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Characterization of *Ralstonia solanacearum* (Smith) Race1, Causing Bacterial Wilt of Brinjal

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***Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the most severe pathogen of Solanaceous crops with a wide host range. *Ralstonia solanacearum* race 1 is responsible for causing bacterial wilt in brinjal. The pathogenic strains were isolated from wilted brinjal plants and isolated on triphenyl tetrazolium chloride (TTC) medium. The strains were confirmed by proving pathogenicity on brinjal plants. Phenotypic characterization revealed that out of five media tested TTC medium was an excellent source for growth and extracellular polysaccaride production (EPS) of *R. solanacearum*. Upon biochemical analysis the *R. solanacearum* strains showed positive reaction for catalase, KOH, citrate utilization and acid production while negative results were observed for Gram staining, starch hydrolysis and fluorescent pigment production. The biovar of the strain was identified by utilization of disaccharides (cellobiose, maltose and lactose) and sugar alcohols (dulcitol, mannitol and sorbitol). The strains of *R. solanacearum* were confirmed to belong to Race 1 based on symptom production on different hosts and hypersensitive response on tobacco plant. Therefore, it is confirmed that *R. solanacearum* race 1 causing bacterial wilt of brinjal belongs to biovar III. PCR amplification of ITS region of the strains of *R. solanacearum* was done and sequenced [MH481663 (ERY1) and MH481664 (VBN1)].**

Key words: Brinjal, *Ralstonia solanacearum,* Race 1, Biovar III

Brinjal (*Solanum melongena* L.) belonging to the family Solanaceae is one of the popular vegetable crop grown across the world. In Tamil Nadu, 14,760 ha area is under brinjal cultivation with an annual production of 196.11 ('000 MT) and with a productivity of 13.29 MT. (Indiastat, 2017). Recently, *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (1995) causing bacterial wilt in brinjal has been identified as an emerging threat to brinjal cultivation. *R. solanacearum* is soil-borne, Gram negative, rod shaped, aerobic and non-spore forming bacteria. It occurs widely in tropical, subtropical and warm temperate regions of the world (Liu *et al*., 2009) and cause heavy yield loss in major crops like tomato, eggplant, potato, tobacco, banana, ginger etc.

Strains of *R. solanacearum* have been grouped into five races (Race 1 to 5) based on ability of bacteria to infect wide host range and create hypersensitive response on tobacco and six biovars (Biovar I to VI) based on the nutritional requirements of the bacteria, particularly carbohydrates and organic acids. Strains belonging to Race-1 have a wide host range and are pathogenic on different solanaceous plants and weed hosts. Race-2 is restricted to triploid banana and *Heliconia*. Race-3 infects potato, whereas Race-4 taints ginger and Race-5 is aggressive specifically on mulberry (Buddenhagen *et al*., 1962).

R. solanacearum affects the brinjal plant by interacting with the plant's xylem vessels by means of colonization and production of extracellular polysaccharides (EPS) which results in blocking of xylem vessels leading to wilting of the entire plant. Infected plants show typical symptoms of sudden green wilting, drooping of leaves and flaccid appearance of shoots resulting in death of the plant. Even after the plant death the bacteria survives in the soil and plant debris which hampers the elimination of inoculum (Hayward, 1991).

Bacterial wilt of brinjal observed worldwide and this emphasizes the need for efficient early identification of disease so that appropriate management stratergies can be adopted. The entire genome of *R. solanacearum* have several regions including Ribosomal genes *viz.,* 16S rDNA sequences and ITS between 16S and 23S. These regions can be used to identify the bacteria using primers targeting these regions. Some specific genes present only in *R. solanacearum* can also be used as an identification tool for confirming *R. solanacearum*. These primers can target polygalacturonase gene (Gillings *et al*., 1993) or *ipxC* gene (Opina *et al.,* 1997).

There is no effective control methods *R. solanacearum* because it has wide host range and genetic diversity. In this concern, present study focuses on identification of race and biovar of *R. solanacearum* strains isolated from brinjal in Tamil Nadu and characterization as it will assist in adopting timely and effective control and quarantine measures.

Material and Methods

Sample collection

Wilted brinjal plants were collected from ten different

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locations of Tamil Nadu. Field diagnosis of infected plant samples was done by critically observing the bacterial wilt symptoms which includes sudden wilting, drooping of young leaves and flaccidity. Ooze test was also performed in the field to distinguish bacterial wilt from fungal wilt.

Isolation of Ralstonia solanacearum pathogen and its pathogenicity testing

The collected plant samples were used for isolation of *R. solanacearum*. The vascular discoloured tissues were cut into small pieces and surface sterilized with 0.5 per cent sodium hypochlorite for 30 sec followed by dipping the tissue in the 70 per cent ethanol for one min and then rinsing the tissues with sterile distilled water for one min to remove trace of ethanol and then put into screw capped vial containing one ml of sterile distilled water. The water becomes turbid upon addition of tissues. The bacterial suspension was streaked on Petri plates containing TTC medium (1 g of casein hydrolysate, 10 g of peptone, 5 g of glucose and 5ml of 1 per cent triphenyl tetrazolium chloride for one litre of distilled water). The streaked plates were incubated at 28 ± 2° C for 48h. The well separated colonies were picked up and purified further by single colony isolation technique and suspended in sterile distilled water in sterile polypropylene tubes and were stored at 4° C (Kelman,1954). After isolation of the bacteria the pathogenicity was performed on one month old brinjal seedlings CO2 through Koch's postulates. The bacterial strains were multiplied in 100 ml CPG broth and incubated for 48h. After incubation, 20ml bacterial suspension (OD 600 nm= 0.20) was drenched in soil carrying the plants with preinjured roots in order to facilitate the entry of bacterial pathogen through roots (Hanson *et al*., 1996).

Cultural characterization

The cultural characters of *R. solanacearum* strains isolated from brinjal were studied by assessing the growth of bacterial cultures on different media *viz.,* King's B medium, nutrient agar medium, semi selective south african medium, casein peptone glucose medium. TTC medium showed the variation in the colony morphology for virulent and avirulent strains. Virulent strains exhibit pink or light red colour with whitish margin and avirulent strains exhibit small, round, dark red without fluidal colonies on TTC medium (Kelman,1954).

Biochemical characterization

Seven biochemical tests *viz.,* Gram staining, potassium hydroxide test, catalase test, indole production, starch hydrolysis, citrate utilization, acid production, gelatin liqeufication and pigment production were performed for confirmation of *R. solanacearum* as described by Rahman *et al*. (2010). Among ten *R. solanacearum* strains VBN1 was selected to perform the biochemical test as it showed greater virulence in pathogenicity test.

Biovar determination

Biovar of *R. solanacearum* are primarily differentiated according to their ability to reduce the disaccharides *viz.*, cellobiose, lactose, maltose and utilize sugar alcohols such as dulcitol, manitol and sorbitol. The biovar determined in mineral medium ($NH_4H_2PO_4$ 1.0g, KCl 0.2g, $MgSO_4.7H_2O$ 0.2g, Difco bacto peptone 1.0g, Agar 3.0g and Bromothymol blue 80.0 mg per litre) containing 1 per cent sugar. Microtitre plates were used to carry out this experiment. Hundred micro litre of basal medium was taken in each well and added with 20 µl of bacterial suspension belonging to ten strains seperately. The inoculated plate was incubated at 30° C and examined after 3 and 7d for change in colour. A yellow colour development indicates the reaction as a positive (Schaad, 1980).

Race identification

The race of *R. solanacearum* was identified by carrying out pathogenicity test on different hosts (Schaad *et al.,* 2001). Among the ten *R. solanaceaum* strains, VBN1 was selected to perform the race identification test as it showed greater virulence in pathogenicity test. Different crops *viz.,* tomato, chilli, banana, potato, ginger and mulberry were raised under glasshouse conditions. The bacterial culture was multiplied in CPG broth and incubated at 28 ± 2° C for 48 h. Bacterial suspension was prepared (OD 0.3 at 600 nm) and cross inoculated on other host by soil inoculation method. Plants inoculated with sterile water kept as control. The inoculated plants were observed at 2 d interval until symptom development. Hypersensitive reaction was assessed on tobacco plant by leaf infiltration technique (Lozano and Sequeira, 1970). Two days old bacterial suspension (OD 0.3 at 600 nm) was injected into the intracellular space of 45 d old tobacco leaf with a hypodermal syringe. Sterile water was used as control. The plants were examined at 2 d interval upto 10 d for expression of wilting of tobacco plant.

Molecular characterization

Bacterial pathogen was multiplied in CPG broth and incubated at 28 ± 2°C for 24 h. Genomic DNA was extracted by following CTAB method with slight modification suggested by Kumar and Anandaraj (2004). Molecular confirmation of *R. solanacearum* was done using universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (ACGGCTACCTTGTTACGACTT-3') with an amplification of 1465 bp that target the 16S rDNA gene of ITS region (Weisburg *et al*., 1991) for the ten strains of *R. solanacearum* DNA was extracted and the PCR was performed with above primers. The PCR products were run on 1% Agarose Gel. Out of ten amplified PCR products two were selected randomly and the partially purified PCR products were sequenced with J.K. Scientific Company, Bangalore, India. BLAST analysis was performed in NCBI for the sequences (www.ncbi.nih.gov/BLAST). Species specific primers *viz.,* Rs-sp Forward (5'-GTCGCCGTCAACTCACTTTCC-3') and Rs-sp Reverse (5'- GTCGCCGTCAGCAATGCGGAATCG-3') were used to amplify the *ipxC* gene of *R. solanacearum*

at the species level with predicted amplicon size of 280 bp (Opina *et al*., 1997). PCR was run using Rs-sp primer and was run on 1% agarose gel.

Results and Discussion

Isolation of Ralstonia solanacearum pathogen and pathogenicity testing

Isolation of bacterium was done from brinjal plant showing characteristics wilt symptoms (Fig. 1a). The vascular bundles of the infected plants showed brown discolouration (Fig. 1b). Infected plants showed milky bacterial streaming from the cut ends of stem hence were ooze test positive (Fig. 1c). The isolated bacterial colonies developed as white, smooth, fluidal, irregularly round and opaque colonies on TTC medium (Fig. 1d). *R. solanacearum* produced fluidal colonies with pink or light red colour colonies on TTC medium after 24 h of incubation was reported previously

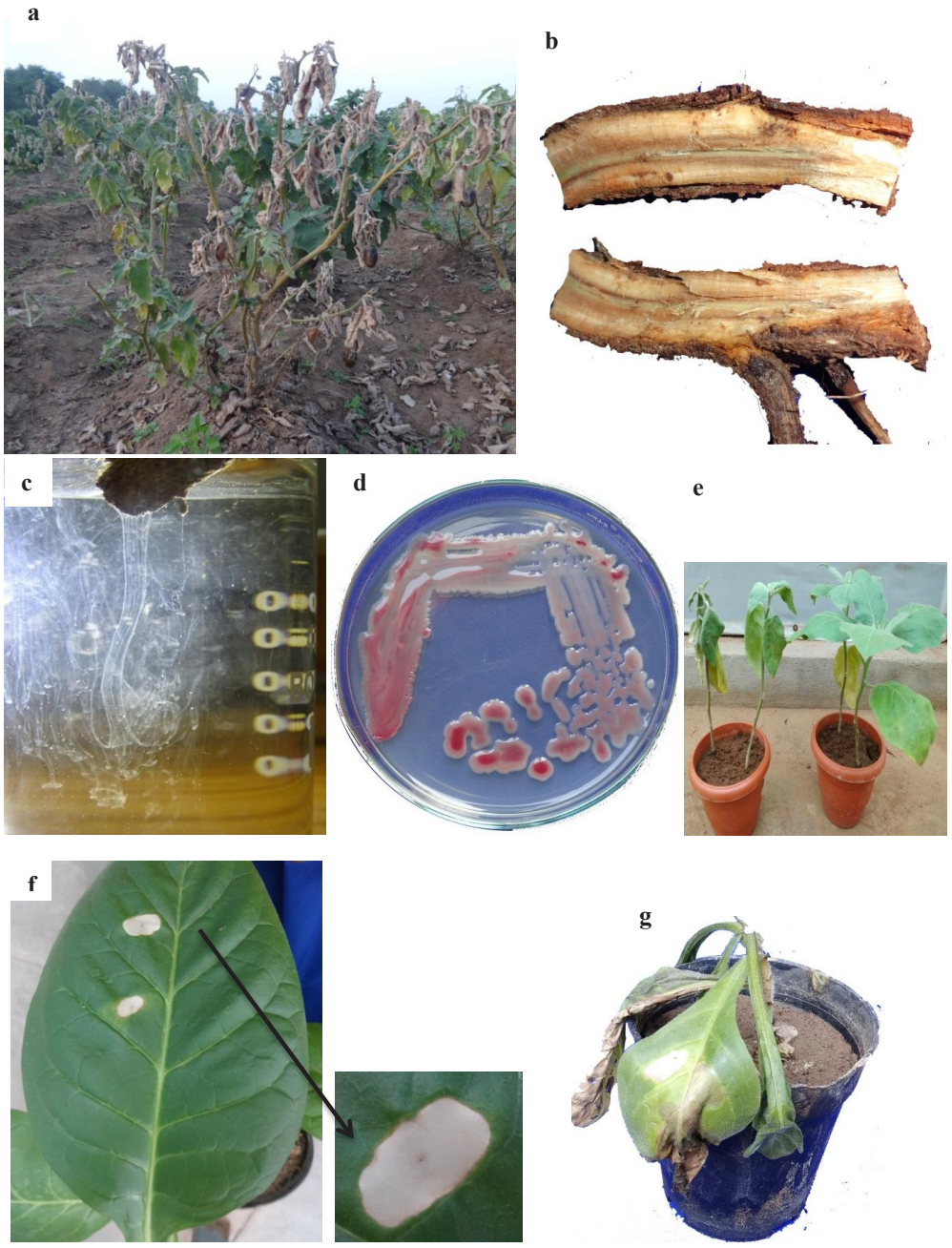


Fig. 1. a- Brinjal plant with symptoms of wilt in the field; b- longitudinal section of brinjal stem showing vascular discolouration; c- white smoke like bacterial streaming and diffusion in water; d- pure culture of *R. solanacearum* on TTC medium; e- pathogenicity test in brinjal inoculated with strain VBN1 showing severe wilt; f-hypersensitive reaction on tobacco leaves; g- Systemic wilt of tobacco plant by leaf infiltration assay.

by Rahman *et al*. (2010). Kelman (1954) also used triphenyl tetrazolim chloride (TTC) medium to distinguish *R. solanacearum* among other bacteria during isolation. The pathogenicity test was performed to prove Koch's postulates. The inoculated seedling reproduced the typical symptoms of sudden wilting and drooping of leaves as observed on the naturally infected plant within 10-15 d of inoculation (Fig. 1e) and also showed intense bacterial streaming from the cut end of stem. The pathogen was re-isolated from inoculated brinjal plant and compared with that of original pure culture of the strains . In control plants inoculated with sterile distilled water without bacterial inoculum, no such symptoms were observed. Zakir Hussain *et al.* (2005) also found that soil drenching method of inoculation was ideal for wilt incidence in brinjal and observed the wilt symptom in brinjal after 20 d of inoculation.

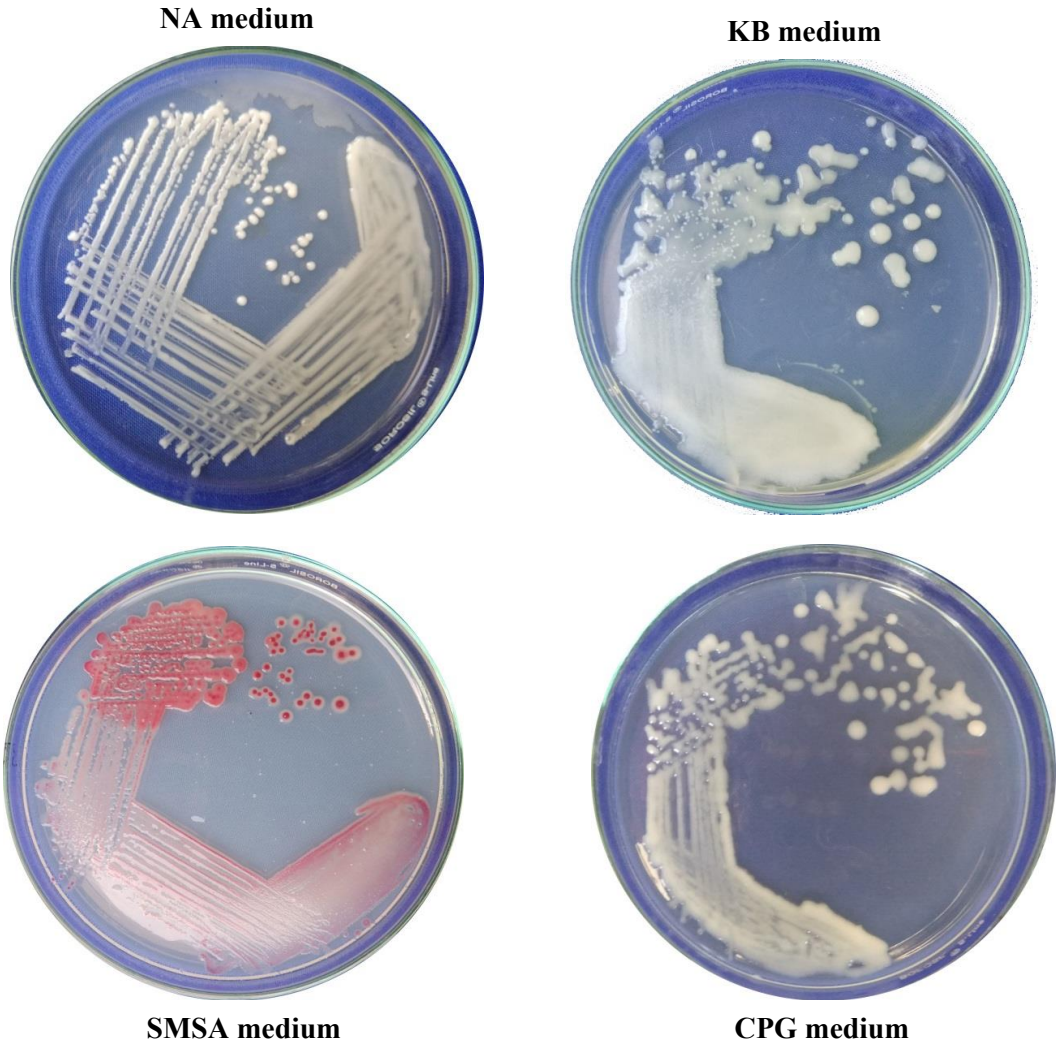
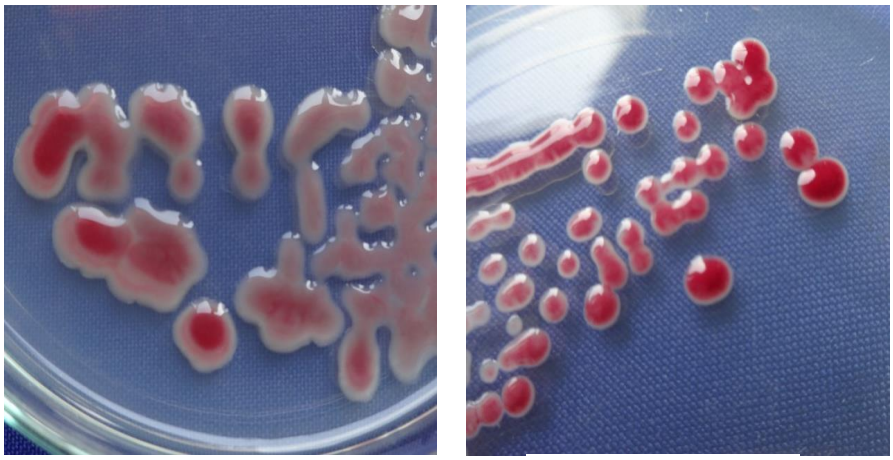


Fig. 2. Colony morphology of *Ralstonia solanacearum* on different media

Cultural and biochemical characterization

Cultural traits on different media are important tools for identification of *R. solanacearum*. In this study, different media were used to characterize the bacteria such as NA, SMSA, CPG, KB and TTC where the colony characters varies from regular to irregular margin, light to dark pink centre, creamy to white colour from small to big size and variation in EPS production were also observed and the results are described in Table 1; Fig. 2. The virulent and avirulent colonies were obtained during culturing of bacteria shown in Fig. 3. Among the different media tested TTC medium was found to be an excellent nutrient medium for growth and EPS production of *R. solanacearum*. According to Rohini *et al*. (2017) TTC medium when used for *R. solanacearum*, it shows the difference between avirulent colonies that look dark red from fluidal virulent that are white with pink centre. In the present study, the colonies were fluidal whitish with a pink center, indicating the isolated bacterium was virulent strains of *R. solanacearum.*

Biochemical characterization helps in genus level identification of the bacteria. Ten strains of *R. solanacearum* isolated from infected brinjal were characterized biochemically based on Gram staining, KOH solubility, catalase test, acid production, gelatin



b. Virulent a. Avirulent

Fig. 3. Virulent and avirulent strains of *Ralstonia solanacearum*

liquefication, starch hydrolysis, fluorescent pigment production and citrate utilization. The isolated bacteria showed positive result for KOH test by producing slime threads. The isolated strains showed gas bubbles upon treating with 3 % H_2O_2 indicating it showed positive result for catalase test. In case of acid production test, the bacterium produced the acid upon addition of methyl red and also positive result for citrate utilization by producing royal blue colour. The isolated bacteria exhibited negative result for Gram staining as it developed pink colour. No halo zone was observed around the culture indicating it showed negative result for starch hydrolysis. The bacterium was unable to liqueify the gelatin medium and also was unable to produce fluorescent pigment, indicating *R. solanacearum* is non-fluorescent. Different biochemical tests were carried out by Pawaskar *et al.* (2014) and they observed that the positive results for gas production, catalase test, citrate utilization and KOH test; negative results for starch hydrolysis, H_2S production, gelatin liquefication and cellulose decomposition tests under *in vitro* condition.

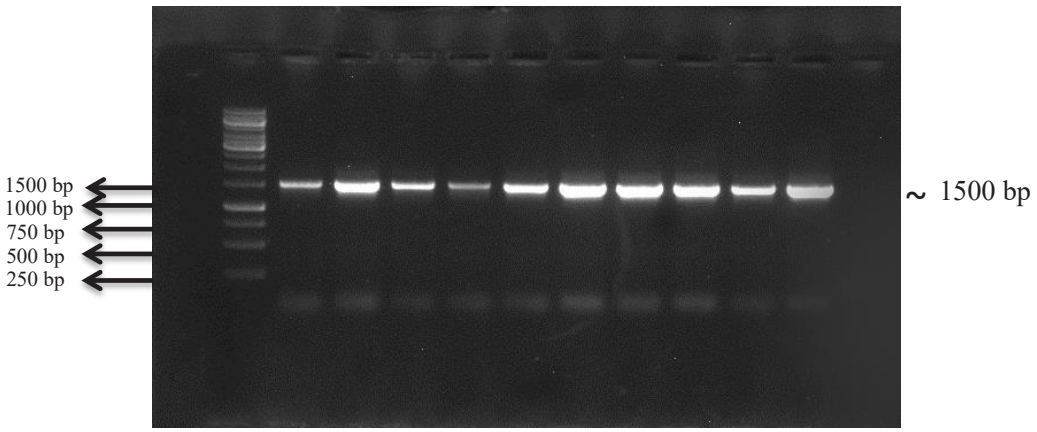


Fig. 4. PCR amplification of fragments of approximately 1500 bp generated by primers FD1& RP2 that refers to the gene of region 16S of the rRNA, from purified DNA from the Strains of *R. solanacearum* obtained from brinjal: L- 1 Kb ladder (Puregene), 1- VBN1, 2- VBN2, 3-ALG, 4- DKM, 5-VKM, 6-ERY1, 7-ERY2, 8-KYR, 9- ATR, 10- MLR and N-negative control

Determination of biovars

The biovar of *R. solanacearum* strains were identified by utilization of disaccharides (cellobiose, maltose, lactose) and sugar alcohols (dulcitol, mannitol and sorbitol). The result showed that all the strains of *R. solanacearum* were utilized the carbon source for their metabolic activity. This was identified by change of colour from bromothymol blue medium to yellow colour due to the production of acid in the medium. The prevalence of race-1 and biovar-III in India was strongly supported on the basis of utilization carbohydrates and sugar alcohols by results obtained by Prasanna Kumar (2004). Rahman *et al.* (2010) also identified the biovar of *R. solanacearum* isolates from brinjal based on the utilization of disaccharides and hexose alcohols. They observed all the seven groups of *R. solanaceraum* isolates oxidized disaccharides (Sucrose, lactose and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) within 3-5 d by the indication of colour change from blue to yellow which was confirmed as biovar III. This indicated that the bacteria isolated from brinjal falls under biovar III.

Identification of races

The racial pattern system groups the strains of *R. solanacearum* according to their ability to infect different host plants *viz.,* Race 1 having wide host range and pathogenic on solanaceous plants and Race 2 is restricted to banana, Race 3 affects potato, Race 4 infects ginger and Race 5 is pathogenic on mulberry. There is no biochemical test for race identification of bacterial wilt pathogen. The races of *R. solanacearum* were identified based on pathogenicity tests on varied host such as chilli, tomato, banana, potato, ginger and mulberry and also by hypersensitive response on tobacco leaves. Characteristics wilt symptoms were observed after 15-20 d of inoculation on tomato and chilli whereas, no symptom was observed in rest of the hosts. In the hypersensitive reaction test, tobacco leaf showed brown necrotic lesions (Fig. 1f) after 24-48 h post infiltration with *R. solanacearum* followed by systemic wilting (Fig. 1g) and complete death of the plant after one week. Dhital *et al.* (2001) also reported similar results indicating that *R. solanacearum* was able to produce HR in tobacco leaves followed by wilting of tobacco after 8 d of inoculation. Chandrashekara *et al.* (2012) reported that all the 57 isolates of brinjal produced typical wilt symptoms on their respective hosts on being inoculated by root injured technique and these isolates were designated as Race-1. In the present study, isolates were collected from the tropical, subtropical and plain regions of Tamil Nadu which was highly favorable for the prevalence of Race-1 of *R. solanacearum*. Hence, based upon the incapability of the isolated strain to cause infection on other hosts and ability to produce HR on tobacco leaves the strain in the present investigation was identified as Race-1.

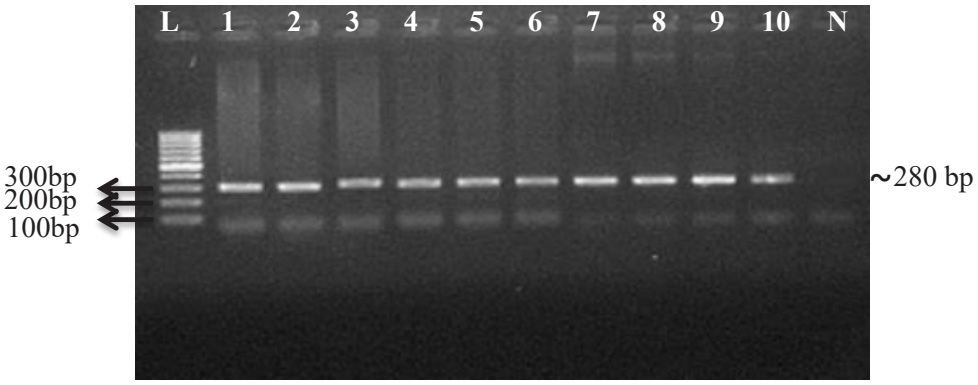


Fig. 5. PCR amplification showing fragments of approximately 280 bp generated by primers species -specific primers that refers to the ipxC (in planta expressed protein)gene of region, from purified DNA from the Strains of *R. solanacearum* obtained from brinjal: L- 100 bp ladder (Puregene), 1- VBN1, 2- VBN2, 3-ALG, 4- DKM, 5-VKM, 6-ERY1, 7-ERY2, 8-KYR, 9- ATR, 10- MLR and N-negative control

Molecular confirmation of Ralstonia solanacearum

Nucleic acid based methods are considered as a powerful tool to identify the microorganisms with high sensitivity and great specificity. PCR amplification of ITS region of ten strains of *R. solanacearum* was performed with universal primers. The amplicon size was approximately 1500 bp (Fig. 4). The species of the genus *Ralstonia* was identified as *R. solanacearum* by sequencing and BLAST search. The strains (ERY1 and VBN1) that showed 100% and 98% homology were submitted in NCBI GenBank (RS1- MH481663 for ERY1 and RS2- MH481664 for VBN1). Similar confirmation with 16S rRNA of *R. solanacearum* isolated from tomato amplified with a size of 1465 bp was reported by Balamurugan *et al.* (2018).

Table 1. Morphology and cultural characters of *R. solanacearum* on different media

Media	Colony morphology
Nutrient Agar medium	Very small colony, creamy white, round, slightly mucoid, translucent, slightly raised surface
King's B medium	Medium sized colonies, highly mucoid, round, opaque, slightly raised surface
Semi Selective South Africa Agar (SMSA) medium	Moderately fluidal, regularly shaped, white colony with dark red coloured center and the colonies were relatively small rigid
Tripheny Tetrazolium Chloride medium	Irregular with smooth margin, highly fluidal, large colonies, pink centered with creamy white border, opaque, raised
Casamino acid-peptone- Glucose (CPG) medium	Large colonies, irregular, creamy white, highly fluidal, opaque, raised surface

Specific primers have been developed earlier for detection of *R. solanacearum* strains using PCR. In this study, all the ten strains of *R. solanacearum* were again confirmed by PCR using specific primer as well which amplified the *ipxC* gene of *R. solanacearum*. All strains amplified with specific primer showed an expected amplicon size of 280 bp (Fig. 5). This result revealed that the bacterial

cultures were *R. solanacearum.* Kumar (2004) used the *R. solanacearum* specific primers (759f and 760r) for amplification of strains from Ginger plants. The amplicon size of about 280 bp was obtained for all the strains which confirms the causal organism of ginger bacterial wilt was *R. solanacearum*. Vanitha and Umesha (2014) reported similarly that the viable cells of *R. solanacearum* were amplified using PCR techniques with specific primers with an amplicon size of 281 bp.

Conclusion

The bacterial strains isolated from ten locations of Tamil Nadu in the study were confirmed to be *Ralstonia solanaceraum* based on phenotypic, biochemical and molecular analysis. All the ten strains were confirmed to belong to Race 1 and Biovar III based on the various phenotypic and biochemical analysis.

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