doddaballapura | UASBR5 |
| 6 | Tomato | Tumkur (Chikkanayakaahalli) | UASBR6 |
| 7 | Tomato | Hassan (Arasikere) | UASBR7 |
| 8 | Chilli | Chikkaballapura | UASBR8 |
| 9 | Capsicum | GKVK, Bengaluru | UASBR9 |
| 10 | Potato | Chikkaballapura | UASBR10 |

**Table 3.** Morphological and bio chemical characteristics of *R. solanacearum* isolated from different hosts.

| Sl. No. | Isolates | Biochemical characteristics | | | | |
|---|---|---|---|---|---|---|
| | | Gram staining | Oxidase | Nitrate agar | Catalase test | KOH solubility |
| 1 | UASBR1 | Negative | + | + | + | + |
| 2 | UASBR2 | Negative | + | + | + | + |
| 3 | UASBR3 | Negative | + | + | + | + |
| 4 | UASBR4 | Negative | + | + | + | + |
| 5 | UASBR5 | Negative | + | + | + | + |
| 6 | UASBR6 | Negative | + | + | + | + |
| 7 | UASBR7 | Negative | + | + | + | + |
| 8 | UASBR8 | Negative | + | + | + | + |
| 9 | UASBR9 | Negative | + | + | + | + |
| 10 | UASBR10 | Negative | + | + | + | + |

**Table 4.** Classification of *R. solanacearum* isolates into biovars.

| Sl. No. | Isolates | Disaccharides | | Sugar alcohols | | | | Biovar |
|---|---|---|---|---|---|---|---|---|
| | | Cellobiose | Maltose | Lactose | Mannitol | Dulcitol | Sorbitol | |
| 1 | UASBR1 | + | + | + | + | - | + | IIIA |
| 2 | UASBR2 | + | + | - | + | - | + | IIIB |
| 3 | UASBR3 | + | + | - | + | - | + | IIIB |
| 4 | UASBR4 | + | + | + | + | + | + | III |
| 5 | UASBR5 | + | + | + | + | + | + | III |
| 6 | UASBR6 | + | + | + | + | - | + | IIIA |
| 7 | UASBR7 | + | + | + | + | - | + | IIIA |
| 8 | UASBR8 | + | + | + | + | + | + | III |
| 9 | UASBR9 | + | + | + | + | - | + | IIIA |
| 10 | UASBR10 | + | + | + | + | + | + | III |

tato with characteristic wilt symptoms in 15- 20 days of inoculation. None of the test isolates were able to infect the other plant species such as banana, ginger, and mulberry. Based on the virulence on different hosts, the test isolates used in the present study were identified as race 1. Similar results were reported by Prasanna Kumar *et al.* (2012) on prevalence of 57 isolates of *R. solanacearum* belonging to race 1 collected from different agro-climatic zones of India and causing wilt on different host plants *viz.* tomato (*Solanum lycopersicum*), brinjal (*Solanum melongena*), potato (*Solanum tuberosum*), bird of paradise (*Strelitzia reginae*), ginger (*Zingiber officinale*), chilli (*Capsicum annuum*), davana (*Artemisia pallens*), and coleus (*Coleus forskohlii*).

Buddenhagen *et al.* (1962) divided the *R. solanacearum* in to three races. Race 1 infects majority of the solanaceous plants including tomato, tobacco, pepper and other plants including some weeds. However, race 2 causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp. Race 3 affects potato and tomato but is weakly virulent on other solanaceous crops. Later, Aragaki and Quinon (1965) reported race 4 from infected ginger in the Philippines. He *et al*. (1983) reported race 5 from mulberry in China. The five races described so far differ in their host range, geographical distri-

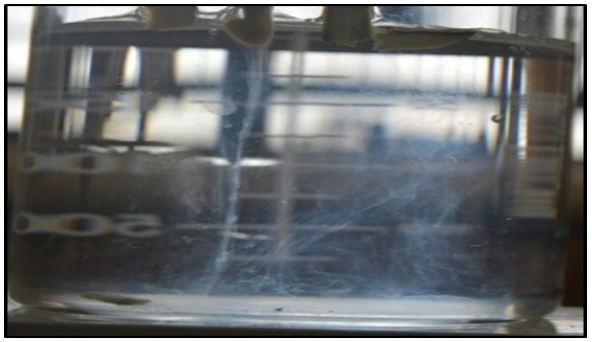

**Plate.1.** *Oozing of bacterial cells from infected tomato plant.*

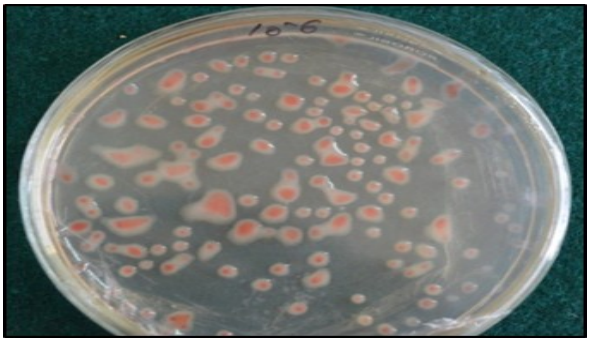

**Plate.2.** *R. solanacearum colonies on TZC medium.*

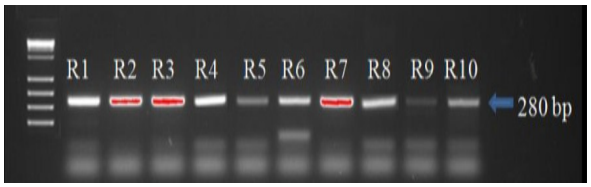


**Plate. 3.** *Identification of isolates of R. solanacearum by using specific primers.*

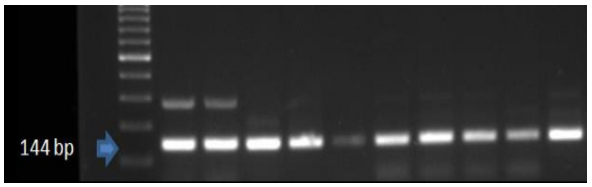


**Plate. 4.** *Phylotype identification of isolates of R. solanacearum.*
Abbreviation: Lane R1: Tomato (GKVK, Bengaluru); Lane R2: Brinjal (GKVK, Bengaluru); Lane R3: Brinjal (Mandya); Lane R4: Tomato (Sadhalli); Lane R5: Brinjal (Doddaballapura); Lane R6: Tomato (Chikkanayakaahalli); Lane R7: Tomato (Arasikere); Lane R8: Chilly (Chikkaballapura); Lane R9: Capsicum (GKVK, Bengaluru); Lane R10: Potato (Chikkaballapura)

bution, and ability to survive under different environmental conditions (French, 1986). Prevalence of race 1 in India is due to its wide adaptability, although race 3 was reported on potato in northern India, it was not reported in southern India.

**Biovar determination:** Hayward (1964) classified different strains of *R. solanacearum* in to five biovars based on their ability to utilize sugars and alcohols. These are essential for bacteria as it contributes to the normal functioning of the cell. The strains of *R. solanacearum* are known to show variation in their ability to utilize alcoholic sugars and disaccharides (Prasanna Kumar, 2004). This variability gives an indication of their host adoptability by utilizing the specific sugars.

The biovar classification of the ten isolates used in the present study was presented in Table.4. In this study, four isolates of *R. solanacearum* (UASBR4, UASBR5, UASBR8, and UASBR10) were categorized into biovar III, which utilized all the six sugars and alcoholic sugars as evidenced by the colour change of bromophenol blue medium to yellow due to the production of acid in the medium (Venkatesh and Khan, 2000; Dhital *et al*., 2001). Prasanna Kumar (2004) also reported the prevalence of biovar III in India. Four isolates *viz.* UASBR1, UASBR6, UASBR7, and UASBR9) were grouped into biovar IIIA, which utilized only three disaccharides and two alcoholic sugars and failed to utilize one alcoholic sugar i.e. dulcitol (Deepa *et al*., 2003; Prasanna Kumar, 2004). The two isolates ,UASBR2 and UASBR3 were grouped in to biovar IIIB as they utilized two disaccharides and two alcoholic sugars and they could not utilize one alcoholic sugar and one disaccharide i.e. dulcitol and lactose, respectively (Prasanna Kumar, 2004; Chandrashekara *et al*., 2012). Different biovars could have lost their ability to utilize dulcitol or dulcitol and lactose or they might be non-essential for the growth and development. This may help the bacteria to survive in minimal nutrient condition, in the absence of hosts.

The inducible sugar utilization is mainly dependent on individual sugar utilization pathways which consists of transporters and catabolic enzymes whose expression is induced in the presence of sugars. The pathway has the feedback of positive and negative mechanisms, the positive feedback emerges from the sugar inducible expression of the transporters and leading to import of sugars while, negative feedback emerges from the sugar inducible expression of enzymes which lead to breakdown of sugars (Afroz *et al*., 2014). Hence, this could be one of the probable reasons for variation in sugar utilization by bacterial isolates, which may not have the particular sugar utilization pathway.

**Identification of *R. solanaearum* by PCR amplification using specific primers:** PCR amplication of isolates *R. solanaearum* using specific universal primers 759/760 yielded 280 bp product encoding 16S rDNA in *R. solanacearum* confirming that all the 10 isolates belonged to *R. solanacearum* (Plate.3).

**Amplification of 16S rDNA:** The Sanger sequencing of 16S rDNA also confirmed the identity

of test isolates to be *R. solanacearum* as compared to their reference strains. Phylogenetic tree of *R. solanacearum* isolates was constructed based on the NCBI Genome Workbench tool (Figure.1). Based on their sequence similarities, the isolates R1, R2, R3, R6, and R10 formed a different branch in the phylogenetic tree, which showed similarity with reference strains BR-5 (KX298844.1) and JL-1(KF668096.1). The isolates R5 and R9 were found to be very distinct which held close homology with reference strain of accession no. KX298858.1 while, R4, R7 and R8 strains showed homology with Tom2 (KM084996.1) reference strain. The isolates R1, R2, R3, R6, and R10 were collected from Bengaluru (tomato), Bengaluru (brinjal), Mandya (brinjal), and Chikkaballapur (potato), respectively. The isolate, R5 was obtained from Doddaballapur (brinjal) whereas, R9 was collected from Bengaluru (capsicum). The isolates, R4 was collected from Devanahalli (tomato), R7 from Hassan (tomato) and R8 from Chikkaballapur (chilly). This results obtained in our present study indicated that the ten isolates collected showed variation with respect to their host and geographical distribution.

**Phylotype characterization:** Based on the origin and evolutionary nature, different strains of *R. solanacearum* were classified into four phylotypes. Phylotype I corresponds to the Asiatic origin, Phylotype II was native to America, Phylotype III contains strains from Africa and the Indian Ocean while, Phylotype IV contains strains from Indonesia. The PCR amplification resulted in a ~144 bp product from all the ten isolates of *R. solanacearum* (Plate.4) indicating that all the isolates belonged to phylotype I. The results of our study were in agreement with Sagar *et al* (2018).

## Conclusion

The bacterial wilt is a widespread disease causing severe yield losses in many economically important solanaceous vegetables in India. Ten isolates of *R. solanacearum* were isolated from different hosts collected from different places of southern Karnataka were characterized through morphological, cultural, biochemical, molecular, and pathogenicity tests. Among the ten test isolates, four isolates were identified as biovar III, four isolates as biovar IIIA, and two isolates as biovar IIIB based on their ability to utilize disaccharides and alcoholic sugars. All ten isolates were identified as race I based on their host specificity. Multiplex PCR confirmed that all isolates collected in the present study belonged to phylotype I.

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