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Cultural, Physiological, Biochemical and Molecular Characterization of *Ralstonia solanacearum* Causing Bacterial Wilt of Tomato in Kerala, India

ABSTRACT

Bacterial wilt is a destructive vascular disease, especially seen in solanaceous vegetable crops, caused by *Ralstonia solanacearum*. It affects tomato and other crops particularly in tropical and subtropical regions. This study aimed to isolate, identify and characterize *R. solanacearum* from bacterial wilt infected tomato plants collected from Kottarakara, Kollam district of Kerala, India. The infected plants exhibited characteristic symptoms, including drooping of young leaves, green wilting and complete plant death. Infected plants showed vascular browning and bacterial ooze streaming from the cut ends of the stem confirmed the presence of the pathogen. The bacterium was cultured on different media such as nutrient agar (NA), king's B agar (KB), casamino acid peptone glucose agar (CPG) and triphenyl tetrazolium chloride agar (TTC) and showed difference in colony colour, margin and texture. On TTC medium, the virulent colonies exhibited fluidal, creamy white bordered margin with pink centre, indicative for excessive exopolysaccharide (EPS) production. Further, physiological tests confirmed its gram negative (G^{-ve}) nature and showed positive for catalase, oxidase and levan production. The bacterial isolate tested positive for nitrate reduction, urease activity, H_2S production, utilization of lysine, ornithine and various sugars (glucose, lactose and arabinose) and sugar alcohols (adonitol and sorbitol). Biovar determination of the isolate confirmed it as, *R. solanacearum* biovar 3. Molecular characterization using 16S rRNA and phylogenetic analysis further confirmed the identity of *R. solanacearum* at the genetic level. The sequence was deposited in the NCBI GenBank under the accession number PV022497. These findings demonstrated the effectiveness of the integrated diagnosis approach, including symptomatology, cultural, physiological, biochemical and molecular methods for accurate identification and characterization of *R. solanacearum*. Understanding the pathogenic and virulence traits of *R. solanacearum* offers insights for the development of better bacterial wilt management strategies in tomato cultivation.

Key words: Tomato, bacterial wilt, Ralstonia solanacearum, cultural characterization, physiological characterization, biochemical characterization, molecular characterization

1. INTRODUCTION

Ralstonia solanacearum is a gram negative, aerobic and soil borne bacterium that causes destructive bacterial wilt disease in solanaceous crops including tomato, brinjal, chilli, potato and tobacco, which significantly limits the crop productivity [15, 12]. The pathogen belongs to the *R. solanacearum* species complex (RSSC), which has genetically diverse strains exhibiting broad host ranges, geographic distribution and phenotypic variability [38].

Conventionally, the pathogen *R. solanacearum* was identified based on races and biovars. The races *of R. solanacearum* were primarily classified based on its infection on different host plants, while the biovars are categorized based on their ability to utilize different disaccharides and sugar alcohols for their biological functions [5, 16, 17]. However, these classifications are often limited to identifying phenotypic variability, and it lacks genetic resolution. More recently, molecular and phylogenetic studies have classified the RSSC into four major phylotypes including phylotype I (Asia), II (America), III (Africa) and IV (Indonesia). These phylotypes are further divided into sequence variants (sequevars) based on partial sequences of conserved genes such as *egl* (endoglucanase), *hrpB* (regulator gene of type III secretion system) and 16S rRNA [14, 38].The integrated approach of pathogen's detection greatly enhanced the understanding of the pathogen's genetic variability and evolutionary relationships, offering an accurate information of phytopathogenic bacteria which is the basis for successful management strategy development.

Fundamental diagnostic tools still serve as foundational methods for the identification of *R. solanacearum*. Colony morphology identification on semi-selective media such as triphenyl tetrazolium chloride (TTC) and selective medium from South Africa (SMSA) often differentiates the virulence and avirulence related characteristics of *R. solanacearum* [20, 7]. Further, physiological and biochemical assays, including gram staining, KOH (potassium hydroxide) solubility test, catalase and Kovac's oxidase tests, levan production, and utilization of carbohydrates and amino acids, are crucial for differentiating *R. solanacearum* from other bacterial phytopathogens [9, 1]. Recent advances in molecular diagnostics, especially the amplification of 16S rRNA have significantly improved accuracy in species level identification of *R. solanacearum*. 16S rRNA sequencing and phylogenetic analysis, enable accurate comparison of the unknown isolate with other strains in the molecular database and confirms its identity and genetic relatedness [2, 10].

Hence, the present study integrates cultural, physiological, biochemical and molecular approaches to confirm the identity of *R. solanacearum* isolated from the bacterial wilt infected tomato plants. The characterization included colony morphology on different media, physiological and biochemical assays, biovar determination through disaccharides and sugar alcohols oxidation profiles, and molecular characterization through 16S rRNA sequencing…... This multi-tiered detection strategy offers a robust structural framework for accurate identification and characterization of *R. solanacearum*, which helps to understand the pathogenic potential of the bacterium, and thus supporting the development of disease management strategies.

2. MATERIALS AND METHODS

2.1 Diseased sample collection

The tomato plants showing bacterial wilt with characteristic symptoms viz., sudden green wilting, water soaked black lesions on stem and leaf axis, and vascular brown discolouration were collected from Kottarakara, Kollam district of Kerala for the isolation of the pathogen, *R. solanacearum*. Standard bacterial ooze test was carried out for the confirmation of bacterial wilt before the sample collection.

2.2 Isolation of *R. solanacearum* from bacterial wilt infected tomato plants

R. solanacearum was isolated from the infected tomato plants using TTC medium (pH 6.8; peptone - 10 g, casein hydrolysate - 1 g, glucose - 5 g, agar - 10 g, 5 mL of 1% 2,3,5-triphenyl tetrazolium chloride, double distilled (dd) water - 1 L). Aseptically, the ooze was collected in sterile double distilled (dd) water from the infected plant samples and 30 µL of the oozed suspension was plated and evenly spread onto the TTC medium. Then the plates were incubated at 27 ± 2 °C with relative humidity (RH) 80 ± 5% for 48 h. The large,

irregular and fluidal creamy white bordered colonies with pinkish centre were maintained on TTC medium for further studies.

2.3 Pathogenicity test on susceptible variety Pusa Ruby

To prove Koch postulates, the pathogenicity of *R. solanacearum* was tested on 35-days old tomato plants var. Pusa Ruby (high yielding but susceptible to bacterial wilt; released from ICAR-Indian Agricultural Research Institute, New Delhi, India) using root clipping method followed by soil inoculation as described by Kumar et al. [24]. A loop full of bacterial colonies was harvested and suspended in TTC broth. Then, the broth was incubated at 27 ± 2 °C and RH 80 ± 5% for 48 h in a shaking incubator (REMI Elektro-technik Ltd, India) at 120 rpm to obtain the desired concentration of the bacterial growth @ 10^8 CFU/mL. Tomato seedlings roots were slightly clipped with scissors and dipped in bacterial inoculum (10^8 CFU/mL), followed by soil inoculation with 5 mL of the inoculum. The inoculated plants were maintained under controlled conditions at 27 ± 2 °C with relative humidity (RH) 80 ± 5% and photoperiod of 12h/12h light and dark, and regularly monitored for the symptom development.

2.4. Cultural characteristics of *R. solanacearum*

Colony characteristics such as colony morphology, colour, margin and texture of *R. solanacearum* were studied on different media, including NA (peptone - 5 g, beef extract - 2.5 g, NaCl - 5 g, agar - 10 g, dd water - 1 L); KB (peptone - 20 g, dipotassium hydrogen phosphate - 1.5 g, magnesium sulphate heptahydrate - 1.5 g, glycerol - 10 mL, agar - 10 g, dd water - 1 L); CPG (glucose - 5 g, peptone - 10 g, casein - 1 g, yeast extract - 1 g, agar - 10 g, dd water - 1 L) and TTC media. Single colony of *R. solanacearum* were streaked onto NA, KB, CPG and TTC media separately. The plates were incubated at 27 ± 2 °C with RH 80± 5% for 48 h to observe the colony characteristics of the bacterium.

2.5 Physiological tests of *R. solanacearum*

The physiological characteristics of *R. solanacearum* were assessed using 48h-old culture following the standard procedure developed by Sharma and Singh [41]. The tests included gram staining, KOH solubility test, catalase oxidase test, Kovac's oxidase test and levan production test.

Gram staining: A bacterial smear was prepared by spreading a loop full of bacterial colony onto a clean glass slide, followed by heat fixation at very low flame. Then the fixed smear was initially stained with crystal violet for 1 min and washed with running tap water. Again, the smear was stained with iodine solution for 30 sec and washed. Then decolourization was done with 95% ethyl alcohol until colour runoff. After washing, the slide was counterstained with safranin for 20 sec and again washed, air dried and observed under a microscope at 1000X magnification using oil immersion.

KOH solubility test: Potassium hydroxide (KOH) solution (3%) was prepared and a drop of the solution was placed on a clean glass slide. Then, a loop full of bacterial colonies were aseptically removed from the culture plate using a sterile inoculation needle and stirred for 10 sec in KOH solution. The formation of slime threads were observed [46].

Catalase oxidase test: A loop full of bacterial culture was mixed with 3% hydrogen peroxide (H_2O_2) on a clean glass side. The production of gas bubbles was observed with naked eye [39].

Kovac's oxidase test: 1% oxidase reagent (tetramethyl-p-phenyl diamine dihydrochloride) was prepared and stored in rubber stopper dark bottle. A piece of filter paper was placed in a petriplate and a drop of reagent was added at the centre of the filter

paper. Then a loop full of bacterial inoculum were rubbed on the filter paper and observed for the development of purple colour within 60 sec [23].

Levan production test: A loop full of bacterial inoculum was streaked on nutrient agar plate supplemented with 5% sucrose. Then the plates were incubated at room temperature (27 ± 2 °C) with RH 80 ± 5% for 48h. The formation of separate, discrete colonies was observed for the levan production [39].

2.6 Biochemical tests of *R. solanacearum*

The white to pinkish, irregular colonies were harvested and suspended in TTC broth. The broth was then incubated at 27 ± 2 °C and RH 80 ± 5% for 48h in a shaking incubator at 120 rpm to obtain the desired concentration (10^8 CFU/mL). The biochemical characteristics of *R. solanacearum* was tested using the KB002 HiAssorted Biochemical Test Kit (Himedia), which consists of 12 wells representing different tests. These tests included citrate utilization (in well no. 1), lysine utilization (well no. 2), ornithine utilization (well no. 3), urease activity (well no. 4), phenylalanine deamination (well no. 5), nitrate reduction (well no. 6), H_2S production (well no. 7), and the utilization of glucose (well no. 8), adonitol (well no. 9), lactose (well no. 10), arabinose (well no. 11), and sorbitol (well no. 12). The kit was aseptically opened under laminar air flow chamber and each well was inoculated with 50 µL of the bacterial inoculum and incubated at room temperature for 24h. After 24h, 3 drops of TDA (tryptophan deaminase) reagent were added to well 5, and 1 drop of sulfanilic acid followed by 2 drops of N, N-dimethyl-1-naphthylamine reagent were added to well 6. The remaining wells were observed for a positive reaction without adding any reagent.

2.7 Biovar identification of *R. solanacearum*

The *R. solanacearum* isolate was differentiated into biovar based on its ability to utilize disaccharides (dextrose, maltose, lactose and sucrose) and sugar alcohols (mannitol and sorbitol) as described by Hayward [16]. 10% of each disaccharides and sugar alcohols was prepared separately and sterilized. A semi solid mineral medium (ammonium dihydrogen phosphate - 1 g, potassium chloride - 0.2 g, magnesium sulphate heptahydrate - 0.2 g, peptone - 1 g, agar - 3 g, bromothymol blue - 80 mg/L, dd water - 1 L) was prepared and 10 mL each of sterilized disaccharides and sugar alcohols were added to the medium separately. Then, 200 µL of the melted medium with each disaccharides and sugar alcohols were dispensed into the wells of a microtiter plate. 20 µL of 48h old bacterial inoculum (10^8 CFU/mL) was added to the microplate wells. Then the plate was incubated at 27± 2 °C and RH 80 ± 5% for 72h. After incubation, the plates were examined for pH changes, indicated by a colour change.

2.8 Molecular characterization of *R. solanacearum*

16S rRNA isolation

The bacterium was grown in nutrient broth (NB) with constant shaking at 120 rpm for overnight at room temperature (27 ± 2 °C). The bacterial pellet was harvested by centrifugation at 10,000 rpm for 10 min. The pellet was resuspended in 600 µLof lysis buffer (9.00 mL of Tris-EDTA (TE) buffer, 600 µL of 10% SDS**,** 60 µL of proteinase K) and vortexed to ensure complete resuspension. The suspension was incubated for 1h at 37°C. After incubation, an equal volume of phenol/chloroform was added and mixed well by inversion. Centrifugation was done at 10,000 rpm for 5 min and the upper aqueous layer was carefully transferred to a new tube. An equal volume of chloroform was added and mixed by inversion, and centrifuged again at 10,000 rpm for 5 min. The upper aqueous layer was transferred to a new tube and equal volume of absolute ethanol was added to precipitate the RNA. The mixture was incubated at -20 °C for 30 min, followed by centrifugation at 11,000 rpm for 15 min at 4 °C. The supernatant was discarded and the RNA pellet was washed with 70% ethanol by centrifugation again at 10,000 rpm for 5 min. Finally, the supernatant was

discarded and the RNA was resuspended in TE buffer and the quality of RNA was checked by using agarose gel electrophoresis.

PCR analysis

PCR amplification was carried out in a 10 µL reaction mixture containing master mix - 5 µL, distilled water - 3 µL, forward primer - 0.5 µL (5'-AGTTTGATCCTGGCTC-3'), reverse primer - 0.5 µL (5'-AAGGACGTGATCCAGCC-3') and template RNA - 1 µL. The PCR amplification was carried out in a PCR thermal cycler (Bio- Rad, USA). The PCR amplification profile of initial denaturation at 95°C for 5.00 min, followed by denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec, extension at 72°C for 60 sec, for 35 cycles, and final extension at 72°C for 7.00 min, and then stored at 4°C for agarose gel electrophoresis.

Agarose gel electrophoresis of PCR products

The PCR products were analyzed using 1% agarose gel electrophoresis. The gel was prepared in 50 mL of 0.5X TBE (Tris-borate-EDTA) buffer with 2 µL of ethidium bromide. The 10 µL of PCR products was loaded into the gel. Electrophoresis was performed at 80V for 45 min using 0.5X TBE buffer, until the bromophenol blue front nearly reached the bottom of the gel. A 1-log DNA ladder was used as the molecular weight standard. The gel was visualized under UV light using a UV transilluminator and the image was captured with a gel documentation system.

Sequence analysis

For this, the PCR products were cut, eluted and purified for sequencing. The resulting amplicon was sequenced using the Sanger sequencing method. The sequence obtained was analyzed using NCBI's BLAST program and deposited in the NCBI GenBank to receive accession number. A phylogenetic tree with the sequence of 16S rRNA of the culture of *R. solanacearum* was then constructed using MEGA11 software.

3. RESULTS AND DISCUSSION

3.1 Symptomatology of bacterial wilt disease of tomato

The bacterial wilt symptoms were observed initially as downward drooping and wilting of young leaves (Fig 1a) along with water soaked blackish lesions on the stem and leaf axes of branching shoots of tomato plants (Fig 1b). In advanced stage, the green wilt symptom of the entire plant was observed which resulted in the complete death of plants (Fig 1c) and the infected stem showed brown vascular discoloration (Fig 1d). The bacterial ooze and its turbidity were observed from cut ends of bacterial wilt affected tomato plant stems which confirms the bacterial wilt of tomato (Fig 1e).The pathogen, *R. solanacearum* was isolated from bacterial wilt infected tomato plants.

This symptomatology is consistent with the findings of Wu et al. [49], who reported the drooping of young leaves, green wilt and vascular browning of infected tomato plants. Green wilting is a primary symptom of bacterial wilt, as *R. solanacearum* inhabits the xylem vessels and produces exoplysaccharides (EPS) that restrict the movement of water and nutrients [28]. In addition, *R. solanacearum* converts plant phenolic compounds into insoluble, dark melanin polymers that cause vascular browning in plants [4]. Similarly, Loreti et al. [27] noticed bacterial wilt symptoms include the collapse of the growing apex with stunted lateral shoots, adventitious root formation on the stem and brown vascular discolouration with dirty white ooze and finally plant became wilted and died. Vanitha et al. [47] observed the flaccid appearance of young leaves, wilting and death of entire plants showing vascular browning with yellowish ooze.

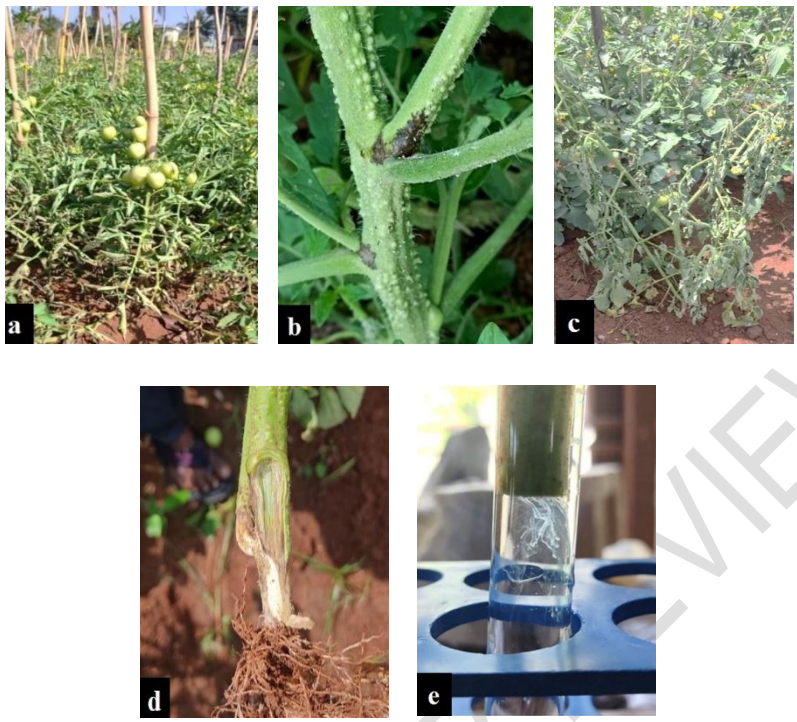


Fig 1: Symptomatology of bacterial wilt of tomato a) initial stage of green wilting, b) water soaked blackish lesions on leaf axis, c) complete wilting of plants (green wilt), d) vascular brown discoloration and e) ooze from the cut ends of infected tomato plants

3.2 Colony characteristics of *R. solanacearum* inciting tomato bacterial wilt

The virulence of *R. solanacearum* can be assessed based on colony colour on TTC medium, where two distinct colony types were observed. Virulent colonies appeared fluidal, irregularly round, and having either entirely white or white with a pink centre. In contrast, avirulent colonies were non-fluidal, smooth and exhibited a deep red colouration (Fig 2). The differential colony formation on TTC medium is influenced by the metabolic activity and EPS production of the bacterial cells. Virulent colonies produce more EPS and have high metabolic activity in localized zones (centre) that result in creamy white bordered with a pinkish centre, while avirulent colonies exhibit a deep red colour due to reduced EPS production and high TTC reduction. Rahman et al. [33] noticed typical virulent fluidal colonies with pink or light red colour center on TTC medium after 24 h of incubation. Similarly, both virulent fluidal colonies with pinkish centre and avirulent deep red colour colonies were observed on TTC medium [20, 6]. Moussa et al. [31] observed that virulent strains form irregular, fluidal colonies with white border and pink centers, whereas avirulent strains produce smaller, round, deep red colonies. Likewise, Liu et al. [26] reported that the reduced EPS production is correlated to down-regulated virulent and avirulent colony phenotypes, and was confirmed through transcriptomic analysis.

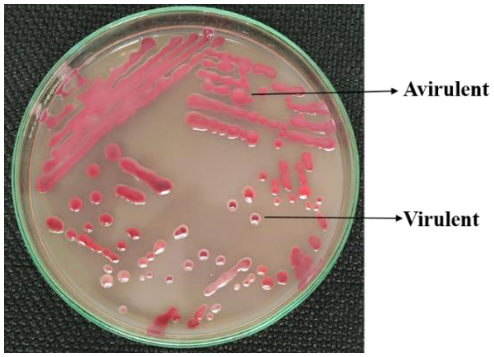


Fig 2: Virulent and avirulent colony characteristics of *R. solanacearum*. Virulent colonies appeared fluidal, irregularly round, and having either entirely white or white with a pink centre, and the avirulent colonies were non-fluidal, smooth with a deep red colouration

3.3 Colony morphology of *R. solanacearum* on different growth media

The colony characteristics, including colour, size and margin of *R. solanacearum* were studied on NA, KB, CPG, and TTC media (Fig 3, Table 1). On NA medium, the colonies were very small, creamy white, round and mucoid, while it was medium sized, highly mucoid and white on KB medium. On CPG medium, the colonies were large, irregular, creamy white and highly fluid. In contrast, on TTC media, irregular large colonies with smooth margins, highly fluid, pink at the centre with a creamy white border were seen. The differences in growth of bacteria suggest that regulation of nutrient availability for EPS production by different media.

These observations are consistent with the findings of Chandrashekara et al. [8] that *R. solanacearum* exhibited small, irregularly round, white and smooth margins on CPG medium. In addition, on TTC and SMSA media, the colonies exhibited a slimy, dull white appearance with a pink to red center, further confirming high metabolic activity by the virulent strain [50, 26].

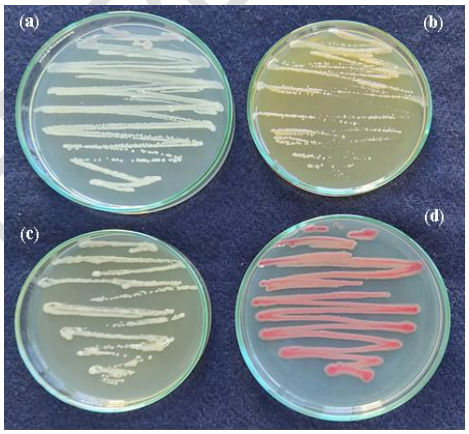


Fig 3: Colony morphology of *R. solanacearum* on different media a) Nutrient agar (NA) medium; b) King's B (KB) medium; c) Casamino acid peptone glucose (CPG) medium and d) Triphenyl tetrazolium chloride (TTC) medium

Table 1: Colony morphology of *R. solanacearum* on different growth media

Sl. No.	Medium used	Colony morphology of *R. solanacearum*
1	Nutrient agar	Small, creamy white, round, slightly mucoid colonies
2	King's B	Medium sized creamy yellow colonies, highly mucoid, round with slightly raised surface
3	Casamino acid peptone glucose	Large colonies, irregular, creamy white, fluidal with raised surface
4	Triphenyltetrazolium chloride	Irregular with smooth margin, highly fluidal, large, pink center with creamy white bordered colonies

3.4 Pathogenicity of *R. solanacearum*

The pathogenicity test revealed that, the isolate *R. solanacearum* was able to produce wilt in 35-days old tomato seedlings (var. Pusa Ruby) by root clipping followed by soil inoculation. The artificially inoculated plants exhibited green wilt symptoms, further leading to complete death of plants (Fig 4). The bacterium was re-isolated from the infected plants and was same of the original isolate. Similarity in morphological characters of isolate with its original culture was also noticed on comparison, thus confirming Koch postulates. Similarly, Balamuruganet al. [3] conducted a pathogenicity test on 30-day old tomato seedlings (cv. Meghdoot) using the soil inoculation method. The observed symptoms included downward drooping of young leaves with a green wilt appearance, followed by complete plant death.

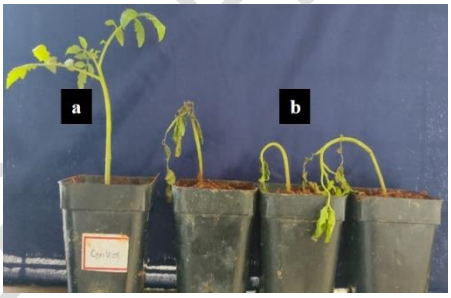


Fig 4: Pathogenicity of *R. solanacearum* on tomato var. Pusa Ruby after 5 days of inoculation. a) Control and b) Inoculated tomato plants

3.5 Physiological characteristics of *R. solanacearum* inciting bacterial wilt of tomato

The physiological characteristics of *R. solanacearum* were studied through gram staining, KOH solubility test, catalase oxidase test, Kovac's oxidase test and levan production test (Table 2). Gram staining results showed that the cells of *R. solanacearum* were red in colour, short, straight rods and G^{-ve} in reaction (Fig 5a). The G^{-ve} nature of *R. solanacearum* is attributed to its thin peptidoglycan layer, which allows the primary stain (crystal violet) to be washed out during decolourization, resulting in red coloured bacterial cells due to safranin staining. Similar morphological and staining characteristics have been previously reported by Khetmalas [21], Venkatesh [48] and Rath and Addy [35].

The KOH solubility test confirmed that *R. solanacearum* is G^{-ve}, as it produced strands of viscid material when its culture was treated with 3% KOH on a glass slide (Fig 5b). The G^{-ve} bacteria have a thin cell wall surrounded by outer membrane and this outer membrane is disrupted by 3% KOH which releases viscous DNA to form slime threads [9,32]. Similarly, the catalase oxidase test further confirmed its G^{-ve} nature by the production of gas bubbles upon exposure to 3% H_2O_2 (Fig 5c). It also confirmed the aerobic nature of *R. solanacearum* by reducing H_2O_2 into water and elemental oxygen [39,22]. In Kovac's oxidase test, *R. solanacearum* colonies developed purple colour within 60 sec of adding the oxidase reagent, indicating a positive result (Fig 5d). This test is useful for distinguishing between aerobic and anaerobic bacteria [23], and also for detecting the presence of cytochrome C oxidase in respiratory chain of bacteria [25]. Recent reports also highlight its utility for differentiating *Enterobacteriaceae* (oxidase-negative) from *R. solanacearum* (oxidase-positive) [34].

The formation of large, white, dome shaped mucoid colonies on NA supplemented with 5% sucrose confirmed the production of levan by *R. solanacearum* (Fig 5e). Levan is an intracellular bacterial polysaccharide produced by the enzyme levan sucrase, which converts sucrose into levan and glucose. This test can be used to differentiate fluorescent bacteria from non-fluorescent bacteria [37]. Mekonnenet al. [30] reported the levan biosynthesis as a phenotypic marker of virulence associated carbohydrate metabolism in *R. solanacearum*.

Table 2: Physiological tests of *R. solanacearum* inciting tomato bacterial wilt

Sl. No.	Physiological tests	Inference
1	Gram staining reaction	+
2	KOH solubility test	+
3	Catalase oxidase test	+
4	Kovac's oxidase test	+
5	Levan production test	+

(+ indicates positive)

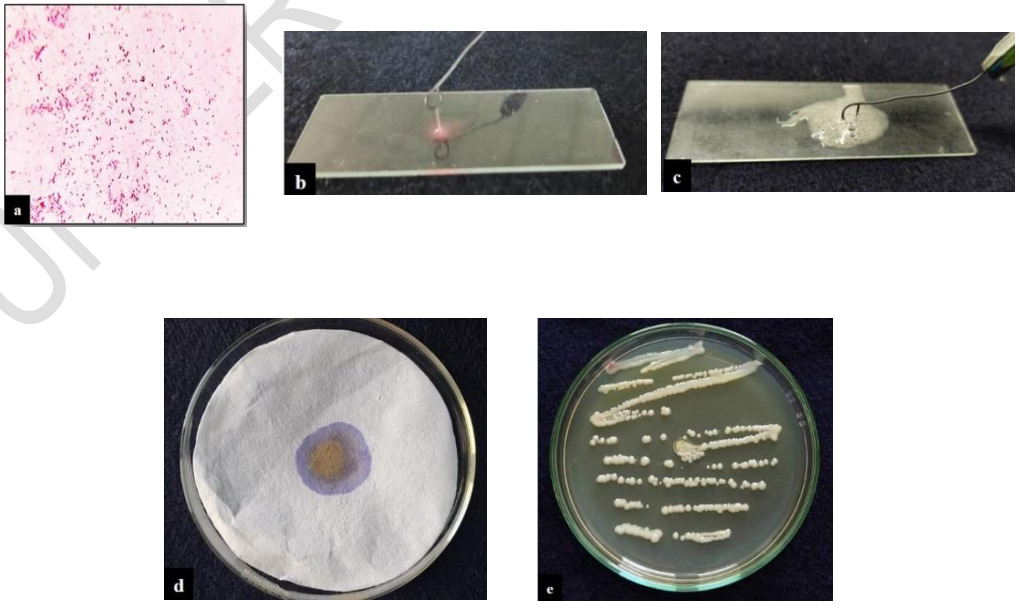


Fig 5: Physiological characteristics of *R. solanacearum*; a) rod shape cells of *R. solanacearum* on 1000X, b) slime thread formation on 3% KOH; c) gas bubble formation on 3% H_2O_2; d) development of purple colour within 60 sec; e) large, white, dome shaped mucoid colonies on NA supplemented with 5% sucrose

3.6 Biochemical characteristics of *R. solanacearum* inciting bacterial wilt of tomato

The biochemical characteristics of *R. solanacearum* were tested using the KB002 HiAssorted Biochemical Test Kit (Himedia, India), which consists of 12 wells representing different tests (Table 3, Fig 6). *R. solanacearum* tested positive for lysine utilization, ornithine utilization, urease activity, nitrate reduction, H_2S production and utilization of glucose, adonitol, lactose, arabinose and sorbitol, whereas it showed negative results to citrate utilization and phenylalanine deamination. These results suggest the ability of *R. solanacearum* to metabolize a wide range of carbohydrates and amino acids, which may support the survival and virulence of bacteria. In comparison, Maji and Chakrabartty [29] reported that, *R. solanacearum* tested positive for nitrate reduction and the utilization of cellulose, lactose and maltose, while it was negative for phenylalanine deaminase, lysine decarboxylase and arginine dihydrolase activities. These differences in biochemical characteristics may reflect strain specific variations and diversity among *R. solanacearum* isolates. Similarly, Dey and Sen [13] reported strain specific variation in sugar metabolism and amino acid decarboxylation, suggesting the role of biochemical characteristics in classification of *R. solanacearum*.

Table 3: Biochemical tests of *R. solanacearum* inciting bacterial wilt of tomato

Well No.	Tests	Original color of the medium	Positive reaction	Inference
1	Citrate utilization	Light yellow	Blue	-
2	Lysine utilization	Light brown	Light to dark purple	+
3	Ornithine utilization	Dark brown	Light to dark purple	+
4	Urease	Orange yellow	Pink	+
5	Phenylalanine deamination	Colourless	Green	-
6	Nitrate reduction	Colourless	Brownish black	+
7	H_2S production	Brownish yellow	Light to dark black	+
8	Glucose utilization	Orange yellow	Yellow	+
9	Adonitol utilization	Orange	Yellow	+
10	Lactose utilization	Dark orange	Yellow	+
11	Arabinose utilization	Orange yellow	Yellow	+
12	Sorbitol utilization	Dark orange	Yellow	+

(+ indicates positive; - indicates negative)

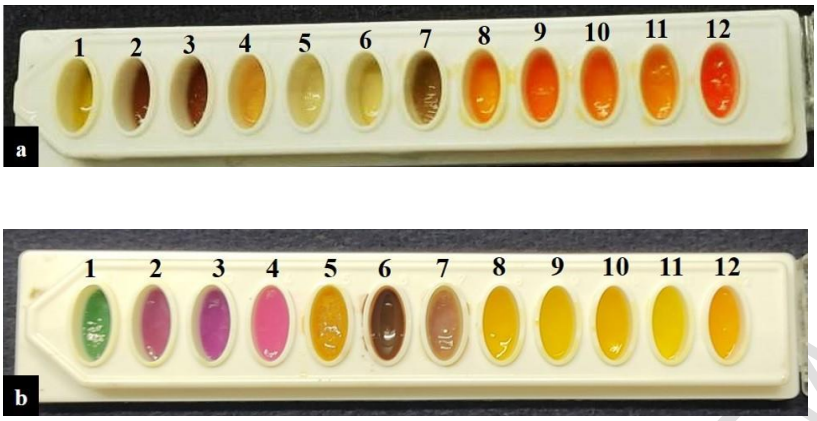


Fig 6: Biochemical characteristics of *R. solanacearum* on KB002 kit; 1) citrate utilization, 2) lysine utilization, 3) ornithine utilization, 4) urease activity, 5) phenylalanine deamination, 6) nitrate reduction, 7) H_2S production, 8) glucose utilization, 9) adonitol utilization, 10) lactose utilization, 11) arabinose utilization and 12) sorbitol utilization; a) control and b) treated with *R. solanacearum*

3.7 Biovar of *R. solanacearum* inciting bacterial wilt of tomato

The biovar test confirmed that the *R. solanacearum* isolate was capable of oxidizing all tested disaccharides (dextrose, maltose, lactose, and sucrose) and sugar alcohols (mannitol and sorbitol) (Table 4). A positive reaction indicated by a colour change from yellow to light green is due to the oxidation of carbohydrates and changes in pH of the medium. This positive reaction confirms the isolate belongs to biovar 3 (Fig 7). Singh et al. [44] reported that out of 65 *R. solanacearum* isolates, 58 were biovar 3, while 7 belonged to biovar 4 based on its utilization of disaccharides and sugar alcohols. Similarly, Denny and Hayward [11] noted that biovar 3 and 4 were prevalent in Asia, causing wilt disease in a wide range of hosts, including solanaceous crops like tomato, potato, chilli and brinjal. Singh et al. [43] and Subedi et al. [45] reported that biovar 3 was dominant among *R. solanacearum* isolates infecting tomato and brinjal in several parts of India and Southeast Asia.

Table 4: Biovar characterization of *R. solanacearum* inciting bacterial wilt of tomato

S. No	Disaccharides/ sugar alcohols	Name of disaccharides and sugar alcohols	Inference
1.	Disaccharides	Dextrose	+
2.		Maltose	+
3.		Lactose	+
4.		Sucrose	+
5.	Sugar alcohols	Mannitol	+
6.		Sorbitol	+

(+ indicates positive)

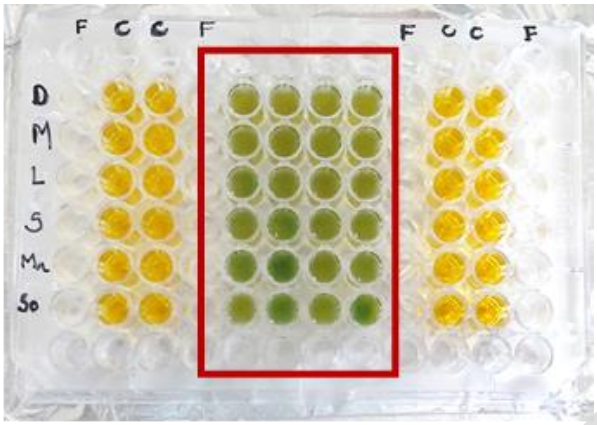
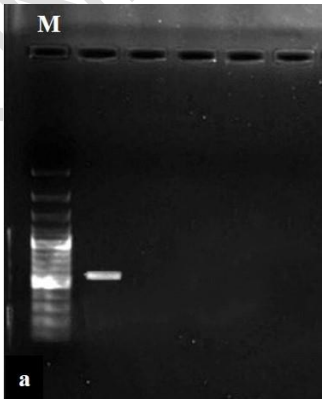


Fig 7: Biovar identification of *R. solanacearum*; Green colour change indicates Biovar 3 of *R. solanacearum*. Disaccharides: D - Dextrose; M - Maltose; L - Lactose and S - Sucrose; Sugar alcohols: Ma - Mannitol and So - Sorbitol; F - Free well and C – Control media without *R. solanacearum*

3.8 Molecular characterization of *R. solanacearum*

16S rRNA region of *R. solanacearum* biovar 3 was amplified using species specific primers and yielded ~540 bp PCR product (Fig 8a). Sanger sequencing of the amplicon confirmed the identity of the isolate, exhibiting high sequence similarity to known *R. solanacearum* strains in the NCBI GenBank database. A phylogenetic tree was constructed using the obtained 16S rRNA sequence along with closely related sequences retrieved from NCBI GenBank (accession numbers: CP034195, CP034197, CP088235, CP034199, CP034194, KC188251, CP088233, KC188236, KC188227, and KC188228). The isolate clustered firmly within the *R. solanacearum* clade, providing strong molecular evidence for species level identification (Fig. 8b). The sequences were deposited in NCBI GenBank under the accession number PV022497. According to earlier studies, bacterial wilt of solanaceous crops is caused by *R. solanacearum* and has been reported by James et al. [19]; Selastin Antony et al. [40]; Rohiniet al. [36]; Hossain, et al. [18]; Sharmaet al. [42].



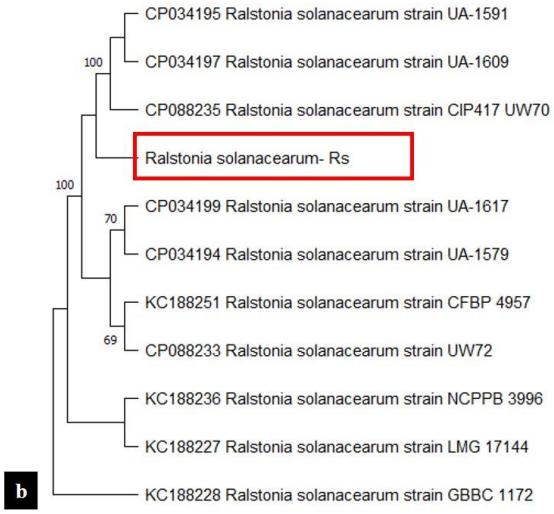


Fig 8: Molecular characterization of *R. solanacearum* a) gel image of amplicon (~540bp; M- 1 log DNA ladder) and b) Phylogenetic tree of *R. solanacearum*-the maximum likelihood tree was computed with Mega 11 with 1000 boot strap replications

4. CONCLUSION

The present study successfully identified and characterized *R. solanacearum* as the causal organism of bacterial wilt of tomato through an integrated approach that included symptomatology, and also with the cultural, physiological, biochemical and molecular characteristics of the pathogen. The characteristic green wilt, vascular browning and bacterial ooze were observed in infected tomato plants. The isolate, *R. solanacearum* showed distinct colony morphology on NA, KB, CPG and TTC media. The physiological characteristics such as gram staining, KOH solubility test, catalase, Kovac's oxidase and levan production tests, further confirmed the nature of *R. solanacearum*. *R. solanacearum* exhibited broad biochemical characteristics, utilizing different carbohydrates and amino acids. The bacterium was identified as Biovar 3 based on utilization of disaccharides and sugar alcohols. Molecular characterization and phylogenetic analysis further confirmed the close relatedness to reference strains of *R. solanacearum*. These findings highlight the phenotypic and genotypic characteristics of *R. solanacearum* Biovar 3 inciting bacterial wilt of tomato and provide a foundation for the precise diagnosis of bacterial wilt pathogen of tomato.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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